Progressive methylation of ageing histones by Dot1 functions as a timer

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Post-translational modifications of histone proteins have a crucial role in regulating gene expression. If efficiently re-established after chromosome duplication, histone modifications could help propagate gene expression patterns in dividing cells by epigenetic mechanisms. We used an integrated approach to investigate the dynamics of the conserved methylation of histone H3 Lys 79 (H3K79) by Dot1. Our results show that methylation of H3K79 progressively changes after histone deposition, which is incompatible with a rapid copy mechanism. Instead, methylation accumulates on ageing histones, providing the cell with a timer mechanism to directly couple cell-cycle length to changes in chromatin modification on the nucleosome core.

Keywords: cell cycle; chromatin; H3K79; H3K4; Set1

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INTRODUCTION

The interaction between histone post-translational modifications (PTMs) and gene regulation has led to the suggestion that stable histone PTMs can function as epigenetic signals to facilitate the propagation of gene expression states (Probst *et al*, 2009; Bonasio *et al*, 2010). Maintenance of cell identity by histone PTMs requires that the daughter cells rapidly re-establish the parental epigenetic patterns following deposition of new, unmodified histones on the duplicated DNA. Some PTMs, such as histone methylation, are relatively stable (Barth & Imhof, 2010), and several copy mechanisms or mitotic-bookmarking strategies seem to be available (Kaufman & Rando, 2010; Zaidi *et al*, 2010). However, the way in which PTMs are reproduced following chromosome duplication and transmitted from one generation to the next is still unclear (Probst *et al*, 2009; Bonasio *et al*, 2010).

Methylation of H3K79 by Dot1 is a mark of many eukaryotic epigenomes. It is located in the structured part of the nucleosome core—predominantly in active chromatin—and is involved in gene regulation and has been linked to cancer (Rusche *et al*, 2003; Krivtsov & Armstrong, 2007; Jones *et al*, 2008). Dot1, which does not have the SET domain found in other known histone lysine methyltransferases, can mono-, di- and trimethylate H3K79 by a distributive or non-processive mechanism (van Leeuwen *et al*, 2002; Frederiks *et al*, 2008). As is the case for many other methylated lysines, the different methylation states of H3K79 might have different functions in gene regulation (Bonasio *et al*, 2010). Indeed, multiple H3K79 methylation is regulated by an evolutionarily conserved pathway; in yeast and human cells, mono-ubiquitination of the histone H2B carboxy-terminus (H2Bub) by Bre1 enhances the activity of Dot1 (Shahbazian *et al*, 2005; Frederiks *et al*, 2008, 2011; McGinty *et al*, 2008; Schulze *et al*, 2009). However, the underlying mechanism of the histone cross-talk is unclear, and the direct and indirect consequences of the different H3K79 methylation states are also unclear (Lu *et al*, 2008;

Fig 1 | A model for H3K79 methylation in dividing cells. (A) Dot1 mono-, di- and trimethylates H3K79 with rate constants k_0 , k_1 and k_2 . The constant k can be interpreted as the specificity constant (pseudo second-order rate constant k_{cat}/K_m). Unmodified histones (K79me0) are synthesized at a rate γ and all forms of H3K79 are diluted with a first order rate constant μ because of an increase in cell volume caused by cell growth. The histone synthesis rate γ was assumed to be constant, reflecting an average over the cell cycle, and specific for each strain or growth condition. (B) Observed (average MS measurement of two independent clonal experimental cultures $\pm$ s.e.m.) and predicted methylated (me1, me2 and me3) and unmethylated (me0) H3K79 fractions in yeast strains expressing different amounts of a galactose-inducible *DOT1* gene (Frederiks *et al*, 2008). (C) Validation of the model using WT strain BY4742 cultured at a different growth rate than that of the strain used to build the model in B (average MS measurement of two independent clonal experimental cultures $\pm$ s.e.m.). (D) Simulation of H3K79 methylation over a range of Dot1 concentrations. The dashed line indicates the approximate WT methylation level. (E) H3K79 methylation in a yeast strain lacking H2Bub (*bre1Δ*). Predicted values were on the basis of three sets of kinetic parameters: (i) those of WT cells; (ii) k_2 was allowed to vary but k_0 and k_1 were set to the values determined for WT cells (best fit k_2); or (iii) k_0 , k_1 and k_2 were all allowed to vary and fitted independently. Similar H3K79 methylation levels were observed in a strain lacking H2BK123ub due to a H2BK123R mutation (see Supplementary Fig S2A online). (F) Predicted rate constants ($\mu\text{M}^{-1} \text{min}^{-1}$) for the three methylation reactions. (G) Immunoblots of H3K79 methylation in *dot1Δ* and *dot1Δbre1Δ* strains carrying an empty vector or expression vectors for WT Dot1 or Dot1Δ2-136. MS, mass spectrometry; SS, sums of squares; WT, wild type.

Frederiks *et al*, 2011). Recent studies indicate that H3K79 methylation states might not only be regulated at specific genomic regions, but also change during the cell cycle (Janzen *et al*, 2006; Schulze *et al*, 2009). To understand the dynamic behaviour of H3K79 methylation and its consequences, we investigated the propagation of methylation of H3K79 by Dot1 in dividing yeast cells.

RESULTS AND DISCUSSION

A mathematical model for *in vivo* H3K79 methylation

We first developed a mathematical model for *in vivo* H3K79 methylation using quantitative proteomics data, growth rates and estimates of nuclear Dot1 and histone H3 levels *in vivo* (Fig 1A,B; supplementary information (model B) and Tables S1, S2 online). This distributive methylation model was developed using a strain with an inducible allele of Dot1 and could accurately predict the histone H3K79 methylation levels in a strain expressing endogenous Dot1 that was grown in normal glucose media (Fig 1C). Our simulations show that the mono-, di- and trimethylation reactions of H3K79 proceed with different kinetics. The rate constants estimated by this model and on the basis of our experimental data (supplementary information online) correspond to $k_0 = 0.055$, $k_1 = 0.019$ and $k_2 = 0.0087 \mu\text{M}^{-1} \text{min}^{-1}$. In agreement with a non-processive mechanism of methylation by Dot1 (Frederiks *et al*, 2008), the second and third methylation events are not faster than the first methylation event: each subsequent reaction is slower than the one before, and the rate constants for the methylation events vary proportionally with the number of free methylation sites on the lysine. These differences might reflect the underlying biochemistry of the methylation reactions; once a methylated lysine is bound in the active site of Dot1, it might not be easily realigned to bring a free methylation site into the correct orientation, leading to non-productive binding events. As expected for a non-processive mechanism of methylation, when H3K79 methylation was simulated over a range of Dot1 concentrations, relative changes in the methylation states were observed (for example, compare ratio of me1 to me3; Fig 1D). By contrast, for a processive mechanism of methylation, all methylation states are expected to change proportionally when the enzyme concentration changes (supplementary Fig S1 online).

To test the predictive value of the model, we first investigated regulation of H3K79 methylation by H2Bub. The observed loss of H3K79me3 in cells lacking H2Bub (*bre1Δ* or H2BK123R) has led to the suggestion that H3K79me2 and H3K79me3 are established independently of each other and that H2Bub specifically promotes trimethylation of H3K79 (Schulze *et al*, 2009). By using rate constants of wild-type cells, our model poorly predicted the H3K79 methylation pattern of cells lacking H2Bub (Fig 1E; supplementary Fig S2A online). This was expected because the differences in methylation could not be explained by differences in growth rate between *bre1Δ* and wild-type cells. However, allowing variations in k_2 (the rate constant of the trimethylation reaction) did not result in a better prediction (Fig 1E). By contrast, when we allowed k_0 , k_1 and k_2 to vary simultaneously, a better fit was obtained (Fig 1E). Thus, the model predicted that the rate constants of all three methylation steps were reduced in *bre1Δ*, as compared with the wild type (Fig 1F), leading to a slower increase in the number of methyl groups per lysine and thereby resulting in

Fig 2 | Cell-cycle progression affects the accumulation of H3K79 methylation. (A) Simulated methylation patterns of three wild-type strains with different growth rates using WT kinetic parameters. (B) Methylation in a WT strain growing at 30 °C in glucose (same sample as in Fig 1C) and growing slowly at 23 °C in galactose media (average MS measurement of two independent clonal experimental cultures $\pm$ s.e.m.). (C) H3K79 methylation in cells in log phase and arrested for 3 h in G1 phase (average MS measurement of two independent clonal experimental cultures $\pm$ s.e.m.). (D) Immunoblot analysis of Dot1 expression in log phase and G1 arrest. MS, mass spectrometry; WT, wild type.

low H3K79me3 and high H3K79me1 levels similar to wild-type cells expressing low levels of Dot1 (that is, a shift to the left in Fig 1D). To verify this prediction experimentally, we examined the effect of H2Bub in cells expressing wild-type Dot1 or the partly active Dot1Δ2-136, which lacks the amino-terminus involved in nucleosome binding (Frederiks *et al*, 2008). Cells expressing Dot1Δ2-136 maintained H3K79me1 and H3K79me2 and lost H3K79me3, similar to cells lacking Bre1 (Fig 1G; Frederiks *et al*, 2008). However, when Dot1Δ2-136—or another independent hypomorphic allele, Dot1-G401A—was combined with *bre1Δ*, H3K79me2 and H3K79me1 were also lost (leading to a further

Fig 3 | Time-dependent changes in H3K79 methylation. (A) Simulation of H3K79 methylation in a single cell starting at S-phase with a cell-cycle length of 120 min (left) or 600 min (right). Methylation levels at the start of S-phase ($t=0$) are identical to the levels at the end of G1 (last time point). (B) Histone H3 was tagged with a RITE cassette. Induction of recombination between LoxP sites (L) by Cre results in a tag switch. (C) Following induction of Cre in log phase, replicating cells were harvested after 0–4 divisions and histone H3–HA–6xHis of different average age was purified under denaturing conditions. By purification of old H3–HA–6xHis after the tag switch, new histones are eliminated, thereby increasing the average age of H3 in the pool. (D) Immunoblot analysis of epitope-tag-switched histone H3 in whole-cell extracts. (E) MS analysis of H3K79 methylation of purified histones of different average age (average of two independent clonal experimental cultures $\pm$ s.e.m.). Zero divisions represent the mix of old and new H3 found in non-switched cells. MS, mass spectrometry; RITE, recombination-induced tag exchange.

shift to the left in Fig 1D; Fig 1G; supplementary Fig S2B–D online). We conclude that H2Bub stimulates the accumulation of methyl groups on H3K79 by enhancing all methylation reactions. This is consistent with the fact that H2Bub promotes mono-methylation (McGinty *et al*, 2008) as well as di- and trimethylation of H3K79 by human Dot1L (Kim *et al*, 2005; Zhu *et al*, 2005; Mohan *et al*, 2010).

A link between cell-cycle length and H3K79 methylation

Having established that predictions of the model can be experimentally validated, we next asked which conditions can influence H3K79 methylation. In the absence of a known demethylase activity that can counteract Dot1 activity, one prediction of the model is that cell-cycle length can affect the average pattern of methylation (Fig 2A). Indeed, slowly growing cells accumulated more methyl groups on H3K79 (for example, the ratio of H3K79me3 to H3K79me1 is increased by twofold; Fig 2B). Furthermore, cells arrested in G1 or G2/M phase also showed more H3K79me3 and less of the lower methylation states than the

log-phase cells (Fig 2C–D; supplementary Fig S3A,B online). Reduced H3K79me2 in G1 has also been shown by immunoblot analyses (Schulze *et al*, 2009). Together, these results suggest that in slowly growing or arrested cells, Dot1 has more time to introduce methyl groups on H3K79.

Methylation on H3K79 accumulates on ageing histones

To determine how H3K79 methylation is propagated during the cell cycle, we developed a single-cell two-phase minimal model for H3K79 methylation, distinguishing S-phase from the rest of the cell cycle (supplementary note online). This model shows a temporary drop in methylation in S-phase when new, unmodified histones are deposited, and then a steady decrease of H3K79me0 accompanied by progressive accumulation of methyl groups on H3K79 during the rest of the cell cycle. Only when the doubling time was substantially increased was a steady state of methylation reached in the model (Fig 3A). The model thus predicts that the degree of H3K79 methylation is dynamic and is determined by the residence time of histones within the chromatin. To investigate

Fig 4 | Role of demethylase activity in accumulation of methyl groups. (A) The inclusion of a demethylase parameter (compare 0.0005 with 0.05 min^{-1} for all demethylation events) has only small effects on the steady-state model (compare with Fig 1B). (B) Demethylase activity can affect the kinetics of methylation in the single-cell model, but the effect depends on the rate of demethylation and cell-cycle length (compare with Fig 3A; supplementary Fig S4 online). (C) H3K4 methylation in a WT strain growing at 30 °C in glucose, growing slowly at 23 °C in galactose media and arrested for 2 h in G1. (D) H3K4 methylation on purified histones of increasing age (Fig 3B–E). Strains expressing H3–HA–6xHis, H3–T7 or a mix thereof were used as controls. Left and right panels represent two independent purifications. gal, galactose; gluc, glucose; WT, wild type.

this possibility, we modified the recently developed recombination-induced tag exchange tool (RITE; Verzijlbergen *et al*, 2010) to biochemically purify histone H3 proteins of different ages (Fig 3B–D; supplementary Fig S3C online). This genetic pulse-chase method enriches for old histone proteins synthesized before induction of the epitope-tag switch under constant growth conditions. Histone age is therefore independent of cellular age. Histone H3 fractions enriched for old proteins had accumulated more methyl groups per H3K79 residue than purified bulk histones—that is, more me3 and less me1—and also showed an increase in the fraction of H3K79 residues that was methylated by any of the three methylation states, as indicated by the decrease in the fraction of H3K79me0 (Fig 3E). Several other lines of evidence confirm the association between histone residence time and H3K79 methylation. Histones from chronologically aged quiescent cells also show a reduction in H3K79me2 and an increase in H3K79me3 levels (MA Osley, personal communication). Furthermore, genes with high relative rates of replication-independent histone turnover—that is, short histone residence time—show low levels of H3K79me3 (Dion *et al*, 2007; Gat-Viks

& Vingron, 2009), and genes in which ‘ancestral’ histones accumulate in replicating cells show higher levels of H3K79me3 (Radman-Livaja *et al*, 2011).

Demethylases and dynamics of methyl marks

Attempts to find a H3K79 demethylase have been unsuccessful (Tu *et al*, 2007). In addition, upon initiation of heterochromatin formation, H3K79 methylation was slowly lost, suggesting that active demethylation did not occur (Katan-Khaykovich & Struhl, 2005; Osborne *et al*, 2009). On the basis of these studies and studies on human Dot1 (Sweet *et al*, 2010; Zee *et al*, 2010), H3K79 demethylation was excluded from the model. If, however, an H3K79 demethylase would be active, one could view the rate constants in the model as the net result of synthesis and removal. Indeed, including a demethylase parameter increased the calculated rate constants for the methylation reactions, but it did not substantially affect the average H3K79 methylation levels that were fitted (Fig 4A; supplementary Table S1 online). However, in the single-cell two-phase model, the combined action of a methylase and demethylase leads to higher turnover of methyl

groups and more-rapid establishment of a steady state following deposition of new, unmethylated histones (Fig 4B; supplementary Fig S4 online).

To investigate the role of demethylases, we investigated the accumulation of methylation on H3K4 by Set1, which can be counteracted by the demethylase Jhd2 (supplementary Fig S1 online; Tu *et al*, 2007). As we could not detect H3K4 peptides by mass spectrometry (supplementary Methods online), changes in H3K4 methylation were analysed by immunoblot assays. No increase in H3K4-methylation levels was detected in arrested or slow-growing cells (Fig 4C). Furthermore, H3K4 methylation did not accumulate on old histones (Fig 4D). In addition, no correlation was found between the locations of old histones and H3K4me2 or H3K4me3 in the yeast epigenome (Radman-Livaja *et al*, 2011). Together, our experimental findings and mathematical model suggest that the presence of a demethylase can counteract the accumulation of methylation on ageing histones. However, differences in the mechanism of multiple methylation (Fig 1; supplementary Fig S1 online) might also contribute to the observed differences between H3K4 and H3K79 methylation.

H3K79 methylation links cell-cycle length to epigenome

Our analysis of the dynamics of H3K79 methylation *in vivo* has several unexpected functional implications. First, in replicating yeast cells, methylation on H3K79 slowly accumulates during the cell cycle on new and old histones (Figs 2,3). A recent study using double SILAC labelling suggests that also in human cells methylation of H3K79 occurs on newly synthesized as well as old histones (Sweet *et al*, 2010). An interesting consequence of these observations is that, after DNA replication, a parental nucleosome with a given H3K79 methylation state (for example, me2) will be further methylated in the daughter cell that received this modified nucleosome (that is, it becomes me3), whereas a new, unmodified histone octamer (that is, me0) is deposited at the same genome position in the other daughter cell, if we assume that histone octamers are segregated randomly between daughter strands (Probst *et al*, 2009; Bonasio *et al*, 2010). In this scenario of ongoing methylation, none of the daughter cells (me0 or me3) precisely recapitulates the parental epigenome (me2). This dynamic behaviour seems incompatible with the idea that methylated H3K79 functions as an epigenetic memory mark at single-nucleosome precision. However, it is possible that methylated H3K79 transmits epigenetic information not by single nucleosomes, but by the average methylation level of larger chromatin regions spanning many nucleosomes, as has been suggested for H3K9 methylation in fission yeast and metazoans. Second, the pattern of H3K79 methylation is affected not only by Dot1 activity, but also by histone dilution due to cell-cycle progression (Figs 2,3).

To investigate possible functional implications, we turned to models for multi-site protein phosphorylation (Salazar *et al*, 2010). Proteins regulated by phosphorylation are frequently phosphorylated on many sites by a single kinase, and the degree of multi-site phosphorylation can be an important determinant of downstream effects (Salazar *et al*, 2010). When modified by a distributive kinase, multi-phosphorylated substrates are expected to appear, and unphosphorylated substrates are expected to disappear, with a certain delay after activation of the kinase. This provides the cell with a mechanism for the timing of molecular events (Salazar *et al*, 2010). Given the similar behaviour of distributive H3K79

Fig 5 | Links between cell-cycle progression, H3K79 methylation and gene regulation. (A) Cells in which *URA3* is silenced can grow on media containing FOA. Fraction of FOA-resistant colonies indicates the degree of silencing of a telomeric *URA3* reporter gene in *dot1Δ* strains carrying Dot1 expression vectors at 23 °C (slow growth) and 30 °C (average of two independent clonal experimental cultures $\pm$ s.e.m.). (B) Immunoblot analysis of Dot1-G401A expression. (C) Model for regulation of H3K79 methylation. All three methylation reactions are stimulated by H2Bub. H3K79 methylation does not reach a steady state but accumulates over time and is counteracted by dilution of methylated histones by unmodified histones. Dynamic H3K79 methylation can link changes in cell-cycle length (replication-dependent histone dilution) to changes in chromatin structure. FOA, 5-fluoroorotic acid; MT, methyltransferase; TEL, telomere; WT, wild type.

methylation—late appearance of me3 and disappearance of me1 (Fig 3A)—this chromatin mark could couple cell-generation time or cell-cycle length—rate of replication-dependent dilution of methylated histones—to a biological response through quantitative changes in chromatin. To test this concept, we examined the quantitative effects of Dot1 on gene silencing. Dot1 promotes targeting of Sir3 to yeast heterochromatin by methylation of H3K79 in euchromatin (Rusche *et al*, 2003; Frederiks *et al*, 2011). Dot1 activity is not limiting for normal silencing, but when its activity is reduced—for example, by a mutation in the active site (Dot1-G401A)—it affects the strength of silencing in a dose-dependent manner (Frederiks *et al*, 2008). When the growth rate was reduced by two independent methods, partial silencing by Dot1-G401A was improved (Fig 5A,B; supplementary Fig S5 online). This finding supports the idea that cell-cycle length can affect gene regulation not only through complex signalling cascades, but also more directly through quantitative time-dependent changes in histone methylation (Fig 5C). Which proteins other than the yeast-silencing protein Sir3 translate the dynamic methylated H3K79 mark into a biological response, and whether other histone methyltransferases function by similar mechanisms are important questions to address in future studies.

METHODS

Yeast strains, plasmids, growth conditions and details of the mathematical model can be found in the supplementary information online. For immunoblotting, home-made rabbit polyclonal antibodies against Dot1, H3K79me1, H3K79me2, H3K79me3 and the C-terminus of H3 were used (Frederiks *et al*, 2008). The H3K4me1 antibody was a gift from Laura O'Neil. Commercially available antibodies that were used in this study are H3K4me2 (07-030, Upstate), H3K4me3 (ab8580, Abcam), Sir2 (sc-6666, Santa Cruz), Pgl1 (A-6457, Invitrogen), T7 (A190-117A, Bethyl or 69522-3, Novagen) and HA (12CA5). For mass spectrometry, histone H3 was purified and analysed as described previously (Frederiks *et al*, 2008). Quantitative reverse transcriptase-PCR was performed as described previously (Verzijlbergen *et al*, 2010). HA-6xHis-tagged (recombination-induced tag exchange) histone H3 was purified from mid-log cultures of strain NK12178, as described in detail in the supplementary Methods online. Briefly, β -estradiol was added at the time of inoculation (1 μ M) to induce an epitope-tag switch to T7 by Cre recombination. After cell lysis by bead beating, HA-6xHis-tagged histone H3 was purified under denaturing conditions (4 M guanidine hydrochloride, 10 mM β -mercaptoethanol, 100 mM sodium phosphate (pH 8) and 250 mM NaCl) using TALON Co²⁺-coated beads (BD Biosciences ClonTech). Bound histones were eluted by incubating the beads for 10 min at 95 °C in 50 μ l buffer H3 (60 mM Tris-HCl, pH 6.8, 200 mM imidazole, 10 mM EDTA and 1% SDS).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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