Published August 4, 2008

Ku80 removal from DNA through double strand break–induced ubiquitylation

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The Ku70/Ku80 heterodimer, or Ku, is the central component of the nonhomologous end joining (NHEJ) pathway of double strand break (DSB) repair. Because Ku forms a ring through which the DSB threads, it likely becomes topologically attached to DNA during repair. The mechanism for its removal was unknown. Using a method to identify proteins recruited to DSBs in *Xenopus laevis* egg extract, we show that DSB-containing DNAs accumulate members of the Skp1–Cul1–F-box complex and K48-linked polyubiquitylated proteins in addition to known repair proteins. We demonstrate that Ku80 is degraded in response to DSBs in a ubiquitin-mediated manner. Strikingly, K48-linked polyubiquitylation, but not proteasomal degradation, is required for the efficient removal of Ku80 from DNA. This removal is DNA length dependent, as Ku80 is retained on duplex oligonucleotides. Finally, NHEJ completion and removal of Ku80 from DNA are independent from one another. We propose that DSB-induced ubiquitylation of Ku80 provides a mechanism to efficiently eliminate Ku from DNA for pre- and postrepair processes.

Introduction

Double strand breaks (DSBs) are a particularly dangerous form of DNA damage, which if not repaired can lead to genome rearrangements and to cancer (Hoeijmakers, 2001). The cell has two major pathways to repair DSBs: homologous recombination, which requires a homologous copy of the DNA for repair, and nonhomologous end joining (NHEJ), which does not (Wyman and Kanaar, 2006). The first component of the NHEJ pathway to bind to a DSB is the Ku70/Ku80 heterodimer, also known as Ku (for review see Downs and Jackson, 2004). Upon binding to DNA, Ku recruits the other factors required for NHEJ, including the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, ligase IV, Cernunnos (also known as XLF), and Artemis (Wyman and Kanaar, 2006). These proteins then process the broken DNA and seal the two ends (Lieber et al., 2003). In addition to repair of nonprogrammed DSBs, Ku is critical for the VDJ recombination pathway of lymphocyte development (Rooney et al., 2004). Ku80 is essential in human somatic cells (Li et al., 2002), but Ku-deficient mice are viable, though small, and display a severe compromised immunodeficiency phenotype resulting from an inability to process VDJ breaks (Nussenzweig et al., 1996; Zhu et al., 1996; Gu et al., 1997).

Although Ku80 requires Ku70 for binding to DNA, Ku70 may display some weak DNA-binding activity on its own (Chou et al., 1992; Griffith et al., 1992). Consistent with its role as a detector of DSBs, Ku binds linear DNA significantly better than closed supercoiled DNA (Blier et al., 1993). Similar to many other proteins involved in DNA metabolism (Hingorani and O'Donnell, 2000), Ku forms a toroid with a central channel that binds DNA (see Fig. 4 B; Walker et al., 2001; Spagnolo et al., 2006). This channel, which makes contacts with B-form DNA, is composed of residues from both subunits of Ku. The channel is thought to be responsible for the heterodimer's recognition of DSBs, as no major structural changes to the ring itself are needed to thread a DNA end through the complex (Walker et al., 2001). A consequence of this mode of end recognition is that Ku and the DNA will become topologically linked on completion of repair (Paillard and Strauss, 1991; Walker et al., 2001).

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Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3-related; DB, double biotin; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, double strand break; ms/ms, tandem mass spectrometry; NHEJ, nonhomologous end joining; SB, single biotin; SCF, Skp1–Cul1–F-box.

The online version of this paper contains supplemental material.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 182 No. 3 467–479
www.jcb.org/cgi/doi/10.1083/jcb.200802146

Supplemental Material can be found at:
http://www.jcb.org/cgi/content/full/jcb.200802146/DC1

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Unlike the replication clamp proliferating cell nuclear antigen and many other ring-shaped DNA-binding complexes, which can be released from linkage to DNA through conformational changes separating two subunits with adjacent domains (Hingorani and O'Donnell, 2000), both subunits of Ku encircle the DNA and form an extended protein–protein interdigitation (Walker et al., 2001). Thus, the two subunits cannot be separated with a simple conformational change, and a removal mechanism analogous to that of proliferating cell nuclear antigen is unlikely. Instead, if the DNA is to remain unbroken, removal of Ku will require the cleavage or large-scale conformational changes of one or both subunits. Because the channel is completely filled by a single duplex of DNA, removal would be necessary for replication to be completed on the repaired DNA.

Using *Xenopus laevis* egg extracts, we find that ubiquitylated proteins are specifically enriched on DNA containing a DSB. In addition, Ku80 bound to DSBs is heavily modified by posttranslational modifications that include polyubiquitin. K48-linked polyubiquitylation is required for the damage-dependent proteasomal degradation of Ku80 and its efficient removal from DNA. However, proteasomal degradation is not necessary for Ku80 removal from DNA. The regulation of Ku80 removal from DNA is DNA length dependent, as Ku80 is not efficiently removed from short double-stranded oligos. Through the use of Ku80 truncation and point mutants, we show that although DSB binding is required for Ku80 ubiquitylation and degradation, the ubiquitin-dependent removal and degradation of Ku80 from DNA is independent of the completion of NHEJ. We propose that DNA binding initiates the polyubiquitylation of Ku80, which is required for its removal. We suggest that ubiquitin-dependent dissociation may be a more general mechanism for the removal of DNA-bound proteins.

Results

Polyubiquitylated proteins accumulate at DSBs

To study processes induced by DSBs, we devised a method to identify proteins accumulated on DNA containing DSBs in *X. laevis* egg extracts. Unless specified, we used extracts arrested at meiotic metaphase II (CSF extracts), which are fully functional for ataxia telangiectasia mutated (ATM) activation in response to DSBs and for Ku-dependent NHEJ (Labhart, 1999; Guo and Dunphy, 2000; Di Virgilio and Gautier, 2005; unpublished data). Either one end or both ends of a linear 3-kb plasmid were biotinylated and bound to streptavidin-coated magnetic beads. Although the former beads leave one DNA end free (single biotin DNA [SB-DNA] beads), both DNA ends of the latter beads (double biotin DNA [DB-DNA] beads) are concealed (Fig. 1 A, left). The DNA of SB-DNA beads resembles a DSB and is rapidly repaired in the CSF extract (Fig. 1 B) as judged by the doubling in DNA length. Several proteins preferentially copurified with SB-DNA beads (Fig. 1 A). Tandem mass spectrometry (ms/ms) revealed that these include previously known DSB-interacting proteins, such as DNA-PKcs, RPA1, Mre11, Ku70, and Ku80 (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200802146/DC1). In contrast, histone H3 copurifies with both SB- and DB-DNA beads (Fig. 1, C and E).

Further ms/ms analysis revealed that all three lanes contained ubiquitin, but the signal was qualitatively strongest in the samples copurified with SB-DNA beads. The presence of ubiquitylated proteins was confirmed by immunoblotting with an antibody against ubiquitin (Fig. 1 C). Whereas both SB- and DB-DNA beads copurified histone H3, SB-DNA beads specifically copurified ubiquitylated proteins. The staining mostly occurred in the high molecular mass region of the gel, with a particularly strong band migrating at ~100 kD.

It has been thought that much of the polyubiquitin at DSBs is linked through lysine (K) 6 (Morris and Solomon, 2004) and K63 (Yang and Zou, 2007), which are not canonical signals for protein degradation. To identify the specific form or forms of polyubiquitylation attached to SB-DNA beads, we took a directed approach using mass spectrometry. We searched the detected peptide masses for those that corresponded to the masses of each of the possible branched ubiquitin peptides, and then confirmed the identities of these peptides using ms/ms. We were able to identify some peptides corresponding to the K63-linked ubiquitin-branched peptide. In addition, nearly the entire gel lane contained K48-branched peptides, which is the proteasomal degradation signal (Pickart and Fushman, 2004). We did not detect any peptides of the other branched isoforms of ubiquitin, including the K6 branch. Additionally, although the previously observed polyubiquitylations were induced on ATM/ATM and Rad3-related (ATR) checkpoint activation, our polyubiquitin signal as detected by immunoblotting was insensitive to caffeine, which can inhibit ATM/ATR activity in egg extracts (unpublished data). We therefore speculated that the K48-linked polyubiquitylation was of a different origin than the previously observed modifications at DSBs, which are thought to be dependent on the BRCA1 and RNF8 ubiquitin ligases (Morris and Solomon, 2004; Polanowska et al., 2006; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007).

Ku80 is ubiquitylated at DSBs

To investigate which proteins bound to a DSB are ubiquitylated, we looked at several candidate proteins that we had identified in the SB-DNA bead purification by ms/ms analysis. Ku80, Ku70, WRN, and Mre11 were labeled with ^{35}S-methionine in reticulocyte lysates, mixed with the egg extract, and incubated with SB-DNA beads. All four proteins were modified to varying degrees when bound to DNA (Fig. 1 D). Ku70 was modified in a very faint ladder, and this was confirmed by immunoblot (unpublished data). Most strikingly, however, DNA-bound Ku80 was heavily modified in a ladder of bands reminiscent of polyubiquitylation. Immunoblotting confirmed that these modifications were present on endogenous Ku80 copurified with SB-DNA beads but not with DB-DNA beads (Fig. 1 E). The majority of the ubiquitylation on SB-DNA beads was Ku dependent (unpublished data). Also, isolation of proteins conjugated with 6-His–tagged ubiquitin from extracts containing linear DNA selectively copurified the modified form of Ku80 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200802146/DC1). Therefore, we concluded that Ku80 itself was ubiquitylated upon binding to the DSB-containing DNA.

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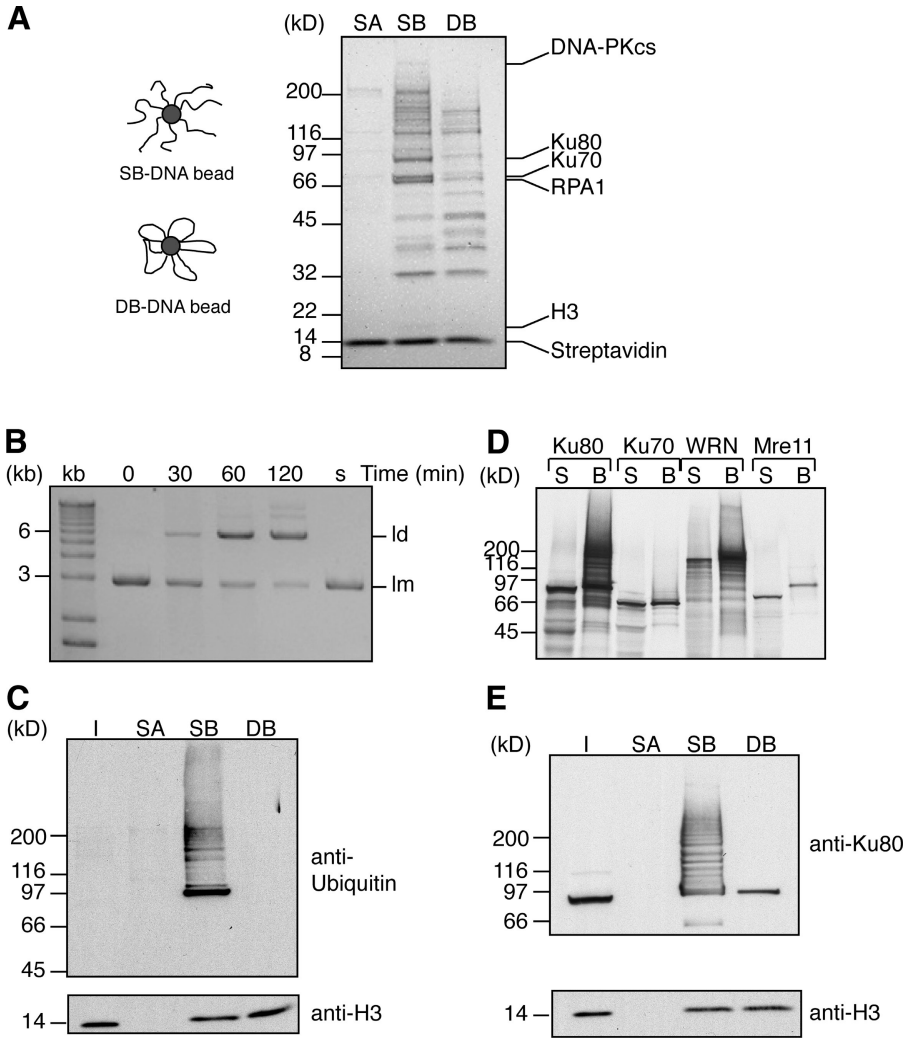

Figure 1. **Polyubiquitylation and Ku80 modification at DSBs.** (A) Streptavidin-coated, SB-DNA, and DB-DNA beads were incubated in egg extract for 30 min, and bound proteins were visualized with colloidal coomassie stain. A similar gel was analyzed by ms/ms, and some proteins identified are indicated. SA, streptavidin beads; SB, SB-DNA beads; DB, DB-DNA beads. (B) After SB-DNA beads were incubated in egg extract for the indicated times, DNA was purified and visualized by ethidium bromide. kb, 1-kb ladder; lm, linear monomer; ld, linear dimer; s, substrate DNA. (C) Proteins coisolated with streptavidin-coated, SB-DNA, and DB-DNA beads after 45-min incubation in egg extract were immunoblotted with antibodies against ubiquitin and histone H3 as a control. I, extract input; SA, streptavidin beads; SB, SB-DNA beads; DB, DB-DNA beads. (D) ^{35}S-methionine–labeled Ku80, Ku70, WRN, or Mre11 were mixed with extract in which SB-DNA beads were incubated for 45 min and visualized by exposure to film. S, supernatant; B, beads. (E) As in C, except the proteins were immunoblotted with antibodies against Ku80 and histone H3.

Components of the Skp1–Cul1–F-box (SCF) complex bind to DSBs in a Ku-dependent manner

We noticed a strong 100-kD band reacting to the antiubiquitin antibody. ms/ms analysis identified the presence of Cul1, a component of the SCF E3 ubiquitin ligase complex (Willems et al., 2004) in this band. By immunoblotting, we confirmed that Cul1 and Skp1 were present in the SB-DNA bead purification, but largely absent from the DB-DNA beads and that the majority of this binding was Ku dependent (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200802146/DC1). In addition, although Ku80 interacted with Ku70 either in the absence or presence of linearized DNA, its interaction with Cul1 was linear DNA dependent (Fig. S2 B). In contrast, other cullin family members (Cul2, Cul3, and Cul4) failed to bind strongly to the SB-DNA beads, although Cul3 did show a slight preference for SB- over DB-DNA beads (Fig. S2 C).

Activated cullin proteins are modified with the ubiquitin-like protein Nedd8 (Pan et al., 2004), which can cross react with antiubiquitin antibodies (Lammer et al., 1998). In fact, antiubiquitin, anti-Nedd8, and anti-Cul1 antibodies recognize the exact same band from the SB-DNA sample after a close examination by two-color Western blots (unpublished data). Therefore, the majority of Cul1 bound to DSBs appears to be neddylated. These results suggest that the SCF is an excellent candidate E3 for the DSB-induced Ku80 ubiquitylation.

Ku80 is degraded by the proteasome in response to DSBs

Because K48-linked ubiquitin was associated with SB-DNA beads, we asked whether exposure to DSBs induced the ubiquitin-mediated degradation of Ku80. To monitor DNA-induced degradation, we added linear DNA to extract containing ^{35}S-labeled Ku80 at a concentration of 10^{12} ends per microliter, or 0.6 mg/ml, in excess of the estimated concentration of Ku80 in the extract ($0.7–2 \times 10^{11}$ molecules per microliter; Labhart, 1999). Within half an hour of the addition of linear DNA, a fraction of Ku80 migrated as a high molecular mass smear (Fig. 2 A and see Figs. 7 E and S4 [available at http://www.jcb.org/cgi/content/full/jcb.200802146/DC1]), whereas Ku80 incubated with supercoiled DNA or buffer did not. We quantified the amount of radioactive signal in the entire lane using a phosphorimager, and found that its intensity decreased over time only when the extract contained linear DNA (Fig. 2 B). In contrast, the abundance of both Ku70 and Mre11 was unchanged when exposed to linear DNA (Fig. 2 C). Immunoblots using an anti-Ku80

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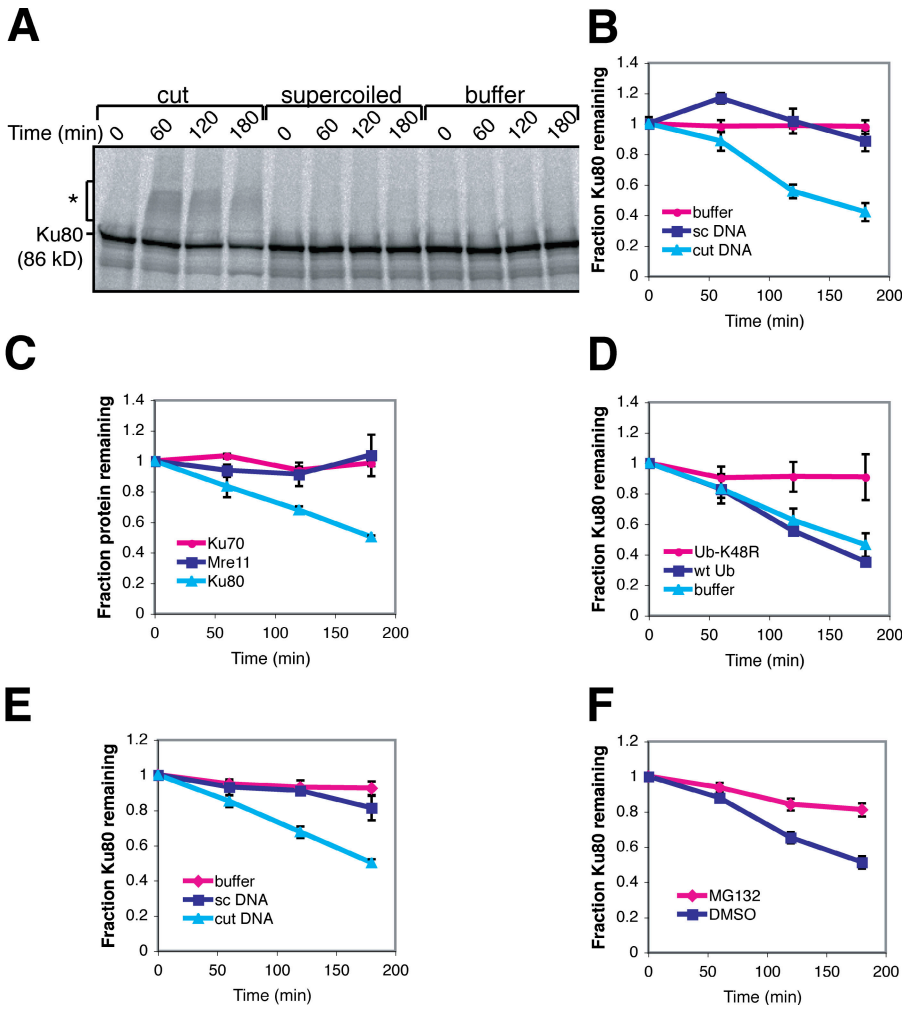

Figure 2. **DSBs lead to the proteasomal degradation of Ku80.** (A) Cut linear DNA, supercoiled circular DNA, or buffer was added to extract containing ^{35}S-labeled Ku80. After incubation at 22°C for the indicated times, samples were taken. The asterisk indicates DSB-dependent modified Ku80. Radioactively labeled protein was visualized using a phosphorimager. (B) Quantification of the radioactivity of the entire lane in A using a phosphorimager (n = 4). (C) Degradation, quantified as in B, of Ku80, Ku70, and Mre11 in the presence of cut DNA (n = 3). (D) Degradation, quantified as in B, of Ku80 in the presence of cut DNA and buffer, 0.5 mg/ml wild-type ubiquitin, or 0.5 mg/ml ubiquitin-K48R (n = 3). (E) Degradation of Ku80 in the presence of buffer, supercoiled DNA, or cut DNA. At indicated time points extract samples were spotted onto glass microfiber filters and precipitated in 10% TCA, and radioactivity was quantified using a liquid scintillation counter (n = 4). (F) Degradation, quantified as in E, of Ku80 in the presence of either DMSO or 100 μM MG132 (n = 4). Error bars denote one standard error of the mean.

antibody revealed that endogenous Ku80 was also modified and lost over time in the presence of DSBs (Fig. S3, A and B). Degradation of Ku80 was observed in response to DSBs in interphase extract as well as CSF-arrested extract (unpublished data).

To accurately quantify the amount of Ku80, including proteins that may be so heavily modified as to not enter the gel, total radioactivity was measured using a liquid scintillation counter after TCA precipitation of protein (Fig. 2 E). These results were nearly identical to those obtained using the phosphorimager.

To confirm that the observed degradation of Ku80 depends on K48-linked polyubiquitin, mutant ubiquitin in which K48 was replaced with arginine (K48R), which can no longer branch at K48, was added to the extract. The mutated ubiquitin acted as a dominant-negative protein and prevented the loss of Ku80 (Figs. 2 D and S4, A and B). Although Ku80 does become modified in the presence of ubiquitin-K48R, these modifications are shifted in favor of lower molecular mass proteins (Fig. S4 A), as would be expected if the mutant was inhibiting ubiquitin branching. In contrast, in the presence of ubiquitin-K6R or -K63R, the ubiquitylation and degradation of Ku80 was unchanged (Fig. S4, A and B; and not depicted), suggesting strongly that only K48-branched ubiquitin was required for Ku80 degradation. Consistently, the proteasome inhibitor MG132 slowed Ku80 degradation, as compared with addition of the solvent DMSO (Fig. 2 F). The decrease in Ku80 in response to DSBs therefore appeared to be caused by its ubiquitin-dependent proteasomal degradation.

Polyubiquitylation, but not proteasomal degradation, leads to the removal of Ku80 from DNA

We next asked whether the ubiquitylation of Ku80 was responsible for its removal from DNA. To address this question, we developed an assay to track the loss of Ku80 from DNA over time (Fig. 3 A). SB-DNA beads were added to extract containing ^{35}S-labeled Ku80. After 30 min, beads were isolated on a magnet and washed with and subsequently resuspended in extracts that did not contain radioactive protein. At 30-min increments, beads were isolated and washed, and the Ku80 remaining on the DNA beads was visualized by autoradiography.

Buffer, ubiquitin, or ubiquitin-K48R was added to both the extract containing and lacking the labeled Ku80. In the presence of buffer or wild-type ubiquitin, the labeled Ku80 initially bound to DNA was heavily modified in a high molecular mass ladder (30 min), and then was rapidly lost after transfer to unlabeled extract (Fig. 3 B). When ubiquitin-K48R was added, however, Ku80 modification was shifted in favor of lower molecular mass species and Ku80 was retained on the DNA even after the

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transfer. Therefore, K48-linked polyubiquitylation is important for Ku80 release from DNA.

This result suggested that proteasomal degradation might be involved in removal of Ku80. To address this question, we tested the effect of MG132 in the release assay. Using a liquid scintillation counter, we measured the levels of radioactive Ku80 that remained on beads and that were released into the supernatant after 30 min in the nonradioactive extract chase (Fig. 3 C, $t = 60$ min). As expected, Ku80 was rapidly lost from the SB-DNA beads in the presence of wild-type ubiquitin. Very little of the dissociated Ku80 was found in the supernatant, indicating that it had been degraded. Consistent with the autoradiography, ubiquitin-K48R largely prevented the removal of Ku80. However, MG132 had no effect on bead retention, although the presence of a large fraction of Ku80 in the supernatant suggests that the drug did effectively inhibit degradation of Ku80 released from DNA. These results demonstrate that K48-linked polyubiquitylation, but not proteasomal degradation, is required for the release of Ku80 from DNA.

Ku80 is inefficiently ubiquitylated and removed from DNA when bound to short oligonucleotide duplexes

As the DNA-binding site of Ku binds B-form DNA, it is capable of binding naked DNA efficiently in contrast to many damage-associated proteins that require chromatin for their activities. To address whether chromatin was required for the DSB-dependent ubiquitylation and degradation of Ku80, we used two short oligonucleotides: one that when annealed formed a 4-nt biotinylated hairpin with a 20-bp base and a 49-bp duplex containing a 3′-biotin on one strand. These were bound to streptavidin-coated magnetic beads and incubated in the extract as previously described. As opposed to SB- and DB-DNA beads, which contain 3 kb DNA, these short oligo DNA beads do not bind histones in extract (unpublished data).

Although oligo DNA beads efficiently bound Ku80 (Fig. 4 A), as expected, the protein was reproducibly modified in a different pattern compared with Ku80 bound to SB-DNA beads. The modifications were shifted to lower molecular mass forms in a pattern reminiscent of that occurring in the presence of ubiquitin-K48R. We hypothesized that the binding of the responsible E3 ubiquitin ligase may be inhibited on the shorter DNAs. Supporting the possibility that the SCF complex is the E3 of Ku80, immunoblotting indicated that components of the SCF complex interacted with oligo DNA beads less efficiently than with SB-DNA beads (Fig. 4 A). Cul1 and Skp1 binding to the oligo DNA beads, normalized to Ku80 binding, was at ∼25–40% of the level of that bound to SB-DNA beads.

Because the pattern of Ku80 ubiquitylation on short DNAs resembled ubiquitylation in the presence of ubiquitin-K48R, we wondered whether the degradation and dissociation from DNA were similarly affected. Indeed, Ku80 was not degraded in the presence of short double-stranded oligonucleotides (unpublished data). In addition, the release of Ku80 from oligo DNA beads was far less efficient than from the SB-DNA beads (Fig. 4, B and C). We conclude that effective ubiquitylation, removal from DNA, and degradation of Ku80 require DNAs longer than 50 bp. Additional DNA-binding sites or nucleosomes may be required.

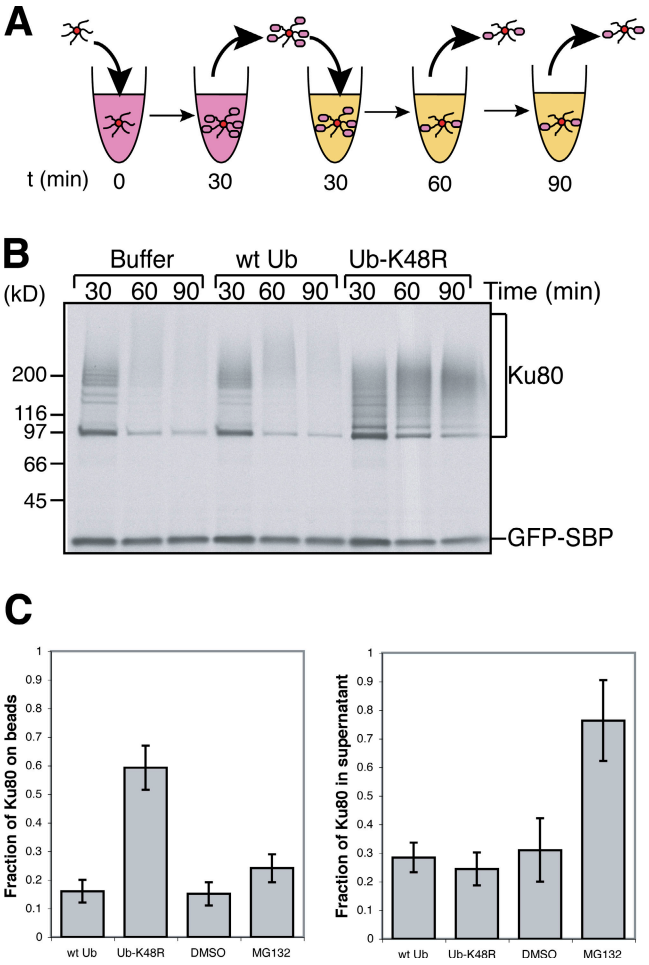

Figure 3. **Polyubiquitylation induces the loss of Ku80 from DNA.** (A) The experimental scheme of the release assay. At time 0, SB-DNA beads and streptavidin-coated beads were added to egg extract containing ^{35}S-labeled Ku80 and either ubiquitin storage buffer, 0.5 mg/ml ubiquitin, 0.5 mg/ml ubiquitin-K48R, DMSO, or 100 μM MG132. Beads were incubated for 30 min, removed from the extract, and subsequently washed and incubated in extract containing the additional factors but lacking the radioactive proteins for an additional 30 or 60 min. As a control for bead recovery, we used the streptavidin beads coated with ^{35}S-labeled GFP fused to the streptavidin-binding peptide (GFP-SBP). (B) The results of the experiment described in A after exposure to film. Extract contained buffer, 0.5 mg/ml ubiquitin, or 0.5 mg/ml ubiquitin-K48R. Polyubiquitylated Ku80 and GFP-SBP, added as a recovery control, are indicated. (C) Extract contained buffer, 0.5 mg/ml ubiquitin, 0.5 mg/ml ubiquitin-K48R, DMSO, or 100 μM MG132. Beads isolated after 30 min in extract containing radioactive Ku80 ($t = 30$ min), along with beads chased for an additional 30 min in nonlabeled extract ($t = 60$ min), were washed, and retained radioactivity was quantified using a liquid scintillation counter. In addition, samples were taken from the supernatant of the 60-min time point and precipitated onto glass microfiber filters with 10% TCA, and radioactivity was quantified using a liquid scintillation counter. The values of Ku80 remaining on beads and Ku80 in supernatant at $t = 60$ min were normalized relative to the radioactivity on beads at $t = 30$ min for each sample. Error bars denote one standard error of the mean; $n = 3$.

DSB-dependent posttranslational modification and degradation of Ku80 require only the DNA-binding region of Ku80

What are the structural requirements for the recognition and ubiquitylation of DNA-bound Ku? The atomic structure of the Ku70/Ku80 heterodimer bound to DNA revealed that the central core domains of both Ku70 and Ku80 form a ring structure that encircles

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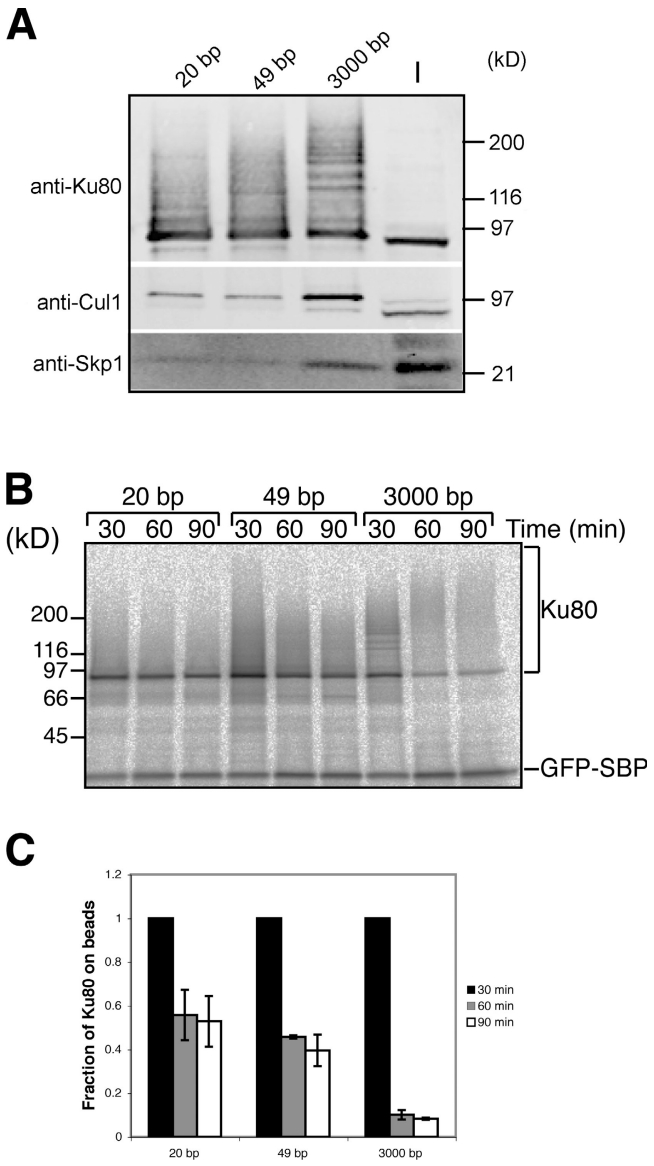

Figure 4. **Ku80 bound to short oligonucleotide duplexes has less polyubiquitylation and is stable on DNA beads.** (A) Steptavidin-coated beads bound to a biotinylated hairpin-containing 20-bp duplex DNA, a 49-bp double-stranded oligo containing a biotinylated 3′ end on one strand, or a 3,000-bp SB-DNA were incubated in extract for 30 min. Binding of Ku80, Cul1, and Skp1 were analyzed by immunoblot. (B) 20- and 49-bp oligo DNA beads and SB-DNA beads were used in the DNA release assay detailed in Fig. 3 A. (C) Quantification of B using a phosphorimager. Error bars denote the range of the data; $n = 2$.

DNA (Walker et al., 2001; Fig. 5 B), whereas N-terminal regions mimic the von Willebrand A domain (Aravind and Koonin, 2001), which is considered to be a module for protein–protein interactions (Fig. 5 A). The very C terminus of Ku80 is important for interaction with DNA-PKcs (Gell and Jackson, 1999; Singleton et al., 1999; Walker et al., 2001; Fig. 5 A) in a manner that is stimulated by DNA (Gottlieb and Jackson, 1993; Suwa et al., 1994). To determine the region that contains the E3 recognition site or the ubiquitin acceptor sites, we made a series of truncations of the N and C termini of Ku80 (Fig. 5 A). Each of these truncations was labeled with ^{35}S and assayed for DNA binding (Fig. 5, A and C) and degradation in the presence of cut DNA (Fig. 5 A).

Consistent with the atomic structure and other previous studies (Cary et al., 1998; Gell and Jackson, 1999; Walker et al., 2001), neither the N nor the C terminus of Ku80 was required to interact with DNA, and we defined a core domain of Ku80 necessary for DNA binding (Fig. 5, residues 244–540). In addition, all truncations that bound DNA also became modified upon binding. We conclude that the E3 recognition site, as well as at least a subset of ubiquitylation sites, resides in the central DNA-binding domain of Ku80.

Next we tested whether these truncations were degraded in extract containing cut DNA. We found that each Ku80 truncation incapable of efficiently binding DNA was stable in the presence of DSBs (Fig. 5 A). Alternatively, truncations that bound DNA were degraded in the presence of DSBs. Some truncations tested, namely amino acids 1–713 and the minimal 244–540, appeared to have significantly longer half-lives than the full-length protein. This slow degradation seemed to correlate with a low translation efficiency of these proteins in the reticulocyte lysate, and we therefore cannot conclude that these truncations are missing regions of the protein required for ubiquitylation and degradation. The fact that the central domain (residues 183–543) is sufficient for binding and effective DSB-dependent degradation (Fig. 5 A and Fig. S3, C and D) indicates that neither the N terminus nor the C terminus of Ku80 is essential for its DSB-induced degradation. Altogether, these results confirm the tight coupling between DNA binding and ubiquitin-mediated degradation of Ku80.

Polyubiquitylation-induced removal of Ku80 from DSBs is not required for the completion of NHEJ

Identification of a Ku80 mutation that inhibits the release from DNA was critical to investigate the biological role of this phenomenon, but we failed to obtain a useful mutant for such studies through the truncation analyses described in Fig. 5. By accident, however, we discovered a point mutation (introduced serendipitously during PCR) of Ku80 that led to inhibition of degradation without affecting its DNA-binding capacity (Figs. 5 A and 6 A). The substitution W275R is at a tryptophan, which is conserved among vertebrates, near the N terminus of the DNA-binding region of Ku80 (Fig. 5 A). This residue appears to make contacts with both Ku70 and with the DNA (Walker et al., 2001), but its function has not been studied.

Although Ku80-W275R was stable in the presence of linear DNA, it became modified in a pattern indistinguishable from the wild-type protein when bound to SB-DNA beads (Fig. 6 B). As we had previously shown that this pattern is dependent on K48-mediated polyubiquitylation (Figs. 3 A and S4 A), we concluded that Ku80-W275R was likely able to be polyubiquitylated normally when bound to DSBs. This led us to speculate that the inhibition of degradation was, instead, mediated by the prevention of its release from DNA. To test this, we used radioactively labeled mutant protein in the release assay detailed in Fig. 3 A. Indeed, Ku80-W275R was stably retained on DNA (Fig. 6, B and C).

A mutant that could bind but was not removed from DSBs gave us an opportunity to begin to test the function of Ku80

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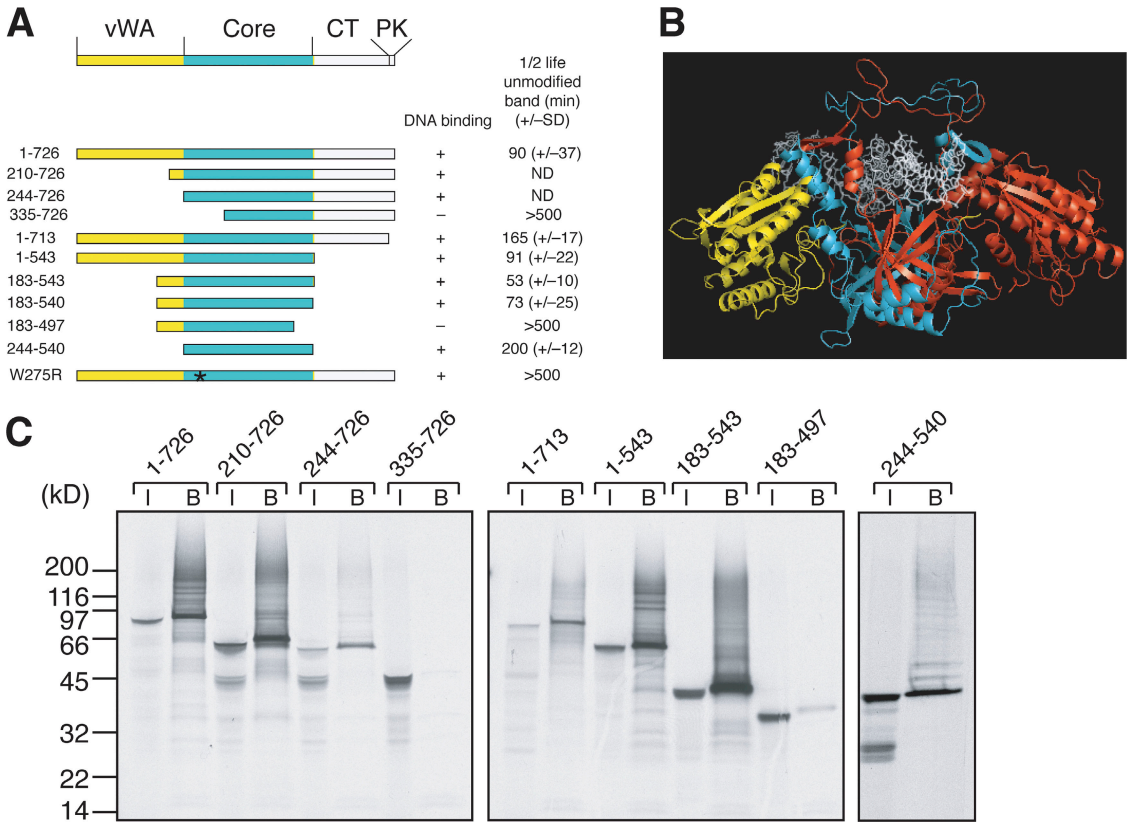

Figure 5. **N- and C-terminal Ku80 truncations are ubiquitylated and degraded in response to binding to DSBs.** (A) Domain organization of Ku80 is modified from a previous review (for review see Downs and Jackson, 2004). Ku80 domains include vWA, N-terminal von Willibrand A domain; Core, central core domain; CT, C-terminal region; PK, DNA-PKcs–interacting peptide (Gell and Jackson, 1999). The core DNA-binding region is indicated in light blue, and the other regions of the protein included in the atomic structure are in yellow. Regions of the protein not included in the structure are white. Numbers to the left indicate amino acids included in the protein. An asterisk indicates the location of amino acid W275. Truncations were generated as indicated and labeled with ^{35}S in rabbit reticulocyte lysate. Proteins were then mixed with egg extract, to which was added either SB-DNA beads to assess DNA binding or cut linear DNA to measure degradation. Half-lives of the unmodified bands after addition of cut DNA, quantified using a phosphorimager, are indicated. (B) The structure of the human Ku70/Ku80 heterodimer bound to DNA (Walker et al., 2001). Residues up to the equivalent of amino acid 543 of the *X. laevis* Ku80 are included in the structure. Red, Ku70; yellow, Ku80; white, DNA. The region of Ku80 required for DNA binding (*X. laevis* residues 244–540) is light blue. (C) Truncations were mixed with extract and incubated with SB-DNA beads for 45 min. Bound proteins were visualized by exposing to film. I, input; B, beads.

removal, and one possibility was that Ku80 must be removed before the completion of NHEJ to allow ligases access to broken DNA ends. To test this model, we examined whether Ku80-W275R could support NHEJ in *X. laevis* egg extracts. We replaced endogenous Ku80 with wild-type or mutant protein by first depleting ~98% of the Ku complex from extract using anti-Ku80 antibodies, and then adding mRNAs encoding Ku80 or Ku80-W275R, as well as Ku70, to translate in the extract (Fig. 6, D and E). The extract translated the Ku mRNAs to ~25% (wild type) and 20% (W275R) endogenous levels. Ku-dependent NHEJ in *X. laevis* egg extract can be monitored by observing the conversion of linearized DNA to circular DNA (Labhart, 1999; Di Virgilio and Gautier, 2005). As expected, mock-depleted extract quickly formed circular DNA (Fig. 6 F), whereas the Ku-depleted extract formed supercoiled circular DNA very inefficiently. In contrast, both wild-type Ku80 and Ku80-W275R were able to rescue NHEJ (Fig. 6 F). Because the point mutant, at least in this simplified assay, appeared to be functional for NHEJ, we conclude that polyubiquitin-mediated removal of Ku80 from DNA is not required for the ligation of DNA ends. Supporting this conclusion, neither ubiquitin-K48R nor MG132 inhibited NHEJ (Fig. S4 C).

Repair through the NHEJ pathway is not required for the ubiquitylation or degradation of Ku80

Because Ku80 removal was not a prerequisite for NHEJ, it was formally possible that the completion of repair, not binding to DSBs itself, was the signal for Ku80 ubiquitylation and removal from DNA. To test this possibility, we looked for a Ku80 mutant that was competent for binding to DNA but was unable to coordinate NHEJ, and monitored whether it can still be ubiquitylated and degraded upon binding to DSBs.

As shown in Fig. 5, the central core domain of Ku80 was essential for DNA binding. In addition, this region alone, when expressed in reticulocyte lysate and added to extract, was ubiquitylated and degraded effectively in response to DSBs. However, this was in the presence of the NHEJ activity of the endogenous protein. We eliminated the potential contribution of endogenous Ku to NHEJ by first depleting at least 97% of it from extracts and then adding back mRNA encoding Ku80 truncations (missing their C terminus or both N and C termini) together with Ku70 mRNA (Fig. 7 A). After translation, Ku70 was produced to near wild-type levels in all three samples, as was the full-length Ku80 (Fig. 7 A). As the ΔC ($Ku80^{1-543}$) and ΔNΔC ($Ku80^{183-543}$)

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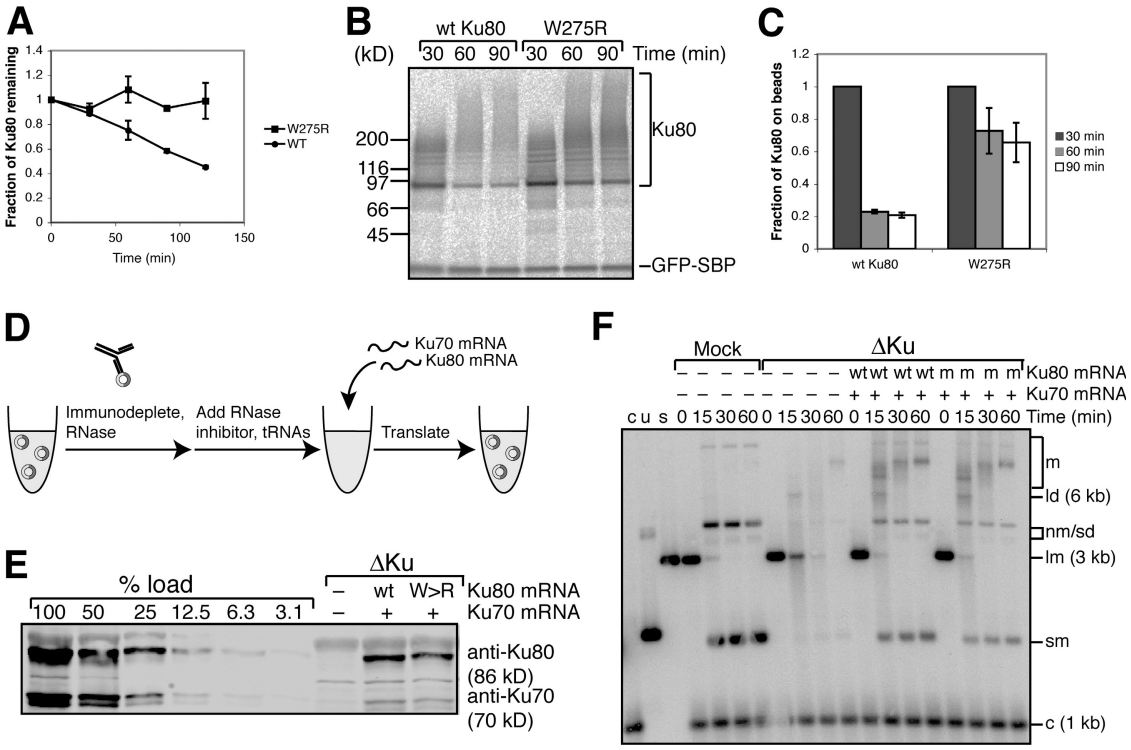

Figure 6. **Ku80-W275R is functional for NHEJ but is not released from DNA.** (A) Radioactively labeled wild-type (WT) Ku80 or Ku80-W275R were mixed with egg extract and linear DNA was added. Samples were taken at the indicated time points, and degradation was quantified using a phosphorimager. Error bars denote the range of data; $n = 2$. (B) Wild-type Ku80 and Ku80-W275R were used in the release assay (Fig. 3 A), and the results were visualized using a phosphorimager. (C) Quantification of results in B, including modified and unmodified Ku80. Error bars denote the range of data; $n = 2$. (D) Schematic of immunodepletion/translation experiment. Extract was immunodepleted using an anti-Ku80 antibody in the presence of RNase A, after which an RNase inhibitor was added along with tRNAs. mRNAs encoding Ku70 and either wild-type or mutant Ku80 were then added and allowed to translate in the extract. (E) Samples of extract after translation were immunoblotted and probed with antibodies against Ku70 and Ku80. For comparison, a dilution series of undepleted extract has been included. (F) After translation, Xmn1-digested pBluescript SK+ DNA was added to the extract. At indicated time points, samples were taken and treated with proteinase K and phenol/chloroform extraction. DNA was run on an agarose gel containing ethidium bromide, Southern blotted, probed with pBluescript DNA, and exposed to film. s, linear substrate; c, internal 1-kb control; u, uncut plasmid; sm, supercoiled monomer; lm, linear monomer; nm/sd, nicked monomer/supercoiled dimer; ld, linear dimer; m, higher order multimers.

truncations were missing the C-terminal epitope of our antibody, we monitored their protein level by adding ^{35}S-methionine to the extract. This revealed that the truncations were produced at similar levels to the full-length Ku80 (Fig. 7 B).

We next tested the ability of Ku80 truncations to coordinate NHEJ reactions. Whereas, as in Fig. 6 F, the mock-depleted extract efficiently formed circular DNA (Fig. 7 C, lanes 1), the Ku-depleted extract did not (Fig. 7 C, lanes 2). This defect in NHEJ was fully rescued by expression of full-length Ku80 and Ku70 (Fig. 7 C, lanes 3). In contrast, expression of the ΔC truncation only partially rescued the depletion (Fig. 7 C, lanes 4), and the ΔNΔC truncation was almost completely nonfunctional (Fig. 7 C, lanes 5), closely mirroring the Ku-depletion phenotype.

To monitor ubiquitylation of Ku80 truncations in the absence of endogenous NHEJ activity, proteins translated in extract in the presence of ^{35}S-methionine were allowed to bind SB-DNA beads (Fig. 7 D). Consistent with our previous results, full-length Ku80, as well as the ΔC and ΔNΔC truncations, became modified when bound to DNA beads, both in the absence and presence of endogenous full-length Ku80 (Fig. 7, compare D with B).

Finally, we examined whether the ΔC and ΔNΔC truncations, which can bind to DNA but have reduced NHEJ activity, can be degraded in response to DSBs. We found that full-length Ku80 was degraded at the same rate as both truncated proteins either in the presence or absence of endogenous wild-type protein (Fig. 7, E and F, left). All three Ku80 varieties were degraded slightly faster in the depleted extract, probably because the radioactive protein is the only Ku present. Consistent with our previous results, the labeled Ku70 was stable in the presence of linear DNA in all samples (Fig. 7, E and F, right). We had previously found that the degradation of the ΔNΔC truncation was dependent on the presence of linear DNA and did not occur on addition of supercoiled DNA (Fig. S3, C and D). We conclude that completion of NHEJ is not required for the damage-dependent degradation of Ku80.

Discussion

The toroidal structure of Ku represents an elegant solution to the problem of DSB recognition, but it necessarily leads to a topological impediment to its removal once repair is complete. We have shown that Ku80 becomes polyubiquitylated when bound to a DSB in *X. laevis* egg extract, and that this leads to its removal from DNA. Although Ku80 is degraded by the proteasome in response to DSBs, its dissociation from DNA does not

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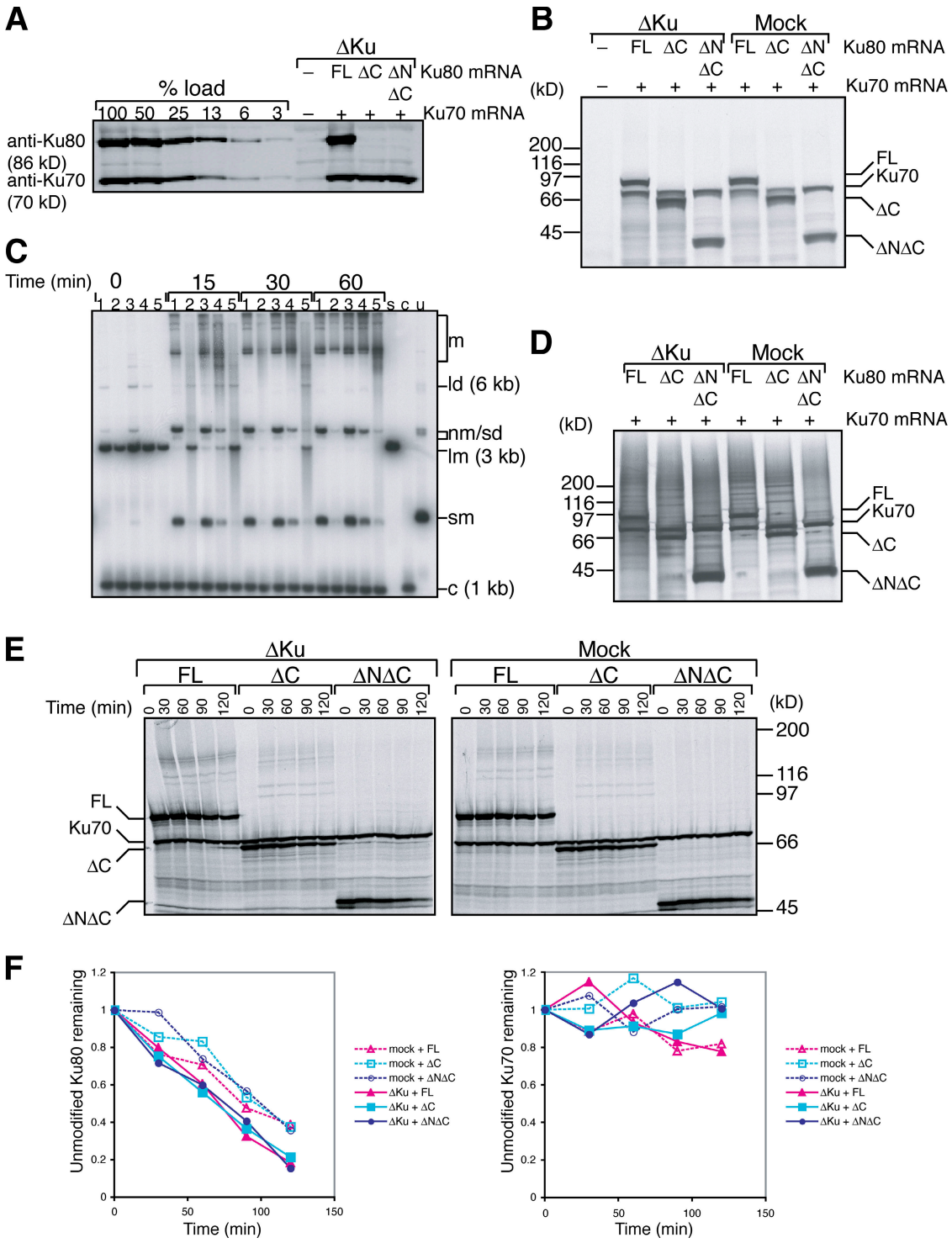

Figure 7. **Nonfunctional Ku80 is ubiquitylated and degraded in response to DSBs.** NHEJ, DNA binding, and degradation of Ku80 using Ku-depleted (ΔKu) extract complemented with full-length or truncated Ku80 and Ku70. After mock or Ku depletion and RNase treatment, mRNAs encoding Ku70 and either full-length Ku80, Ku80$^{1\text{-}543}$ (ΔC), or Ku80$^{183\text{-}543}$ (ΔNΔC) were translated in the extract. ^{35}S-methionine was added as indicated to radioactively label the translated proteins. (A) Extracts were immunoblotted with antibodies against Ku80 and Ku70. C-terminally truncated Ku80 cannot be detected with the anti-Ku80 antibody. (B) Autoradiograph of extracts labeled with ^{35}S-methionine visualizing the C-terminally truncated Ku80 as well full-length Ku80 and Ku70. (C) NHEJ assay. Xmn1-digested pBluescript SK+ was added to mock- or Ku-depleted extracts after translation. At time points indicated, samples were removed, and DNA was purified by proteinase K digestion and phenol extraction. The resulting Southern blot was probed with pBluescript SK+ and exposed to film. Lanes 1, mock-depleted extract; lanes 2, ΔKu extract; lanes 3, ΔKu extract + Ku80 + Ku70; lanes 4, ΔKu extract + Ku80 ΔC + Ku70; lanes 5, ΔKu extract + Ku80 ΔNΔC + Ku70. s, linear substrate; c, internal 1-kb control; u, uncut plasmid; sm, supercoiled monomer; lm, linear monomer; nm/sd, nicked monomer/supercoiled dimer; ld, linear dimer; m, higher order multimers. (D) Autoradiograph of labeled proteins copurified with SB-DNA beads, revealing the binding of Ku80 truncations to SB-DNA beads and their modifications. (E) Degradation assay. Xmn1-digested pBluescript SK+ was added to extracts after translation in the presence of ^{35}S-methionine. Autoradiograph of extract samples taken at the indicated time points is shown. (F) Quantification of results in E, using a phosphorimager. Left, loss of Ku80 (unmodified band); right, Ku70.

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require proteasomal degradation. Our results are in contrast to a previous observation that both Ku70 and Ku80 are ubiquitylated and degraded in response to apoptosis, which showed no specific requirement for DSBs (Gama et al., 2006). The evidence obtained in this study leads us to a model for Ku removal from DNA (Fig. 8). In this model, binding of Ku to DNA induces a signal recognized by an E3 ubiquitin ligase, which then polyubiquitylates Ku80. Polyubiquitylation leads to either a conformational change in Ku, which releases it from DNA, or, more likely, to the recruitment of an additional factor that actively dissociates Ku80 from the DNA. After dissociation, Ku80 is recognized and degraded by the proteasome.

Our proposition that Ku80 ubiquitylation is responsible for its removal from DNA is the simplest model and is consistent with all of our observations: that Ku80 becomes polyubiquitylated when bound to DNA; that the global inhibition of K48-linked polyubiquitylation inhibits the removal of Ku80 from DNA; that global inhibition of proteasomal degradation does not inhibit the removal of Ku80 from DNA; that removal is required for the proteasomal degradation of Ku80; and that under conditions in which specifically Ku80 ubiquitylation is inefficient, such as on oligo DNA beads, its removal and degradation are inhibited as well. However, it remains possible that the K48-linked polyubiquitylation of an additional protein contributes to the release of Ku80 from DNA.

The altered modification ladder of Ku80 in the presence of ubiquitin-K48R or short oligonucleotides is probably caused by the mono- or diubiquitylation of multiple lysines on the protein. This will result in a ladder that is shifted toward lower molecular mass forms. The striking DNA release and degradation phenotypes in the presence of this modestly shifted ladder are not surprising. The proteasome requires chains of at least four K48-linked ubiquitin subunits to recognize its substrate (Pickart and Fushman, 2004). The factor or factors required to remove Ku80 from DNA likely have similar ubiquitin chain length requirements.

Although the E3 ubiquitin ligase for this process remains unknown, a tantalizing possibility is that it is the SCF complex, which we found binds specifically to DSBs in a Ku-dependent manner. Interestingly, a role for the SCF complex in postrepair processes has been observed. Cell cycle recovery from the DNA replication checkpoint response depends on the SCF-mediated degradation of claspin, whose phosphorylation by Plk1 is recognized by the F-box protein βTrCP (Mailand et al., 2006; Peschiaroli et al., 2006). However, unlike the majority of Cul1 and Skp1, βTrCP does not bind to DSBs in a Ku-dependent manner (unpublished data), suggesting that βTrCP is not the F-box protein responsible for Ku-dependent DSB binding of the SCF complex.

The signal for Ku80 polyubiquitylation remains unknown. As we have shown that only the DNA- and Ku70-binding region of Ku80 is required for ubiquitylation and degradation, the E3 ligase–binding site is predicted to reside there. Alternatively, it is possible that Ku70 recruits the ligase. Many ubiquitin ligases, such as the SCF complex, recognize their substrates through posttranslational modifications, often phosphorylations. Ku80 is phosphorylated by DNA-PKcs, and three phosphorylation sites on human Ku80 have been identified by mass spectrometry

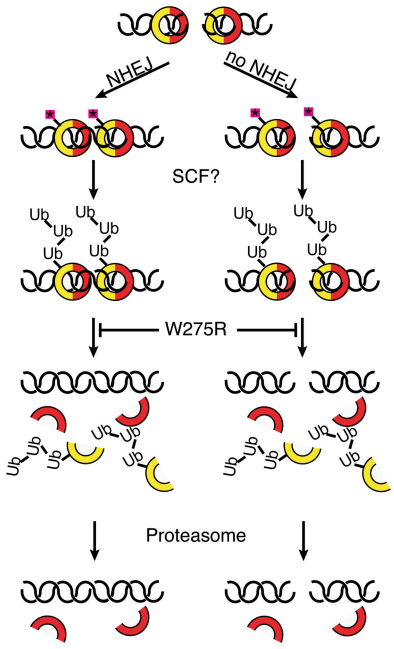

Figure 8. **A model for Ku removal from DNA.** Ku70 (red) and Ku80 (yellow) form a ring that encircles a DSB. The heterodimer either recruits other NHEJ proteins, repairing the break (left), or fails to do so (right). Binding to DNA causes Ku to become posttranslationally modified, possibly through phosphorylation (pink boxes). An E3 ubiquitin ligase such as the SCF complex recognizes this modification and polyubiquitylates Ku80. This leads to dissociation from DNA, which is inhibited by the W275R mutation. Dissociation is followed by proteasomal degradation.

(Chan et al., 1999). None of these sites are in the DNA-binding region we have identified, although it is possible that additional sites exist.

We have shown, through point and truncation mutants, that completion of NHEJ is uncoupled from ubiquitin-dependent removal and degradation. First, Ku80-W275R is functional for NHEJ coordination, seemingly becomes ubiquitylated, yet is not removed from DNA or degraded. This result implies that removal from DNA, although not required for NHEJ, is a prerequisite for proteasomal degradation. W275 is a residue in the DNA-binding domain of Ku80, making contacts with Ku70 and with DNA. A tryptophan to arginine mutation at this site is likely to result in a significant change in local structure. It is possible that this structural modification prevents the large-scale conformational change required for Ku80 removal or that it inhibits the recognition of a factor that actively removes Ku80 from DNA.

Complimentarily, experiments using truncation mutants have demonstrated that the DNA-binding region alone, which is insufficient for NHEJ, is enough for the ubiquitylation and degradation of Ku80. Therefore, Ku80 may be removed from DNA before the completion of repair, a fact seemingly in contradiction with its function. One possible explanation is that the ubiquitylation of Ku80 induces the removal only of inactively bound protein. However, the rate of degradation of the nonfunctional N- and C-terminally truncated protein was identical to that of wild-type protein, suggesting that inactively bound protein is removed and degraded no more quickly than actively bound protein. Another possibility is that this mechanism is in competition with

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the completion of NHEJ. If the two break sites can be synapsed and ligated before the removal of Ku80, repair will be completed through the NHEJ pathway. If not, this process will perhaps make way for repair proteins involved in different pathways. Indeed, Ku is inhibitory to repair through the homologous recombination pathway at unique I-SceI–generated DSBs in chicken and mammalian cells (Fukushima et al., 2001; Pierce et al., 2001) and at RAG1/2-generated DSBs in mouse embryonic stem cells (Weinstock and Jasin, 2006). Interestingly, FRAP studies indicate that Ku is very dynamic on DSBs in mammalian cells, with a half-life on the order of a few minutes (Mari et al., 2006), which is similar to what we see in our release assays.

Recently, an increasing appreciation for the role of polyubiquitylation at DSBs has emerged. A critical DSB-binding protein, BRCA1, is itself an E3 ubiquitin ligase that adds K6-linked polyubiquitin onto substrate proteins. One such substrate, CtIP, which is thought to promote end resection at DSBs (Sartori et al., 2007), binds chromatin while ubiquitylated (Yu et al., 2006). In addition, recent data have shown that the DSB-binding E3 ubiquitin ligase RNF8, along with the E2 Ubc13, polyubiquitylates histones H2A and H2AX, and that this leads to the recruitment of BRCA1 and other damage response proteins to DSBs (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Zhao et al., 2007). Interestingly, the recruitment of BRCA1 and RNF8 to DSBs, and thus their DSB-localized ubiquitylations, both require the ATM/ATR checkpoint pathways, whereas the ubiquitylation of Ku80 that we observe does not. In addition, neither of these pathways is known to result in the degradation of a target protein, whereas Ku80 is degraded.

Whereas investigations of repair protein localization have thus far focused on recruiting proteins to DSBs, the problem of protein removal from DNA is equally important. DNA must be cleared either to allow other repair proteins access to the DNA or to allow postrepair processes. Although Ku is particularly interesting in this context because of its likely topological attachment to DNA after repair, the regulated removal of proteins from DNA is a broad problem that currently is not well understood. Ubiquitin-mediated removal may be a general mechanism for protein dissociation from DNA. A similar case is the Aurora B protein, part of the mitotic chromosomal passenger complex, that must be removed from chromatin after anaphase and before nuclear envelope reformation. The ubiquitin-dependent chaperone protein p97/VCP was recently shown to be required for the removal of K48-polyubiquitylated Aurora B from chromatin (Ramadan et al., 2007). It is possible that ubiquitylated Ku80 is removed via a similar process and that this is a general mechanism for the controlled removal of proteins from DNA.

Materials and methods

Frog egg extract

Meiotic metaphase II–arrested (CSF) *X. laevis* egg extract was prepared as previously described (Murray, 1991).

Antibodies, proteins, and Western blots

Polyclonal rabbit antibodies were generated against the C termini of *X. laevis* Ku80, Ku70, Skp1, and Cul1 using the following peptides: Ku70, CKKQELVDALVEYFKKN; Ku80, CMEDEGDVDDLLDMM; Cul1, CDILIEKEYLERVDGEKDTYSYLA; Skp1, EEEEAQVRKENQWCEEK. Mouse antiubiquitin (P4D1; Santa Cruz Biotechnology, Inc.), and rabbit anti-H3 (1791; Abcam) were used. Wild-type recombinant ubiquitin was purchased from Sigma-Aldrich, and recombinant ubiquitin-K48R, -K6R, and -K63R were purchased from Boston Biochem. An expression plasmid encoding 6-His–tagged ubiquitin was obtained from T. Hunt (Cancer Research UK, London Research Institute, South Mimms, UK), and the protein was purified as previously described (Klotzbucher et al., 1996). The Odyssey Infrared Imaging System (Li-Cor) was used for detection and quantification of Western blots.

Plasmids

ESTs encoding wild-type *X. laevis* Ku70 (IMAGE: 4033219), Ku80 (IMAGE: 4032122), Mre11 (IMAGE: 7391659), WRN (IMAGE: 5077986), and Cul1 (IMAGE: 6642922) were purchased from Open Biosystems. Ku80 truncations were cloned by PCR into pCS2+, and Ku80-W275R was generated by site-directed mutagenesis. To translate SBP-GFP, GFP was cloned into a vector supplied by S. Sampath (Rockefeller University, New York, NY) containing SBP under the SP6 promoter.

Protein isolation on DNA beads

DNA beads (Heald et al., 1996) were made using pBluescript SK+. For SB-DNA beads, the plasmid was digested with NotI and BamHI (New England Biolabs, Inc.), and for DB-DNA beads it was digested with BamHI. Overhangs were filled in with the Klenow fragment of DNA polymerase I (New England Biolabs, Inc.), biotin-dATP and biotin-dUTP, and bound to streptavidin M280 dynabeads (Invitrogen) at 1 µg DNA/3 µl bead slurry (SB-DNA) or 1 µg DNA/10 µl bead slurry (DB-DNA). Oligonucleotides for oligo DNA beads were purchased from Integrated DNA Technologies (20-bp hairpin: GAGGATCTGGAGCTAAGCTGGT/iBiodT/ACAGCTTAGCTCCAGATCCTC [underlined regions, base pair]; 49-bp duplex: GGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGC and GCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCC/3Bio/). Annealed biotinylated oligos were bound to steptavidin M280 dynabeads at a concentration of ~1 µg DNA/15 µl bead slurry.

To isolate DNA-bound proteins, beads were incubated in egg extract containing 100 µg/ml cycloheximide, 10 µg/ml nocodazole, and additional proteins and drugs as indicated at 22°C. 50 µl of extract was used for every 1 µg DNA (SB- and DB-DNA beads) or 0.5 µg DNA (oligo DNA beads). Additional naked streptavidin-coated beads were included to make bead isolation easier and to bind SBP-GFP. Beads were removed from extract with a magnetic particle separator and washed in XB buffer (10 mM KCl, 10 mM Hepes, pH 8, 50 mM sucrose, and 1 mM $MgCl_2$) plus 0.05% Triton X-100 (Fig. 1) or in 30SDB2 (10 mM Hepes, 10 mM β-glycerophosphate, 50 mM NaF, 20 mM EGTA, 2 mM EDTA, 0.5 mM spermin, 30% sucrose, and 0.05% Triton X-100; for all other experiments). Beads were either resuspended in SDS sample buffer and run on a denaturing acrylamide gel or resuspended in scintillation fluid (ScintiVerse; Thermo Fisher Scientific) and quantified using a liquid scintillation counter (1209 Rackbeta; LKB Wallac).

For DNA release assays, phosphorimager (Fujifilm) quantification of modified and unmodified Ku80 was normalized to an adjusted GFP-SBP signal for that lane. As GFP-SBP binding does not reach equilibrium during the course of the experiment, the signals for each time point were adjusted accordingly; each was normalized to a mean of the signals for that time point.

Protein degradation

pBluescript SK+ digested with PvuII and SspI (Figs. 2, 5, and 6, and supplemental figures [available at http://www.jcb.org/cgi/content/full/jcb.200802146]) or XmnI (Fig. 7) was purified by phenol extraction and resuspended in 10 mM Hepes, pH 8, and 50 mM KCl, at 6 mg/ml. A typical degradation experiment contained 16 µl of egg extract containing 100 µg/ml cycloheximide; 2 µl total in vitro translated protein in rabbit reticulocyte lysate (Promega), additional proteins, drugs, and/or mRNA; and 2 µl of 6 mg/ml DNA or buffer (10 mM Hepes and 150 mM KCl). Samples were either TCA precipitated and counted using a scintillation counter or were resolved by SDS-PAGE electrophoresis and quantified using a phosphorimager. To simplify experiments, only the intensity of the unmodified band was quantified (Figs. 5 A and 7 F). For all other figures, the intensity of the entire lane was quantified.

Immunodepletion of Ku and translation in RNase-treated extract

Ku was immunodepleted using anti-Ku80 antibodies bound to protein A dynabeads (Invitrogen). For RNase treatment, we used a previously described method (Murray, 1991; Kelly et al., 2007). Ku70 and Ku80 mRNAs

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were diluted in the RNase-treated extract to a final concentration of 210 ng/µl each. Translations proceeded at 22°C for 90 min. After 90 min, translations were stopped with the addition of 100 µg/ml cycloheximide.

NHEJ assays

NHEJ was assayed using a modification of previously described methods (Di Virgilio and Gautier, 2005). 50 ng of Xmn1-linearized pBluescript SK+ was added to 50 µl of egg extract. At indicated times, 10 µl was diluted into 200 µl of Stop solution (0.3 M sodium acetate, pH 7, 10 mM EDTA, 50 mM Tris-Cl, pH 7.5, 1% SDS, 7.5 µg/ml glycogen, and 1 mg/ml proteinase K). 10 ng of a 1-kb PCR fragment of pBluescript SK+ was added to each reaction at this time as an internal control. Proteinase K digestions proceeded at 55°C for at least 2 h, and DNA was purified by phenol extraction. DNAs were separated on agarose gels containing 0.5 mg/ml ethidium bromide, which were either visualized by UV or Southern blotted and probed with pBluescript SK+.

Mass spectrometry

The samples were analyzed using a modular mass spectrometry tool, essentially as described previously (Blethrow et al., 2007). In brief, proteins were digested with trypsin (Roche) at 37°C for 7 h. Peptides were then extracted from the gel slices with a slurry of POROS R2 20 reverse-phase resin (Applied Biosystems) in 5% formic acid and 0.2% trifluoroacetic acid at 4°C for 6 h. The resin was transferred to C18 Ziptips (Millipore) and washed with 0.1% trifluoroacetic acid. Peptides were eluted on a metal plate with saturated α-cyano-4-hydroxycinnamic acid in two parts water, one part acetonitrile. Peptides were analyzed by matrix-assisted laser desorption ionization mass spectrometry using a PrOTOF 2000 mass spectrometer (PerkinElmer) and vMALDI-LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific). Proteins were identified using the Xproteo search engine.

Online supplemental material

Fig. S1 shows that Ku80 is coisolated with 6-His–tagged ubiquitin in the presence of DSBs. Fig. S2 shows that Cul1 and the SCF complex interact specifically with DSBs in a Ku-dependent manner. Fig. S3 shows that endogenous full-length Ku80 and Ku80$^{183\text{-}543}$ are degraded in response to DSBs. Fig. S4 shows that K48-dependent polyubiquitin is uniquely required for Ku80 degradation, but is not required for NHEJ. Table S1 shows the mass spectrometry data for damage-binding proteins bound to SB-DNA beads. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200802146/DC1.

We thank T. Hunt and S. Sampath for reagents and E. Arias, S. Jackson, J.R. Hogg, W.M. Michael, D. Shechter, D. Weinstock, H. Yamano, and members of the Funabiki laboratory for their comments on the manuscript.

L. Postow was supported by the Kimberly Lawrence-Netter Cancer Research Discovery Fund and a postdoctoral fellowship from the Leukemia and Lymphoma Society of America. E.M. Woo was supported by a Howard Hughes Medical Institute Predoctoral fellowship. B.T. Chait was supported by National Institutes of Health grant RR00862. H. Funabiki was supported by the Rockefeller University and the Irma T. Hirschl/Monique Weill-Caulier Trust.

Submitted: 25 February 2008
Accepted: 3 July 2008

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