# Transcription-Dependent Polyubiquitination of RNA Polymerase II Requires Lysine 63 of Ubiquitin†

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ABSTRACT: Lysine-63-linked polyubiquitin chains are not thought to signal protein degradation but instead signal for a variety of cellular processes including some types of DNA repair. RNA polymerase (Pol) II is polyubiquitinated following DNA damage or upon treatment of nuclear extracts with the transcription inhibitor α-amanitin. Here, we report, using a reaction *in vitro*, that transcription-dependent polyubiquitination of RNA Pol II consists of lysine-63-linked chains. This modification is specific for RNA Pol II engaged in active transcription and arrested by α-amanitin.

Transcription-coupled DNA repair ensures that actively transcribed genes are repaired more efficiently than the rest of the genome (*1*−*3*). This specialized repair pathway is important for removing lesions that stall transcription and is, in fact, initiated by the arrest of RNA polymerase (Pol)[1] II at the site of DNA damage (*4*−*7*). Repair factors are consequently recruited to the DNA lesion. Among these is the transcription factor (TF)IIH complex composed of many subunits including xeroderma pigmentosum (XP) proteins XPB and XPD (*8*, *9*). It is known that mutations in Cockayne syndrome (CS) proteins CSA and CSB, which lead to deficiencies in transcription-coupled repair (*10*, *11*), cause severe developmental problems in patients (*12*). Ultraviolet (UV) radiation and cisplatin, which induce DNA lesions preferentially repaired by transcription-coupled repair, also trigger ubiquitination of RNA Pol II (*13*, *14*). This does not occur in CSA and CSB cells, suggesting a role for this modification in signaling for DNA repair (*13*). Further, a direct link between Pol II ubiquitination and transcription arrest was demonstrated when ubiquitination of Pol II was induced *in vitro* by the addition of cisplatin-damaged DNA in a transcription-dependent manner (*15*).

Proteins that undergo ubiquitination are targeted for different cellular destinies (*16*, *17*). The majority of polyubiquitin chains occurs via lysine 48 of ubiquitin, and this targets proteins for proteasome-mediated degradation (*18*, *19*). Polyubiquitination on lysine 63, which is less-studied, has a nondegradative role and is involved in alteration of protein function or recruitment of proteins for different cellular processes (*20*), such as in error-free postreplicative DNA repair by proliferating cell nuclear antigen (PCNA) (*21*, *22*) and in the activation of IκBα kinase in the NFκBα signaling pathway (*23*, *24*). Interestingly, breast cancer susceptibility protein (BRCA)1, a ubiquitin ligase of substrates including Pol II *in vitro* (*25*, *26*), does not form the conventional lysine-48-linked polyubiquitin chains (*27*) but auto-ubiquitinates via lysine-6-linked chains (*28*).

The function of Pol II ubiquitination is not clear. It has been hypothesized that ubiquitination could promote DNA repair by targeting the Pol for degradation to allow access of repair factors to the lesion (*13*). Alternatively, it could facilitate degradation and hence the removal of Pol II in the absence of DNA repair (*29*). Evidence from yeast revealed that Pol II is ubiquitinated and degraded in the absence of Rad26 (CSB homolog)-mediated transcription-coupled repair (*30*). In contrast, the level of Pol II in normal or XP cells lacking global genome repair (*14*) but not in CS cells lacking transcription-coupled repair (*31*) decreased an hour after UV irradiation. These and other conflicting lines of evidence between yeast and human cells with respect to transcription-coupled repair suggest a more complex biochemistry for Pol II ubiquitination, which may not be conserved between the two species. CSA, for which there is currently no yeast homologue, has recently been identified to be a ubiquitin ligase (*32*).

We have previously demonstrated that Pol II is ubiquitinated *in vitro* in response to transcription inhibition by α-amanitin and cisplatin-damaged DNA (*15*). This reaction was most active in nuclear extracts prepared from S-phase cells arrested by aphidicolin treatment (*15*). Degradation of ubiquitinated Pol II was not observed *in vitro* (*15*), although

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[1] Abbreviations: Pol, polymerase; TF, transcription factor; XP, xeroderma pigmentosum; CS, Cockayne syndrome; UV, ultraviolet; PCNA, proliferating cell nuclear antigen; BRCA, breast cancer susceptibility protein; Ub, ubiquitin; WT, wild type; PCR, polymerase chain reaction; HA, hemaglutinin; LB, Luria−Bertani; OD, optical density; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; VHL, von Hippel-Lindau; E2, ubiquitin-conjugating enzyme; SM, starting material.

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treatment of cells with α-amanitin induced Pol II degradation *in vivo* (*33*). To investigate the cellular fate of ubiquitinated Pol II and its potential role in signaling for transcription-coupled repair, we examined the nature of the polyubiquitin chains formed on Pol II upon transcription inhibition, using a previously established *in vitro* assay. Because both α-amanitin and globally cisplatinated DNA induced transcription-dependent ubiquitination of Pol II to a similar extent (*15*), we used α-amanitin, which inhibits Pol II during elongation (*34*, *35*), to induce a consistent amount of ubiquitination. We found that amanitin-dependent ubiquitinated Pol II is polyubiquitinated on lysine 63 of ubiquitin, suggesting a nondegradative signaling role during transcription inhibition. We propose that lysine-63-linked polyubiquitin chains may be involved during DNA damage in signaling for downstream factors that alleviate the transcriptional blockage, such as repair proteins.

## EXPERIMENTAL PROCEDURES

*Plasmids.* Histidine-tagged ubiquitin (Ub) His-K48RUb and His-K63RUb were constructed by subcloning the open-reading frame of ubiquitin into pQE30 (Qiagen), which contains a $His_6$ tag. The cDNAs encoding wild type (WT) Ub and mutant K48R Ub were amplified by polymerase chain reaction (PCR) from the parental vectors pDG268 and pDG279, respectively, kind gifts of Dr. D. Gray (*36*). The primers for both sequences were forward primer, 5′-GCG GAT CCC AGA TCT TCG TGA AAA CC-3′, and reverse primer, 5′-CGT CAA GCT TAA CCA CCT CTC AGA CG-3′

The PCR products were restricted with *Bam*HI and *Hind*III, ligated into the respective restriction sites in pQE30, and designated as pQE30-HisUb and pQE30-HisK48RUb.

His-K63RUb was constructed from pQE30-HisUb. The nucleotide sequence encoding a truncated region of ubiquitin where a *Sal*I restriction site naturally occurs was amplified by the same forward primer as above and the following reverse primer, introducing the lysine 63 mutation: 5′-GCA GGG TCG ACT CGC GCT GGA TGT TGT AGT C-3′

The PCR product was digested with *Bam*HI and *Sal*I and ligated into the larger fragment of pQE30-HisUb excised by the same enzymes, to give pQE30-HisK63RUb.

HA-tagged WT and mutant ubiquitin were amplified from pQE30-WTHisUb, pQE30-HisK48RUb, and pQE-HisK63RUb with the following primers: forward primer incorporating a hemaglutinin (HA) tag and *Nde*I restriction site, 5′-CGG AAT TCA TAT GTA CCC ATA CGA TGT TCC AGA TTA CGC TCA GAT CTT CGT GAA AAC C-3′, and reverse primer incorporating a *Bam*HI site, 5′-GCG GAT CCT TAA CCA CCT CTC AGA CG-3′.

The PCR products were digested with *Nde*I and *Bam*HI and ligated into similar restriction sites in pET3a (Novagen). All plasmids were verified by DNA sequencing.

*Expression and Purification of His-Tagged and HA-Tagged Ubiquitin Proteins.* pQE30 expression vectors were transformed into *Escherichia coli* DH5 α strains, whereas the pET3a vectors were transformed into BL21 DE3 strains. Luria−Bertani (LB) media were inoculated with bacteria harboring the various plasmids, and cultures were grown to an optical density $(OD)_{600}$ of 0.6 prior to induction of protein expression with 0.5 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG). Cultures were grown for another 2 h before harvesting. His-tagged proteins were isolated using Ni-NTA agarose, following the native protein purification protocol from Qiagen. HA-tagged proteins were isolated using anti-HA-affinity agarose and eluted with a HA peptide (Roche). Proteins were quantified using the Pierce BCA kit and confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blot against the His and HA tags.

*Ubiquitination Assays.* Nuclear extracts were prepared from cells arrested in the G1/S phase by aphidicolin (*15*). Ubiquitination assays were performed essentially as described (*15*). Nuclear extracts (60−70 μg) were preincubated in the presence or absence of 5 μM α-amanitin (Calbiochem) at 30 °C for 15 min in 10 mM HEPES-KOH (pH 7.9), 10% glycerol (v/v), 60 mM KCl, 7 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 12 μg/mL polyI·C, 2 μg/mL polydG·dC containing 7 mM dithiothreitol, and 1 μg of DNA template (pG5MLP-G380 containing a major late promoter and a 380-nucleotide G-less cassette). Reactions were incubated for a further 20, 30, or 40 min after addition of 1 mM ATP, 10 mM creatine phosphate, C/G/UTP mix (200 μM each), and 1.25 μg of His-tagged ubiquitin. The preincubation step was included to allow sufficient binding of α-amanitin to Pol II for a complete inhibition of transcription *in vitro*. A total of 20 μM protease inhibitor lactacystin (Calbiochem) or 5 μM ubiquitin aldehyde (Calbiochem) was added to the reactions during the preincubation step where indicated. For inhibition assays, various concentrations of recombinant HA-tagged or untagged versions (Calbiochem) of WT/mutant ubiquitin or methylated ubiquitin (Calbiochem) were added to the reaction in the presence of His-tagged ubiquitin.

Upon completion of the reactions, His-ubiquitinated proteins were isolated by incubating at 4 °C for 1 h with 20 μL of Ni-NTA agarose (Qiagen) in a final volume of 200 μL in buffer A [50 mM sodium phosphate (pH 7.9), 0.3 M NaCl, and 0.05% Tween 20 (v/v)] containing 10 mM imidazole. The Ni-NTA agarose was preblocked with 1 mg/mL bovine serum albumin prior to use. After low-speed centrifugation (735*g*), the Ni-agarose beads containing His-ubiquitinated proteins were washed twice with 1 mL of buffer A containing 50 mM imidazole. Ni-bound proteins were eluted by boiling agarose in SDS-loading buffer [20 mM Tris-HCl (pH 6.8), 10% glycerol (v/v), 100 mM 2-mercaptoethanol, 1% SDS (w/v), and 0.02% bromophenol blue (w/v)] containing 0.1 M EDTA (pH 7.0). The supernatants were analyzed by 7.5% SDS−PAGE, followed by electrotransfer to Immobilon-P membranes (Millipore). The protein content was determined by immunoblotting against either the N terminus of RNA Pol II (N20 polyclonal antibody from Santa Cruz Biotechnology), or the His tag (mouse ascite fluid from Amersham Pharmacia).

To test the transcription dependence of ubiquitination, the assay was performed as described above but with 1 μg of anti-TFIIB (monoclonal affinity purified, Transduction Laboratories) or anti-*c*-myc (monoclonal affinity purified, Upstate Biotechnology) added at the preincubation step. Anti-TFIIB suppresses transcription at the preinitiation stage (*37*), whereas α-amanitin inhibits transcription elongation by blocking the transition in Pol II conformation on DNA, after the formation of a phospho-diester bond (*34*, *35*).

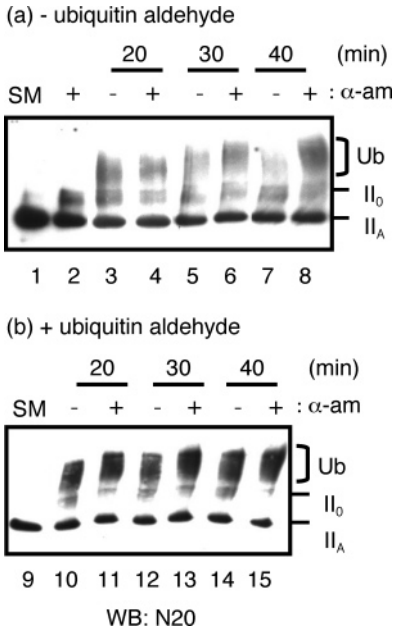


FIGURE 1: Ubiquitination of Pol II is a dynamic process. The *in vitro* ubiquitination assay was conducted in the (a) absence and (b) presence of ubiquitin aldehyde. Reactions were preincubated with and without α-amanitin as indicated, followed by a further incubation with His-tagged ubiquitin for 20 (lanes 3, 4, 10, and 11), 30 (lanes 5, 6, 12, and 13), and 40 min (lanes 7, 8, 14, and 15). Ubiquitinated proteins were then selected on Ni-NTA agarose and analyzed by SDS−PAGE, followed by immunoblotting against the N terminus of Pol II (antibody N20, Santa Cruz Biotechnology). Lanes 1 and 9, starting material (SM); and lane 2, ubiquitination with α-amanitin in the absence of His-Ub.

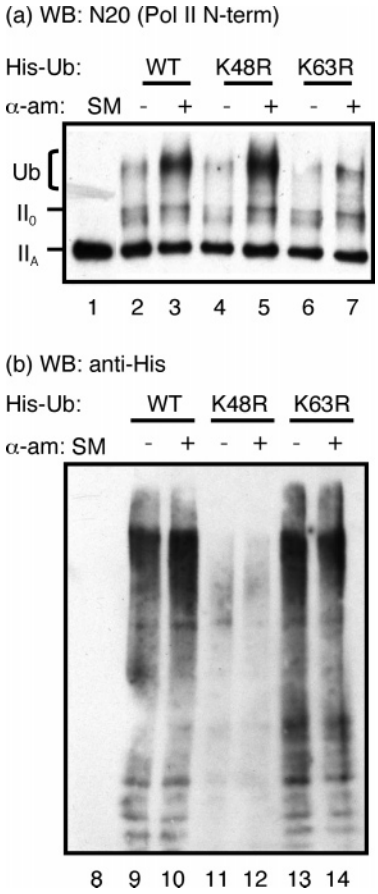


FIGURE 2: K63R ubiquitin mutant suppresses Pol II polyubiquitination. After a preincubation in the absence and presence of α-amanitin as indicated, nuclear extracts were incubated for 40 min with His-tagged WT ubiquitin (lanes 2, 3, 9, and 10) and His-K48R (lanes 4, 5, 11, and 12) and His-K63R mutants (lanes 6, 7, 13, and 14) in the presence of a proteasome inhibitor, lactacystin. The Ni-selected proteins were analyzed by SDS−PAGE and Western blot against (a) an antibody to Pol II and (b) an antibody against the His tag. Lanes 1 and 8, SM.

## RESULTS

*Transcription-Dependent Ubiquitinated Pol II Is Stable.* Pol II levels have been observed to decrease upon UV irradiation of cells. We therefore investigated the stability of Pol II upon ubiquitination *in vitro*. Using nuclear extracts prepared from aphidicolin-treated cells, reactions were preincubated with α-amanitin and then incubated for a further time interval. At 20 min, Pol II was ubiquitinated to a similar degree in the presence (lane 4 of Figure 1a), or absence (lane 3 of Figure 1a) of α-amanitin, as indicated by the low-mobility diffuse bands. These bands were not observed when His-tagged ubiquitin was not included in the reaction (lane 2 of Figure 1a), confirming ubiquitination. As observed previously (*15*), in the absence of His-tagged ubiquitin (lanes 1 and 2 of Figure 1a), nonspecific binding of Pol II onto Ni-NTA beads was also detected.

In the presence of amanitin, the level of ubiquitinated Pol II persisted and was stable over the 30- and 40-min incubations (lanes 6 and 8 of Figure 1a, respectively). However, in the absence of amanitin, the level of ubiquitinated Pol II declined after 30 and 40 min (lanes 5 and 7 of Figure 1a). This decrease in the level of ubiquitinated Pol II was probably a result of deubiquitination activity because it was inhibited by addition of aldehyde-treated ubiquitin. Under these conditions, the amount of Pol II ubiquitinated in the absence (lanes 10, 12, and 14 of Figure 1b) and presence (lanes 11, 13, and 15 of Figure 1b) of amanitin did not change over time.

*Pol II Is Polyubiquitinated on Lysine 63 of Ubiquitin.* We next characterized the nature of the stable species of amanitin-dependent ubiquitinated Pol II. All subsequent experiments were performed with an incubation time of 40 min under *in vitro* transcription conditions. We tested the effect of different His-tagged ubiquitin mutants on Pol II ubiquitination in the presence of lactacystin, a proteosome inhibitor. In comparison with WT ubiquitin (lane 3 of Figure 2a), addition of K48R ubiquitin had no effect on the level of Pol II ubiquitinated upon α-amanitin treatment (lane 5 of Figure 2a). On the other hand, addition of K63R ubiquitin virtually abolished Pol II ubiquitination (lane 7 of Figure 2a). This suggests that lysine 63 is required for amanitin-dependent ubiquitination of Pol II. Addition of either ubiquitin mutant had a negligible effect on ubiquitination in the absence of amanitin (lanes 4 and 6 of Figure 2a). We note that the early, amanitin-independent species was not inhibited by either lysine 48 or lysine 63 or double mutations of ubiquitin (not shown).

To rule out the possibility that K63R ubiquitin may be a nonfunctional protein, we analyzed ubiquitinated material from the same reactions after selection on Ni agarose by immunoblotting against the His tag. The levels of ubiquitinated proteins in reactions containing WT ubiquitin (lanes 9 and 10 of Figure 2b) and K63R ubiquitin (lanes 13 and 14 of Figure 2b) were similar, confirming that K63R ubiquitin

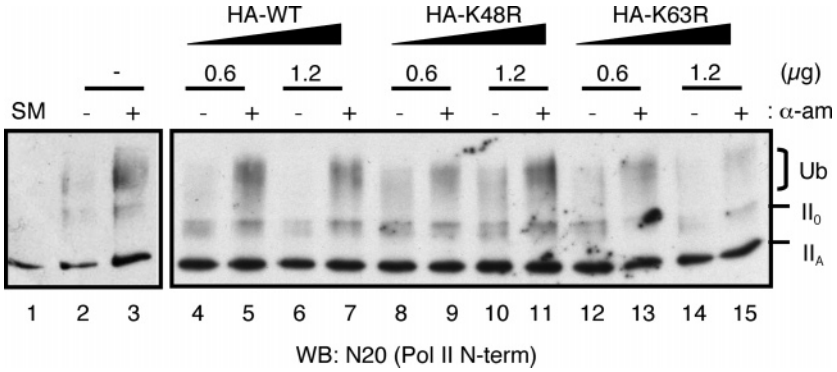


FIGURE 3: Inhibition assay. Effect of HA-WT, HA-K48R, and HA-K63R ubiquitin on modification of Pol II with His-tagged ubiquitin. The *in vitro* ubiquitination assay was set up with His-WT ubiquitin and lactacystin, essentially as described in Figure 2. Different amounts of HA-tagged WT (lanes 4−7), HA-K48R (lanes 8−11), HA-K63R (lanes 12−15) ubiquitin, or none (lanes 2 and 3) were also added to the reactions. His-ubiquitinated proteins were selected on Ni-NTA agarose and analyzed as described in Figure 1. An immunoblot against RNA Pol II is shown here. Lane 1, SM.

was used as efficiently as WT ubiquitin in modification of most endogenous proteins. Overall ubiquitination with the K48R mutant was greatly diminished (lanes 11 and 12 of Figure 2b), indicating that, unlike Pol II, most proteins required lysine 48 for polyubiquitination.

To test whether the K63R-ubiquitin mutant acted as a polyubiquitin chain terminator, we studied the effect of adding HA-tagged ubiquitin mutants on the ubiquitination of Pol II, in the presence of His-tagged WT ubiquitin. Either a half or an equal weight amount of the HA-tagged mutant was added to the reactions to test whether it would inhibit modification by His-tagged WT ubiquitin. Under these conditions, the mutant protein should only inhibit as a chain terminator and not as a competitor. As a positive control for the experiment, HA-tagged WT ubiquitin was observed to have no effect on ubiquitination of Pol II with His-tagged ubiquitin (lanes 4−7 of Figure 3), when compared to a reaction containing only the His-tagged protein (lanes 2 and 3 of Figure 3). Similarly, addition of HA-tagged K48R ubiquitin did not inhibit Pol II ubiquitination (lanes 8−11 of Figure 3). In contrast, addition of HA-tagged K63R ubiquitin reduced Pol II ubiquitination at 0.6 μg (lanes 12 and 13 of Figure 3) and almost completely inhibited ubiquitination at 1.25 μg (lanes 14 and 15 of Figure 3). This indicates that K63R was an effective polyubiquitin chain terminator. Consistent with this, adding similar amounts of methylated ubiquitin, which has no free lysine side chains for polyubiquitination, inhibited Pol II ubiquitination (lanes 8−11 of Figure 4). As expected, the addition of an untagged version of the K48R mutant, a control reaction, had no effect on the level of ubiquitination (lanes 4−7 of Figure 4).

*A Transcriptionally Active Pol II Is Targeted by Lysine-63-Linked Polyubiquination.* We investigated whether lysine-63-linked polyubiquitination of Pol II was dependent upon transcription. Because polyubiquitination by WT and K48R ubiquitin is presumably linked through lysine 63, we tested the effect of suppressing transcription with an antibody to TFIIB, a general TF involved in preinitiation complex assembly. Reactions containing K63R were included as controls. As we have shown previously (*15*), anti-TFIIB abolished *in vitro* transcription in the nuclear extracts efficiently (not shown). Consistent with previous results (*15*), it also abrogated Pol II ubiquitination with WT (compare lanes 2 and 3 with lanes 4 and 5 of Figure 5a) and K48R

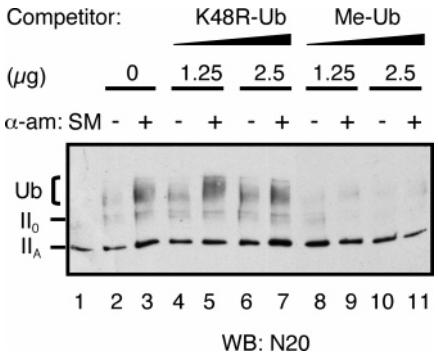


FIGURE 4: Methylated ubiquitin inhibits the formation of ubiquitinated Pol II. Reactions were conducted as described in Figure 3, except that untagged versions of K48R ubiquitin mutant (lanes 4−7), methylated ubiquitin (lanes 8−11), or none (lanes 2 and 3) were added in the presence of His-tagged ubiquitin. Shown is an immunoblot of Ni-selected ubiquitinated proteins, using an antibody against RNA Pol II. Lane 1, SM.

(lanes 6 and 7 of Figure 5a) ubiquitin. As a control, we observed that anti-*c*-myc had no effect on polyubiquitination of Pol II by either WT (lanes 11 and 12 of Figure 5b), K48R (lanes 13 and 14 of Figure 5b), or K63R (lanes 15 and 16 of Figure 5b) ubiquitin. The small amount of ubiquitination that was present in a reaction containing K63R ubiquitin and anti-*c*-myc (lanes 15 and 16 of Figure 5b) was completely abolished by anti-TFIIB (lanes 8 and 9 of Figure 5a), indicating that this low level of ubiquitination is dependent upon transcription.

## DISCUSSION

RNA Pol II is polyubiquitinated upon treatment of mammalian cells with either UV radiation or cisplatin (*13*, *14*). A potentially related reaction, polyubiquitination of Pol II, which is dependent upon transcription inhibition by either α-amanitin or cisplatin−DNA lesions, has been observed in reactions *in vitro* (*15*). The biological role or roles of polyubiquitination of Pol II is, however, unclear. It has been speculated to be involved in signaling for DNA repair (*13*) and/or targeting Pol II for degradation to relieve transcription blockage (*29*, *30*).

To gain insight into these processes, we have investigated the nature of the polyubiquitination added to Pol II when transcription is inhibited by α-amanitin *in vitro*. In previous studies, this drug, which inhibits Pol II during an elongation

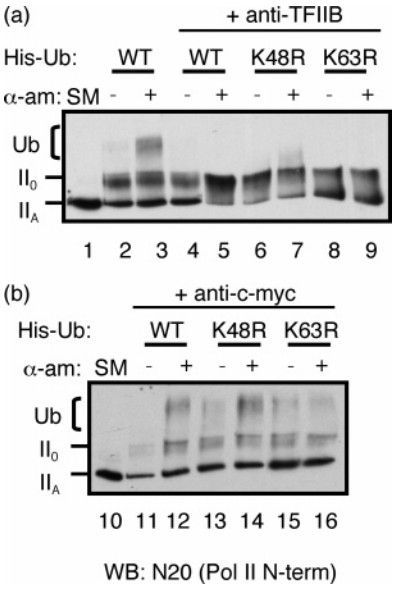


FIGURE 5: Transcription dependence of Pol II ubiquitination. The effect of (a) an antibody against TFIIB (lanes 4−9) or (b) a control antibody *c*-myc (lanes 11−16), on ubiquitination of Pol II with WT and mutant ubiquitin (K48R and K63R) was examined. Experimental conditions were as described in Figure 2. The material selected on Ni-NTA agarose was analyzed by SDS−PAGE and immunoblotted against Pol II. Lanes 1 and 10, SM; and lanes 2 and 3, reactions with WT ubiquitin in the absence of an antibody.

step, stimulated polyubiquitination of Pol II in a fashion identical to that observed when cisplatin-modified DNA was added to the reaction (*15*). We performed the assay with nuclear extracts that were prepared from cells arrested at the G1/S-phase boundary and previously determined to show transcription- and amanitin-dependent ubiquitination (*15*). We report that in these reactions, Pol II is modified with lysine-63-linked polyubiquitin chains in a transcription-dependent manner. This novel finding indicates that ubiquitination of Pol II during transcription may serve a signaling role in contrast to a degradative role typical of the more common lysine-48-linked polyubiquitination.

Pol II ubiquitination is a dynamic process in these nuclear extracts. In the absence of α-amanitin, ubiquitination of Pol II was initially observed but disappeared with further incubation because of the activity of ubiquitin proteases. This polyubiquitination reaction was not dependent upon the addition of α-amanitin. The α-amanitin-dependent species, on the other hand, was stable and persisted. Synthesis of the latter species was inhibited if transcription was blocked by addition of an antibody to the TFIIB factor. Further, addition of mutant ubiquitin in which lysine 63 was substituted by arginine potently inhibited this amanitin-dependent reaction, while addition of ubiquitin with lysine 48 substituted with arginine did not. Ubiquitination with the K63R mutant was inhibited by chain termination and not competition. These results strongly indicate that, in these reactions, transcription-dependent Pol II polyubiquitin chains are linked through lysine 63.

Although Pol II is a substrate for many ubiquitin ligases *in vitro*, including CSA (*32*), BRCA1 (*25*) and the von Hippel-Lindau (VHL) protein (*38*), the ubiquitin ligase responsible for ubiquitination of Pol II in this transcription-dependent reaction is not obvious. There was little effect on amanitin-induced polyubiquitination of Pol II in nuclear extracts prepared from cells in which either CSA, CSB, or BRCA1 was knocked down by RNA interference, although low levels of each protein remained in these extracts (unpublished data). Interestingly, studies in yeast showed that Pol II can be ubiquitinated by Def1, which forms a complex with Rad26 (homologue of CSB) (*30*). Def1 initiates rapid degradation of Pol II in response to DNA damage in the absence of Rad26-mediated repair (*30*). Pol II levels also declined in human fibroblasts upon UV damage, consistent with an induction of a degradative form of polyubiquitinated Pol II (*14*). Two proteins, CSA and CSB, which are important for transcription-coupled DNA repair, are probably related to this decline of Pol II in mammalian cells. Ubiquitination of Pol II decreases in CSA and CSB mutants (*13*). Consistent with this, hyperphosphorylated Pol II levels persisted in cells lacking CSA and CSB, and this was also observed in WT cells treated with a proteosome inhibitor (*31*). These results suggest that CSB might interact with a stalled Pol II and, in some manner, promote a degradative form of ubiquitination. This would not be inconsistent with past (*39*, *40*) and recent results (*41*).

Ubiquitination of Pol II resulting in degradation is probably signaled through linkage at lysine 48. This may be one of the prevalent biochemical pathways in response to DNA damage in the G1 phase, the part of the cell cycle where characterization of the CSA and CSB mutants was done (*13*, *31*). In contrast, the nuclear extracts used in these studies were prepared from cells arrested early in the S phase by treatment with aphidicolin. It is possible that polyubiquitination of Pol II through lysine 63 is predominant in the S phase because of the activity of the ubiquitin-conjugating enzyme (E2) Ubc13, which mediates ubiquitination through this site (*42*). We note that lysine-63-linked polyubiquitination of a ribosome subunit was observed predominantly in the S phase of yeast cells (*43*). Perhaps an increased level of DNA-damage checkpoints in the S phase may require a signaling role for ubiquitination of Pol II via lysine 63 chains (which is amanitin-dependent), in addition to a degradative function via lysine 48 chains in G0/G1-phase cells, as observed by Ratner et al. (*14*). Consistent with this idea, nuclear extracts from unsynchronized cells (G0/G1) exhibited little of the amanitin-dependent ubiquitination of Pol II (*15*), which is presumably a characteristic of the lysine 63 polyubiquitin chain species. A similar model has been proposed for the different roles of PCNA, which could be regulated by monoubiquitination, polyubiquitination via lysine 63, or sumoylation (*21*, *22*). The role of polyubiquitination through lysine 63 by the Ubc13 pathway in error-free postreplicative repair involving PCNA is not known. Yeast mutants defective in Ubc13 or the associated protein Mms2, a ubiquitin-conjugating enzyme variant (Uev), are defective in error-free postreplicative DNA repair (*21*, *42*, *44*, *45*). This process is probably conserved in mammals because a mammalian Ubc13 cDNA will complement the corresponding yeast mutation (*46*). The possibility that Pol II arrested during elongation is recognized by a pathway dependent upon lysine 63 polyubiquitination is provocative. This modification is not thought to signal degradation but can have a role in subcellular localization (*47*). Replication foci and DNA repair foci form in S-phase cells (*48*, *49*), and it seems plausible that an arrested RNA Pol could be targeted to specific foci by polyubiquitination.

## ACKNOWLEDGMENT

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## REFERENCES

1. Bohr, V. A., Smith, C. A., Okumoto, D. S., and Hanawalt, P. C. (1985) DNA repair in an active gene: Removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall, *Cell 40*, 359−369.
2. Mellon, I., Spivak, G., and Hanawalt, P. C. (1987) Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene, *Cell 51*, 241−249.
3. Mellon, I., and Hanawalt, P. C. (1989) Induction of the *Escherichia coli* lactose operon selectively increases repair of its transcribed DNA strand, *Nature 342*, 95−98.
4. Hanawalt, P. C. (1994) Transcription-coupled repair and human disease, *Science 266*, 1957−1958.
5. Tornaletti, S., and Hanawalt, P. C. (1999) Effect of DNA lesions on transcription elongation, *Biochimie 81*, 139−146.
6. Hoeijmakers, J. H. J. (2001) Genome maintenance mechanisms for preventing cancer, *Nature 411*, 366−374.
7. Svejstrup, J. Q. (2002) Mechanisms of transcription-coupled DNA repair, *Nat. Rev. Mol. Cell Biol. 3*, 21−29.
8. Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J. H. J., Chambon, P., and Egly, J. M. (1993) DNA repair helicase−A component of BTF2 (TFIIH) basic transcription factor, *Science 260*, 58−63.
9. Schaeffer, L., Moncollin, V., Roy, R., Staub, A., Mezzina, M., Sarasin, A., Weeda, G., Hoeijmakers, J. H. J., and Egly, J. M. (1994) The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor, *EMBO J. 13*, 2388−2392.
10. Leadon, S. A., and Cooper, P. K. (1993) Preferential repair of ionizing radiation-induced damage in the transcribed strand of an active human gene is defective in Cockayne syndrome, *Proc. Natl. Acad. Sci. U.S.A. 90*, 10499−10503.
11. Venema, J., Mullenders, L. H., Natarajan, A. T., van Zeeland, A. A., and Mayne, L. V. (1990) The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA, *Proc. Natl. Acad. Sci. U.S.A. 87*, 4707−4711.
12. Nance, M. A., and Berry, S. A. (1992) Cockayne syndrome: Review of 140 cases, *Am. J. Med. Genet. 42*, 68−84.
13. Bregman, D. B., Halaban, R., van Gool, A. J., Henning, K. A., Friedberg, E. C., and Warren, S. L. (1996) UV-induced ubiquitination of RNA polymerase II: A novel modification deficient in Cockayne syndrome cells, *Proc. Natl. Acad. Sci. U.S.A. 93*, 11586−11590.
14. Ratner, J. N., Balasubramanian, B., Corden, J., Warren, S. L., and Bregman, D. B. (1998) Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair, *J. Biol. Chem. 273*, 5184−5189.
15. Lee, K. B., Wang, D., Lippard, S. J., and Sharp, P. A. (2002) Transcription-coupled and DNA damage-dependent ubiquitination of RNA polymerase II *in vitro*, *Proc. Natl. Acad. Sci. U.S.A. 99*, 4239−4244.
16. Varshavsky, A. (1997) The ubiquitin system, *Trends Biochem. Sci. 22*, 383−387.
17. Hershko, A., and Ciechanover, A. (1998) The ubiquitin system, *Annu. Rev. Biochem. 67*, 425−479.
18. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein, *Science 243*, 1576−1583.
19. van Nocker, S., and Vierstra, R. D. (1993) Multiubiquitin chains linked through lysine 48 are abundant *in vivo* and are competent intermediates in the ubiquitin proteolytic pathway, *J. Biol. Chem. 268*, 24766−24773.
20. Pickart, C. M. (2000) Ubiquitin in chains, *Trends Biochem. Sci. 25*, 544−548.
21. Ulrich, H. D., and Jentsch, S. (2000) Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair, *EMBO J. 19*, 3388−3397.
22. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO, *Nature 419*, 135−141.
23. Deng, L., Wang, C., Spencer, E., Yang, L. Y., Braun, A., You, J. X., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain, *Cell 103*, 351−361.
24. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK, *Nature 412*, 346−351.
25. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination, *Proc. Natl. Acad. Sci. U.S.A. 96*, 11364−11369.
26. Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohta, T. (2001) The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation, *J. Biol. Chem. 276*, 14537−14540.
27. Chen, A., Kleiman, F. E., Manley, J. L., Ouchi, T., and Pan, Z. Q. (2002) Autoubiquitination of the BRCA1-BARD1 RING ubiquitin ligase, *J. Biol. Chem. 277*, 22085−22092.
28. Wu-Baer, F., Lagrazon, K., Yuan, W., and Baer, R. (2003) The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin, *J. Biol. Chem. 278*, 34743−34746.
29. Svejstrup, J. Q. (2003) Rescue of arrested RNA polymerase II complexes, *J. Cell Sci. 116*, 447−451.
30. Woudstra, E. C., Gilbert, C., Fellows, J., Jansen, L., Brouwer, J., Erdjument-Bromage, H., Tempst, P., and Svejstrup, J. Q. (2002) A Rad26−Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage, *Nature 415*, 929−933.
31. McKay, B. C., Chen, F., Clarke, S. T., Wiggin, H. E., Harley, L. M., and Ljungman, M. (2001) UV light-induced degradation of RNA polymerase II is dependent on the Cockayne's syndrome A and B proteins but not p53 or MLH1, *Mutat. Res. 485*, 93−105.
32. Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A. F., Tanaka, K., and Nakatani, Y. (2003) The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage, *Cell 113*, 357−367.
33. Nguyen, V. T., Giannoni, F., Dubois, M. F., Seo, S. J., Vigneron, M., Kedinger, C., and Bensaude, O. (1996) *In vivo* degradation of RNA polymerase II largest subunit triggered by α-amanitin, *Nucleic Acids Res. 24*, 2924−2929.
34. Vaisius, A. C., and Wieland, T. (1982) Formation of a single phosphodiester bond by RNA polymerase B from calf thymus is not inhibited by α-amanitin, *Biochemistry 21*, 3097−3101.
35. de Mercoyrol, L., Job, C., and Job, D. (1989) Studies on the inhibition by α-amanitin of single-step addition reactions and productive RNA synthesis catalysed by wheat-germ RNA polymerase II, *Biochem. J. 258*, 165−169.
36. Tsirigotis, M., Zhang, M., Chiu, R. K., Wouters, B. G., and Gray, D. A. (2001) Sensitivity of mammalian cells expressing mutant ubiquitin to protein-damaging agents, *J. Biol. Chem. 276*, 46073−46078.
37. Lee, T. I., and Young, R. A. (2000) Transcription of eukaryotic protein-coding genes, *Annu. Rev. Genet. 34*, 77−137.
38. Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C., and Czyzyk-Krzeska, M. F. (2003) von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination, *Proc. Natl Acad. Sci. U.S.A. 100*, 2706−2711.
39. Tantin, D., Kansal, A., and Carey, M. (1997) Recruitment of the putative transcription-repair coupling factor CSB/ERCC6 to RNA polymerase II elongation complexes, *Mol. Cell. Biol. 17*, 6803−6814.
40. van Gool, A. J., Citterio, E., Rademakers, S., van Os, R., Vermeulen, W., Constantinou, A., Egly, J. M., Bootsma, D., and Hoeijmakers, J. H. (1997) The Cockayne syndrome B protein, involved in transcription-coupled DNA repair, resides in an RNA polymerase II-containing complex, *EMBO J. 16*, 5955−5965.

41. Tremeau-Bravard, A., Riedl, T., Egly, J. M., and Dahmus, M. E. (2004) Fate of RNA polymerase II stalled at a cisplatin lesion, *J. Biol. Chem. 279*, 7751−7759.
42. Hofmann, R. M., and Pickart, C. M. (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair, *Cell 96*, 645−653.
43. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D. (2000) Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain, *Cell 102*, 67−76.
44. Broomfield, S., Chow, B. L., and Xiao, W. (1998) MMS2, encoding a ubiquitin-conjugating-enzyme-like protein, is a member of the yeast error-free postreplication repair pathway, *Proc. Natl. Acad. Sci. U.S.A. 95*, 5678−5683.
45. Xiao, W., Chow, B. L., Fontanie, T., Ma, L. B., Bacchetti, S., Hryciw, T., and Broomfield, S. (1999) Genetic interactions between error-prone and error-free postreplication repair pathways in *Saccharomyces cerevisiae*, *Mutat. Res. 435*, 1−11.
46. Ashley, C., Pastushok, L., McKenna, S., Ellison, M. J., and Xiao, W. (2002) Roles of mouse UBC13 in DNA postreplication repair and Lys63-linked ubiquitination, *Gene 285*, 183−191.
47. Galan, J. M., and HaguenauerTsapis, R. (1997) Ubiquitin Lys63 is involved in ubiquitination of a yeast plasma membrane protein, *EMBO J. 16*, 5847−5854.
48. Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. M. (1997) Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage, *Cell 90*, 425−435.
49. Chen, J. J., Silver, D. P., Walpita, D., Cantor, S. B., Gazdar, A. F., Tomlinson, G., Couch, F. J., Weber, B. L., Ashley, T., Livingston, D. M., and Scully, R. (1998) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells, *Mol. Cell 2*, 317−328.

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