Function and regulation of the histone methyltransferase Dot1
Frederiks, F.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER 3

Non-processive methylation by Dot1 leads to functional redundancy of histone H3K79 methylation states

Floor Frederiks¹, Manuel Tzouros³, Gideon Oudgenoeg³, Tibor van Welsem¹, Maarten Fornerod², Jeroen Krijgsveld³ and Fred van Leeuwen¹

¹ Divisions of Cellular Biochemistry and ² Tumor Biology, Netherlands Cancer Institute, Amsterdam, 1066 CX, The Netherlands
³ Department of Biomolecular Mass Spectrometry, Faculty of Science, Utrecht University, Utrecht, 3584 CA, The Netherlands

ABSTRACT

While mono-, di- and trimethylation states of lysines on histone proteins typically have specific functions in chromatin, no specific functions have been attributed so far to the different methylation states of histone H3K79 generated by Dot1. Here we determined that Dot1, in contrast to other known histone methyltransferases, introduces multiple methyl groups on lysines via a non-processive mechanism. The kinetic mechanism implies that the H3K79 methylation states cannot be generated independently, suggesting functional redundancy. Indeed, gene silencing in yeast, which is dependent on Dot1, depended on global H3K79 methylation levels and not on one specific methylation state. Furthermore, we found that the previously observed critical role of histone H2B ubiquitination in H3K79 trimethylation can be explained by reduced synthesis of all H3K79 methylation states. Our results suggest that non-processive multiple methylation of H3K79 leads to a binary code, which is expected to limit the possibilities for regulation by putative demethylases or binding proteins.
INTRODUCTION

Lysine methylation of histone proteins affects various aspects of chromosome biology such as silencing, transcription, DNA recombination, and response to DNA damage (3). A single lysine residue can be mono-, di- or trimethylated and several lines of evidence suggest that different methylation states can have distinct biological functions. For example, the different methylation states of several lysines often show distinct distribution patterns in a gene-specific or genome-wide manner (4). These patterns are generated by SET-domain methyltransferases that can specifically introduce one, two, or three methyl groups on specific lysine residues (7). The methylation patterns can subsequently be modified by histone demethylases that can remove one or two methyl groups (8;9). Finally, the effects of histone methylation are often mediated by proteins that preferentially bind to one or two methylated states of a certain lysine residue (10).

All known histone lysine methyltransferases contain a SET domain, with the exception of Dot1, which has a conserved catalytic core similar to arginine methyltransferases (2;11). Dot1 is conserved from trypanosomes to humans and catalyzes mono-, di-, and trimethylation of histone H3K79, a residue located on the nucleosome core (1). In yeast, flies and mammals, Dot1 has been shown to affect gene expression. In mammals Dot1 is involved in activation of Hox genes in certain types of leukemia (12), as well as Ras-induced gene silencing (13) and aldosterone-induced gene repression (14). In budding yeast Dot1 is critical for Sir protein-mediated heterochromatic gene silencing, the DNA damage response, and the pachytene checkpoint in meiosis (1;15-20).

The functions of the different methylation states of histone H3K79 are currently unknown. In contrast to other known methylated lysines of histones, all methylated states of H3K79 seem to localize similarly along the length of a gene, suggesting that their functions might overlap (21). In addition, there are no indications that H3K79 methylation is reversible (22-24) and no protein has been identified that unambiguously binds specifically to a methylated state of H3K79 (25;26). Interestingly, efficient di- and trimethylation of H3K79 in yeast requires monoubiquitination of histone H2B on K123 (H2BK123ub1) by the Rad6/Bre1 complex (21;27-29). Similarly, in humans, dimethylation of H3K79 is stimulated by ubiquitination of H2BK120 (30). It has been suggested that the ubiquitin moiety on H2B might interact with the catalytic site of Dot1 and thereby influence the processivity of the methylation reaction (21). However, based on the crystal structure of yeast Dot1 (2), and its comparison with that of human Dot1 (11), it
Dot1 methylates H3K79 using a distributive mechanism

has been proposed that a processive mechanism is incompatible with the structure of the active site of Dot1 (2). All known (SET-domain) histone lysine methyltransferases of which the kinetic mechanism has been examined are processive in vitro, with one possible exception (see Discussion). Arginine protein methyltransferases, which, like Dot1, contain a class-I methyltransferase domain, also function as processive enzymes, probably because the active enzymes are homo-dimers that can sequentially add two methyl groups to the substrate arginine (7). Here we show that Dot1, in contrast to other known protein lysine methyltransferases, is a non-processive methyltransferase in vitro and in vivo, and that this kinetic mechanism has direct consequences for the regulation and function of the different methylation states of histone H3K79.

RESULTS

Dot1 is a distributive enzyme

To understand the function and regulation of the different methylation states of H3K79, we first investigated the kinetic mechanism of methylation by Dot1. Multiple methylation of a single lysine can occur through two mechanisms. Processive enzymes perform consecutive rounds of methylation without substrate dissociation. Distributive or 'hit-and-run' enzymes dissociate and re-associate to allow exchange of co-factor and rotation of the substrate lysine to achieve multiple rounds of methylation. In wild-type yeast cells ~90% of H3K79 is methylated by Dot1 and consists of ~22% K79me1, 26% K79me2, and 44% K79me3 (1;21). Such a complex methylation pattern (generated by single enzyme) suggests that Dot1 is either a distributive enzyme, a processive enzyme which transfers methyl groups very slowly, or a processive enzyme of which the degree of processivity is regulated by assembly of the enzyme into a putative higher-order complex, as has been found for SET-domain methyltransferases (31-34).

We first analyzed the kinetic mechanism of H3K79 methylation in vitro using recombinant Dot1 purified from bacteria. Since Dot1 is not active on free histone H3 (1;2;6;11;15), chromatin isolated from a yeast strain lacking endogenous Dot1 was used as substrate. The H3K79 methylation levels of reactions with different amounts of Dot1 were determined by immunoblot analysis (Fig. 1A) and mass spectrometry (Fig. 1B). When the incubation time is kept constant, two possible outcomes can be predicted. First, different amounts of a processive enzyme are expected to produce different amounts of only one
methylation state (in the absence of putative associating factors) or to produce patterns of methylation of which the relative abundance of the different methylation states is invariant. Second, a distributive enzyme is expected to produce all three methylation states but to monomethylate multiple lysines before it re-associates with monomethylated lysines to introduce a second methyl group. Similarly, trimethylation is expected to be preceded by accumulation of
Dot1 methylates H3K79 using a distributive mechanism. Therefore, the relative abundance of the three methylation states is expected to depend on the amount of a distributive enzyme (see below). In our in vitro assay, at low concentrations of recombinant Dot1, only H3K79me1 was found (Figs. 1A-B). When the concentration of Dot1 was increased, we observed first accumulation of H3K79me2 and then accumulation of H3K79me3 (Figs. 1A-B). Although the total amount of methylation achieved under the in vitro conditions was not as high as in vivo, the production of all three methylation states, the order of appearance of the methylation states, and the change in relative abundance strongly suggested that Dot1 was distributive. We incubated chromatin from a wild-type strain and a dot1Δ strain without recombinant Dot1 to confirm the lack of H3K79 demethylase activities and Dot1-independent methyltransferase activities in the assay (Fig. 1C).

Under in vitro conditions Dot1 might lack factors or (self) interactions necessary for normal activity. Therefore, we next examined the kinetic mechanism of Dot1 in vivo by replacement of the endogenous DOT1 gene by a tagged version of DOT1 expressed from a galactose-inducible promoter. The expression level of Dot1 was controlled by altering the ratio of galactose/glucose in the media (Fig. 2A), similar to previously described protocols (35). When the amount of enzyme becomes limiting, a processive enzyme is expected to methylate fewer histones but carry out its same processive cycles on the few lysines that it does methylate. In contrast, when a distributive enzyme is present in limiting amounts, it is expected to produce less of the higher methylation states and more of the lower methylation states. The methylation levels under steady state conditions were determined by immunoblot analysis and mass spectrometry (Figs. 2A-B). At very low levels of Dot1, mainly H3K79me1 was found, at intermediate Dot1 levels mainly H3K79me1 and -me2 were found, and at higher levels all H3K79 methylation states were present, like in wild-type cells. These results are inconsistent with a processive mode of action and suggest that Dot1 is a distributive enzyme in vivo. Also, the in vivo behavior of Dot1 resembles the activity in vitro (Fig. 1). We next compared the in vivo experimental data (Fig. 2C) to a theoretical distribution of methylation states (Fig. 2D). In this simplest version of a distributive model, all methylation events on H3K79 are equally probable and independent of each other. Surprisingly, considering the simplicity of this model, the observed change in methylation states across the different Dot1 expression levels resembled the theoretical model. In particular the decline of H3K79me0, the increase in H3K79me3, and the occurrence of local maxima in H3K79me1 and -me2 with increasing methylation probability take place in a similar way. This further strengthens the idea that Dot1 acts in a "methylate-and-
run” rather than a multi-step processive fashion. A notable deviation from the simplest distributive model is that H3K79me2 and me3 accumulate faster than predicted in vivo as well as in vitro (Figs. 1 and 2). This could be explained by assuming that the later methylation steps proceed more efficiently. It will be interesting to determine the binding affinities of Dot1 for the different H3K79 methylation states and to determine the ability of Dot1 to associate, dissociate,
and re-associate with its substrates. These studies are complicated, however, by the fact that Dot1 shows no activity towards peptide substrates (1;15;16). Dot1 methylates histone H3 in the context of the nucleosome (1;15;16) and binding to different sites of this protein-DNA complex is required for full activity (2;6;11;36).

Another deviation is that at the highest Dot1 expression levels, the unmethylated fraction (me0) remained substantial (~10%, see Fig. 2B). This deviation most likely represents regions of the genome that are refractory to methylation by Dot1, such as the silent chromatin compartment repressed by the Sir complex (estimated to be ~1%) where Sir3 and Dot1 have been suggested to compete for the same binding site on a basic patch on the N-terminal tail of histone H4 (6;36). Since this part of H4 interacts with several other chromatin proteins (6;36), it is possible that chromatin proteins other than Sir3 affect Dot1 activity outside the Sir-domains. Together, our results identify Dot1 as the first known distributive protein lysine methyltransferase in vivo.

A Dot1 mutant with compromised methyltransferase activity

The distributive mechanism of multiple methylation suggests that the different H3K79 methylation states cannot be generated independently. For example, the lower methylation states are obligatory transient intermediates in the synthesis of higher methylation states. Therefore, we hypothesized that the different methylation states of H3K79 might show functional overlap and thus lack specific functions. To test this hypothesis, we investigated the functional consequences of altered methylation spectra. We did not use the inducible GAL1 promoter because carbon sources in the media are known to influence many phenotypes, including gene silencing (37). As an additional means of manipulating the activity of the Dot1 enzyme, we searched for mutants of Dot1 with altered catalytic activity.

Glycine 401 (G401) is a highly conserved residue in the Dot1 active site and is involved in binding of the methyl donor S-adenosyl-methionine (AdoMet) (2;11). Mutation of the equivalent glycine in the arginine methyltransferase Hmt1 to arginine or alanine has been shown to result in complete or partial loss of activity, respectively (38). We previously showed that the G401R mutant of Dot1 is inactive in vitro and that G401R and G401A mutants have lost the Dot1 silencing functions when expressed from a plasmid in vivo (1). However, when the Dot1-G401A mutant was re-examined, we found that it retained partial catalytic activity in vitro, in contrast to the G401R mutant, which was completely inactive (Fig. 3A). Dot1-G401A exhibited greatly reduced cross-linking to the cofactor AdoMet (Fig. 3B and Fig. S3), suggesting that the reduced catalysis of Dot1-G401A was caused
Figure 3. Dot1-G401A: an active site mutant with reduced catalytic activity. (A) The \textit{in vitro} activity of recombinant Dot1, Dot1-G401A and Dot1-G401R towards chromatin from a \textit{dot1} strain (NKI3006) was analyzed by monitoring the incorporation of radio-labeled 3H-S-adenosylmethionine. Reaction mixtures were separated by SDS-PAGE and 3H-labeled methylated histone H3 was detected using autoradiography (1). The total level of histone H3 was analyzed by immunoblot and the amount of recombinant Dot1 was assessed by Coomassie Blue staining. (B) Binding of AdoMet to Dot1 and Dot1-G401A was determined by UV cross-linking followed by SDS-PAGE and transfer to PVDF membrane by electro blotting. 3H-AdoMet cross-linked to Dot1 was detected by autoradiography (1). Dot1 was detected by immunoblot analysis. (C) Dot1 expression and H3K79 methylation were compared in strains expressing Dot1 or Dot-G401A from the endogenous locus or from a single-copy plasmid. A single-copy plasmid without insert (pRS315) was transformed into a wild type (NKI3023), a \textit{dot1.Δ} (NKI3024) and a \textit{dot1-G401A} (NKI3025) strain. Single copy plasmids pDOT1 and pdot1-G401A were transformed into a \textit{dot1.Δ} strain (NKI3024). All strains were grown under equal conditions in selective synthetic media lacking leucine. Whole-cell extracts of log-phase cultures were analyzed by immunoblot. The asterisk indicates a non-specific band recognized by the H3K79me2 antibody.
Dot1 methylates H3K79 using a distributive mechanism by impaired AdoMet binding. The Dot1-G401A mutant was reintroduced into yeast by replacement of the wild type chromosomal DOT1 gene by homologous recombination to express the mutant from the endogenous DOT1 promoter and at the normal genomic location to ensure equal expression levels. When the H3K79 methylation levels were compared to wild type Dot1 on immunoblots (Fig. 3C) and by mass spectrometry (Fig. 3D), the Dot1-G401A mutant showed undetectable H3K79me3, partial loss of H3K79me2, and increased H3K79me1. The levels of H3K79 methylation were further modified by expression of wild-type Dot1 and Dot1-G401A from the endogenous DOT1 promoter on a single-copy plasmid in a dot1Δ strain, which resulted in lower expression levels of Dot1 when compared to the endogenous genes (Fig. 3C) and thereby reduced methylation levels (Figs. 3C-D). The changes in relative methylation levels observed under the different conditions confirmed the distributive methylation mechanism of Dot1. The G401A mutant did not have a specific defect in trimethylation because normal methylation levels could be restored by expressing this mutant at very high levels (see below). The mutation in the AdoMet-binding site did not affect binding to chromatin because the binding of Dot1 and Dot1-G401A to the coding sequences of the ACT1 and GAL1 genes was indistinguishable (Fig. S4). Thus the Dot1-G401A mutant provides a genetic tool to compare phenotypes of strains with different H3K79 methylation levels but grown under the same conditions.

**Functional redundancy of the H3K79 methylation states in silencing**

We and others have previously suggested that one function of the abundant methylated H3K79 in euchromatin is to restrict the limiting Sir2/3/4 silencing proteins to regions of silent chromatin, by preventing non-specific binding to euchromatin (1;18). The disruption of telomeric silencing in dot1Δ cells or cells carrying a mutation in H3K79 is probably caused by loss of this targeting mechanism (39). To determine the role of the degree of H3K79 methylation in gene silencing, silencing of a telomeric URA3 reporter gene was investigated by a quantitative growth assay (Fig. 4A). The degree of silencing in the strains described in Fig. 3C-D was correlated to the methylation state of H3K79 as determined by mass spectrometry (Fig. 3D). In summary, there was no correlation

(D) The relative levels of H3K79me0, -me1, -me2 and -me3 were measured of the following strains: wild type (BY4742), dot1Δ (NKi3010), dot1-G401A (NKi3010), dot1Δ (NKi3024) transformed with pDOT1 or pdot1-G401A. The strains are isogenic or nearly isogenic to the strains described in panel 3C. All strains were grown on glucose media at 30°C and H3K79 methylation was analyzed using mass spectrometry as described in Fig. 2B. Each experiment was performed in duplicate and error bars represent the spread of the data.
between the level of any of the specific methylation states and the degree of telomeric silencing, which is illustrated by the following examples. First, H3K79me1 levels were very similar in strains expressing plasmid-encoded \textit{DOT1} and plasmid-encoded \textit{dot1-G401A}, while silencing was nearly completely lost in the latter strain. Second, H3K79me2 levels were twice as high in strains
expressing plasmid-encoded \textit{DOT1} when compared to the endogenous \textit{dot1-G401A} strain, while silencing was nearly identical. In addition, H3K79me2 levels in strains expressing endogenous \textit{DOT1} and plasmid-encoded \textit{DOT1} were similar while silencing was lower in the latter strain. Third, H3K79me3 is absent in endogenous \textit{dot1-G401A} strains and present at high levels in strains expressing \textit{DOT1} from a plasmid, while the two strains showed very similar silencing properties. These results show that silencing did not correlate with any of the specific methylated states of H3K79. However, when the sum of all methylation states was plotted against the degree of silencing of the \textit{URA3} reporter gene, a strong positive correlation between the overall level of methylation and gene silencing was observed (Fig. 4B), confirming that all methylated states contributed to silencing. These observations support the idea that the non-processive mode of modification by Dot1 leads to different methylation states that have redundant functions.

While silencing positively correlates with the overall degree of H3K79 methylation, when Dot1 is overexpressed at very high levels (Fig. 4C), silencing is disrupted (Fig. S5 and [1]). At these high expression levels, Dot1 methylates 99\% of histone H3 in euchromatin and silent chromatin (Fig. 4D and data not shown), suggesting that Sir3, which competes with Dot1 for binding to the N-terminal tail of histone H4 (6;36), is unable to prevent H3K79 methylation in regions of silent chromatin. These results show that Dot1 activity is limiting in the cell and that a decrease as well as an increase in Dot1 activity can lead to changes in gene silencing. Overexpression of Dot1-G401A resulted in normal silencing (Fig. S5) and levels of H3K79 methylation similar to wild-type strains expressing endogenous levels of Dot1 (Fig. 4D), suggesting that this catalytically compromised enzyme was unable to methylate H3K79 in silent chromatin. The observation that lack of H3K79me3 by this mutant (Figs. 3C-D) was restored by overexpression (Fig. 4D), shows that Dot1-G401A does not have an intrinsic trimethylation defect and is consistent with a distributive mechanism of methylation by Dot1.

**H3K79me3 is not required for DNA damage checkpoint activation**

Besides its role in gene expression, Dot1 has been shown to be required for activation of the DNA damage checkpoint upon exposure to UV irradiation (19;20). Strains lacking Dot1 show slow activation of the central checkpoint kinase Rad53 (19;20). This checkpoint function of Dot1 is mediated by H3K79 methylation because strains harboring a mutation in H3K79 also show defects in
activation of Rad53 (19;20). To determine the role of the degree of H3K79 methylation in DNA damage checkpoint activation we examined phosphorylation of Rad53 after re-introduction of plasmids expressing Dot1 or Dot1-G401A into a dot1Δ strain of the W303 background, in which the checkpoint defect of dot1Δ is more apparent than in the S288C background that we used in our other studies (Chapter 7). As expected, Rad53 phosphorylation was undetectable in dot1Δ strains carrying the empty vector and restored by introduction of wild-type Dot1 (Fig. 5). Rad53 phosphorylation was restored albeit partially in the strain expressing Dot1-G401A (Fig. 5). Since strains expressing this mutant protein completely lack detectable H3K79me3 (Fig. 3D), we conclude that H3K79me3 might partially contribute to DNA damage checkpoint activation but the other methylation states of H3K79 are also involved. This is in agreement with observations that overexpression of Dot1, which leads to increased levels of H3K79me3 and reduced levels of H3K79me1 and me2 (Fig. 4D), does not affect Rad53 phosphorylation (19). Together, these results indicate that the overall level of methylation rather than the level of one specific methylation state determines the efficiency of DNA damage checkpoint activation. Due to the nature of the assays used, we have not been able to verify this in a more quantitative manner.

**Regulation of multiple methylation by H2B ubiquitination**

Thus far, our results show that the different methylation states of H3K79 show functional overlap, and that H3K79me3 is not required for gene silencing and DNA damage checkpoint activation as long as sufficient amounts of H3K79me1 and me2 are present. We propose that the functional overlap is a consequence of the non-processive mechanism of methylation. However, the lack of specific functions

![Figure 5. Trimethylation of histone H3K79 is not required for DNA damage signaling.](image-url)

Activation of the central checkpoint kinase Rad53 after irradiation of cells arrested in G1/S with 100 J/m² UV was analyzed in a dot1Δ strain (UCC7014) transformed with single-copy plasmid pDOT1, empty vector (pRS315) or single-copy plasmid pdot1-G401A. Rad53 is activated by phosphorylation, which is visible as an upward shift of the protein on immunoblot. Whole-cell extracts from cultures arrested in G1/S were analyzed using antibodies against Rad53 and Dot1. Antibodies against Pgk1 were used as a loading control.
Dot1 methylates H3K79 using a distributive mechanism

of H3K79me3 was unexpected because H3K79me3 is the most abundant methylation state of H3K79 and is dependent on monoubiquitination of lysine 123 of histone H2B (H2BK123ub1) by the E2/E3 complex Rad6/Bre1, suggesting some form of selective regulation of the higher methylation state (21). Indeed, strains lacking H2BK123 ubiquitination due to deletion of BRE1 or mutation of H2BK123 showed a strong reduction in H3K79me3 and an increase in H3K79me1 and unmethylated H3K79 (Fig. 6A and [21]). How might Bre1 affect multiple methylation of H3K79? It has been suggested that the ubiquitin moiety on H2B might interact with a putative ubiquitin-binding domain at the N-terminus of Dot1 (2) and thereby influence the processivity of the methylation reaction (21). However, our data show that methylation of H3K79 does not occur in a processive manner and offer an alternative explanation. A shift towards lower and unmethylated states of H3K79, as seen in strains lacking monoubiquitination of H2B (Fig. 6A) was also found when the general activity of Dot1 was reduced (Figs. 1-3). Therefore, we investigated the possibility that H2B ubiquitination might not specifically affect trimethylation but might rather affect the general activity of the distributive Dot1 enzyme. To verify this model we investigated the role of BRE1 in strains expressing plasmid-encoded Dot1-G401A, which already lack H3K79me3 but show reduced H3K79me2 and increased H3K79me1 (Fig. 3D). Deletion of BRE1 in this strain led to a loss of H3K79me1 and H3K79me2 (Fig. 6B), showing that Bre1 does not specifically regulate H3K79me3 but instead enhances overall catalysis of H3K79 methylation.

H2B ubiquitination and deubiquitination are in dynamic equilibrium and the steady state levels of H2BK123ub1 (~10%) are much lower than the levels of H3K79 methylation (~90%) in vivo. It has been proposed that H3K79 methylation might be enhanced when H2B is transiently ubiquitinated within chromatin (27). The mechanism by which H2B ubiquitination affects catalysis of Dot1 remains unknown. One possibility is that the ubiquitin moiety directly affects the active site of Dot1 (21). However, the aggravation of the methylation defect of active-site mutant Dot1-G401A by deletion of BRE1 suggested the possibility that Bre1 might act a different level. Another possibility is that H2B ubiquitination affects the in vivo chromatin substrate such that H3K79 on the nucleosome core or the basic patch on the N-terminal tail of histone H4, which might both be inaccessible in the stacked nucleosomes of chromatin fibres (40), become more accessible for interaction with Dot1 (41). Ubiquitinated H2B might lead to a reconfigured chromatin structure through the action of the proteasomal ATPases Rpt4 and Rpt6, which bind to ubiquitinated H2B and are required for the effect of this
Figure 6. H2B ubiquitination regulates all H3K79 methylation states and does not act via the putative ubiquitin-binding domain of Dot1. (A) The relative levels of H3K79me0, -me1, -me2, and -me3 in an htb1-K123R strain (NKI3028) and a bre1Δ strain (UCC7370) were measured using mass spectrometry as described in Fig. 2B. (B) The effect of deletion of BRE1 on H3K79 methylation by Dot1 and Dot1-G401A in vivo was analyzed by immunoblot using antibodies against H3K79me1, -me2, -me3, and total histone H3. A dot1Δ (UCC7183) and a dot1Δ bre1Δ strain (UCC7371) were transformed with an empty vector (-) or single-copy vectors pDOT1 (wt) or pdot1-G401A (A). Protein extracts were isolated from cells exponentially growing in synthetic media lacking leucine. (C) The activity of recombinant Dot1, Dot1-G401A, and Dot1-G401R towards histone H3 in chromatin from a dot1Δ strain (UCC7183) and a dot1Δ bre1Δ strain (UCC7371) was analyzed by in vitro methylation assays followed by immunoblot analysis. Wild-type strain UCC7164, incubated without enzyme, was used as a reference for endogenous H3K79 methylation levels. An antibody against H3K4me2 was used as a control for the absence of BRE1 and an antibody against total histone H3 was used as a loading control.
modification on multiple methylation of H3K79 (41). We favor this latter model for two reasons. First, when short chromatin fragments isolated from a dot1Δ strain were used as substrate, recombinant Dot1 carried out di- and trimethylation of H3K79 in vitro (Figs. 1A and 6C). The level of H2BK123ub1 in these chromatin substrates is unknown but we expect it to be low or absent. To exclude any effects of residual H2B ubiquitination, chromatin was isolated from a dot1Δbre1Δ strain. In vitro H3K79 methylation of chromatin from a dot1Δbre1Δ strain was indistinguishable from that of a dot1Δ BRE1 strain (Fig. 6C). These results show that Dot1 has the intrinsic ability to carry out mono-, di-, and trimethylation and that H2BK123 ubiquitination is not required for multiple methylation by Dot1 under these in vitro conditions where short chromatin fragments are used a substrate. Second, when a series of deletion mutants of the N-terminus of Dot1 were analyzed (Fig. 6D), we found that disruption of the putative ubiquitin-binding domain of Dot1 (Dot1Δ2-86) only weakly reduced H3K79 methylation and did not have a detectable effect on gene silencing (Figs. 6E-F). This is in sharp contrast to the loss of H3K79me3 and reduction in H3K79me2 found in bre1Δ strains, which lack H2B ubiquitination (Fig. 6A). When deletions were extended into the charged lysine-rich region of the N-terminus of Dot1, methylation was progressively lost (Fig. 6E). This was expected because this positively charged region of Dot1 and a similar region in human Dot1 are known to be involved in binding to DNA and nucleosomes (2;6;11). Based on these in vivo results we conclude that the putative ubiquitin-binding domain of Dot1 is not critical for H3K79 methylation, which suggests that the effect of H2BK123ub1 on multiple methylation of H3K79 is not mediated via this domain.

(D) Schematic representation of deletion mutants of Dot1. In addition to the conserved methyltransferase core (2), Dot1 contains a C-terminal acidic patch that interacts with the histone H4 tail (H4) (5;6), and an N-terminal domain that is required for nucleosome binding (2;6). The N-terminal domain contains a putative ubiquitin-binding domain (Ub?; 40-100) (2), a Lysine-rich domain (K-rich; 105-172) (2), and a short stretch that has sequence similarity to a part of human Dot1 that is required for activity of human Dot1 (cons; 87-144) (2;11). (E) Full length Dot1 and deletion mutants contained an N-terminal FLAG tag (f) and were expressed by the endogenous DOT1 promoter from a single-copy plasmid in a dot1Δ strain (UCC7183). Dot1 expression and H3K79 methylation were examined by immunoblot analysis. The asterisks indicate non-specific bands. Antibodies against Pgk1 and unmodified H3 were used as loading controls. (F) Telomeric silencing of the strains described in panel 6E was analyzed as described in Fig. 4A.
DISCUSSION

Dot1 is a distributive histone methyltransferase

SET-domain histone methyltransferases can transfer up to three methyl groups to a lysine substrate. The structures of SET-domain enzymes suggest that co-factor exchange can take place at the surface of the proteins without dissociation of the substrate, thereby allowing processive methylation (7). The degree of methylation that a SET-domain enzyme can achieve, however, is limited by the structure of the active site (7) and can be modulated by associating factors (31;32;34). The kinetic mechanism of multiple methylation of a number of unrelated SET-domain histone methyltransferases from different organisms has been studied in vitro. Most of these enzymes, such as murine G9a, Neurospora DIM-5, and pea LSMT were shown to carry out multiple methylation in a processive manner (42-46). In contrast, Drosophila and human H3K9 methyltransferases SU(VAR)3-9 and SUV39H1, respectively, have been suggested to be distributive based on in vitro studies on short peptides (47;48). However, the kinetic mechanism of these enzymes is still under debate (46) because human SUV39H1 was studied by radioactive assays without direct examination of multiple methylation states (48), and fly SU(VAR)3-9 enzyme seems compromised in vitro since it is not very active on nucleosomes (47). It has recently been argued that it is unlikely that enzymes catalyzing the same reaction with a very highly conserved structural motif would use different kinetic mechanisms (46). Thus it seems likely that most if not all SET-domain methyltransferases are processive in vivo. Protein arginine methyltransferases have also exclusively been reported to act as processive enzymes (7;49).

Dot1 is a unique histone lysine methyltransferase. It does not contain a SET domain but has a structure similar to protein arginine methyltransferases (2;11). The effect of H2B ubiquitination on H3K79me3 in vivo has led to the suggestion that Dot1 is a processive enzyme (21). This possibility was also suggested based on the crystal structure of human Dot1, in which the putative lysine-binding channel and the entry of the binding pocket for AdoMet are physically separated (11). However, based on the crystal structure of yeast Dot1 (2), and its comparison with that of human Dot1 (11), it has been proposed that a processive mechanism is incompatible with the structure of the active site of Dot1 because AdoMet and the reaction product S-adenosyl-homocysteine (AdoHcy) are buried in a deep pocket inside Dot1, suggesting that exchange of AdoHcy for AdoMet after each round of methylation requires a conformational change of the
active site and release of the substrate lysine (2). Our results are in agreement with the latter model. We now show that Dot1 methylates H3K79 in a distributive manner in vitro and in vivo, which makes this enzyme the first known example of a distributive protein lysine methyltransferase. Interestingly, this uncommon mechanism puts restrictions on the function, recognition, and regulation of H3K79 methylation.

**A link between synthesis and function of different methylation states of H3K79**

There is ample evidence that the different methylation states of lysines can have specific functions. However, the distributive mechanism of Dot1 suggests that H3K79me1 and -me2 are found in chromatin as obligatory intermediates in the synthesis of H3K79me3. This idea is supported by the observation that, in contrast to the different states of other methylated lysines, all three H3K79 methylation states co-localize across a gene (21;50). In addition, we found that while H3K79 methylation is essential, H3K79me3 was not required for activation of the DNA damage checkpoint in yeast. Furthermore, all methylation states of H3K79 are involved in telomeric silencing and none of the specific methylation states is unique. Rather, it is the sum of all the methylation states of H3K79 that determined the extent of silencing (Fig. 4B). Our results are in agreement with a recent study in which the N-terminal part of Sir3 was found to interact in vitro with peptides representing a region of histone H3 surrounding H3K79 (36). While the Sir3 fragment bound to the unmodified peptide, all three methylation states disrupted the binding of Sir3 (36). Whether the different H3K79 methylation states have specific roles in other processes such as transcription or the pachytene checkpoint remains to be determined.

Since the structures of the yeast and human Dot1 methyltransferase domains are very similar, H3K79 methylation in other organisms is likely to be carried out through a distributive mechanism, like in yeast. This suggests that the different methylation states of H3K79 in yeast as well as other organisms might have redundant roles. One exception might be the protozoan parasite *Trypanosoma brucei*, which expresses two Dot1-like proteins. DOT1A is responsible for genome-wide dimethylation of H3K76 (the *T. brucei* residue synonymous to H3K79), whereas DOT1B catalyzes only the conversion from di- to trimethylation (51). The two enzymes have distinct functions, suggesting that in a situation of two Dot1-like enzymes with different catalytic properties, the different methylation states may have non-redundant functions.
The kinetic mechanism of Dot1 has implications for regulation, recognition, and resetting of methylated H3K79

Methyl-binding proteins are usually specific for one or two methylation states of a certain lysine residue (10). Our results show that all three H3K79 methylation states are involved in telomeric silencing. Therefore, if H3K79 methylation affects silencing by recruitment of a methyl-H3K79 binding-protein, the protein must have the unusual property of binding to all methylation states but not to unmethylated H3K79. In addition, since Dot1 is a distributive enzyme, a protein that would bind to H3K79me1 and/or -me2 would also interfere with synthesis of H3K79me3. To date no protein has been identified that unambiguously binds specifically to a methylated state of H3K79 (15;16). Therefore, one possibility is that methylated H3K79 does not recruit proteins to regulate silencing but affects silencing directly. Our results are consistent with the model that H3K79 methylation acts as an anti-binding mark that prevents Sir-protein binding in euchromatin and thereby enhances targeting of the limiting Sir proteins to domains of silent chromatin.

Recently, histone demethylases have been identified that can alter the methylation state of lysines by removal of one or two methyl groups (8;9). Demethylases can reset specific methylation states established by processive SET domain containing methyltransferases. Thus far, there are no indications that H3K79 methylation is reversible (22), no demethylases have been identified for H3K79 (9), and none of the putative demethylases in yeast is known to affect H3K79 methylation (23;24). If silencing and possibly other functions of H3K79 methylation are modulated by demethylation, the demethylase must have the unusual ability to remove all three methyl groups to reset H3K79me3 to H3K79me0. In addition, because of the non-processive mode of synthesis, demethylation of lower methylation states of H3K79 would not only reverse lower states but also prevent new synthesis of higher methylation states, underscoring the interdependence of the methylation states. This raises the intriguing possibility that H3K79 methylation in yeast might be an irreversible histone modification.

The unusual kinetic mechanism of Dot1 not only affects how the H3K79 methyl marks are written and read but also how they are regulated. Ubiquitination of H2B is required for H3K79 trimethylation in yeast (21) and normal levels of H3K79 dimethylation in humans (30), which suggested that the higher methylation states might have specific functions. However, our results show that H3K79me3 in yeast is not specifically involved in gene silencing or DNA
Dot1 methylates H3K79 using a distributive mechanism. Furthermore, we now find that ubiquitination of H2B does not specifically regulate trimethylation of H3K79 but affects overall catalysis and thereby synthesis of all methylation states. Recently, loss of higher methylation states of H3K79 has also been observed when the interaction between yeast Dot1 and the N-terminal tail of histone H4 is disrupted (6;36) and in temperature-sensitive mutants of the Dot1-associating protein Cps35/Swd2, which is also a member of the Set1 complex COMPASS (52). The observed H3K79 methylation defects are consistent with a model in which these Dot1 interactions affect the general activity of Dot1.

What could be the biological role of multiple methylation of H3K79 if the different methylation states show functional overlap? Because of the distributive mechanism of methylation by Dot1, the degree of methylation of H3K79 might be an indicator of accessibility of H3K79 and thereby of overall chromatin structure. As such H3K79 methylation might be a memory mark of where H2B was transiently ubiquitinated and chromatin transiently remodeled (41). This might explain the observed biases in genomic localization of the different H3K79 methylation states (21;50). Alternatively, the number of methyl groups on lysines, which is expected to accumulate over time, might serve as a molecular clock, for example to measure time after deposition of newly synthesized and unmodified histones during DNA replication. However, the biological relevance of these putative and speculative functions remains unknown.

**MATERIALS AND METHODS**

**Yeast strains, plasmids and media**

Yeast strains and plasmids are described in Table 1. Silencing assays were performed using media containing 1 g/l 5-fluoroorotic acid and media were described before (1). To gradually increase DOT1 expression from the GAL1 promoter, the following combinations of carbon sources were used: galactose/glucose/raffinose 0/2/0%, 0.4/1.1/1.0%, 0.8/0.8/1.0% and 1.4/0.4/1.0%. The single-copy pLEU2-DOT1 vector (pRS315-DOT1) and the multicopy GAL1-promoter pTRP1-DOT1 plasmid (pTCG-DOT1) were described previously (1). Mutations in glycine 401 in the DOT1 gene were introduced by Quick Change (Stratagene) and verified by sequencing. Deletion mutants of DOT1 were generated by PCR. A single FLAG tag was introduced at the N-terminus of DOT1 and the clones were reintroduced in pLEU2-DOT1 to drive expression by the DOT1 promoter.

**In vitro methyltransferase assays**

Full-length wild type or mutant DOT1 was cloned into pET16b (Novagen) and expressed as a 10xHIS fusions in *E. coli* BL21(DE3)pLysS (Novagen). Recombinant Dot1 proteins
were purified using Talon Metal Affinity Resin (Clontech) as described (1). Fractions were analyzed for protein content by Coomassie staining. Yeast nuclear extracts were prepared according to the protocol by the Steve Hahn lab (www.fhcrc.org/science/labs/hahn). Nuclei from 500 ml cultures (1.5 x 10^7 cells/ml) were resuspended in 1 ml buffer N (25 mM K_2SO_4, 30 mM HEPES pH 7.6, 5 mM MgSO_4, 1mM EDTA, 10% glycerol, 0.5% NP-40) and used as a substrate for the methyltransferase reactions. In vitro methyltransferase reactions were carried out at 30°C in 10 mM Tris-HCl pH 7.9 using ~1 μg of recombinant protein, 1-2 μl nuclear extract and 32 μM S-adenosyl-methionine in a final volume of 15 μl for 1-2 hrs. Reactions were terminated by addition of 5 μl 5x SDS-PAGE loading buffer and analyzed by immunoblot analysis and mass spectrometry. UV cross-linking of AdoMet to Dot1 was performed as previously described (2).

**Histone purification and mass spectrometry**

Histone H3 was isolated from nuclear extracts prepared from log phase cultures. Twenty microliters of nuclear extract or in vitro reaction mixture was separated on a 15% SDS-PAGE gel and proteins were stained with Coomassie Blue. The band corresponding to histone H3 was excised from the gel and shrunk twice with 100 μl acetonitrile. The proteins in the gel band were digested overnight at 37°C with 500 ng of Endoproteinase Arg-C (Roche Applied Science) in 85 μl buffer (10 mM CaCl_2, 100 mM Tris pH 7.6) and 10 μl activation solution (50 mM DTT, 5 mM EDTA, Roche Applied Science). The supernatant was collected and vaporized using a Speedvac (New Brunswick Scientific). Prior to analysis, samples were desalted using the stop and go extraction (Stage) procedure as described (53). Briefly, the samples were dissolved in 20 μl of 0.1% trifluoroacetic acid, loaded on the C18 material and washed with 20 μl of the same solution. The peptides were eluted with 2 μl of 50% acetonitrile and acidified with 18 μl of 0.6% acetic acid. The presence of histone H3 was first confirmed by nanoLC-MS/MS using an LTQ mass spectrometer (Thermo Scientific) operated as described (54). The levels of methylation of H3K79 were then determined by multiple reaction monitoring (nanoLC-MRM) using a 4000 Q TRAP mass spectrometer (Applied Biosystems). The MRM transitions and parameters were chosen and optimized using a set of synthetic peptides with the sequence EIAQDFK*TDLR (K*=K-me0, -me1, -me2, and -me3).

**Antibodies**

For immunoblots, antibodies purchased from commercial suppliers as well as rabbit polyclonal antibodies produced in-house were used with identical results. The sequence of the peptide used for the H3K79me1 antibody was C-IAQDFK*TDLRF-C (K*=K-me1) and for the H3K79me2 and H3K79me3 antibodies IAQDFK*TDLRF (K*=K-me2 or K-me3 respectively). Specificity of these antibodies was tested by spotting 10 μg and 1 μg of H3K79me0, -me1, -me2 and -me3 peptides on a nitrocellulose membrane and analyzing how well these peptides were recognized by the different antibodies (Fig. S1). The sequence of the peptide used for the antibody against the C-terminus of yeast histone H3 was C-QKKDIKLRRARRGER. The rabbit polyclonal antibody against Dot1 has been described before (1). Commercially available antibodies that were used in this study are H3K79me1 (ab2886, Abcam), H3K79me2 (ab3594, Abcam), H3K79me3 (ab2621, Abcam),
H3K4me2 (07-030, Upstate), H3 (ab1791, Abcam), Rad53 (sc-6749, Santa Cruz), Pgk1 (A-6457, Invitrogen) and Myc (9E10, Roche Applied Science).

**DNA damage checkpoint activation**

Exponentially growing cells (OD ~0.5) were arrested in G1/S with 5 μg/ml α-factor for 2 hrs, spun, resuspended in fresh media containing α-factor and plated on 14 cm Petri-dishes. The plates were irradiated with 100 J/m² UV light of 254 nm using a Stratalinker (Stratagene). Trichloroacetic acid protein extracts were prepared 30 minutes after irradiation and compared to non-irradiated controls.

**Binomial modeling**

The binomial distribution model of H3K79 methylation was calculated using the probability mass function

\[
 f(k; n, p) = \binom{n}{k} p^k (1-p)^{n-k}
\]

where \( f(k; n, p) \) is the probability of a methylation state and \( p \) is the probability of methylation for \( k = 0, 1, 2, 3 \) (representing me0, me1, me2 and me3) and \( n=3 \), representing the maximum number of methyl groups on H3K79.

**TABLE 1. Yeast strains and plasmids**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742</td>
<td>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>(55)</td>
</tr>
<tr>
<td>NK13002</td>
<td>BY4742, dot1Δ::NatMX</td>
<td>This study</td>
</tr>
<tr>
<td>NK13006</td>
<td>BY4742, dot1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>NK13009</td>
<td>BY4742, dot1Δ::dot1-G401R</td>
<td>This study</td>
</tr>
<tr>
<td>NK13010</td>
<td>BY4742, dot1Δ::dot1-G401A</td>
<td>This study</td>
</tr>
<tr>
<td>NK13023</td>
<td>BY4742, URA3-TEL-VIII</td>
<td>This study</td>
</tr>
<tr>
<td>NK13024</td>
<td>BY4742, dot1Δ::NatMX URA3-TEL-VIII</td>
<td>This study</td>
</tr>
<tr>
<td>NK13025</td>
<td>BY4742, dot1Δ::dot1-G401A URA3-TEL-VIII</td>
<td>This study</td>
</tr>
<tr>
<td>NK13087</td>
<td>BY4742, dot1Δ::DOT1-9myc-HphMX</td>
<td>This study</td>
</tr>
<tr>
<td>NK13088</td>
<td>BY4742, dot1Δ::dot1-G401A-9myc-HphMX</td>
<td>This study</td>
</tr>
<tr>
<td>UCC7164</td>
<td>MATα ade2Δ::HisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 ade2-TEL-VR URA3-TEL-VIII</td>
<td>This study</td>
</tr>
<tr>
<td>UCC7183</td>
<td>UCC7164, dot1Δ::KanMX</td>
<td>This study</td>
</tr>
<tr>
<td>UCC7370</td>
<td>UCC7164, bre1Δ::KanMX</td>
<td>This study</td>
</tr>
<tr>
<td>UCC7371</td>
<td>UCC7164, dot1Δ::NatMX bre1Δ::KanMX</td>
<td>This study</td>
</tr>
<tr>
<td>UCC7366</td>
<td>Isogenic to UCC7164</td>
<td>This study</td>
</tr>
<tr>
<td>UCC7356</td>
<td>UCC7366, dot1Δ::NatMX</td>
<td>This study</td>
</tr>
<tr>
<td>NK11059</td>
<td>UCC7366 dot1Δ::KanMX-Pgal1-3HA-DOT1</td>
<td>This study</td>
</tr>
<tr>
<td>NK13028</td>
<td>MATα lys2Δ0 trp1Δ63 his3Δ200 ade2Δ::HisG ura3Δ0</td>
<td>This study</td>
</tr>
</tbody>
</table>
Deletion strains, strains bearing an epitope-tagged DOT1 allele and a strain expressing DOT1 from the galactose inducible GAL1 promoter were made by PCR-mediated gene replacement (55;56). Strains expressing DOT1 mutants from the endogenous promoter were constructed by replacement of DOT1 in strain BY4742 by URA3 using homologous recombination (yielding NKI3006), followed by replacement of URA3 by the various mutant alleles using homologous recombination and selection on 5-fluoroorotic acid. NKI3028 (htb1-K123R) was generated by replacement of plasmid pRS317-HTA1-HTB1 in UCC7315 (57) with pRG423 (a gift from dr. R. Gardner, derived from pRG422 (57)). All strains were verified by PCR or Southern blot and immunoblot analysis.

**ACKNOWLEDGEMENTS**

We thank Dan Gottschling and Richard Gardner for plasmids and reagents, Henk Hilkmann for peptide synthesis, Kitty Verzijlbergen for the antibody against the C-terminus of histone H3 and Evy Battaglia for help with initial experiments. We thank Xiaodong Cheng, Dan Gottschling, Piet Borst, Titia Sixma, Bas van Steensel, and members of the van Leeuwen lab for critical reading of the manuscript and helpful discussions. FvL was a Special Fellow of the Leukemia and Lymphoma...
Dot1 methylates H3K79 using a distributive mechanism

Society and was supported by NOE The Epigenome of the EU 6th framework program. This work was supported by the Netherlands Proteomics Centre.

REFERENCES


Dot1 methylates H3K79 using a distributive mechanism


**SUPPLEMENTARY INFORMATION**

Figure S1. Specificity of the antibodies raised against H3K79me1, -me2 and -me3.

The specificities of the H3K79me1, H3K79me2 and H3K79me3 antibodies generated for this study were tested using dot blots. 10 μg or 1 μg of unmethylated, mono-, di- and trimethylated peptides (sequence EIAQDFK*TDLR, where K* is H3K79 with 0, 1, 2 or 3 methyl groups) were spotted on a nitrocellulose membrane and the blots were incubated with the indicated antibodies. The antibodies show different affinities for the respective substrates. However, results obtained by immunoblotting of chromatin samples were in agreement with quantitative results obtained by mass spectrometry, confirming that this panel of antibodies gives a good indication of the H3K79 methylation state.
Dot1 methylates H3K79 using a distributive mechanism

Figure S2. Expanded version of Fig. 2A.

The endogenous DOT1 gene of a wild-type strain was replaced by an N-terminally 3xHA-tagged DOT1 gene expressed from the inducible GAL1 promoter (strain NKI1059). Media containing different ratios of galactose/glucose were used to achieve a stepwise increase in Dot1 levels. DOT1 expression was compared to that of strains in which DOT1 or dot1-G401A were expressed from the endogenous DOT1 promoter (UC7366 and NKI3025, respectively) and a dot1Δ strain (UCC7356). Dot1 protein levels, H3K79 methylation states, and total histone H3 in log-phase whole-cell lysates were analyzed by immunoblot analysis by running all the samples on the same gel.

Figure S3. Dot1 does not self-methylate.

Transfer of radiolabel from 3H-AdoMet to Dot1 without UV cross-linking was examined in the presence or absence of a chromatin substrate as described in panels 3A and 3B to demonstrate that Dot1 does not self-methylate.
Figure S4. Binding of Dot1 to genes is unaffected by the G401A mutation.

ChIP analysis of the binding of 9xMyc-tagged wild type Dot1 (NK13087) and Dot1-G401A (NK13088) to the GAL1 and ACT1 genes in cells grown on galactose or glucose. Bound DNA fragments were analyzed by multiplex PCR and signals were normalized to input. The experiment was performed in duplicate and error bars represent the spread of the data. ChIP was carried out as described previously (1) with anti-Myc. The amount of Myc whole serum used per IP was 100 μl. Primer sequences for GAL1 were 5′-GGCCCTGGTTATCATATGTC-3′ and 5′-TCGATGCCGGATTCAATATCG-3′; for ACT1 5′-CCAATTGCTCGAGAGATTTC-3′ and 5′-CATGATA-CCTTGTTGCTTTG-3′. The amplified DNA fragments were separated by 2% agarose gel electrophoresis stained with ethidium bromide and imaged using a GeneFlash (Syngene). Data were quantified using the TINA 2.09 software (Raytest). Dot1 binding was assessed using 9myc-tagged versions of Dot1 because our Dot1 antibody does not work in ChIP.

Figure S5. Overexpression of wild-type Dot1 disrupts silencing and overexpression of Dot1-G401A restores silencing in a dot1Δ strain.

DOT1 overexpression (OE) plasmids (pTCG-DOT1 and derivatives thereof) were transformed into a wild type (UCC7164) and dot1Δ strain (UCC7183). The strains were spotted in tenfold dilution series on selective media (YC-Trp+Gal) to induce expression from the GAL1 promoter and in the presence or absence of 5-fluoroorotic acid (± FOA) to measure silencing of the telomeric URA3 reporter gene. Cells in which URA3 is silenced are resistant to FOA and cells that express URA3 are sensitive to FOA.