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Histone H2B mono-ubiquitylation maintains genomic integrity at stalled replication forks

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ABSTRACT

Histone modifications play an important role in regulating access to DNA for transcription, DNA repair and DNA replication. A central player in these events is the mono-ubiquitylation of histone H2B (H2Bub1), which has been shown to regulate nucleosome dynamics. Previously, it was shown that H2Bub1 was important for nucleosome assembly onto nascent DNA at active replication forks. In the absence of H2Bub1, incomplete chromatin structures resulted in several replication defects. Here, we report new evidence, which shows that loss of H2Bub1 contributes to genomic instability in yeast. Specifically, we demonstrate that H2Bub1-deficient yeast accumulate mutations at a high frequency under conditions of replicative stress. This phenotype is due to an aberrant DNA Damage Tolerance (DDT) response upon fork stalling. We show that H2Bub1 normally functions to promote error-free translesion synthesis (TLS) mediated by DNA polymerase eta (Polη). Without H2Bub1, DNA polymerase zeta (Polζ) is responsible for a highly mutagenic alternative mechanism. While H2Bub1 does not appear to regulate other DDT pathways, error-free DDT mechanisms are employed by H2Bub1-deficient cells as another means for survival. However, in these instances, the anti-recombinase, Srs2, is essential to prevent the accumulation of toxic HR intermediates that arise in an unconstrained chromatin environment.

INTRODUCTION

Chromatin primarily functions to package and protect the genome. While often thought of as an inert, repressive entity, chromatin is now known to exist in a dynamic state of flux. It is being continually remodeled to regulate the access of cellular machineries to DNA, which conduct essential biological functions including transcription, DNA repair, recombination and replication (1,2). These processes are tightly regulated by histone chaperones, which are instructed by histone post-translational modifications. Defective chromatin remodeling negatively impacts these processes and is associated with genomic instability and aberrant gene expression (3–5).

Important for the restoration of chromatin structure during transcription elongation is the mono-ubiquitylation of histone H2B (H2Bub1) (6–8). In budding yeast, H2Bub1 is catalyzed by the E2-ubiquitin-conjugating enzyme, Rad6 and the E3-ubiquitin-ligase, Bre1 (9,10). In higher organisms, genes that encode Rad6 and Bre1 are highly conserved. Human homologs include hRad6 and two Bre1 homologs, RNF20 and RNF40 (11,12). During transcription, H2Bub1 promotes the reassembly of displaced nucleosomes on actively transcribed genes. In the absence of H2Bub1, incomplete chromatin reassembly reveals cryptic promoters to the transcriptional machinery, which results in the accumulation of aberrant, internally initiated transcripts (6). It is thought that H2Bub1 functions primarily to prevent such anomalies by stabilizing newly assembled immature nucleosomes behind the transcription machinery (13).

In addition to its role in transcription elongation, our previously published study indicated that H2Bub1 is also an important regulator of chromatin restoration and replication fork progression during DNA replication in yeast (14). In the absence of H2Bub1, the chromatin on nascent DNA behind the replication fork is incompletely assembled. This results in a myriad of replication defects. Specifically, yeast lacking H2Bub1 have an extended S-phase, are sensitive to DNA damaging agents, and the replisome is slowed/destabilized under conditions of replicative stress. Additionally, we observed evidence of reduced rate of fork progression near active origins in yeast lacking H2Bub1. Various connections between chromatin assembly during DNA replication and the rate of fork progression have been well established. Several studies have shown that when nucleosome assembly at the replication fork is incomplete, the replisome responds by reducing the rate of replication and stalling frequently (15–19). Such mechanisms ensure that the chromatin structure is re-established before additional template DNA is revealed by unwinding at the replication fork and subjected to genomic insult.

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Frequent fork stalling also has the potential to initiate DNA damage tolerance (DDT) pathways. In yeast, DDT is mediated by the *RAD6* epistasis group of genes, consisting of at least two parallel mechanisms (20–22). The first, translesion DNA synthesis (TLS), is regulated by the mono-ubiquitylation of PCNA on Lys 164 by Rad6/Rad18 at stalled replication forks (23,24). During TLS, DNA lesions are replicated by specialized DNA polymerases, including Pol-Zeta (Polζ), Pol-Eta (Polη) and others (25–29). However, these polymerases overcome fork stalling at the expense of elevated mutagenesis (30–32). Therefore, TLS is typically referred to as the 'error-prone' arm of DDT. In contrast, damage avoidance mechanisms of DDT may also be utilized that involve template switching (21,33). These mechanisms utilize the sister chromatid as a template for high fidelity DNA synthesis past replication blockages and are therefore error-free. Template switching is regulated by poly-ubiquitylation of PCNA at Lys 164 by Mms2/Ubc13/Rad5 (34–36). While the mechanism of template switching is still not fully understood, it is thought to involve the action of helicases such as Rad5 and Mph1 to promote fork regression and/or strand invasion. PCNA can also be sumoylated at Lys 164 by Ubc9/Siz1, where it functions to prevent the initiation of homologous recombination during an unperturbed S-phase (21,36). Sumoylated PCNA stabilizes the anti-recombinase, Srs2, at the replication fork (37,38). There, Srs2 can disrupt Rad51 nucleoprotein filaments via its helicase activity (39,40).

Here, we demonstrate that yeast lacking H2Bub1 accumulate replication-associated mutations at a higher frequency than WT cells when treated with agents that cause fork stalling. This likely contributes to the tumor suppressive function of H2Bub1 in humans (41–43). In response to UV damage, H2Bub1 functions to promote error-free translesion synthesis (TLS) mediated by DNA polymerase eta (Polη). In the absence of H2Bub1, TLS is dominated by the error-prone DNA polymerase zeta (Polζ). In congruence with a role of H2Bub1 in regulating TLS, we show that yeast lacking H2Bub1 have elevated PCNA-ub1 levels, and genetic analyses place H2Bub1 as an important regulator of TLS in response to replication stress. While H2Bub1 does not appear to regulate other DDT pathways directly, H2Bub1-deficient cells depend heavily on alternative 'error-free' DDT mechanisms for their survival upon exposure to exogenous DNA damaging agents. However, the absence of H2Bub1 demands that these mechanisms be strictly regulated by the anti-recombinase, Srs2, to prevent the accumulation of toxic HR intermediates that arise in an unconstrained chromatin environment.

MATERIALS AND METHODS

Yeast strains

Yeast stains used in this study are listed in Table 1.

Measurement of the spontaneous mutation rate and HU- and UV-induced mutation frequency

The rates of spontaneous mutation and frequencies of induced mutation were determined by fluctuation analysis (44,45). At least eighteen 5- to 6-ml cultures were started for each strain from single colonies and grown to stationary phase in liquid yeast extract peptone dextrose (YPD) medium. Cells were diluted and plated onto synthetic complete medium containing L-canavanine (60 mg/l) and lacking arginine (SC-Arg + CAN) for the canavanine resistant (Can^r) mutant count and onto synthetic complete (SC) medium for the viable count. The Can^r mutant frequencies were calculated by dividing the Can^r mutant count by the viable cell count. The Drake equation was applied to the mutation frequencies to calculate the mutation rates (46). The median mutation rates and frequencies were used to compare spontaneous and induced mutagenesis in different strains. The 95% confidence limits for the median mutation rates and frequencies were determined as described (45). The hydroxyurea (HU)-induced mutation frequencies were measured using a similar procedure. However, the cultures were started from $\sim 10^4$-cells inoculum rather than from single colonies and grown in YPD medium containing 20 mM HU (45). The median frequency of Can^r mutants was used to compare HU-induced mutagenesis in different strains. For UV-induced mutagenesis, yeast strains were grown to stationary phase from single colonies in liquid YPD medium and plated after dilutions onto SC medium for viability count and onto SC + CAN for the Can^r mutant count. The cells were irradiated with 254 nm UV-C light (15 J/m^2) immediately after plating and incubated at 30°C for 3 days. The mutant frequencies were calculated by dividing the Can^r mutant count by the viable cell count.

Measurement of the sister chromatid exchange recombination rate and UV-induced frequency

Haploid yeast strains (YD122 and YD122 *asf1Δ*) containing the 3′Δ-*his3* 5′Δ-*his3* sister chromatid exchange (SCE) substrate that forms a functional *HIS3* upon recombination were kindly provided by Jessica Tyler (47). The rate of SCE was determined by fluctuation analysis, as described in the previous section. At least nine 5-ml cultures were started for each strain from single colonies and grown to stationary phase in YPD medium. Cells were plated onto SC medium lacking histidine (SC-His) and onto SC medium for viable count. The UV-induced (15 J/m^2) SCE frequency was calculated by dividing the His^r count by the viable cell count.

HU and MMS sensitivity assay

Five-fold serial dilutions of each strain were spotted onto YPD plates containing no drug or the indicated concentrations of HU or methyl methane sulfonate (MMS). The plates were incubated for three days at 30°C and photographed.

Immunoblot analysis of yeast extracts

Yeast strains were grown to logarithmic phase in liquid YPD medium. For analysis of MMS-treated cells, MMS was present in the medium at a concentration of 0.02% during the last 90 min of the culture growth. The cells were collected by centrifugation, resuspended in an equal volume of lysis buffer containing 150 mM NaCl, 50 mM Tris–Cl, pH 7.4, 1 mM EDTA, 10 mM β-mercaptoethanol and protease inhibitors (Sigma P2714), and disrupted by vortexing

Table 1. Yeast strains used in this study

Haploid Strain	Genotype	Source
MAO479	*MAT**a**, hta1-htb1::LEU2 HTA2-GAL1/GAL10- HTB2 ura3–1 trp1–1 leu2–3,-112 his3–11 ade2–1 can1–100 <pZS145 (HTA1-Flag-HTB1 HIS3)>*	M.A. Osley
MAO480	*MAT**a**, hta1-htb1::LEU2 HTA2-GAL1/GAL10- HTB2 ura3–1 trp1–1 leu2–3,-112 his3–11 ade2–1 can1–100 <pZS146 (HTA1-Flag-htb1-K123R HIS3)>*	M.A. Osley
MAO695	*MAT**a**(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 ura3-52,1 leu2Δ1 lys2Δ1 lys2-128Δ his3Δ200 trp1Δ63 HTA1-HTB1(HIS3)*	M.A. Osley
MAO696	*MAT**a**(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 ura3-52,1 leu2Δ1 lys2Δ1 lys2-128Δ his3Δ200 trp1Δ63 HTA1-HTB1(HIS3) htb1-K123R*	M.A. Osley
MAO747	MAO695*; bre1Δ::kanMX*	M.A. Osley
KMT034	*MAO695; rev3Δ::kanMX*	This study
KMT042	*MAO696; rev3Δ::kanMX*	This study
KMT061	*MAO695; rad5Δ::hphNTI*	This study
KMT095	*MAO696; rad5Δ::hphNTI*	This study
KMT054	*MAO695; rad18Δ::hphNTI*	This study
KMT074	*MAO696; rad18Δ::hphNTI*	This study
KMT057	*MAO695; ubc13Δ::hphNTI*	This study
KMT093	*MAO695; bre1Δ::kanMX ubc13Δ::hphNTI*	This study
KMT076	*MAO696; ubc13Δ::hphNTI*	This study
KMT052	*MAO695; rad51Δ::hphNTI*	This study
KMT071	*MAO696; rad51Δ::hphNTI*	This study
KMT090	*MAO695; bre1Δ::kanMX rad51Δ::hphNTI*	This study
KMT063	*MAO695; mph1Δ::hphNTI*	This study
KMT097	*MAO695; bre1Δ::kanMX mph1Δ::hphNTI*	This study
KMT081	*MAO696; mph1Δ::hphNTI*	This study
KMT067	*MAO695; srs2Δ::hphNTI*	This study
KMT101	*MAO695; bre1Δ::kanMX srs2Δ::hphNTI*	This study
KMT087	*MAO696; srs2Δ::hphNTI*	This study
KMT118	*MAO695; rad30Δ::hphNTI*	This study
KMT119	*MAO696; htb1-K123R rad30Δ::hphNTI*	This study
YD122	*MAT**a**trp1::his3- Δ3′ his3- Δ5′ URA3*	J. Tyler
YD122 *asf1Δ*	*MAT**a**trp1::his3- Δ3′ his3- Δ5′ URA3 asf1Δ::kanMX*	J. Tyler
KMT106	*MAT**a**trp1::his3- Δ3′ his3- Δ5′ URA3 bre1Δ::kanMX*	This study

at 4°C with an equal volume of 0.5-mm zirconium oxide beads (Next Advance). The lysate was then incubated with 0.2% Triton X-100 and 0.1% SDS for 10 min on ice, and cell debris was pelleted by centrifugation. The clarified extracts were loaded onto a 10% polyacrylamide gel (Invitrogen) in a loading buffer containing 8 M urea, subjected to electrophoresis and transferred to a PVDF membrane (GE Healthcare). The blots were probed with rabbit polyclonal antibodies to yeast PCNA (gift from Paul Kaufman) and goat anti-rabbit secondary antibodies conjugated to Cy5 (GE Healthcare). The bands were visualized using a Typhoon 9410 imaging system.

RESULTS

Loss of H2Bub1 results in elevated mutagenesis upon replicative stress

Our previous studies demonstrated that H2Bub1 levels were maintained on chromatin near replication origins in a replication dependent manner. Specifically, we showed that the E3 ubiquitin ligase, Bre1, was recruited to active origins, where it rapidly ubiquitylated H2B incorporated into nucleosomes on nascent DNA (14). We determined that H2Bub1 in this context was important for restoring chromatin structure near origins, perhaps by stabilizing newly assembled nucleosomes. Such a role would be consistent with previous studies indicating that H2Bub1 functions to stabilize assembled nucleosomes during transcription elongation (6,13). The incomplete chromatin structure on nascent DNA as a consequence of H2Bub1 loss resulted in a number of replication phenotypes. Firstly, reduced replication rates near origins were observed, which was reflective of a general defect in replisome stability soon after origin firing. As a result, S-phase was significantly delayed in cells lacking H2Bub1, and this delay was largely independent of any indirect transcriptional irregularities (14).

We hypothesized that the incomplete chromatin structures resulting from H2Bub1 dysfunction could give rise to enhanced genomic insult by cellular nucleases and/or other sources. Likewise, reduced fork progression might reflect more frequent fork stalling, which would initiate DNA-damage tolerance (DDT) mechanisms. Therefore, we first asked whether there was enhanced mutagenesis in cells lacking H2Bub1. To do this, we measured the spontaneous acquisition of canavanine resistance in yeast that carried the *CAN1* gene as previously described (45). For this, we utilized a yeast strain in which the only expressed copy of the histone H2B gene carried a point mutation at its ubiquitylation site (*htb1-K123R*). We also generated another strain in which the *BRE1* gene was deleted as another means to eliminate H2B ubiquitylation. Both strains have undetectable levels of H2Bub1 by Western blot (9,48,49) (data not shown). As seen in Figure 1A and Table 2, we did not observe an increase in the spontaneous mutation rate of *htb1-K123R* or *bre1Δ* cells relative to an isogenic WT control. Rather, although not statistically significant, the spontaneous mutation rate was reduced in the absence of H2Bub1. Whereas, WT cells exhibited a spontaneous mutation rate of 2.5 per 10^7 survivors, *htb1-K123R* and *bre1Δ*

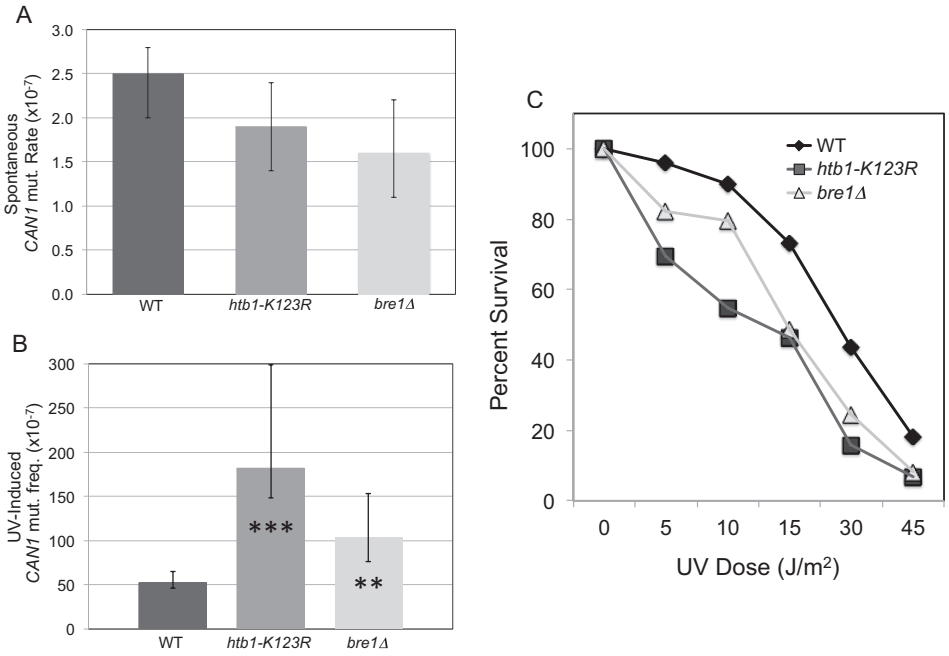

Figure 1. Loss of H2Bub1 promotes mutagenesis under conditions of replicative stress. (**A**) The graph shows the rates of spontaneous Can^r mutation for wild-type (WT; black bar), *htb1-K123R* (grey bar) and *bre1Δ* (light grey bar) cells. Rates are given as ($\times 10^{-7}$) and are medians for 45 independent cultures (from 5 experiments). 95% confidence intervals are shown for each strain. (**B**) The frequencies of UV-induced (15 J/m^2) Can^r mutation are indicated for WT (black bar), *htb1-K123R* (grey bar) and *bre1Δ* (light grey bar) cells. Frequencies are given as ($\times 10^{-7}$) and are medians calculated from 18 independent cultures (from two experiments). 95% confidence intervals are shown for each strain as well as asterisks representing *P*-values of $\leq$0.01 (**) or $\leq$0.001 (***) relative to WT controls. (**C**) Percent survival of WT (black diamond), *htb1-K123R* (grey box) and *bre1Δ* (light gray triangle) cells over a range of UV doses (0, 5, 10, 15, 30 and 45 J/m^2).

mutants exhibited rates of 1.9 and 1.6 per 10^7 survivors, respectively.

Our previous study indicated that H2Bub1-deficiency generated DNA replication-associated defects that were more pronounced under conditions of replicative stress (14). Therefore, we asked whether UV irradiation could influence the *CAN1* mutation frequency of H2Bub1-deficient yeast. Using a low dose of UV-C irradiation (15 J/m^2) to induce replication fork blockages, we noted a significant (~3.5-fold) increase in the mutagenic frequency in the *htb1-K123R* mutant relative to WT cells (Figure 1B and Table 2). WT cells exhibited a Can^r frequency of 52.3 $\times$ 10^{-7}, compared to 181.9 $\times$ 10^{-7} for *htb1-123R* cells. Interestingly, deletion of *BRE1* only produced a ~2.0-fold increase in the frequency of mutagenesis relative to WT cells, suggesting that other targets of Bre1-mediated ubiquitylation may counteract the elevated mutagenic frequency observed in the *htb1-K123R* mutant. Alternatively, or in addition, there may be some undetectable levels of H2Bub1 in the absence of Bre1, due to the functional redundancy of ubiquitin ligases.

The UV-induced mutation frequencies in both *htb1-K123R* and *bre1Δ* cells inversely correlated with their survival over a broad UV-C dose range (Figure 1C and data not shown). At 15 J/m^2 of UV-C, 73.3% of WT cells survive UV treatment, whereas only 46.3% and 48.8% of *htb1-K123R* and *bre1Δ* cells, respectively, can withstand the same dose. Together, these results suggest that loss of H2Bub1 suppresses survival from UV-induced DNA damage due to an elevated mutagenic mechanism.

In addition to UV, enhanced mutagenic frequencies were also observed in *htb1-K123R* cells relative to WT in response to HU and MMS-induced replicative stress (Table 2 and Supplementary Figure S1). Whereas, HU treatment produced a mutagenic frequency of 20.0 $\times$ 10^{-7} in WT cells, *htb1-K123R* mutants sustained a mutagenic frequency of 50.1 $\times$ 10^{-7}, a 2.5-fold increase. A low acute dose of MMS (0.002% for 90 min) produced a more modest increase in the mutagenic frequency of *htb1-K123R* cells relative to WT (1.6-fold increase; Supplementary Figure S1).

Interestingly, elevated mutagenesis negatively impacts the ability of H2Bub1-deficient cells to survive HU treatment. Whereas, 92.6% of WT cells survive HU treatment, only 68.8% of *htb1-K123R* mutants do (Table 2). This suggests that the elevated mutagenic frequencies that occur in *htb1-K123R* mutants are also associated with replication forks that are stalled without any exogenous physical lesions, such as those imparted by UV light or MMS.

Elevated mutagenic frequencies in cells lacking H2Bub1 are dependent upon TLS

Lesion bypass is commonly accomplished by the sequential action of two or more TLS polymerases. One polymerase inserts nucleotides opposite the damaged template, and the other extends from those inserted nucleotides. Y-family polymerases, such as Polη, contribute to the initial nucleotide insertion in a largely error-free manner (30,32,50). Much of the mutagenesis associated with TLS *in vivo* is attributed to the actions of Polζ, which primarily functions

Table 2. *CAN1* mutation rates and frequencies

Strain	Spontaneous Can^r rate ($\times 10^{-7}$)	15 J/m^2 UV light Can^r frequency ($\times 10^{-7}$); viability	20 mM hydroxyurea Can^r frequency ($\times 10^{-7}$); viability
WT	2.5 [2.0–2.8]#	52.3 [46.8–64.8] ; 73.3%	20.0 [18.5–18.8] ; 92.6%
htb1-K123R	1.9 [1.4–2.4]	181.9*** [148.3–299.0] ; 46.3%	50.1** [36.2–48.3] ; 68.8%
bre1Δ	1.6 [1.1–2.2]	103.8*** [76.3–153.0] ; 48.8%	N/A
rev3Δ	1.3 [0.7–2.1]	1.9*** [1.4–3.6] ; 12.1%	12.0 [7.2–22.8] ; 76.3%
htb1-K123R rev3Δ	1.1 [0.6–3.7]	2.3*** [0.0–8.1] ; 17.5%	1.0** [5.7–7.0] ; 39.2%
rad30Δ	3.1 [1.9–5.7]	163.9*** [112.2–261.4] ; 33.0%	N/A
htb1-K123R rad30Δ	1.7 [0.8–2.8]	157.2*** [117.1–202.3] ; 23.6%	N/A

#Values in [ ] represent 95% confidence intervals calculated as described in methods.
**P values versus WT $\leq$ 0.01; ***P values vs. WT $\leq$ 0.001.

as an extender after a base has been inserted across from a lesion (51–56).

The elevated mutagenic phenotype of cells lacking H2Bub1 would be consistent with frequent stalling of replication forks and the initiation of an elevated or abnormally mutagenic TLS response. To ask whether the elevated HU-induced mutation frequencies observed in *htb1-K123R* cells were dependent upon Polζ, we deleted the *REV3* catalytic subunit gene in both WT *HTB1* and *htb1-K123R* backgrounds.

As mentioned before, WT cells acquired HU-induced canavanine resistance at a frequency of 20.0 per 10^7 survivors. In the absence of *REV3*, however, that frequency fell 40% to 12.0 per 10^7 survivors (Figure 2A and Table 2). Despite an elevated initial mutagenic frequency of 50.1 per 10^7 survivors in the *htb1-K123R* background, subsequent deletion of *REV3* in the *htb1-K123R rev3Δ* double mutant resulted in a virtually undetectable Can^r frequency (1.0 per 10^7 survivors). Therefore, HU-induced *CAN1* mutagenesis is completely dependent upon Polζ in the absence of H2Bub1. The greater dependence of *htb1-K213R* cells on Polζ relative to WT cells was also reflected by reduced fitness in response to replication stress. Whereas, 68.8% and 76.3% of *htb1-K123R* and *rev3Δ* cells, respectively, survive HU treatment in these experiments, only 39.2% of double mutants can withstand such stress (Table 2).

We observed very low UV-induced *CAN1* mutagenic frequencies in both WT and *htb1-K123R* cells when *REV3* was mutated (Table 2; 1.9 and 2.3 per 10^7 survivors). Therefore, regardless of the H2B ubiquitylated state, Polζ plays an essential role in the generation of TLS-dependent mutations in response to UV-induced replication fork stalling. In contrast to HU, TLS contributes largely to the viability of both H2Bub1-proficient and H2Bub1-deficient cells. Only 12.1% of *rev3Δ* and 17.5% of *rev3Δ htb1-K123R* double mutants survive UV treatment compared to 73.3% and 46.3% of WT and *htb1-K123R* cells (Table 2).

We hypothesized that the elevated TLS-dependent mutagenesis observed in the absence of H2Bub1 could be due to aberrant TLS polymerase usage. Because Polη is associated with 'error-free' TLS, we asked whether it could be regulated in an H2Bub1 dependent manner. This idea was influenced by the fact that Polη has a UBZ ubiquitin recognition motif whose function is still not fully understood (57,58). Deletion of the *RAD30* catalytic subunit of Polη resulted in a UV-induced hypermutable phenotype very similar to the *htb1-K123R* mutant (163.9 and 181.9 per 10^7 survivors respectively; Table 2). Interestingly, the *htb1-K123R rad30Δ* double mutant had a UV-induced *CAN1* mutagenic frequency of 157.2 per 10^7 survivors, indicating that these two mutations are epistatic. This would be consistent with the idea that H2Bub1 is important for Polη recruitment and its role in 'error-free' TLS in response to UV damage. In the absence of H2Bub1, Polζ may play a more dominant role in both nucleotide insertion across from a lesion and extension. Given that *REV3* is similarly required for mutagenesis in both WT and H2Bub1-deficient cells, the enhanced UV-induced mutagenesis observed in *htb1-K123R* mutants would be consistent with *i)* reduced Polζ fidelity, *ii)* the generation of longer Polζ tracts within a relaxed chromatin environment, and/or *iii)* more frequent use of Polζ due to more frequent fork stalling.

To address these possibilities, we performed DNA sequencing of the *CAN1* locus from Can^r isolates from both WT and *htb1-K123R* cells in response to 0 and 15 J/m^2 UV treatment. With or without prior UV treatment, both WT and *htb1-K123R* strains displayed similar numbers of total mutations, as well as similar numbers of base substitutions, frameshift mutations, and complex type mutations (Supplementary Figure S2AB; top panels). The mutagenic rates/frequencies were then calculated for each of the individual base substitutions (Supplementary Figure S2AB; lower panels). We observed a general increase the entire spectrum of base substitutions in *htb1-K123R* cells relative to WT upon UV treatment, consistent with an elevated usage of TLS polymerases. However, due to our small sample size (~50 DNA sequences per condition), we were unable to discern a specific TLS polymerase signature.

Contrary to UV, loss of Polη did not significantly elevate the mutagenic frequency of cells treated by HU, irrespective of the H2Bub1 state (Table 2 and Supplementary Figure S3). The primary role of Polη is to insert nucleotides across from a physical lesion in a largely error-free manner, and its absence may result in more frequent insertion by the more error-prone Polζ. Interestingly, HU does not generate physical lesions, yet loss of *RAD30* suppressed the mutagenic phenotype of *htb1-K123R* mutants, suggesting that Polη is at least partially responsible for the elevated HU-induced mutagenesis in the absence of H2Bub1.

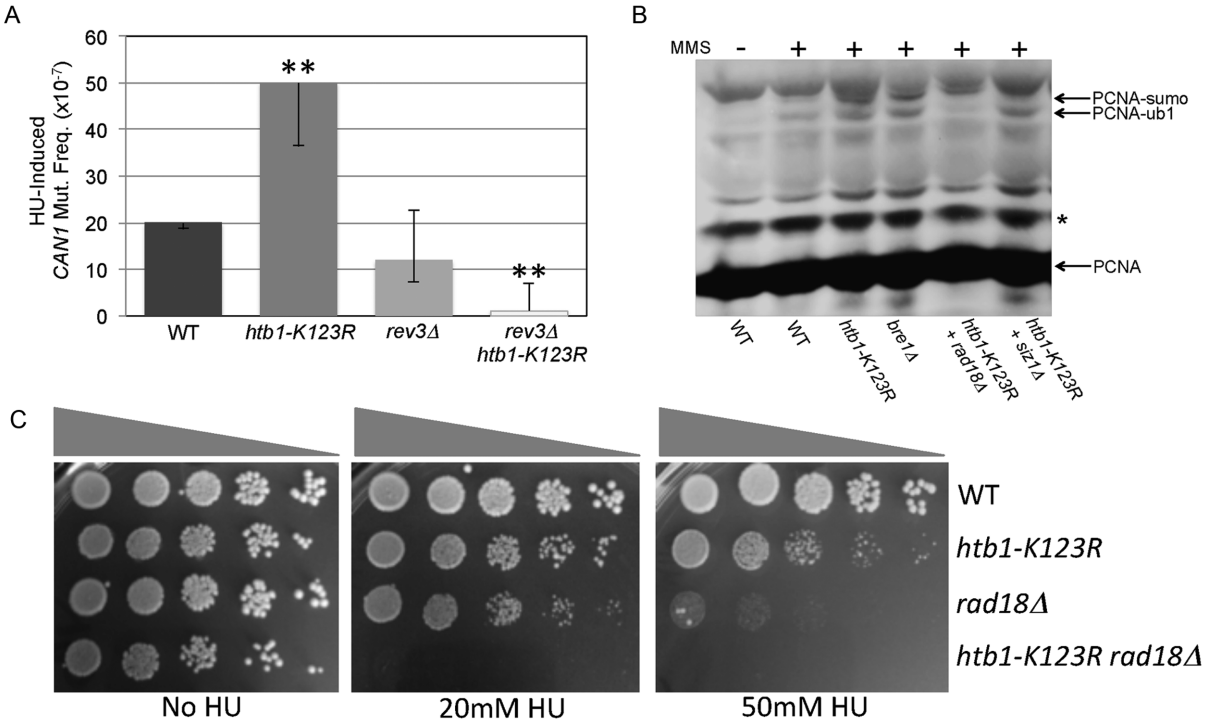

Figure 2. TLS contributes to the recovery of stalled replication forks in an H2Bub1 directed manner. (**A**) The graph shows the frequencies of HU-induced Can^r mutation for wild-type (WT; black bar), *rev3Δ* (grey bar), *htb1-K123R* (light grey bar) and *rev3Δ htb1-K123R* (white bar) cells. Frequencies are given as ($\times 10^{-7}$) and medians for nine independent cultures (from one experiment). 95% confidence intervals are shown for each strain as well as asterisks representing *P*-values of $\leq$0.01 (**) relative to WT controls. (**B**) Detection of ubiquitylated and sumoylated PCNA in whole-cell extracts of WT and the indicated mutant strains by Western blot analysis with antibodies against PCNA. The positions of unmodified (PCNA), mono-ubiquitylated (PCNA-Ub1) and mono-sumoylated (PCNA-SUMO) PCNA are indicated. Mid-log phase cultures were treated with 0.02% MMS for 90 min are indicated by a + symbol above the gel image. Non-specific bands indicated by an * symbol were used as a loading control. (**C**) Serial dilutions of WT and the indicated mutant strains were spotted onto YPD plates containing 0 mM, 20 mM and 50 mM HU.

Yeast lacking H2Bub1 have elevated levels of post-translationally modified PCNA in response to MMS treatment

The post-translational state of PCNA is recognized as a master regulator of DDT mechanisms (22). Specifically, PCNA mono- and poly-ubiquitylation determine whether error-prone or error-free mechanisms are initiated to help overcome replication fork blockages. PCNA mono-ubiquitylation on lysine 164 (PCNA-ub1) is catalyzed by Rad6/Rad18 and is necessary for TLS (21,23,33). We hypothesized that should *htb1-K123R* cells be undergoing TLS at a higher frequency than WT cells, we would see a relative increase in global PCNA-ub1 levels in response to DNA damage. To test this, we generated denatured whole cell extracts from log-phase cultures treated with 0.02% MMS for 90 minutes. These extracts were subjected to Western blot analysis using antibodies against PCNA (gift from Dr Paul D. Kaufman). As seen in Figure 2B, both *htb1-K123R* and *bre1Δ* extracts exhibited elevated PCNA-ub1 levels relative to WT extracts. Note that the PCNA-ub1 band is absent in the *rad18Δ htb1-K123R* control lane and is greatly diminished in the WT lane without MMS treatment. An asterisk marks a non-specific band used as a loading control. These data are consistent with the idea that loss of H2Bub1 results in an elevated TLS response upon replication fork stalling.

Interestingly, we also noted a significant increase in the levels of sumoylated PCNA (PCNA-SUMO) in extracts derived from cells lacking H2Bub1 and upon exposure to MMS (Figure 2B). PCNA-SUMO prevents HR during S-phase by stabilizing Srs2 (37,38). Srs2, in turn, disrupts Rad51 nucleoprotein filaments via its helicase activity (39,40). Deleting the *SIZ1* gene in the *htb1-K123R* background eliminated the PCNA-SUMO band on Western blots, confirming the identity of this slow migrating form of PCNA.

Deletion of *RAD18* eliminates both error-free and error-prone arms of DDT by preventing the initial mono-ubiquitylation of PCNA. Therefore, *rad18Δ* mutants are very sensitive to agents that cause replicative stress, including HU. We tested *rad18Δ*, *htb1-K123R*, and *rad18Δ htb1-K123R* double mutants for genetic interactions by observing their HU sensitivity phenotypes in spotting assays. While the single mutants each conferred some sensitivity, the double mutant was very sensitive to even low doses of HU (Figure 2C). This phenotype would be consistent with cell survival in the absence of H2Bub1 being dependent upon DDT mechanisms, such as TLS and template switching (fork regression and/or strand invasion of sister chromatid).

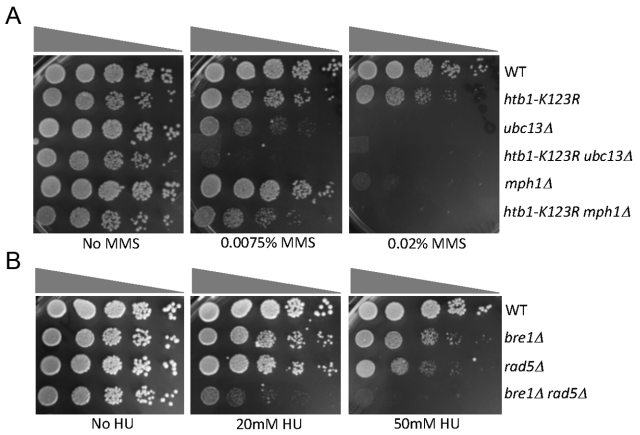

Figure 3. H2Bub1 deficient cells rely on error-free DNA-damage tolerance pathways. (**A**) Serial dilutions of wild-type (WT) and the indicated mutant strains were spotted onto YPD plates containing 0.0%, 0.0075% and 0.02% MMS. (**B**) Serial dilutions of WT and the indicated mutant strains were spotted onto YPD plates containing 0 mM, 20 mM and 50 mM HU.

Template switching mechanisms participate in the survival of H2Bub1-deficient cells upon replicative stress

Poly-ubiquitin chains can be further extended from PCNA-ub1 by the action of Ubc13/Mms2 to initiate error-free damage avoidance mechanisms that involve template switching. In order to determine whether template-switching mechanisms are functional in the absence of H2Bub1, we generated a series of single and double mutant strains and analyzed their sensitivity to DNA damaging agents. Deletion of *UBC13* resulted in a profound sensitivity to low doses of MMS (Figure 3A). This sensitivity was much more severe than that conferred by the *htb1-K123R* mutation alone, and the *htb1-K123R ubc13Δ* double mutant was synergistically sensitive, indicating that these mutants are not epistatic (Figure 3A).

The mechanisms by which stalled replication forks can either regress or invade nascent DNA for the purpose of template switching are not fully understood. However, it has been proposed to involve the actions of at least two enzymes, Rad5 and Mph1. Rad5 interacts with Rad18 and PCNA at sites of DNA damage and is important for the recruitment of Ubc13-Mms2. Rad5 is an ATPase, which may function as a helicase to unwind replication forks into 'chicken foot' structures for error free DDT. Similar to our *UBC13* analysis, we observed a non-epistatic relationship between *rad5Δ* and *bre1Δ* with regard to HU sensitivity (Figure 3B).

The DNA motor protein and FANCM homolog, Mph1, has also been implicated as a regulator of replication fork regression (59–63). In spotting assays, we find that *mph1Δ* cells are modestly sensitive to MMS, as are *htb1-K123R* cells. However, *htb1-K123R mph1Δ* double mutants are more sensitive than either mutant alone (Figure 3A). Together with the *UBC13* and *RAD5* analyses, we conclude that template-switching mechanisms are not only functional in the absence of H2Bub1, but that H2Bub1-deficient cells depend considerably upon template switching as a means for survival upon replication associated stress.

H2Bub1 deficiency initiates survival mechanisms that depend upon Srs2 guarded homologous recombination

Elevated levels of PCNA-sumo (Figure 2B) were the first indication that H2Bub1-deficient cells may be running into some sort of HR crisis. As discussed, the main function of PCNA-sumo is to recruit Srs2, whose helicase activity is required to prevent HR during S-phase. While replication fork regression and template switching can facilitate continuity of replication, it can also lead to the generation of toxic recombination intermediates (64–66). Therefore, we hypothesized that Srs2 would be a necessary component for H2Bub1-deficient cell survival. Indeed, this is the case. While single *srs2Δ* mutants are not sensitive to low doses of HU, deletion of *SRS2* in either an *htb1-K123R* or *bre1Δ* background rendered them highly sensitive (Figure 4A). These results indicate that upon replicative stress, H2Bub1-deficient cells depend heavily upon Srs2 for survival, presumably to prevent the accumulation of toxic HR intermediates.

If the main role of Srs2 is to prevent HR, one might predict that HR mutants might suppress the HU sensitivity of *htb1-K123R* or *bre1Δ* mutants. However, spotting assays revealed that both *htb1-K123R rad51Δ* and *bre1Δ rad51Δ* double mutants are synergistically sensitive to HU (Figure 4B). Together, we conclude that upon replicative stress, H2Bub1-deficient cells depend upon HR mechanisms for survival, and that those HR mechanisms must be carefully regulated by Srs2. In support of this, deletion of *RAD51* partially suppressed the HU sensitivity of *htb1-K123R srs2Δ* double mutants, indicating that HR mechanisms contribute to their reduced viability (Supplementary Figure S4).

In order to measure HR in vivo, we generated strains with a specific substrate (*3′Δ-his3 5′Δ-his3*; gift from Jessica Tyler, MD Anderson Cancer Center), which measures sister chromatid exchange (SCE). Only upon SCE can these markers form a functional *HIS3* gene. By selection on media lacking histidine, we can score for SCE events. We introduced a *bre1Δ* mutation into this strain and compared it to both WT and *asf1Δ* controls. *ASF1* encodes a histone chaperone important for replication-coupled nucleosome assembly. As previously published, *asf1Δ* mutants displayed an elevated spontaneous SCE rate (47). Specifically, *asf1Δ* mutants produced 8.4 His^+ revertants per 10^6 cells, compared to 2.5 and 2.2 for WT and *bre1Δ* cells, respectively (Figure 4C). Upon UV-induction, there was no appreciable difference in the SCE frequencies of the three strains (Figure 4D). Therefore, it does not appear that H2Bub1-deficiency has any affect on the levels of this type of replication associated HR. However, it is yet to be seen whether these HR events are more likely to be mutagenic in the absence of H2Bub1.

DISCUSSION

In summary, we have found that loss of H2Bub1 results in an aberrant DDT response to replication fork blockages. Elevated PCNA-ub1 levels in H2Bub1-deficient cells in response to MMS treatment suggests that H2Bub1 functions to regulate TLS. Indeed, we show that upon frequent fork stalling, mutant yeast lacking H2Bub1 undergo a particularly error-prone version of TLS, in which the infidelity of

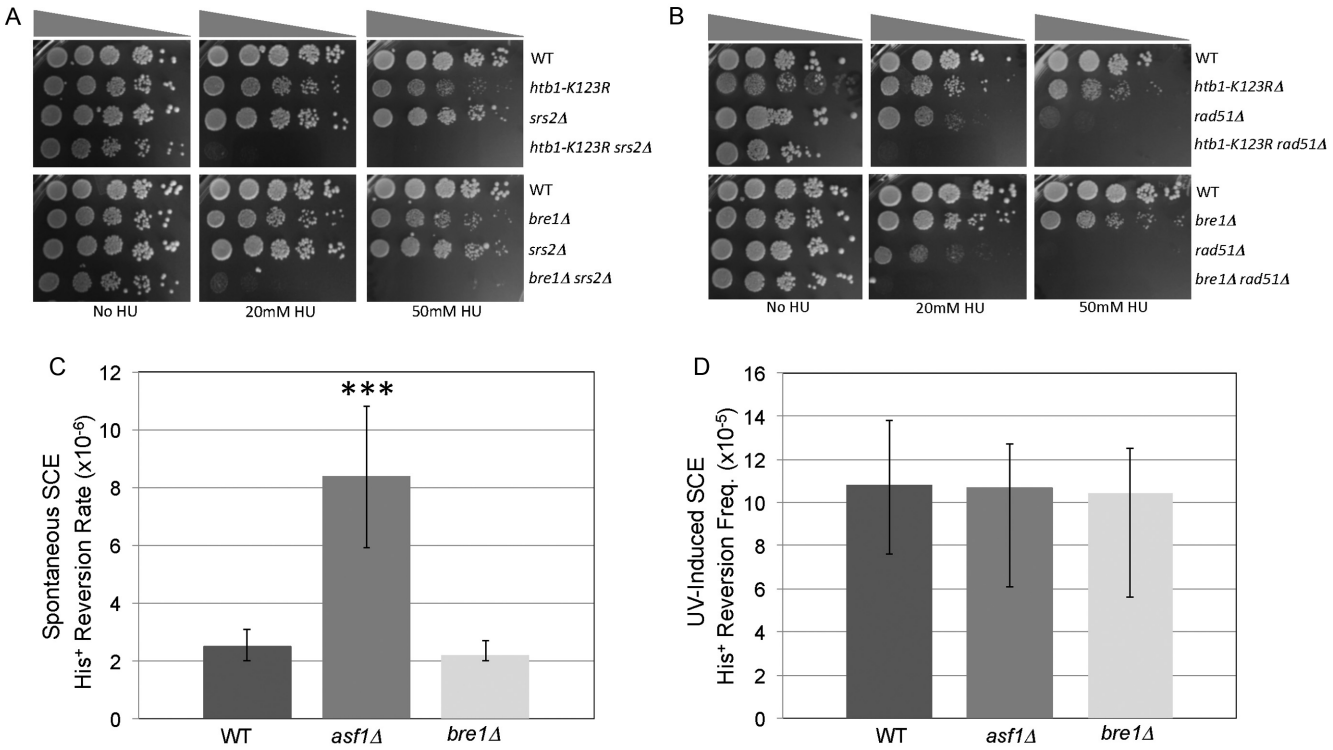

Figure 4. Srs2 is an essential regulator of HR-mediated DDT pathways in the absence of H2Bub1. (**A** and **B**) Serial dilutions of wild-type (WT) and the indicated mutant strains were spotted onto YPD plates containing 0 mM, 20 mM and 50 mM HU. (**C**) The graph shows the rates of spontaneous sister chromatid exchange (-His reversion via SCE) for WT, *asf1Δ* and *bre1Δ* cells. Rates are given as ($\times 10^{-6}$) and are medians for 18 independent cultures (from two experiments). 95% confidence intervals are shown for each strain as well as asterisks representing *P*-values of $\leq$0.001 (***) relative to WT controls. (**D**) The graph shows the frequencies of UV-induced (15 J/m^2) sister chromatid exchange (-His reversion via SCE) for WT, *asf1Δ* and *bre1Δ* cells. Frequencies are given as ($\times 10^{-5}$) and are medians for nine independent cultures (from one experiment). 95% confidence intervals are shown for each strain.

DNA damage bypass depends exclusively upon the action of Polζ. This is true not only for UV induced damage, but also for HU induced replication fork blockage, indicating that the mutagenic mechanism is independent of an initiating physical lesion.

Interestingly, H2Bub1 mutants (*htb1-K123R*) are epistatic to *rad30Δ* (Polη) mutants with regard to an elevated UV-induced mutagenic phenotype, suggesting that the error-free arm of TLS is impaired. Rad30 contains a UBZ, ubiquitin recognition motif, whose function is unclear. While a previous study did indicate that this domain is dispensable for PCNA-ub1 interaction, it does enhance Rad30:PCNA interactions mediated by the adjacent Rad30 PIP domain (58). In 2007, the Prakash lab carried out a careful mutagenic analysis of the Polη UBZ domain with regards to its TLS function. Several key conserved amino acid residues were mutated and found to confer UV sensitivity. None of these mutations appeared to impact polymerase activity *in vitro*. However, one mutant allele (*rad30-D570A*) failed to fully complement the mutagenic phenotype of *rad30Δ* cells, suggesting that it may not be properly recruited to stalled replication forks (57). Our ongoing studies will determine if Rad30 indeed interacts with H2Bub1, and whether this conserved residue is necessary for that interaction as a means for its recruitment. Regardless of the mechanism, our data is consistent with a model by which loss of H2Bub1 prevents the function of Polη in error free TLS at stalled replication forks.

While H2Bub1 does not appear to regulate 'error-free' mechanisms of DDT (fork regression or strand invasion) *per se*, our genetic analyses clearly show that H2Bub1-deficient cells depend upon these mechanisms for their survival. H2Bub1 deficient mutants are synergistically sensitive to agents that cause replicative stress when combined with *ubc13Δ*, *rad5Δ*, or *mph1Δ* mutations. Therefore, while an elevated TLS response contributes to the survival of H2Bub1-deficient cells, other DDT mechanisms are also being employed.

A somewhat enigmatic finding is that both the anti-recombinase, Srs2, and the recombinase, Rad51, are also required for the survival of H2Bub1-deficient cells upon replicative stress. While fork reversion mechanisms do not appear to require HR, it is likely that HR is required for the strand invasion step necessary for template switching. Regardless of whether fork regression or strand invasion mechanisms are employed, branch migration of the resulting Holliday junctions, if left unrestrained, may have the potential to generate toxic HR intermediates (67–69). Given that H2Bub1-deficient yeast already have an incomplete chromatin structure at stalled replication forks (14), it conceivable that unconstrained HR mechanisms are contributing to genomic instability through the generation of toxic intermediates. In support of this, we show that loss of *RAD51*

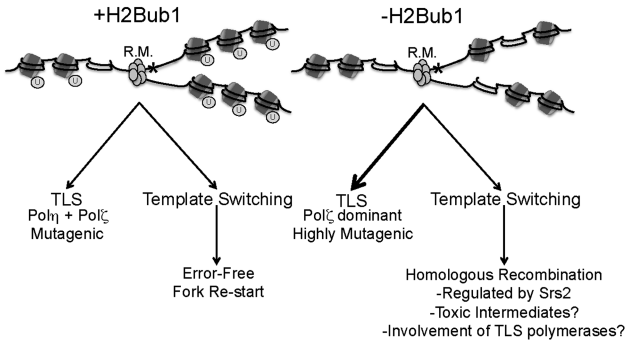

Figure 5. Model for DNA damage tolerance mechanisms with and without H2Bub1. When replication forks are challenged with a physical lesion (*), H2Bub1 (indicated by circled 'U') promotes proper chromatin assembly and TLS mediated by Polζ/Polη. In the absence of H2Bub1, an incomplete chromatin structure encourages a Polζ dominant and highly mutagenic form of TLS. In addition, the error-free arm of DDT (i.e. template switching) becomes reliant upon the strict regulation of Srs2, presumably to help prevent the accumulation of toxic HR intermediates. In addition, TLS polymerases may be contributing to mutagenesis during HR-associated DNA synthesis.

partially suppresses the HU sensitivity of *htb1-K123R srs2Δ* double mutants (Supplementary Figure S4). Therefore, we believe that Srs2 becomes an essential factor to limit HR in an environment unconstrained by a relaxed chromatin structure. Failure to keep HR mechanisms at bay results in the generation of toxic intermediates, which explains why *srs2Δ htb1-K123R* and *srs2Δ bre1Δ* double mutants are so severely sensitive to even small doses of HU (Figure 5).

During DNA double-strand break repair, Polη can function to extend 3′ strands, which have invaded to form a D-loop HR intermediate (70,71). Whether Polη plays a similar role in extension of processed replication forks is unclear, but such a mechanism may also be dependent upon H2Bub1. Loss of H2Bub1 could shift the balance to a Polζ dominant and error-prone variety of template switch.

Several studies have revealed a tumor suppressor role of H2Bub1 in mammals (72,73). The RNF20 gene is mutated in cancers of the colon, ovary, head/neck squamous cell carcinoma, and melanoma (41). Aberrant expression of RNF20 is associated with testicular and breast cancer (74,75). Most recently, it was reported that reduced H2Bub1 expression is a poor prognostic biomarker for colorectal cancer (43). Consistent with that finding is another study that implicates the ubiquitin protease, USP22, which targets H2Bub1 for turnover in malignant colon carcinoma (76). Interestingly, despite a conserved role in transcription, the absence of H2Bub1 significantly impacts the expression levels of only a subset of genes, the identities of which can only partially explain H2Bub1's function as a tumor suppressor (42,73,77,78). It has been suggested that the transcription-independent roles of H2Bub1 in DNA repair and DNA replication may play a major role in preventing malignant transformation. Because the molecular players in both H2Bub1 maintenance at the replication fork and for mediating DDT pathways are highly conserved amongst eukaryotes, we anticipate that our findings in yeast will lend great insight as to the function of H2Bub1 in mammals.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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