Connecting the dots
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Modeling distributive histone modification by Dot1 methyltransferases: from mechanism to biological insights

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3.1 Abstract

Methylation of histone proteins plays a crucial role controlling genome activity. To understand how the responsible histone methyltransferases are regulated it is important to know their fundamental biochemical properties within the cell and relate these to cellular methylation dynamics. Repeated experiment-modeling cycles have led to insights into the in vivo dynamics of methylation of lysine 79 on histone H3 (H3K79) by the methyltransferase Disruptor of Telomeric Silencing 1 (Dot1). Genetic perturbation in yeast, quantitative measurements and computational modeling were combined to show that Dot1 employs an uncommon, distributive methylation mechanism. A steady-state in vivo methylation model using this information has provided validated explanations for methylation defects in mutants. Subsequent single-cell models have provided insights into the dynamics of H3K79 methylation throughout the cell cycle and uncovered a role for histone protein ageing. These integrated modeling approaches will aid in understanding how regulatory mechanisms influence Dot1’s role in gene expression, cell cycle progression and cancer and can be applied to other methylation systems.
### 3.2 Introduction

Histone protein methylation is intimately involved in many chromatin-mediated regulatory functions of the genome (Greer and Shi, 2012). Methylation of histones predominantly occurs on lysines and arginines and the exact site of modification determines the downstream consequences. Methylation of histone H3K79 by Dot1 (in mammals called DOT1-Like or DOT1L), is an evolutionary conserved modification system with important functions (Farooq et al., 2016; McLean et al., 2014; Nguyen and Zhang, 2011; van Leeuwen et al., 2012; Vlaming and van Leeuwen, 2016). Dot1 was originally discovered in budding yeast where it affects gene silencing, DNA damage response and repair, and meiotic checkpoint signaling. In flies, worms and mammals DOT1L is known to be involved in gene transcription. In mammals DOT1L and H3K79 methylation have been shown to act as a barrier against reprogramming (Xu et al., 2016) and to mediate tumorigenesis by certain oncogenic fusions of the Mixed Lineage Leukemia (MLL) 1 gene (*MLL1*) (Brien et al., 2016). Importantly, DOT1L inhibitors are currently in clinical trials for treatment of human MLL-rearranged leukemia, in which DOT1L misregulation is responsible for oncogenic transformation (Brien et al., 2016). Finally, in African trypanosomes two Dot1 proteins together set up a dynamic methylation pattern that ensures proper cell cycle progression and antigenic variation (Dindar et al., 2014; Figueiredo et al., 2008; Frederiks et al., 2010; Gassen et al., 2012; Janzen et al., 2006). Although a few proteins have been identified that can mediate the effect of methylation of H3K79 or ‘read’ its methylation state, how Dot1 and H3K79 methylation control chromatin structure and function is still poorly understood. More information on the function and regulation of Dot1/DOT1L can be found in several review papers (Farooq et al., 2016; McLean et al., 2014; Nguyen and Zhang, 2011; van Leeuwen et al., 2012; Vlaming and van Leeuwen, 2016).

H3K79 methylation by Dot1/DOT1L has also received special attention because of its uncommon properties (Nguyen and Zhang, 2011; Vlaming and van Leeuwen, 2016). One important feature is the location of H3K79 on the globular surface of the nucleosome core (Figure 3.1), which contrasts with most other methylation sites, which are situated on the unstructured histone tails (Feng et al., 2002; Ng et al., 2002; Van Leeuwen et al., 2002). Indeed, Dot1 methylates H3K79 only in the context of a nucleosome, which precludes the use of simple peptide substrates in methyltransferase assays (McGinty et al., 2008; Sawada et al., 2004; Van Leeuwen et al., 2002). Therefore, analyzing Dot1's binding and activity towards synthethic peptides with defined methylation states is not possible. Another interesting feature that distinguishes Dot1 from other histone lysine methyltransferases is that is does not have a SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain. This is unusual because the SET domain constitutes the catalytic part of the vast majority of histone lysine methyltransferases (Cheng et al., 2005; Couture and Trievel, 2006; Del Rizzo and Trievel, 2011). Instead Dot1 contains a type-I methyltransferase domain (Min et al., 2003; Sawada et al., 2004; Yu et al., 2012). This results in distinct mechanisms
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Figure 3.1: Dot1 methylates histone H3K79 on the surface of the nucleosome core. Structure of the yeast nucleosome (PDB 1HD3 (White et al., 2001), visualized using Chimera (Pettersen et al., 2004)) in front view (A) and side view (B). Histone tails are absent from the crystal structure.

of methylation and regulation between methyltransferases (Cheng et al., 2005; Cheng and Zhang, 2007; Smith and Denu, 2009). Finally, a single Dot1 protein is responsible for mono-, di-, and trimethylation of H3K79, although in some species this task is split between multiple Dot1 proteins, as will be discussed below. Generally, the control of the different H3K79 methylation states and their specific functions are still poorly understood. A few mechanisms have been identified that promote H3K79 methylation, including ubiquitination of the C-terminal tail of histone H2B (H2Bub) along transcribed regions and recruitment of DOT1L to genes via its interaction with AF9, AF10 and other protein partners (Vlaming and van Leeuwen, 2016). However, how H2Bub influences the activity of Dot1 at a molecular level is not well understood (Chatterjee et al., 2010; Fierz et al., 2012; Holt et al., 2015; Vlaming and van Leeuwen, 2016; Zhou et al., 2016). An important first step in explaining the changes in methylation seen when regulatory pathways are compromised is to understand how the (multiple) methylation states are established, i.e. knowing the mode of methylation and the kinetic parameters.

Another outstanding issue is how dynamic the cellular H3K79 methylation landscape is. Histone modifications together with other mechanisms define the epigenetic identity of a cell. This does not mean, however, that histone modifications are static. Some modifications are subject to dynamic addition and removal by modifying and demodifying enzymes, providing opportunities for adjustments by signaling to these opposing activities (Greer and Shi, 2012). To date, demethylases for H3K79me have not been reported (Black et al., 2012), suggesting that other mechanisms may be at play to counteract the Dot1 enzymes. DNA replication and the concomitant duplication of chromatin is by definition a process that disrupts the methylation pattern by the dilution of modified chromatin-bound histones with newly synthesized histones that are unmodified, or modified by so called ground-state modifications (Alabert and Groth, 2012; Corpet and Almouzni, 2009; Probst et al., 2009). Whereas some histone modifications such as histone acetylation and ubiquitination show high turnover and can be rapidly re-established following S phase, histone methylation is generally more stable and only slowly re-established (Alvarez et al., 2011; Barth and Imhof, 2010; Pesavento et al., 2008; Scharf et al., 2009a; Scharf and
Imhof, 2011; Zee et al., 2010a). Whether and how cells re-establish the modification pattern that existed prior to S phase is a fundamental question that lies at the heart of epigenetics as determinant of cell identity. Understanding the emergence of histone methylation patterns throughout the cell cycle requires a detailed understanding of the intrinsic biochemical properties of methyltransferases like Dot1.

Given the limitations of and challenges with in vitro methylation assays we have undertaken a combination of genetic engineering in yeast, quantitative measurements and mathematical modeling to extract information on the basic properties of Dot1 in the living cell (Figure 3.2). Here we describe our efforts to determine the mechanism of methylation by Dot1 and how this feeds into the regulation of the H3K79me landscape in the cell. This question is highly relevant because of the role of Dot1 in very fundamental cellular processes (Farooq et al., 2016; McLean et al., 2014; Nguyen and Zhang, 2011; Vlaming and van Leeuwen, 2016; Wang et al., 2016).

Figure 3.2: Schematic representation of the alternating model-experiment interactions throughout the described studies. Models I–V (in red) correspond to distinct systems of differential equations (not just parameter differences). Model I represents the population (steady state) models with distributive kinetics. Model II represents the so called single cell (cycle) models. Model III and IV represent the steady state and cell cycle models, respectively, with reversible reactions. Finally, Model V is the single cell model adapted for Trypanosoma (i.e. with three distinct cell cycle phases). Along the systems biology cycle each model(ing) step is preceded by a (symbolic) research question (underlined). Outgoing arrows point to model output. In blue fonts experimental validation of the preceding model is indicated. Detailed explanations can be found in the main text.
3.3 The methylation mechanism of Dot1

The first requirement for understanding the mechanisms of regulation of H3K79 methylation is knowing the intrinsic mechanism of methylation. Dot1 mono-, di- and trimethylates H3K79. In general, such a multiple methylation reaction can occur processively or distributively (Figure 3.3). In processive methylation, the methyltransferase remains bound to the substrate histone and performs its subsequent methylation reactions before it releases the final methylation product. A new round of methylation requires that the reaction byproduct S-adenosyl-L-homocysteine (SAH) is exchanged for the methyl donor S-adenosyl-L-methionine (SAM), without releasing the substrate histone. In this situation, the concentration of the intermediate states never exceeds that of the enzyme. In distributive methylation, the intermediate methylation states and SAH are released after each addition of a methyl group. Binding of a SAM-bound enzyme to intermediate states is needed for subsequent methylation events. The methylation mechanism is important for understanding the regulation and function of histone methylation states because the two mechanisms result in a different relation between the amount of enzyme activity and the absolute and relative abundance of the methylation states. Whereas in processive methylation all methylation states are expected to increase proportionally with increasing amounts of enzyme, in distributive methylation the relative amounts of the methylation states show characteristic changes. For example, when starting from an unmethylated substrate, monomethylation has to accumulate to sufficient levels before a distributive enzyme is likely to encounter it among the many unmethylated substrates and convert it into a dimethylated state and so on. Eventually, the buildup of di- and trimethylated lysines will be at the cost of un-, and monomethylated states.

To determine the mechanism of methylation by Dot1, we measured the changes in H3K79 methylation as a function of changes in Dot1 protein levels in yeast. To that end, Dot1 was expressed from different promoters and the four methylation states of H3K79 (no, mono-, di-, and tri-methylation, or me0, me1, me2 and me3, respectively) were quantified by immunoblot or mass spectrometry. As described in Frederiks et al. (2008), measurements of methylation fractions as a function of Dot1 levels showed peaks of the fractions of methylation states, as outlined in Figure 3.4. For a distributive mechanism with equally probable binding of different methylation products one would expect such a progressive methylation with succession of characteristic peaks as the Dot1 amount increases. For a processive model the unmethylated form alone would enter the processing or production line and exit with a specific probability for each methylation state (see Figure 3.4). Consequently, increasing the probability of enzyme (Dot1) encounters would only increase the number of events and not the exit probabilities. Briefly, a simple processive mechanism should lead to characteristic methylation fraction ratios which should be identical for each different amount of Dot1. The distributive mechanism was supported with a basic statistical model in Frederiks et al (Frederiks et al., 2008). In this model
H3K79 methylation is represented as \( N=3 \) discrete reaction trials (so called Bernoulli trials) with a certain methylation probability \( p \) that is equal for all methylation states in accordance with such a distributive model. The number of successful methylation events \( K \) after \( N \) trials then represents the actual methylation state (0, 1, 2, or 3). The probability of a methylation state for a certain probability of methylation \( p \) (equivalent to the total fraction of methylation) is then given by the binomial probability distribution:

\[
f(K; N; p) = \binom{N}{K} \cdot p^K \cdot (1 - p)^{N-K}
\]

(3.1)

Plotting that function illustrated the expected succession of peaks of methylation fractions. The lack of a quantitative fit to the data was attributed to a potential difference in binding probabilities of the different methylation states.

Subsequently, with in vitro experimental data under controlled conditions available kinetic models were set up to describe how a fixed amount of histone H3 would be gradually methylated in a processive or distributive way (Appendix). However, contrary to model simulations, a large range (1000-fold) of added Dot1 only produced relatively small differences in overall methylation. Available data suggested that Dot1 activity was higher in vivo and could result in pronounced differences in methylation.

Because a more classical and controlled in vitro assay did not deliver data of sufficient quality for fitting to a kinetic model, we applied a modeling approach using quantitative information on in vivo parameters (De Vos et al., 2011). Mathematical models for processive and distributive methyltransferases were developed that simulate histone H3K79 methylation in cells to determine the kinetic mechanism and obtain estimates of the rate constants of the different methylation steps (Figure 3.4). Data on different steady-state expression levels of Dot1 in yeast were obtained using an inducible and tunable promoter. The different Dot1 expression levels were estimated in a semi-quantitative manner from immunoblots by comparing them to the endogenous expression level,
Figure 3.4: Models for in vivo H3K79 methylation. A) Distributive model. Dot1 places the first, second or third methyl group on H3K79 with rate constants $k_0$, $k_1$ and $k_2$. Unmodified histones (K79me0) are synthesized at a rate and all forms of H3K79 are diluted with a first order rate constant $\mu$ because of an increase in cell volume caused by cell growth. B) Processive model. Dot1 mono-, di- and trimethylates unmodified H3K79 with rate constants $k_0$, $k_0'$ and $k_0''$. Synthesis and dilution are as in A. C/D) Simulation of distributive and processive H3K79 methylation over a range of Dot1 concentrations. The dashed line indicates the approximate WT methylation level. A, B, C represent high, low and very low levels of Dot1 activity. Panels A–C were modified from De Vos et al. (2011).

which had previously been estimated to be 2000 copies per cell (Ghaemmaghami et al., 2003). The methylation states were measured by sensitive and accurate quantitative mass spectrometry methods. The substrate histone concentration in yeast was set to 200,000 copies per cell based on the size of the yeast genome and semi-quantitative protein abundance measurements (De Vos et al., 2011; Gavin et al., 2002; Ghaemmaghami et al., 2003) and for each strain and experimental condition the growth rate was determined. This set of data was used as input for modeling the in vivo methylation of H3K79.

Living cells represent a much greater complexity than in vitro conditions, with homeostasis and balanced processes playing an important role. The regulation of Dot1 activity in vivo is likely to be complex and non-uniform across the genome. However, building a mathematical model requires simplification and abstraction. Several assumptions were made to build the initial model. First, we assumed that the in vivo H3K79 methylation state was entirely controlled by Dot1 activity, histone synthesis rate and cell growth, without co-regulation by other factors, such as the SAM levels. Second, the influence of the substrate/co-factor SAM was assumed to be constant. Third, we considered all histones in the cell as equal substrates, i.e. we assumed the methylation to occur in one compartment without specific chromatin domains with altered properties such as Dot1 accessibility or Dot1 targeting. Finally, a growing population of yeast cells was represented by a single model compartment. At steady state the as-
sumption of a constant concentration of the histone H3 pool then holds as a result of the production of new histones balanced by continuous growth. The cells were supposed to be asynchronous and the methylation states were assumed to be in a (population-scale) steady state, as was confirmed repeatedly by temporal sampling.

Ordinary differential equations (ODEs; Appendix) were used to describe dynamic changes in the four methylation states (represented by the variables Me0, Me1, Me2, Me3) based on the two main mechanisms (Figure 3.3). In all cases the histone production rate $\gamma$ is a constant term that, together with the growth dilution process (represented by the dilution rate constant $\mu$) is acting on all states proportional to their abundance. This leads to a constant, steady-state total histone concentration. For a processive mechanism (Appendix: Eq. 3.5) the unmethylated state can undergo three different fates corresponding to mono-, di-, and trimethylation (followed by product release). In a distributive mechanism each methylation state has the potential to interact with the methylase and a methylation event is initiated from any free substrate (Eq. 3.2: $v_i$ terms represents the methylation rates from the respective states $i$; details can be found in the Appendix). Upon that basic reaction stoichiometry, different types of kinetic expressions can be superimposed. Importantly, the level of kinetic detail (and number of associated parameters) needs to be tailored to the available data. A general assumption was that reaction rates were proportional to the total Dot1 concentration ($dot$ in Eq. 3.3), thus assuming that it reflects free Dot1 concentration. Comparing the most simple (1-parameter) processive and distributive models (Appendix: Eqs. 6, 8) confirmed that the distributive model more successfully predicted the in vivo data from Frederiks et al. (2008).

In De Vos et al. (2011) this model selection approach of comparing the quality of fit (visually and quantitatively using the sum of the squared errors as a measure) was extended to more complicated kinetic expressions (such as Appendix: Eqs. 9-11). A distributive model based on simple mass-action (proportional) kinetics was found to give the best fit with the lowest number of parameters (Eq. 3.3). Based on these results, it could be concluded that Dot1 is a distributive methyltransferase in vivo. This mechanism is supported by a crystal structure of yeast Dot1, which suggests that exchanging SAH for SAM requires active-site remodeling and release of the substrate lysine (Sawada et al., 2004).

$$\begin{align*}
\frac{d(me0(t))}{dt} &= \gamma - \mu \cdot me0(t) - v_0 \\
\frac{d(me1(t))}{dt} &= -\mu \cdot me1(t) + v_0 - v_1 \\
\frac{d(me2(t))}{dt} &= -\mu \cdot me2(t) + v_1 - v_2 \\
\frac{d(me3(t))}{dt} &= -\mu \cdot me3(t) + v_2
\end{align*}$$

(Eq. 3.2)

$$\begin{align*}
v_0 &= k_0 \cdot dot \cdot me0(t) \\
v_1 &= k_1 \cdot dot \cdot me1(t) \\
v_2 &= k_2 \cdot dot \cdot me2(t)
\end{align*}$$

(Eq. 3.3)
Through the model also rate constants for the three transitions were estimated ($k_0$, $k_1$, $k_2$ in Eq. 3.3). Rate constants in these models represent the best estimations of the $k_{cat}/Km$ ratio for each methylation event. The kinetic constants in this study therefore represent apparent rate constants. More complicated enzyme kinetic equations can be considered but they had too many parameters to allow a unique fit to the available experimental data. The estimated rate constants of yeast Dot1 decreased as the number of methyl groups on the substrate increased (De Vos et al., 2011). One possible explanation for this trend is that the chance of properly orienting a free hydrogen on the terminal amino group of the lysine is reduced when fewer free hydrogen atoms are available, i.e. when one or two hydrogen atoms have already been replaced by methyl groups. Finally, a similar but semi-quantitative strategy was applied to methyltransferase Set1 (SET-domain containing 1), which mono-, di-, and trimethylates histone H3K4. Simulations of the **in vivo** results strongly suggest a processive mechanism for this SET-domain methyltransferase (De Vos et al., 2011).

### 3.4 A distributive mechanism of methylation provides an explanation for trans-histone crosstalk phenotypes

Ubiquitination of the C-terminal tail of histone H2B promotes H3K79 methylation (Vlaming and van Leeuwen, 2016). The knowledge of the methylation mechanism of Dot1 has helped to provide insight into this evolutionary conserved pathway of trans-histone crosstalk. Mutants of the H2B ubiquitination machinery show a striking loss of H3K79me3, which led to the suggestion that it might act by altering the processivity of Dot1 or specifically affecting the trimethylation activity of Dot1 (Schulze et al., 2009; Shahbazian et al., 2005). By starting from the wild-type model, the methylation state of a strain without H2Bub was predicted after adjustment of the specific growth rate (De Vos et al., 2011). In accordance with the hypothesis that trimethylation is specifically affected by ubiquitination, first only the $k_2$ parameter, describing the rate of conversion of H3K79me2 to me3 was fitted based on the data. As this did not significantly improve the data fit, and a good fit was only produced by changing all the parameters, the initial hypothesis could be rejected (De Vos et al., 2011). To validate the prediction that all transitions were compromised in the absence of H2Bub in the cell, we performed a genetic epistasis test: partial-loss-of-function alleles of DOT1, showing loss of H3K79me3 and gain of H3K79me1 (in agreement with the distributive mechanism) were combined with inactivation of the H2Bub machinery, which also partially compromises Dot1 activity. If H2Bub specifically promotes H3K79me3 formation, no additive effects would be expected. If H2Bub promotes all transitions, it would further decrease the methylation in a background with a partial-loss-of-function allele of DOT1. The combination of mutations led to a loss of all methylation states, confirming the
mechanism predicted by the mathematical model (De Vos et al., 2011; Frederiks et al., 2008). That H2Bub regulates all H3K79 methylation transitions is nicely in agreement with observations in fly and mammalian cells, in which H3K79me3 is present at very low levels and H2Bub promotes synthesis of H3K79me1 and H3K79me2 (Vlaming and van Leeuwen, 2016).

3.5 Histone dilution as a mechanism to counteract the distributive build-up of H3K79 methylation

The steady-state model includes growth as a measure for histone dilution. A wave of dilution occurs during S phase when chromatin is duplicated by assembling a complement of newly synthesized, unmethylated histones. However, histone dilution can also occur outside S phase by so-called replication-independent (RI) histone turnover (Venkatesh and Workman, 2015; Zentner and Henikoff, 2013). Likely the histone production and methylation rates are under-estimated because of that. A prediction of the steady-state model is that in the absence of a demethylase, dilution of histones (be it replication-coupled or -independent) may be the main mechanism to counteract the slow build-up of H3K79me. To test the prediction that RI histone turnover negatively regulates H3K79 accumulation, we examined our dataset on histone inheritance, which can be considered as the inverse of histone turnover (Radman-Livaja et al., 2011). On a genome-wide scale, histone inheritance is not equal for each gene and a positive correlation was observed between high histone inheritance (i.e. low turnover) and H3K79me3 (Radman-Livaja et al., 2011). This observation is in agreement with the prediction of the model that histone turnover negatively regulates H3K79me3 in the cell.

3.6 A single-cell model of H3K79 methylation to describe the dynamics during the cell cycle

The steady-state model has been extremely helpful in understanding the kinetic mechanism of Dot1. To get more insight into the dynamics of H3K79 methylation throughout the cell cycle and specifically how the methylation pattern is re-established following chromatin duplication, a different approach was needed. Instead of a time-averaged or population perspective this new model zoomed in on the cellular, more specifically the nuclear scale (De Vos et al., 2011). The previously obtained distributive kinetic model served as the starting point. However, for a single cell model the assumption of continuous histone production breaks down since it is known that histone synthesis is tightly connected with DNA duplication during S phase. To gain insight in the effect of periodic changes in histone production rate on the methylation state of a typical cell, two main phases were distinguished: a phase representative of the S phase and a phase representative of the G2-M-G1 phases combined (Figure 3.5). The
S phase was assumed to take 20% of the total cell cycle time. Histone production was only taking place in the S phase, yet at a 5-fold higher rate to obtain the same overall histone production rate. Without an explicit variable for volume growth, a growth dilution is constantly acting on the histones (Dot1 concentration is a fixed parameter). Since according to the assumptions of the model methylation fractions are homogeneously distributed over the nuclear compartment, it follows that mitosis will not do more than just partition the original volume into two identical compartments. Since that does not alter the concentrations of the methylation states, cell division was not simulated. Since we further assumed that consecutive cell generations would pass on their methylation state, we repeated the single cell cycle simulation until a stable oscillatory pattern of methylation was reached, taking that as representative for the population (Figure 3.5).

The single-cell model predicted that Dot1 is slow, and that the degree of H3K79 methylation is dynamic and is determined by the residence time of histones within the genome (De Vos et al., 2011). To validate this experimentally, we performed methylation analysis of biochemically-purified old or aged histones from a cell. Histones that were synthesized at least 4 cell divisions prior to the purification showed substantially higher H3K79me3 and lower H3K79me0/1/2 levels than histones of young average age. Histones of an average intermediate age showed an intermediate increase in H3K79me (De Vos et al., 2011). These results are in line with the idea that H3K79 methylation levels slowly accumulate over time. Of note, the accumulation progresses over multiple cell generations, indicating that the build-up of methylation is not finished at the end of a cell cycle just before a new S phase initiates. Slow accumulation of H3K79me on ageing histones spanning multiple cell divisions has also been observed in human cells by independent pulse-chase proteomics methods (Sweet et al., 2010). Interestingly, these dynamics suggest what has been referred to as ‘scrambling’ of epigenetic information and is incompatible with epigenetic memory models that assume exact duplication of the methylation patterns from mother to daughter cells (De Vos et al., 2011; Sweet et al., 2010). Instead, the accumulation of H3K79me on aging histones or in aging cells could in principle act as a molecular timing mechanism. However, the physiological relevance of such a timer remains to be established.

3.7 Extending the single-cell model with demethylation

The modeling described above does not include demethylation. To date there is no record of the existence of a H3K79 demethylase but demethylases have been found for other histone lysine methylation sites (Cheng and Zhang, 2007; Kooistra and Helin, 2012). To examine the influence of a reverse reaction, a demethylase activity was included in the next modeling phase. Extending the steady state and single cell models in De Vos et al. (2011) was straightforward;
it suffices to mirror each methylation reaction with a demethylation reaction (Appendix: Eqs. 12,13). The reaction rate was again assumed proportional to the enzyme (demethylase) concentration and to the substrate concentration. Simulations were done as before. In this model, the absence and presence of demethylases are both compatible with the same steady-state patterns. However, introducing a reverse process must be balanced by faster forward kinetics. An interesting consequence of this adjustment is a faster establishment of a steady state. Our previously described experiments in yeast showed that growth arrest or histone ageing led to increased H3K79 methylation (De Vos et al., 2011), which is inconsistent with fast establishment of an equilibrium and suggests that a H3K79 demethylase is absent or only a minor contributor. In contrast, growth-dependent methylation and accumulation of methylation on older histones did not happen in the case of the Set1 methyltransferase (De Vos et al., 2011), which is known to operate in the presence of a demethylase activity that modulates the H3K4me1/3 levels (Huang et al., 2015; Liang et al., 2007; Osborne et al., 2009; Tu et al., 2007).

3.8 Modeling H3K76 methylation by Dot1 in African trypanosomes

Having established the models to study the mechanism, regulation and cell cycle dynamics of H3K79 methylation in yeast, we applied this toolbox to examine the regulation of H3K76 methylation in African trypanosomes, the causative agents of sleeping sickness. In this unicellular parasite, two Dot1-like proteins, Dot1A and Dot1B are responsible for H3K76 methylation (Dindar et al., 2014; Gassen et al., 2012; Janzen et al., 2006). Together they set up a strictly controlled cell cycle pattern of H3K76me2 and H3K76me3, as nicely demonstrated by the Janzen lab using immunofluorescence (Dindar et al., 2014; Gassen et al., 2012; Janzen et al., 2006). This dynamic methylation pattern ensures proper
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cell cycle progression and modulates antigenic variation in African trypanosomes (Dindar et al., 2014; Figueiredo et al., 2008; Frederiks et al., 2010; Gassen et al., 2012; Janzen et al., 2006). To understand the mechanistic basis for the observed cell cycle pattern, quantitative information on the Dot1A and Dot1B activities was obtained by expressing the proteins individually in yeast under the control of promoters of different strengths (Stulemeijer et al., 2015). This approach was not possible in trypanosomes since misregulation of these enzymes is not tolerated (Gassen et al., 2012; Janzen et al., 2006). We subjected Dot1A and Dot1B to a computational analysis using the same strategy as described for yeast Dot1. Simulations with the distributive model resulted in a better fit of the experimental data than the processive model (Stulemeijer et al., 2015). Together with the observed characteristic changes in methylation this strongly suggested that Dot1A and Dot1B, like yeast Dot1, methylate with a distributive mechanism. Very elegant *in vitro* assays from the Janzen lab using recombinant enzymes and recombinant unmodified chromatin templates provided independent evidence for the distributive mechanism of Dot1A and Dot1B (Dindar et al., 2014). The two enzymes have very distinct estimated rate constants. For example, Dot1A displays a very low trimethylation rate (low $k_2$). In contrast, Dot1B has a high rate of di- and trimethylation (high $k_1$ and $k_2$), which led to the prediction that when expressed at sufficient levels, it can result in a binary switch: when a monomethylated state has been produced, Dot1B will very rapidly convert it into dimethylation and subsequently trimethylation (Stulemeijer et al., 2015). This prediction could be experimentally validated by measuring the appearance of methylation states on newly synthesized histones using a genetic pulse-chase assay in yeast. Indeed, in the presence of Dot1B, newly synthesized histones rapidly accumulated the trimethylation state without the detectable appearance of mono- and dimethyl intermediates (Stulemeijer et al., 2015). This assay also qualitatively agreed with the estimated rate constants for Dot1A.

### 3.9 A two-enzyme single-cell model to describe the cell cycle control of H3K76 methylation in trypanosomes

Next we addressed the question whether dynamic Dot1 isoform expression patterns during the trypanosome cell cycle can explain the observed methylation dynamics. To that end we built a kinetic model that simulates the trypanosome cell cycle using previous models developed for yeast, but now taking into account a division of labor between the two distributive enzymes and the 13-hour trypanosome cell cycle profile, which is very different from the 90-minute cell cycle of yeast (Gassen et al., 2012). The trypanosome cell cycle was divided into three phases based on the observed fluctuations of the methylation states: an S phase (20%), G2/M phase (40%) and G1 phase (40%). The estimated rate constants of the steady-state models were used to parameterize a model that represents H3K76 methylation in trypanosomes (Figure 3.5). However, impor-
stantly, here we could not assume that Dot1A and Dot1B activity are constant throughout the cell cycle. Instead we fitted a cell cycle-regulated activity profile based on fluctuations of the three methylation states observed by immunofluorescence data from the Janzen lab (Gassen et al., 2012; Janzen et al., 2006; Stulemeijer et al., 2015). In this model Dot1A and B are inactive during S phase when a complement of new histones is assembled. Dot1A becomes active just after S phase, leading to the sequential appearance of H3K76me1 and -me2 (Figure 3.5). Dot1B is initially tightly repressed but activated at the start of G1, after which all H3K76 residues rapidly ‘switch’ to the trimethyl state. The resulting two-enzyme cell cycle model provides a resource that can be used to gain more insight into how the cell cycle-regulated pattern is generated and how sensitive it is to perturbation. It also offers the possibility to predict the changes in methylation upon Dot1B overexpression or loss of Dot1A, two conditions that have been described to be lethal in trypanosomes (Gassen et al., 2012; Janzen et al., 2006). The two conditions have in common that H3K76me1 is predicted to be virtually absent, while in wild-type cells it is predicted to peak in the G2/M phase (Stulemeijer et al., 2015). It is tempting to speculate that H3K76me1 represents a biologically important signal in trypanosomes. Addressing this prediction by experimental validation will require the development of tools to specifically control this methylation state in trypanosomes.

3.10 Future developments and applications of the models

The approach of subsequent phases of genetic perturbation, quantitative measurements, model building, and experimental validation (Figure 3.2) has provided very valuable insights into the regulation of H3K79 methylation by Dot1. One interesting result is that relatively straightforward and simple mathematical models and knowledge about the distributive mechanism very well explain the methylation defects seen in regulatory mutants. Although the methylation changes can be complex or seemingly specific for one methylation state, most phenotypes can be simply explained by altered overall activity of the distributive Dot1 enzyme. As such, the combined results and models provide a useful resource for future studies on regulation of Dot1. Indeed, they have been invaluable for interpreting H3K79 methylation levels seen in engineered H2Bub crosstalk strains (Vlaming et al., 2014). Several assumptions were made to build the initial versions of the models. In the future it will be important to refine the models. One aspect that deserves further attention is the non-homogeneous H2Bub landscape: H2Bub is mainly found in transcribed regions and is absent in promoters, regulatory regions and silent chromatin (Schulze et al., 2011, 2009). It will be interesting to represent this dichotomy in the models to obtain rate constants for the activity of Dot1 on the two types of substrates. Interestingly, the general model is qualitatively in agreement with the methylation differences between the two chromatin compartments: low H2Bub goes together with low
H3K79me3 and high H3K79me1 (like activity B in Figure 3.4C), while high H2Bub correlates with high H3K79me3 and low H3K79me1 (like activity A in Figure 3.4C) (Schulze et al., 2009; Vlaming et al., 2014). Not surprisingly, the in vivo situation includes additional layers of complexity, such as the recruitment of Dot1 to specific sites in the genome by its binding partners (Vlaming and van Leeuwen, 2016). At such sites Dot1 may methylate by a pseudo-processive mechanism if efficient targeting would keep Dot1 close to its histone substrate so that it could methylate the same histone multiple times. Finally, the models described here can in principle also be applied to other histone methylation systems. Recently, the dynamics of several histone methylation marks has been measured in different biological contexts (Alabert et al., 2015; Alvarez et al., 2011; Barth and Imhof, 2010; Pesavento et al., 2008; Scharf et al., 2009a; Scharf and Imhof, 2011; Scharf et al., 2009b; Sweet et al., 2010; Zee et al., 2010a,b). These studies can provide a good starting point for model development. The availability of CRISPR-Cas9 systems to repress and activate genes will further facilitate altering the levels of activity of methyltransferases in the cell system of interest. Modeling methylation systems at other sites will likely face several challenges, such as the control of a specific methylation event by multiple enzymes, the involvement of demethylases, the targeting or activation of methyltransferases by protein-protein interactions, and the influence of histone crosstalk. On the other hand, in vitro assays on defined peptide substrates may be available. Nevertheless, fundamental knowledge on the methylation mechanism, estimated rate constants, and simplified in vivo models can provide an important framework for understanding the regulation and dynamics of histone methylation in the cell.

3.11 Acknowledgments

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3.12 Appendices

Glossary

**Histone methylation:** a post-translational modification (PTM) of histone proteins that predominantly occurs on arginine and lysine residues; arginines can be mono- and dimethylated, lysines can be mono-, di-, and trimethylated.

**Methyltransferase domain:** histone lysine methylation is carried out by proteins containing as the catalytic part a Su(var)3-9, Enhancer-of-zeste, Trithorax (SET) domain or a Class I methyltransferase domain, which is present in many other types of methyltransferases.

**Distributive mechanism:** mechanism in which the enzyme releases from its substrate after one reaction step, requiring new binding for subsequent reactions.

**Processive mechanism:** mechanism in which the enzyme remains bound to its substrate during several reaction steps, only releasing from its final product.

**Trans-histone crosstalk:** one histone PTM controls another histone PTM on a different histone.

**Dot1/DOT1L:** a distributive histone lysine methyltransferase of histone H3 lysine 79, harboring a Class I domain and promoted by trans-histone crosstalk.

**Abbreviations and acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Dot1</td>
<td>yeast Disruptor of Telomeric Silencing 1</td>
</tr>
<tr>
<td>DOT1L</td>
<td>Dot1-like</td>
</tr>
<tr>
<td>H2Bub</td>
<td>C-terminal H2B ubiquitination</td>
</tr>
<tr>
<td>H3K79</td>
<td>histone H3 lysine 79</td>
</tr>
<tr>
<td>H3K79me</td>
<td>histone H3 lysine 79 methylation</td>
</tr>
<tr>
<td>$k_0/k_1/k_2$</td>
<td>apparent rate constant for mono/di/trimethylation</td>
</tr>
<tr>
<td>me0</td>
<td>unmethylated</td>
</tr>
<tr>
<td>me1/2/3</td>
<td>mono/di/trimethylated</td>
</tr>
<tr>
<td>MT</td>
<td>methyltransferase</td>
</tr>
<tr>
<td>ODE</td>
<td>ordinary differential equation</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosyl-L-homocysteine</td>
</tr>
<tr>
<td>SET</td>
<td>Su(var)3-9, Enhancer-of-zeste, Trithorax</td>
</tr>
<tr>
<td>Set1</td>
<td>yeast SET domain-containing 1</td>
</tr>
<tr>
<td>$\mu$</td>
<td>specific growth/dilution rate constant</td>
</tr>
</tbody>
</table>
Parameter estimation and formal mathematical description of the models

Parameter estimation

Parameters are an essential part of most models and determining parameter values can be the primary reason to build a model in the first place. In many cases no accurate parameter estimates are available for ODE models and so called regression methods are used to obtain estimates (Ashyraliyev et al., 2009). It is important to realize that it can be impossible to determine a parameter simply because of the form of the mathematical expressions or because of data that are not providing sufficient information. Since such an unidentifiable parameter can compromise estimation of other parameters it is preferably fixed. Parameter fixing can also be required if strong correlations between parameters exist. The question whether parameter values can be uniquely determined is studied in the mathematical field of system identification. Briefly said a good, i.e. information-rich, experiment or experimental design elicits a specific system response through each underlying process (and its associated parameter(s)). If a good data series is available (sometimes after statistical outlier removal or data smoothing techniques), the challenge is still to find that optimal solution in parameter space starting from an initial guess of parameter estimates. The success of that search will critically depend on two factors: the fitting criterion and the search algorithm.

The fitting criterion or objective function value (also called cost or fitness function) is a measure for how well the simulated data correspond with the experimental data. The maximum likelihood estimator (MLE) is arguably the most widely used measure and is based on the assumption that the most likely parameter vector (or value) is the one that maximizes the probability or likelihood of the observed data. Importantly, if the assumption holds that the errors (differences between measured and predicted data) are independent and normally distributed, then the MLE estimate is the same as the parameter estimate that minimizes the sum of the squares. In other words in many cases the traditional method of minimizing the sum of the squared errors provides an acceptable criterion for data fitting.

Due to the often complex behavior of solutions of systems of ODEs as a function of parameters, the effect on the objective function (one can image this as a landscape with hills and valleys in the case of two parameters) can be very unpredictable. The risk is that the search method gets stuck in a local depression or minimum. The distinction is often made between local and global search methods. Methods of the first class (which is further divided into direct methods and gradient-based methods depending on the use of derivatives) can be proved to lead to a (local) minimum. They are typically fast, yet, cannot guarantee to find the global minimum, i.e. the lowest point in the landscape. Global methods on the other hand will not get stuck and over time find lower
objective function values. However, the search process is very slow and in most cases not guaranteed to converge to the global minimum. Since in biological studies good initial estimates of parameters are the exception, a good strategy is to start with a global search to identify promising areas in the fitness landscape and then switch to a local search method for rapid convergence. That was also the strategy adopted in De Vos et al. (2011) as we will further discuss below.

In De Vos et al. (2011) data fitting is actually used not only for parameter estimation but also as a model selection technique as detailed in the main section. The fraction of the different H3K79 methylation states in 4 yeast strains expressing different amounts of a galactose-inducible DOT1 gene (and growing generally at different rates) served as the experimental data used for determining parameters $k_0$, $k_1$, and $k_2$ of the Dot1 distributive model for a wild-type strain. The other parameters in the model were assumed to be known. Therefore the differences in methylation states are assumed to reflect differences in the kinetic parameters that are fitted by the regression method and experimentally determined specific growth rates.

For the fitting criterion the ordinary simple least squares measure was used with the Mathematica (6.0) NMinimize function for nonlinear global optimization (Wolfram Research, Inc, 2007). We used the ‘Automatic’ mode, which uses global search methods (‘RandomSearch’, ‘Simulated Annealing’, ‘DifferentialEvolution’) in combination with the direct local search method ‘Nelder-Mead’. As discussed above this is a good strategy to avoid getting stuck in a local minimum while still allowing fast convergence. Starting from a random set of parameter values (within a predetermined parameter space) the cost function value was calculated based on the methylation state of the model after convergence to its steady state value (typically 6000 minutes). Repeating that procedure yielded consistent parameter estimates (to a high precision). As a control the cost function value as a function of the kinetic parameters $k_0$, $k_1$, and $k_2$ was plotted to demonstrate that it is a sensitive and smooth (monotone) function of the parameter values in the neighborhood of the respective estimates. Such a relationship enables accurate parameter estimation.

As an extra test of the robustness of the fitting procedure random (Gaussian) noise was added to the original methylation data to a degree that exceeded the experimental noise on those data. Re-fitting consistently led to parameter estimates close to the reference estimates (from data without added noise). Moreover, this also provided a way to estimate the uncertainty of the parameter estimates. A modification of the classical bootstrapping approach (cf. for instance Efron (Efron and Tibshirani, 1994)) was used. Instead of random resampling from a set of experimental data replicates, random Gaussian noise (with sigma = ± 2.5% methylation) was added to the reference data set, before running the fitting routine on the ‘resampled’ data. The standard deviation of the re-fitted parameters then served as estimate of the parameter error. A series of randomized data sets were generated and fitting results were used for statistical analysis. The average of the parameter estimates and the cost function were plotted as a function of the number of runs until their values sufficiently converged. The standard deviation corresponding to that set of runs
was then used as a rough estimate of the parameters’ standard error. Since (artificial) experimental errors are in this approach strictly mapped to the errors in the three fitted parameters the corresponding error estimates are potentially over-estimates. Indeed, unlike in reality in the model other parameters (and processes) do not contribute to variation in the methylation states.

Formal mathematical description of the models

Diverse ways exist to construct a mathematical model that describes a biochemical process. As in many other cases, for the studies described here systems of ordinary differential equations (ODEs) were used. ODEs can accurately represent time dependent changes in well-mixed (homogenous) fixed-volume compartments such as approximately found in cells or organelles. All systems described below express how the four states of the histone H3K79, represented by the (concentration) variables $me_0, me_1, me_2,$ and $me_3,$ change over time. Two main classes of models can be distinguished with the first aimed at representing the steady state methylation over a population of cells. The second class represents the temporal changes in methylation state in a typical single cell.

I. Kinetic models for steady state methylation

Initially, a set of ODE models was defined for fitting to in vitro data from nuclear extracts incubated with different amounts of recombinant Dot1 (Fred-eriks et al., 2008). This did not produce satisfactory results (see main text) and therefore the models were adapted for in vivo data. More specifically, the originally fixed concentration of histone substrate was replaced with a constant histone production rate ($\gamma$) and first order dilution/degradation rates (with rate constant $\mu$). In this way a steady state total histone concentration $H_{tot}$ is reached over time with:

$$\gamma = \mu \cdot (me_0 + me_1 + me_2 + me_3) = \mu \cdot H_{tot} \quad \text{(3.4)}$$

a. Processive mechanism

$$\begin{cases} 
\frac{d(me_0(t))}{dt} = \gamma - \mu \cdot me_0(t) - v_0 - v_0' - v_0'' \\
\frac{d(me_1(t))}{dt} = \mu \cdot me_1(t) + v_0 \\
\frac{d(me_1(2))}{dt} = \mu \cdot me_2(t) + v_0' \\
\frac{d(me_3(t))}{dt} = \mu \cdot me_3(t) + v_0'' 
\end{cases} \quad \text{(3.5)}$$

The $v_0$ terms represent different conversion steps starting from non-methylated H3K79 and leading to different methylation end products. The following kinetic expressions were used for model comparison:

Same conversion rate constants $k$ for all steps (dot is the parameter for Dot1
concentration):

\[ \begin{align*}
  v_0 &= k \cdot \dot{\text{me}}_0(t) \\
  v_0' &= k \cdot \dot{\text{me}}_0(t) \\
  v_0'' &= k \cdot \dot{\text{me}}_0(t)
\end{align*} \]

(Different rate conversion constants \( k_0, k_0', k_0'' \):)

\[ \begin{align*}
  v_0 &= k_0 \cdot \dot{\text{me}}_0(t) \\
  v_0' &= k_0' \cdot \dot{\text{me}}_0(t) \\
  v_0'' &= k_0'' \cdot \dot{\text{me}}_0(t)
\end{align*} \]

b. Distributive mechanism

\[ \begin{align*}
  \frac{d(\text{me}_0(t))}{dt} &= \gamma - \mu \cdot \text{me}_0(t) - v_0 \\
  \frac{d(\text{me}_1(t))}{dt} &= -\mu \cdot \text{me}_1(t) + v_0 - v_1 \\
  \frac{d(\text{me}_2(t))}{dt} &= -\mu \cdot \text{me}_2(t) + v_1 - v_2 \\
  \frac{d(\text{me}_3(t))}{dt} &= -\mu \cdot \text{me}_3(t) + v_2
\end{align*} \]

For a distributive mechanism \( \text{me}_0, \text{me}_1, \text{me}_2 \) are substrates for methylation as represented by the terms \( v_0, v_1 \) and \( v_2 \). Below the main tested rate expressions for those conversion steps are presented.

One rate constant \( k \) for all simple mass-actions steps:

\[ \begin{align*}
  A \quad & v_0 = k \cdot \dot{\text{me}}_0(t) \\
  & v_1 = k \cdot \dot{\text{me}}_1(t) \\
  & v_2 = k \cdot \dot{\text{me}}_2(t)
\end{align*} \]

Different rate constants \( k_0, k_1, k_2 \) for all mass-actions steps:

\[ \begin{align*}
  B \quad & v_0 = k_0 \cdot \dot{\text{me}}_0(t) \\
  & v_1 = k_1 \cdot \dot{\text{me}}_1(t) \\
  & v_2 = k_2 \cdot \dot{\text{me}}_2(t)
\end{align*} \]

This model can be simulated and analyzed interactively via the JWS online model database on: http://jjj.biochem.sun.ac.za/models/devos/.

Michaelis-Menten kinetics with the same \( K_m \) and different catalytic constants \( k_{cat,0}, k_{cat,2}, k_{cat,2} \):

\[ \begin{align*}
  C \quad & v_0 = k_{cat,0} \cdot \dot{\text{me}}_0(t) \cdot \frac{\text{me}_0(t)}{K_m} \frac{1}{1 + \frac{\text{me}_0(t)}{K_m}} \\
  & v_1 = k_{cat,1} \cdot \dot{\text{me}}_1(t) \cdot \frac{\text{me}_1(t)}{K_m} \frac{1}{1 + \frac{\text{me}_1(t)}{K_m}} \\
  & v_2 = k_{cat,2} \cdot \dot{\text{me}}_2(t) \cdot \frac{\text{me}_2(t)}{K_m} \frac{1}{1 + \frac{\text{me}_2(t)}{K_m}}
\end{align*} \]
Michaelis-Menten kinetics with the same $k_{\text{cat}}$ and different constants $Km_0$, $Km_1$, and $Km_2$ constants:

\[
D \begin{cases}
    v_0 = k_{\text{cat}} \cdot \dot{D} \cdot \frac{m_0(t)}{Km_0} \\
    v_1 = k_{\text{cat}} \cdot \dot{D} \cdot \frac{m_1(t)}{Km_1} \\
    v_2 = k_{\text{cat}} \cdot \dot{D} \cdot \frac{m_2(t)}{Km_2} 
\end{cases}
\]

Michaelis-Menten kinetics with product inhibition:

\[
E \begin{cases}
    v_0 = k_{\text{cat},0} \cdot \dot{D} \cdot \frac{m_0(t)}{Km_0} + \frac{m_1(t)}{Km_1} + \frac{m_2(t)}{Km_2} + \frac{m_3(t)}{Km_3} \\
    v_1 = k_{\text{cat},1} \cdot \dot{D} \cdot \frac{m_1(t)}{Km_1} + \frac{m_2(t)}{Km_2} + \frac{m_3(t)}{Km_3} \\
    v_2 = k_{\text{cat},2} \cdot \dot{D} \cdot \frac{m_2(t)}{Km_2} + \frac{m_3(t)}{Km_3} \\
\end{cases}
\]

### c. Reversible reactions (distributive)

In case of reversible kinetics, terms were added to the system of Eq. 3.2 that represent demethylation of $m_1$, $m_2$, and $m_3$ ($v_1^\prime$, $v_2^\prime$, and $v_3^\prime$, respectively; Eq. 3.12). Demethylase activity was integrated into the apparent rate constant $k_{r,\text{app}}$ (Eq. 3.13).

\[
\begin{aligned}
    \frac{d(m_0(t))}{dt} &= \gamma - \mu \cdot m_0(t) - v_0 + v_1^\prime \\
    \frac{d(m_1(t))}{dt} &= -\mu \cdot m_1(t) + v_0 - v_1 - v_1^\prime + v_2^\prime \\
    \frac{d(m_2(t))}{dt} &= -\mu \cdot m_2(t) + v_1 - v_2 - v_2^\prime + v_3 \\
    \frac{d(m_3(t))}{dt} &= -\mu \cdot m_3(t) + v_2 - v_3^\prime \\
\end{aligned}
\]

\[
\begin{cases}
    v_1^\prime = k_r \cdot \text{dem} \cdot m_1(t) = k_{r,\text{app}} \cdot m_1(t) \\
    v_2^\prime = k_r \cdot \text{dem} \cdot m_2(t) = k_{r,\text{app}} \cdot m_2(t) \\
    v_3^\prime = k_r \cdot \text{dem} \cdot m_3(t) = k_{r,\text{app}} \cdot m_3(t) 
\end{cases}
\]

### II. Single cell model for temporal methylation during the cell cycle

The system of Eq. 3.2, with the kinetic expressions of Eq. 3.3, also forms the basis of the single cell cycle models for yeast and trypanosomes. As explained in
the main text two and three cell cycle phases are defined for them, respectively, with histone synthesis restricted to the first phase (S). For the yeast model Dot1 activity is assumed constant during the cell cycle. For the trypanosome model(s) different expression profiles for the A and B isoforms of Dot1 over the three phases are proposed. Detailed information, including the corresponding parameter sets, can be found in (De Vos et al., 2011; Stulemeijer et al., 2015).