

# Flexibility in crosstalk between H2B ubiquitination and H3 methylation in vivo

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**Running title:** Plasticity of histone crosstalk in vivo

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**Two sentence summary:** Various tethered ubiquitins, expressed as linear histone-ubiquitin fusions, can substitute for native H2BK123ub in the crosstalk to H3 methylation and in the DNA damage response. This reveals a remarkable plasticity in H2Bub signaling in vivo.

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Histone H2B ubiquitination is a dynamic modification that promotes methylation of histone H3K79 and H3K4. This crosstalk is important for DNA damage response and has been implicated in cancer. In engineered yeast strains, ubiquitins tethered to every nucleosome promoted H3K79 and H3K4 methylation from a proximal as well as a more distal site, but only if in a correct orientation. This plasticity indicates that the exact location of the attachment site, the native ubiquitin-lysine linkage and ubiquitination cycles are not critical for trans-histone crosstalk in vivo. The flexibility in crosstalk indicates that other ubiquitination events may also promote H3 methylation.

## Introduction

The DNA in the nucleus is wrapped around octamers of histone proteins. Histones can be modified and these post-translational modifications (PTMs) regulate most processes that occur at the DNA. In turn, histone PTMs are regulated by signaling events in the cell, such as other histone PTMs. This crosstalk can occur between PTMs on the same histone, but also between histones.

An evolutionary-conserved trans-histone crosstalk is that between ubiquitin on the C-terminus of histone H2B and methylation of histone H3 on lysine 79 (H3K79) and lysine 4 (H3K4) (Fig 1A). In yeast, H2B is ubiquitinated on lysine 123 (H2BK123) by the Rad6/Bre1 complex, which enhances the overall activity of Dot1 (Fig 1B) and activates the Set1 complex (COMPASS) ([1] and references therein). Whereas H2Bub is a transient mark, H3K4 and especially H3K79 methylation are more stable and will remain after H2Bub has been removed [2,3]. In mammalian cells, ubiquitination of H2B by RNF20/40 promotes H3K79 methylation by DOT1L, and H3K4 methylation by several Set1/MLL complexes [4,5]. As proliferation in MLL-rearranged leukemias depends on RNF20, DOT1L and MLL1, this trans-histone crosstalk may have a role in cancer [6–8].

In general, despite intensive studies, how monoubiquitin acts as a signal often remains unknown [9]. For the role of ubiquitinated H2B (H2Bub) in promoting Dot1 activity, two non-exclusive models have been suggested: a wedge model and a bridge model (reviewed in [10,11]). The wedge model, in which the ubiquitin moiety disrupts chromatin structure and enables Dot1 to access the nucleosome core surface, is supported by *in vitro* experiments that showed that ubiquitin on H2B disrupts chromatin compaction [12]. However, since the crosstalk can occur on mononucleosomes [13,14], the opening of the chromatin cannot be the only mechanism behind the crosstalk. Moreover, *in vivo*, H2Bub may actually lead to chromatin compaction [15–17]. In the bridge model, ubiquitin functions as a bridge to recruit or activate Dot1. However, Dot1 has a similar affinity for both ubiquitinated and unmodified nucleosomes [13] and the only putative ubiquitin-binding domain in Dot1 [18] is not required for the crosstalk [19].

Although the molecular mechanisms remain elusive, *in vitro* studies have revealed that there is some plasticity in this remarkable histone crosstalk. Ubiquitin confers crosstalk when linked to various residues in the vicinity of H2BK123, and the ubiquitin-like modifier Nedd8 can stimulate Dot1 as strongly as ubiquitin itself [14]. Whether similar rules apply to the crosstalk *in vivo* is an important next question that remains to be addressed. However, studying the function of one specific ubiquitination event on one particular target *in vivo* is challenging [20]. We took advantage of the possibility to engineer complex histone mutants in budding yeast, to learn which molecular features of ubiquitinated H2B are important for the trans-histone crosstalk to H3 methylation *in vivo*. Our experiments using ubiquitins tethered to the nucleosome reveal that the crosstalk from ubiquitination to methylation has a high degree of plasticity *in vivo*. Our study leads to new insights about the molecular determinants and provides support for a new model for the crosstalk towards H3K79 methylation.

## Results

### Ubiquitin tethered to the N-terminus of H2A confers crosstalk to H3K79 methylation

To specifically determine the features of H2Bub that promote histone methylation at H3K79 and H3K4 *in vivo*, we designed yeast strains in which the endogenous H2Bub pathway was eliminated by deletion of *BRE1* and in which a ubiquitin moiety was specifically re-introduced on the nucleosome. We used a plasmid shuffle assay to create strains in which an engineered H2A gene was the only source of H2A protein (Supplementary Fig S1A and B). Ubiquitin (ub) was tethered via a short HA tag to H2A at S1 or at S19, in close proximity to H2BK123 (Fig 1A). The terminal glycine of ub was removed to prevent ub-H2A cleavage (Supplementary Fig S1D). Strains expressing HA-S1-H2A, ubHA-S1-H2A or ubHA-S19-H2A were viable (Supplementary Fig S1B and D), suggesting that these

fusions showed no gross defects in histone transport, assembly, and chromatin packaging. The HA-S19-H2A-expressing *bre1Δ* strain was not viable and was eliminated from further analyses, see Supplementary Fig S1B. Immunoblot analysis confirmed that the H2A fusion proteins were expressed, that no wild-type H2A was present, and that the majority of the fusions were full-length (Fig 2B). ChIP-qPCR experiments confirmed that tagged H2A was incorporated into chromatin (Supplementary Fig S1E).

Multiple methylation by Dot1 occurs by a distributive mechanism, which leads to a characteristic shift in the relative abundance of the H3K79 methylation states when the activity of Dot1 increases or decreases (Fig 1B) [19,21]. The ubiquitin moiety at H2AS19, close to the native position of ubiquitination, caused a shift from H3K79me0/me1 to H3K79me2/me3, while Dot1 expression was not increased (Fig 2C; Supplementary Fig S1F). Unexpectedly, the more distal ubiquitin at S1 of H2A was also able to promote H3K79 methylation, albeit with a somewhat lower efficiency (Fig 2C). This did not require the formation of ubiquitin branches, since KO mutants of ubiquitin could still promote H3K79 methylation (Supplementary Fig S1G).

H3K79 methylation is not distributed uniformly across the genome, but is very low in heterochromatin, intermediate in intergenic regions, and high in coding sequences (see Fig 2D) [22]. In a *bre1Δ* strain a shift to lower methylation states was observed at all loci (in line with the distributive mechanism, see Fig 1B). Tethered ubiquitins increased methylation at all tested loci. The H3K79 methylation in strains expressing histone-ubiquitin fusions was more evenly distributed than in wild-type cells with native H2Bub (Fig 2D), confirming that this crosstalk contributes to the establishment of the H3K79 methylation pattern. However, since the pattern was still partially maintained, additional mechanisms of regulation must exist, such as histone turnover [19,23] or protection of nucleosomes by binding of the Sir complex [24].

Together, these findings show that a ubiquitin constitutively tethered to the N-terminus of H2A by a linear fusion confers trans-histone crosstalk *in vivo*, throughout the genome. To eliminate indirect effects of the ub-H2A on H3K79 methylation, we performed *in vitro* methyltransferase assays using chromatin templates isolated from the engineered yeast strains. Ubiquitin tethered to the H2A N-terminus promoted the activity of yeast Dot1 as well as human DOT1L *in vitro*, suggesting that it has a direct effect on Dot1 activity (Fig 2E and Supplementary Fig S1H).

### **Ubiquitin tethered to the C-terminus of H2A or H2B confers modest crosstalk to H3K79 methylation**

To find the limits of the observed plasticity, and thereby determine which features are important for the crosstalk, we created C-terminal fusions between histone H2A or H2B and ubiquitin (Supplementary Fig S2A). The C-terminal glycine of ub was changed to valine to avoid conjugation of the fusion protein to ubiquitination targets. Yeast strains expressing an H2A-ub or H2B-ub fusion protein were viable in the absence of untagged versions of the respective histones (Supplementary Fig S2B). Expression of the fusion proteins and the absence of untagged histone protein was confirmed by immunoblot analysis (Fig 3B).

In *bre1Δ* strains, lacking endogenous H2Bub, ubiquitins tethered to the C-terminus of H2A or H2B led to more H3K79me1/me2 and less H3K79me0, while Dot1 expression was not increased (Fig 3C; Supplementary Fig S2C). However, they bypassed the lack of endogenous H2Bub only partially; ubiquitin tethered to the H2A or H2B C-terminus was much less potent than ubiquitin tethered to the N-terminus of H2A (see Fig 2C).

### **Ubiquitin crosstalk to H3K4 methylation**

Having established that ubiquitin tethered to the nucleosome can take over the role of native H2Bub in promoting Dot1 activity, we asked whether they could also be used to gain insight into the crosstalk from H2Bub to H3K4 methylation. Using the engineered yeast strains we found that ub-H2A fully restored H3K4me1 and me2 and partially restored H3K4me3 in the absence of endogenous H2Bub (Fig 4A). H2A-ub did not promote H3K4 methylation and H2B-ub had a modest

effect (Fig 4A). Taken together, the findings indicate that also the crosstalk to H3K4 methylation is plastic and that Dot1 and Set1 require overlapping but also distinct structural features of chromatin containing H2Bub (Supplementary Table 1).

### **Tethered ubiquitins substitute for native H2B ubiquitination in the DNA damage response.**

H2B ubiquitination and H3K79 methylation are required for a timely induction of the DNA damage response [25]. However, whether dynamic changes in H2Bub or histone methylation are required is unknown. Two general models have been proposed: i) due to global H2Bub and H3K79me, checkpoint proteins are at the DNA and can be phosphorylated upon DNA damage, ii) a local increase in H2Bub and H3K79me at the site of lesion recruits and/or activates checkpoint proteins [26–28]. We investigated this problem, using phosphorylation of the central kinase Rad53 as the readout of checkpoint activation after UV irradiation of G1-arrested cells. Whereas *bre1Δ* cells poorly phosphorylated Rad53 upon UV irradiation (Fig 4B), tethered ubiquitins restored Rad53 phosphorylation in *bre1Δ* cells (visible as an upward shift of the protein, Fig 4B and Supplementary Fig S3A). Since the constitutive ubiquitin moieties on each nucleosome are sufficient for checkpoint activation after UV, we conclude that a local accumulation of H2Bub specifically at the site of damage is not required. Instead, the data suggest a role of global chromatin modification in modulating the DNA damage checkpoint. In addition, since the ubiquitin tethers are static, our results demonstrate that cycles of ubiquitination and deubiquitination are not required for DNA damage checkpoint activation.

## **Discussion**

The crosstalk between H2B ubiquitination and H3K4 and H3K79 methylation is evolutionary conserved from yeast to metazoans. Since many other chromatin proteins are also subject to ubiquitination, an important question is which molecular features of ubiquitinated H2B are important for the trans-histone crosstalk in vivo. We have shown that various tethered ubiquitins, expressed as linear histone-ubiquitin fusions, can substitute for native H2BK123ub in the crosstalk to H3K79 and H3K4 methylation. This reveals an unexpected plasticity in the crosstalk in vivo.

The extent of the plasticity in the crosstalk in vivo is remarkable. Previously, in vitro experiments already revealed some plasticity in the site of ubiquitin attachment [14,29]. However, the in vivo plasticity of the crosstalk observed here goes beyond what has so far been observed in vitro. This allows us to draw several conclusions about the features of H2Bub that are important for crosstalk to H3 methylation.

First, the precise location of ubiquitin attachment to the nucleosome core surface is not critical for nucleosome crosstalk since H3K79 and H3K4 methylation were enhanced by ubiquitin tethered to various positions on the nucleosome. This is in line with the finding that ubiquitination of H2BK34 by MSL2 stimulates H3K79 and H3K4 methylation in human cell lines and in vitro [30]. In contrast to the previous finding that a short linker between ubiquitin and H2B decreases the crosstalk considerably in vitro [14], ubiquitin tethered to H2AS1 was approximately as effective as ubiquitin on H2AS19. Note that the ubiquitin tethered to H2AS1 is attached to the flexible H2A N-terminus and thereby not in itself located on the nucleosome core surface.

Second, the specific branched structure of ubiquitin linked to a lysine side chain via an isopeptide bond is not a critical feature, since the genetically-encoded ubiquitin fusions represent linear proteins and also lack the terminal GG motif. Therefore, the specificity must originate from other parts of ubiquitin.

Third, modification-demodification cycles of H2Bub are not required for crosstalk in vivo. Steady-state levels of H2Bub are the result of ongoing ubiquitination by Bre1/Rad6 and deubiquitination by Ubp8 and Ubp10 [3]. It has been proposed that turnover of histone

modifications could be an important determinant of epigenetic regulation [31,32]. However, since our non-cleavable ubiquitins could promote H3K79 and H3K4 methylation, we conclude that H3 methylation can be promoted without H2Bub turnover.

Fourth, Bre1 activity towards non-nucleosomal substrates is not a requirement for the crosstalk, since ubiquitins tethered to the nucleosome confer crosstalk to H3 methylation in the absence of Bre1. It has been suggested that the Bre1/Rad6 ubiquitination machinery, rather than H2BK123ub, promotes maximal crosstalk, potentially by ubiquitinating Swd2 [33,34]. However, this mechanism is still under debate [35]. In our strains the presence of ubiquitin on the nucleosome is uncoupled from Bre1 activity. Our finding that tethered ubiquitins can promote H3 methylation in the absence of Bre1 argues against a critical role of the ubiquitination machinery. However, since even the most efficient tethered ubiquitins we engineered did not fully rescue H3K4me3 and H3K79me3, it is still possible that the Bre1-dependent ubiquitination of a non-histone protein has an additional effect.

Fifth, the orientation of the ubiquitin seems to be a critical structural feature. The C-terminus of H2B and the N-terminus of H2A are at approximately the same distance from H2BK123 (Supplementary Fig S3B and C). Yet, ubiquitin tethered to H2AS1 was much more effective in histone crosstalk. One key structural difference is that the ubiquitin on the N-terminus of H2A is presented in the same orientation as the native ub on H2BK123 whereas ubiquitin on the C-terminus of H2B is in the opposite orientation.

We believe that our *in vivo* results, together with previous *in vitro* findings, are most parsimonious with a model in which ubiquitin can interact with the nucleosome surface and thereby increase the chance of a productive encounter of Dot1 with H3K79 on the nucleosome. This could be achieved by creating a physical road block or crash barrier that coaches Dot1 in the right direction (see Fig 5). A similar fold-back mechanism has previously been suggested for ubiquitin on PCNA [36]. Ubiquitin tethered to the N-terminus of H2A, both at S19 and S1, would be able to fold back just like the native H2Bub. The ubiquitins tethered to the C-termini, which are in the opposite orientation, would not be able to do this. The modest crosstalk that is still observed for these tethers may occur through another mechanism, possibly as proposed in the wedge or bridge model.

The finding that ubiquitin moieties at locations other than H2B can promote histone methylation suggests that nucleosome crosstalk could also be affected by other ubiquitination events in the cell. This is consistent with the crosstalk that has been observed between H2BK34ub and H3 methylation [30], but the site of ubiquitination does not have to be limited to H2B or even the nucleosome core. It will be interesting to determine whether the extensive chromatin ubiquitination during DNA damage response in mammalian cells [25], especially the ubiquitination at the N-terminus of H2A [37], influences the methylation of resident or newly-assembled histones during the process of DNA repair. The tethered ubiquitins that we describe here are genetically encoded and amenable to genetic manipulation. Therefore, they will provide opportunities to further delineate the role of ubiquitin in the control of histone methylation and other aspects of chromatin structure and function.

## Experimental procedures

**Strains, plasmids and growth conditions.** Yeast media and growth conditions, and strains and plasmids are described in the Supplementary Methods.

**Immunoblotting and mass spectrometry.** Histone methylation and expression was determined by immunoblotting and mass spectrometry as described previously [19]. See Supplementary Methods.

**In vitro methyltransferase assays.** Recombinant 10xHis-Dot1 proteins were incubated with yeast nuclear chromatin templates and analyzed by immunoblot analysis as described previously [21]. See

Supplementary Methods.

**DNA damage checkpoint activation.** Yeast cells were arrested in G1 for two hours, exposed to 100J/m<sup>2</sup> UV irradiation and recovered for 30 minutes before protein extracts were made, as described previously [21]. See Supplementary Methods.

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### Author contributions

HV and FvL designed the studies, analyzed the data, and wrote the paper. HV and TvW performed the plasmid and strain constructions, yeast manipulations and methyltransferase assays. ELdG, AFMA and AJRH were responsible for mass spectrometry measurements. DO constructed conditional ub-tag cassettes under supervision of PSS.

### Conflict of interest

The authors declare that they have no conflict of interest.

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## Figure legends

### Figure 1 - Location of H2B ubiquitin and its role in H3K79 methylation.

A Nucleosome core particle in which H2BK123, H3K79 and H3K4 are indicated as well as the sites of linear ubiquitin attachment described below. Most of the unstructured N- and C-terminal histone tails are absent from this structure; the histone ends fused to ubiquitin (see below) are indicated with dashed triangles.

B Dependence of H3K79me levels on Dot1 activity, modified from [19]. Dashed lines indicate approximate H3K79me levels in WT or *bre1Δ* cells. Arrow indicates the direction of the changes expected upon positive histone crosstalk.

### Figure 2 - Ubiquitin on the H2A N-terminus promotes H3K79 methylation in vivo and in vitro.

A Cartoon of H2A/H2B in the used strains.

B Immunoblot analysis shows tagged H2A and the absence of endogenous wild-type H2A. Asterisk indicates non-specific signal. 'HA indicates ubHA-H2A proteins that lost the ub moiety due to the activity of ubiquitin-specific proteases.

C MS analysis of H3K79 methylation levels in strains shown in panel A and B (mean and individual data points of two biological replicates). See also Supplementary Figure S1F.

D ChIP-qPCR data of the different methylation states at three loci. Values for each methyl antibody were normalized to H3, after which all values were normalized per methyl antibody to wildtype at the *HML* locus. Primer sets against *HML*, the promoter of the *GAL1* gene and the coding sequence of *SPA2* were used. Mean +/- SD of three biological replicates is shown.

E Immunoblot analysis of the in vitro methyltransferase activity of purified 10xHis-tagged yeast or human Dot1 protein towards chromatin templates isolated from yeast strains lacking Dot1 and Bre1 and expressing wild-type H2A or N-terminal fusions of H2A. See also Supplementary Figure S1H.

### Figure 3 - Ubiquitins tethered to the C-terminus of H2A and H2B can modestly promote H3K79 methylation in vivo.

A Cartoon of H2A/H2B in the used strains.

B Immunoblot analysis of tagged and untagged histones. Asterisk indicates non-specific signal.

C MS analysis of H3K79 methylation levels in strains expressing H2A or H2B C-terminal ub fusions (mean and individual data points of two biological replicates). See also Supplementary Figure S2C.

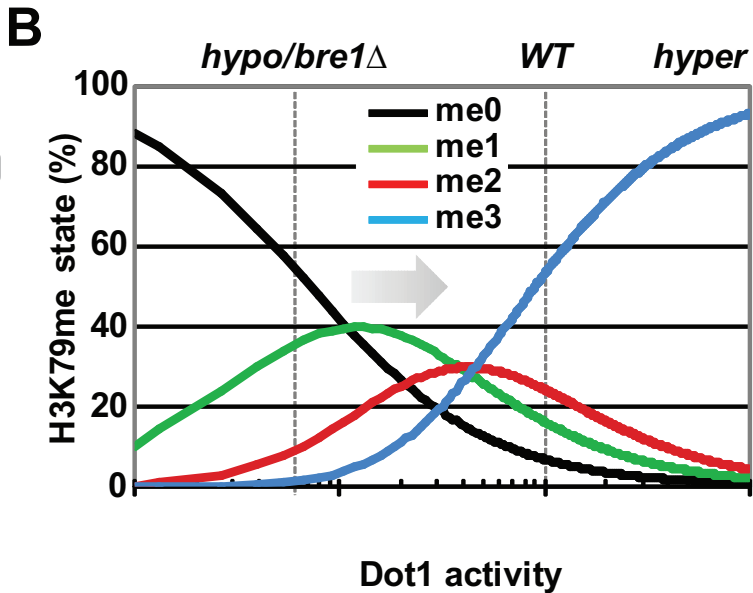
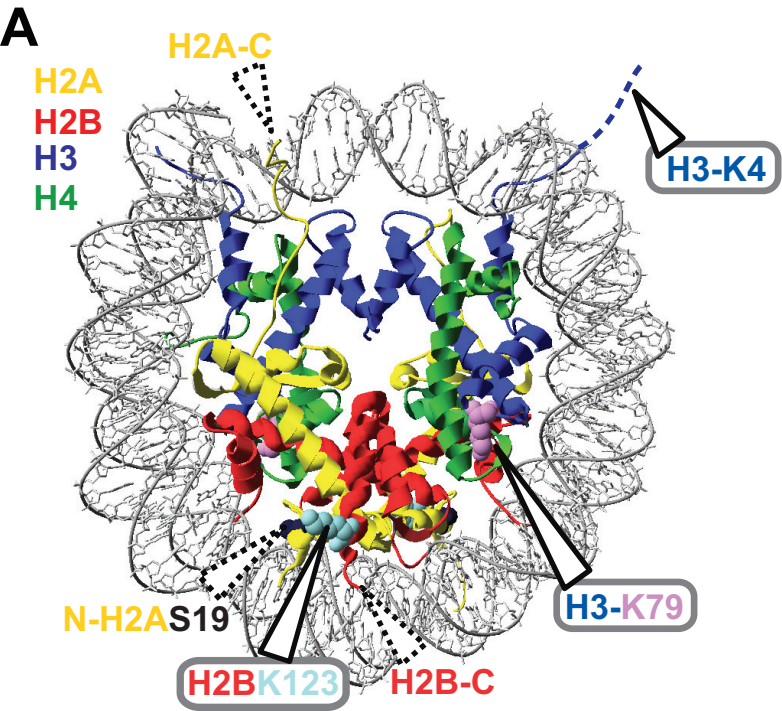
### Figure 4 - Tethered ubiquitins can promote H3K4 methylation and DNA damage checkpoint activation.

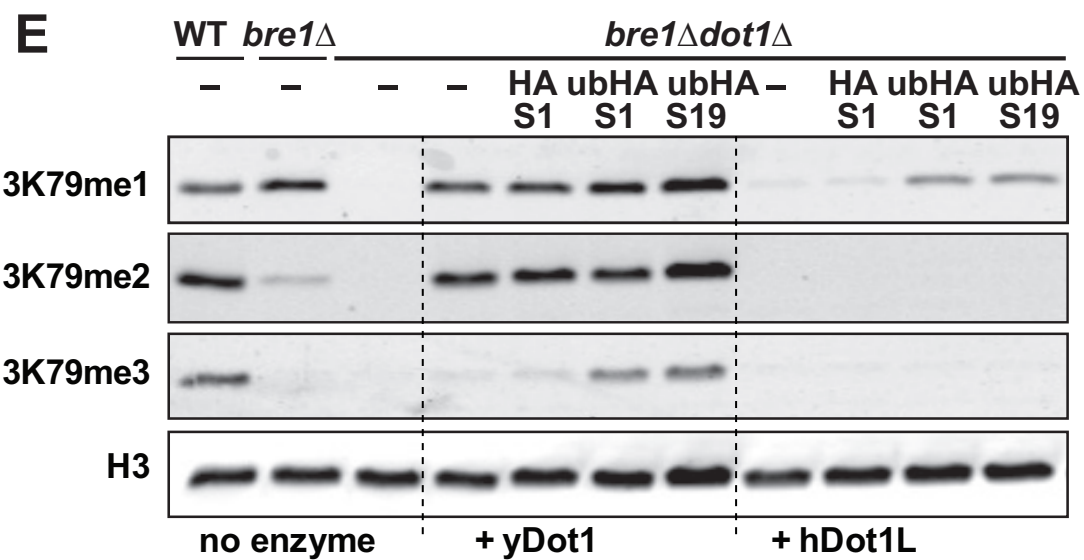
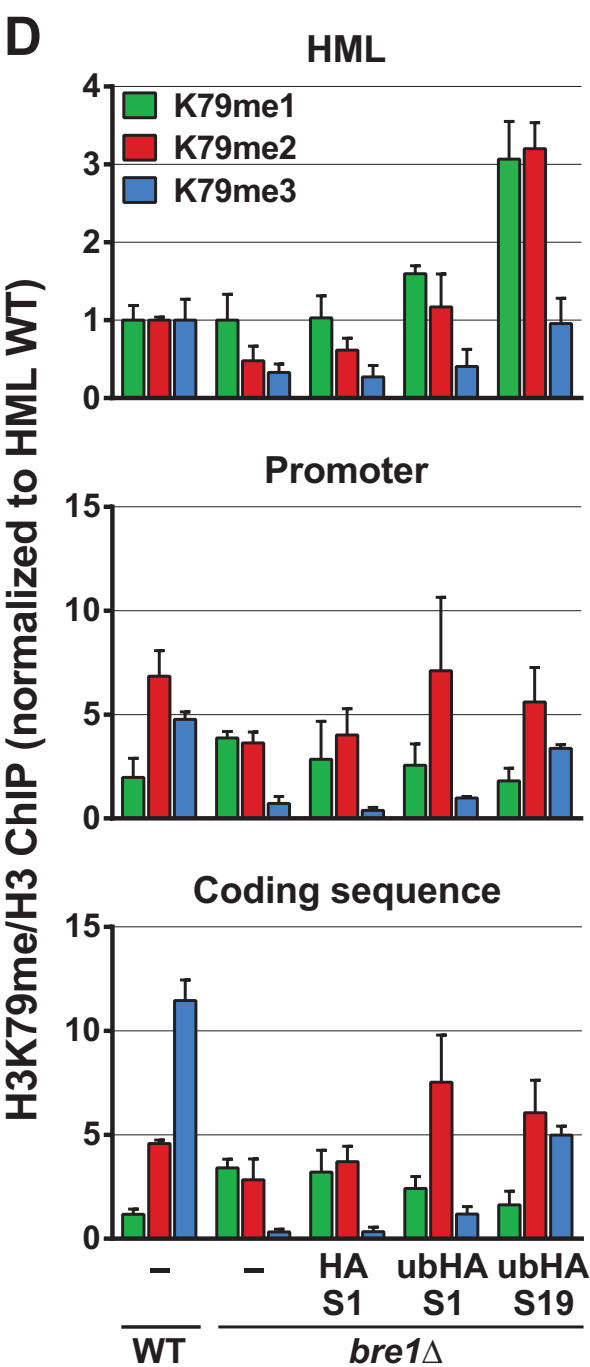
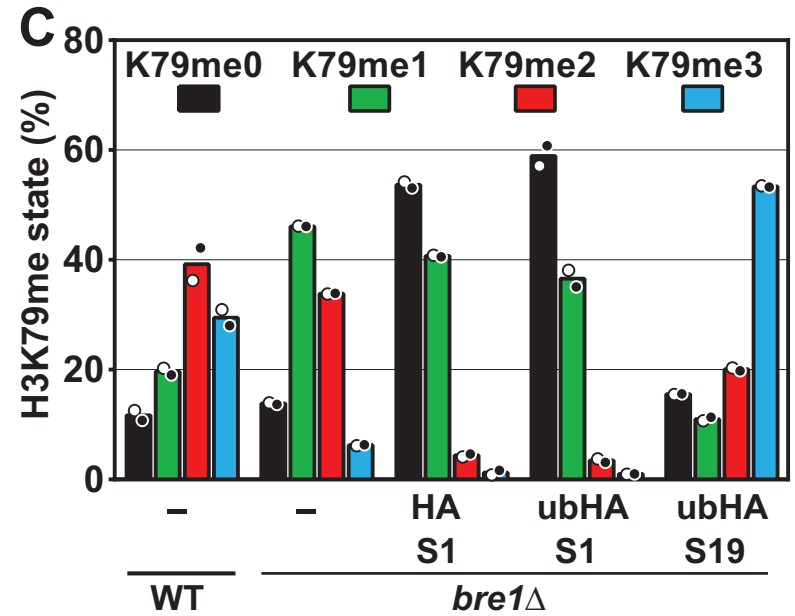
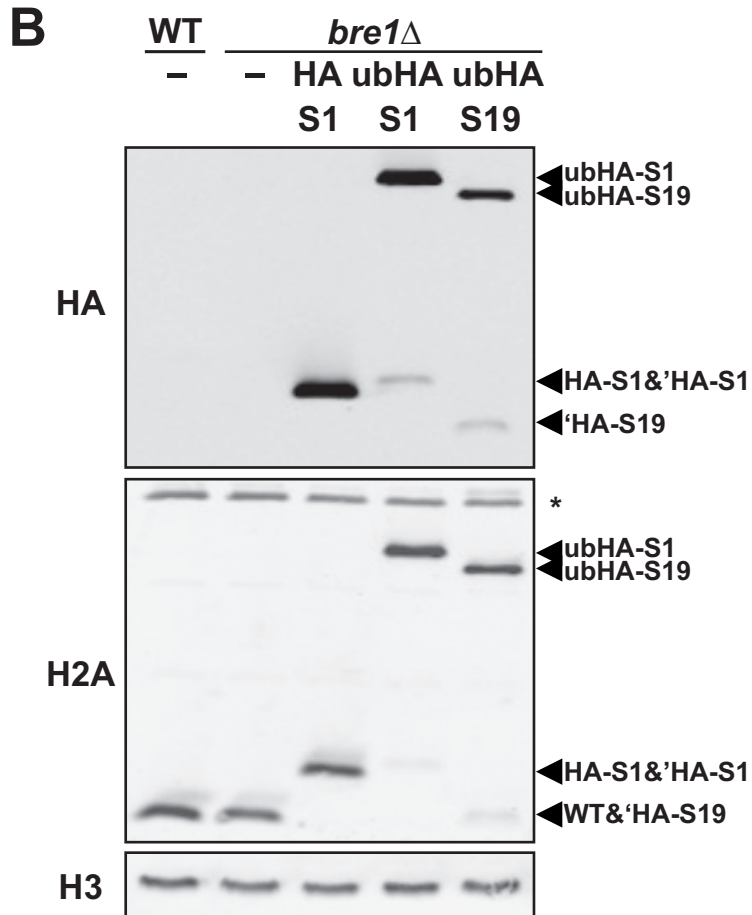
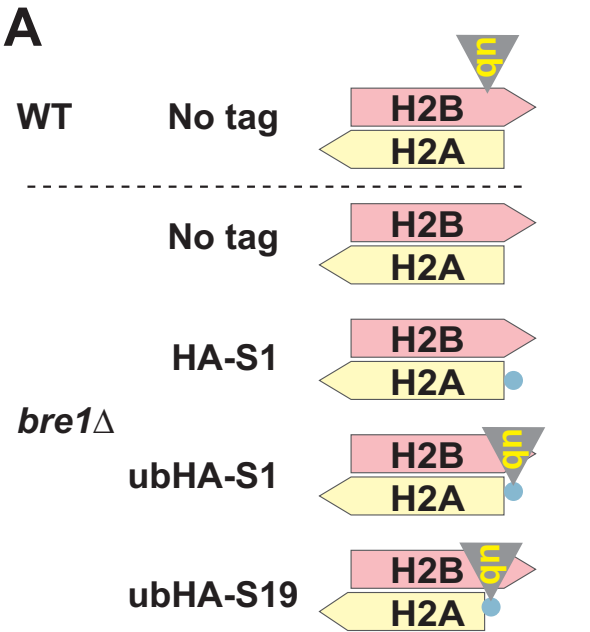
A Immunoblot analysis of H3K4 methylation in strains expressing various histone-ubiquitin fusions.

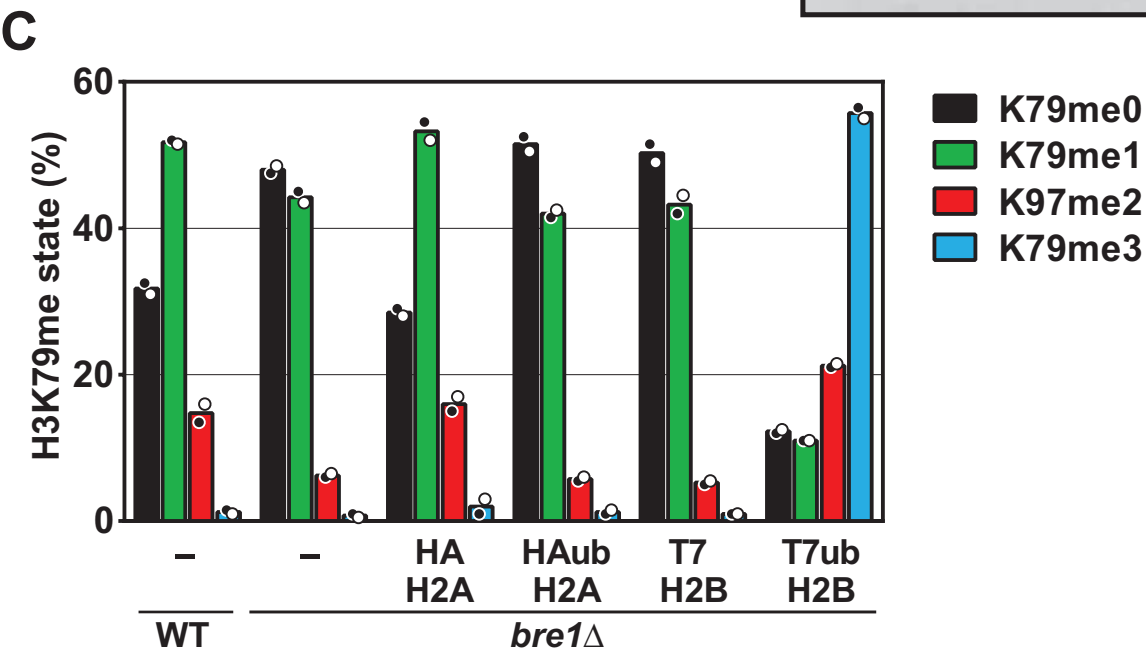
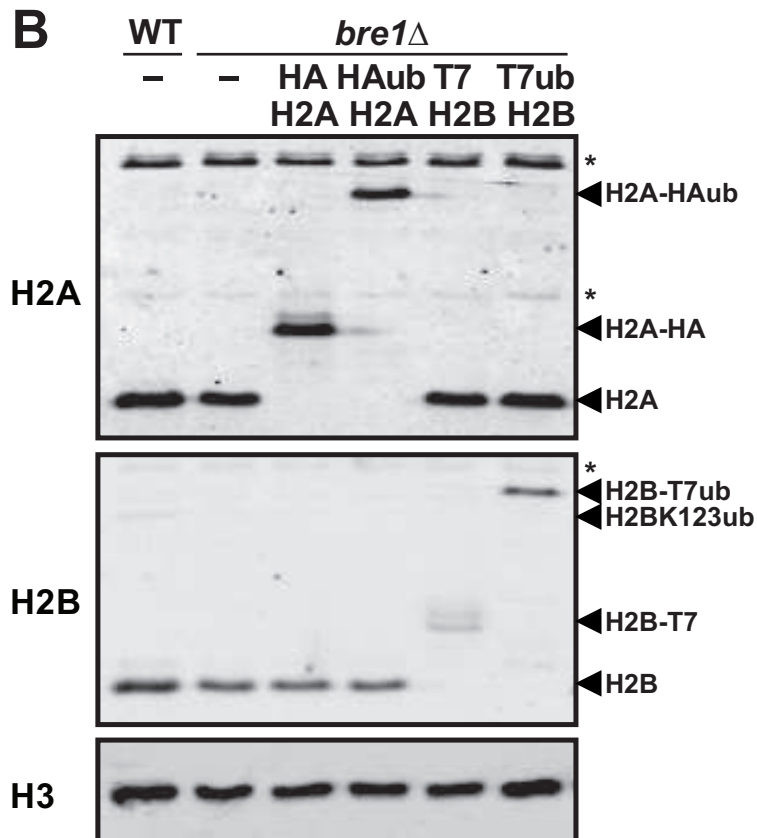
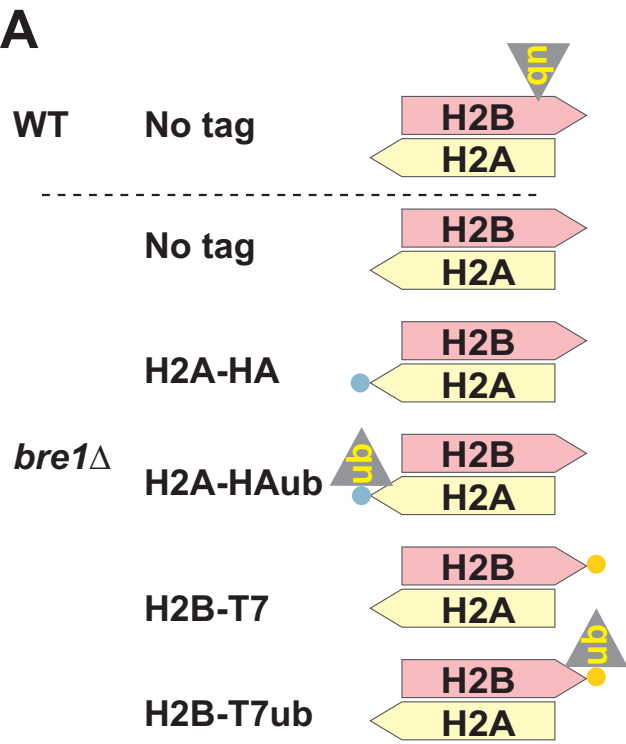
B Immunoblot analysis of activation of the central DNA damage checkpoint kinase Rad53 upon exposure of yeast cells arrested in G1/S to 100 J m<sup>-2</sup> UV irradiation. Rad53 is activated by phosphorylation, which leads to a mobility shift.

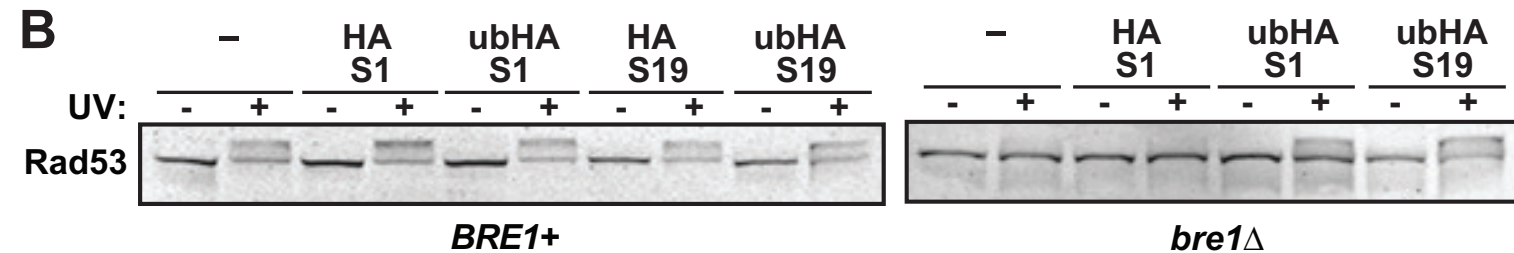
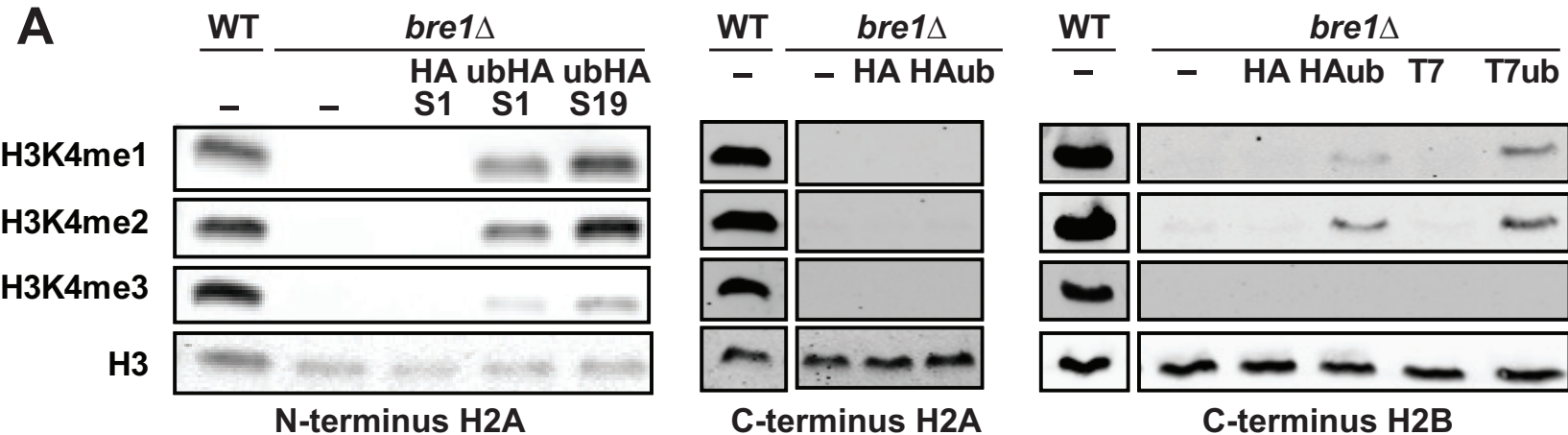
### Figure 5 - Schematic representation of a crash barrier model for the role of H2Bub in promoting histone methylation.

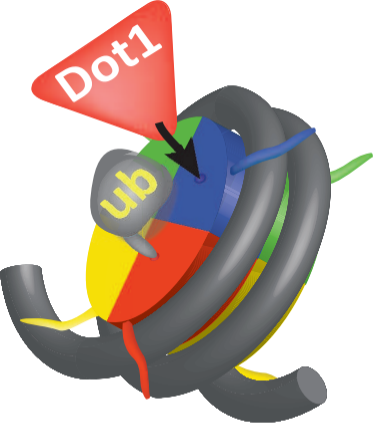
Ubiquitin, if in the correct orientation, can interact with the nucleosome and coach Dot1 towards H3K79, thereby increasing Dot1's chances of a productive encounter. See text.



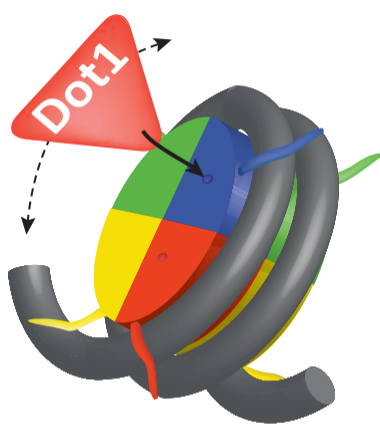








**H2BK123ub**



**No ubiquitin**



**ub-H2A fusion**