RESEARCH PAPER

Epigenetics 10:4, 293–302; April 2015; Published with license by Taylor & Francis Group, LLC

Cooperative stabilization of the SIR complex provides robust epigenetic memory in a model of SIR silencing in *Saccharomyces cerevisiae*

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Keywords: budding yeast, epigenetic inheritance, heterochromatin, mathematical modeling, nucleosome modifications, positive feedback

How alternative chromatin-based regulatory states can be made stable and heritable in order to provide robust epigenetic memory is poorly understood. Here, we develop a stochastic model of the silencing system in *Saccharomyces cerevisiae* that incorporates cooperative binding of the repressive SIR complex and antisilencing histone modifications, in addition to positive feedback in Sir2 recruitment. The model was able to reproduce key features of SIR regulation of an *HM* locus, including heritable bistability, dependence on the silencer elements, and sensitivity to SIR dosage. We found that antisilencing methylation of H3K79 by Dot1 was not needed to generate these features, but acted to reduce spreading of SIR binding, consistent with its proposed role in containment of silencing. In contrast, cooperative inter-nucleosome interactions mediated by the SIR complex were critical for concentrating SIR binding around the silencers in the absence of barriers, and for providing bistability in SIR binding. SIR-SIR interactions magnify the cooperativity in the Sir2-histone deacetylation positive feedback reaction and complete a double-negative feedback circuit involving antisilencing modifications. Thus, our modeling underscores the potential importance of cooperative interactions between nucleosome-bound complexes both in the SIR system and in other chromatin-based complexes in epigenetic regulation.

Introduction

Epigenetic gene regulation allows transient signals to create long-lived gene expression states, enabling cells to retain a memory of past environments and to pass this memory to their descendants. The best understood mechanism involves positive feedback circuits among diffusible regulators.[1-3] An alternative class of mechanisms involves modifications to the DNA itself, such as methylation of CpG,[4,5] or of histone proteins intimately associated with it.[6-9] Such chromatin-based epigenetic memory is less well understood but is believed to underlie a large proportion of long-lived gene expression states, both in health and disease.[10,11] Due to its *cis*-acting nature, chromatin-based epigenetic memory has the unique property of allowing 2 identical DNAs to remain in different expression states in the same cell, as seen in X-chromosome inactivation[12] or genomic imprinting.[13]

Epigenetic memory requires mechanisms that can generate bistability (at least 2 alternative states that are stable over time) and heritability (each state must be able to persist through DNA replication and cell division). Alternative states of diffusible regulators are inherited by the distribution of soluble cell components to both daughter cells, while DNA methylation is inherited by the distribution of one parental DNA strand to each daughter cell. Inheritance of nucleosome modification states is possible because parental nucleosomes are distributed to each daughter chromosome.[14,15] Achieving bistability is not trivial; it requires positive feedback with cooperativity (more precisely, ultrasensitivity)[2] and both states must be stable enough to survive fluctuations due to the noisy environment inside cells. In the case of nucleosome-based epigenetic memory, positive feedback is thought to be provided by a mechanism in which a histone post-translational modification recruits the enzyme that creates the same modification on nearby nucleosomes.[6,7] Indeed, a number of complexes involved in epigenetic regulation contain 'reader' and 'writer' modules for the same nucleosome modification.[16-18]

The Sir silencing system of *Saccharomyces cerevisiae*[19-21] is the best characterized model system for epigenetic regulation by nucleosome modification. SIR silencing appears simpler than

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Submitted: 12/18/2014; Revised: 01/26/2015; Accepted: 02/02/2015
http://dx.doi.org/10.1080/15592294.2015.1017200

analogous systems in more complex eukaryotes and is most clearly nucleosome-based, free of DNA methylation and RNAi, which contribute to heritable bistability in other systems. The Sir system prevents expression of a few kilobases of DNA at 2 loci, *HML* and *HMR*, which encode alternative mating-type genes. Repression is dependent on recruitment of the Sir2, Sir3, and Sir4 proteins by proteins bound to silencer DNA elements located on each side of these loci[19]. Most models of Sir silencing center around a positive feedback loop in which deacetylation of H4K16 by Sir2 creates high affinity nucleosomal binding sites for the Sir2-Sir3-Sir4 complex (SIR), allowing it to bind across the *HM* loci.[20,21]

The *HM* loci are stably silenced in wild-type strains, but weakening of the silencers can produce bistable behavior in which the locus alternates between active and inactive states, each of which can persist for tens of generations, generating variegated expression patterns.[22] Under these conditions, one *HM* locus can be active while the other is silenced,[23] confirming that the epigenetic state is encoded on the DNA. The Sir system also silences genes close to telomeres and can generate variegated expression of reporter genes near telomeres.[24]

We and others have developed mathematical models of the SIR system.[25-27] Mathematical approaches provide a rigorous way to check that the proposed mechanisms produce the observed behaviors, to identify critical and perhaps general features of the system, and to suggest modifications and further tests of existing models. Mathematical models necessarily make simplifying assumptions but can nevertheless provide general insights.[28] For example, a common feature of these models is the need for positive feedback that is non-local (that is, works beyond neighbor nucleosomes) and is cooperative.[7]

Our most recent SIR model was able to reproduce the bistable behavior of an idealized *HM* locus, its dependence on the silencers, and the ability of simple barriers to inhibit spreading of silencing modifications.[26] However, the behavior of the model was not robust, being very sensitive to small changes in the parameters. It also employed a somewhat complicated recruitment of Sir2 activity, and inhibition of spreading required special properties for the DNA outside the *HM* locus, specifically, the presence of multiple antisilencer elements.

Here, we developed a more realistic model of an *HM* locus by explicitly including binding of the SIR complex, and by considering the multiple nucleosome modification states that arise from the dimeric nature of the nucleosome and from the presence of ubiquitous antisilencing nucleosome modification by Dot1.[29] We find that simple interactions between SIR complexes bound to separate nucleosomes aid bistability and can provide localization of SIR around the silencers in the absence of barriers.

Results and Discussion

Modeling approach and rationale

Two-step deacetylation. Our previous model simplified the system by defining only 2 relevant nucleosome modification states, acetylated (active) and unacetylated (silenced), with a one-step deacetylation-acetylation interconversion reaction.[26] We now incorporate the fact that the nucleosome has 2 copies of each histone, giving 3 nucleosome types created by the acetylated-unacetylated state of H4K16 (**Fig. 1**). This makes the positive feedback in the Sir2 deacetylation reaction a multistep process that can potentially provide the cooperative positive feedback needed for bistability,[7] removing the need for an intrinsically cooperative Sir2 enzyme.[26]

Dot1 methylation. In vitro studies indicate competition between Dot1 methylation of H3K79 and Sir binding to nucleosomes. H3K79 methylation inhibits Sir3 binding to isolated nucleosomes[30] and also reduces binding of the Sir2-3-4 complex on polynucleosomal templates.[31] Nucleosome methylation by Dot1 is also blocked by Sir3.[30,32] This seems to be the extent of the interaction, since there is no evidence that H3K79me inhibits Sir2 deacetylation of the same nucleosome, and Dot1 itself does not seem to compete with Sir binding, as overexpression of catalytically inactive Dot1 does not inhibit silencing *in vivo*.[33] Also, Sir2 deacetylation does not seem to directly inhibit Dot1 action, which is insensitive to the status of the H4 tail lysines.[30] In our model, H3K79me inhibits Sir binding and Sir binding inhibits methylation of H3K79 (**Fig. 1**). No enzyme for demethylation of H3K79 has been identified; therefore, loss of methylation seems to occur through nucleosome or histone replacement during DNA replication[34] or exchange reactions.

Cooperative binding of the Sir complex. The Sir2, Sir3, and Sir4 proteins form a complex in solution[35] that interacts with isolated nucleosomes and polynucleosomes.[16,36] However, since the protein-protein and protein-DNA contacts involved in assembling the SIR-nucleosome complex are only partially understood, we do not attempt to model SIR complex assembly in detail, but make 2 assumptions about SIR binding: (1) A SIR complex binds to a single nucleosome only if the nucleosome is fully deacetylated and demethylated (UU/uu; **Fig. 1**), a property that could be achieved biochemically if SIR complex binding to each half-nucleosome were highly cooperative and these modifications strongly inhibit binding; (2) A SIR complex bound to one nucleosome can be stabilized by cooperative contacts with other SIR-bound nucleosomes.

Such intra- and inter-nucleosomal contacts are similar to those proposed for the HP1/SWI6 protein.[37] Sir3 and Sir4 both have nucleosome binding activity as well as interaction interfaces that could potentially mediate such cooperative intra- and inter-nucleosome contacts. Sir3 appears to be the primary histone modification-dependent nucleosome binding component.[16,38] Crystal structures of the Sir3 N-terminal BAH domain bound to the nucleosome show interactions with the H4 N-terminal tail (including H4K16) and the region including H3K79.[39,40] Sir3 dimerizes via its C-terminal winged-helix (wH) domain, and loss of the wH domain reduces Sir3 binding to nucleosome arrays *in vitro*.[36] This Sir3-Sir3 interaction is critical, since removal of the wH domain abolishes silencing, and this defect can be restored by a heterologous dimerization domain.[36] Sir4 also interacts with nucleosomes, though in a modification-independent manner *in vitro*,[16] and dimerizes via its C-terminal coiled-coil dimerization domain.[41] The Sir4 C-terminal domain also interacts with Sir3,

most likely within the Sir3 AAA-like domain,[42] potentially bridging between Sir3 molecules.[16] The Sir4-Sir3 interaction is critical for silencing,[35,42] but since this interaction is needed to recruit Sir2 to the nucleosome-bound complex, it is not clear whether it also forms important contacts that stabilize Sir3 or Sir4 binding to nucleosomes. The existence of SIR-mediated intra-nucleosome contacts is supported by electron microscopic observations of filamentation or clumping of multi-nucleosome arrays by SIR *in vitro*.[16,38]

In the model, inter-nucleosome contacts have the effect of reducing the rate of SIR unbinding from the nucleosome, a feature we term cooperative stabilization. There are a number of different ways to model this feature, depending on the higher order structures allowed, which are currently unknown. We thus model cooperative stabilization simply, by allowing each SIR-bound nucleosome a chance to interact either with the nearest SIR-bound nucleosome or silencer to its left (along the DNA) as well as with the nearest SIR-bound nucleosome or silencer to its right, essentially assuming 2 interaction interfaces per SIR-bound nucleosome (**Fig. 1**). The probability of interaction is assumed to be inversely proportional to the distance to these nucleosomes. Thus, SIR will more often dissociate from a nucleosome with no other SIR-bound nearby than it will if the nucleosome is in a cluster of SIR-bound nucleosomes.

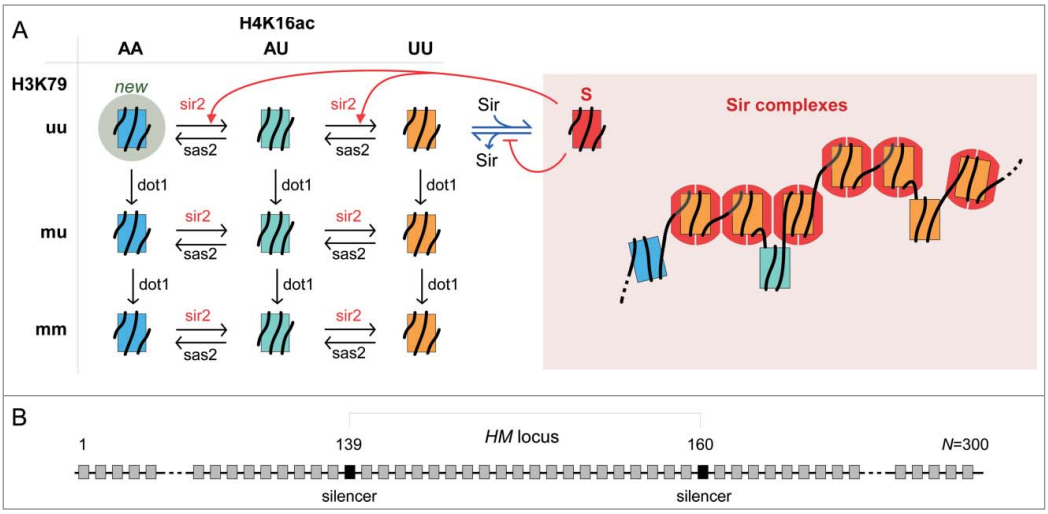

Figure 1. Model for nucleosome states and their interconversion in Sir silencing. (**A**) Nine modification states are produced by acetylation of both, one or neither of the 2 H4K16 positions per nucleosome, combined with methylation of neither, one or both H3K79 positions. The rate of the methylation reaction is determined by the parameter *dot1*; demethylation is assumed to be absent. The rate of acetylation is determined by the parameter *sas2*. Some deacetylation occurs constitutively (parameter *hdac*). None of these reactions are affected by modification state. Only unmodified nucleosomes may bind the SIR complex (Sir2-Sir3-Sir4), giving the tenth nucleosome state (S), with the rate of binding dependent on the free SIR concentration. A SIR-bound nucleosome recruits Sir2 that can deacetylate any other nucleosome in the system with a probability that is inversely proportional to the linear distance (number of nucleosome steps) between them. SIR-bound nucleosomes are resistant to methylation or acetylation. A SIR complex bound to one nucleosome may interact with 0, 1, or 2 other S nucleosomes in a distance-dependent manner, with the probability of dissociation reducing with increasing number of contacts (see text). Thus, S nucleosomes tend to inhibit the dissociation of SIR complexes from other S nucleosomes (red barred arrow). Non-S nucleosomes are subject to an exchange reaction (parameter *exch*), where they are replaced with a 'new' AA/uu nucleosome. During DNA replication each nucleosome in the system is, with a 50% probability, replaced with an AA/uu nucleosome. Any S nucleosome that is not replaced is converted to a UU/uu (unmodified) nucleosome, i.e., SIR complexes are removed by replication. (**B**) The *HM* genomic region in the model consists of 300 positions: 298 nucleosomes, and 2 silencers located 20 nucleosomes apart. Silencers are effectively permanently unmodified nucleosomes and can thus always bind SIR complexes. However, the probability of SIR dissociation from silencers (determined by the *siloff* parameter) can be lower than from nucleosomes. SIR-bound silencers act like S nucleosomes in their SIR-SIR interactions. SIR is removed from the silencers during replication but silencers are not subject to nucleosome exchange.

SIR binding to the silencers. In our previous model, the silencers were positions that recruited Sir2.[26] Here, we allow the silencers to also serve as nucleation points for SIR complex formation, consistent with their known mode of action.[43,44] Effectively, silencers act in the same way as nucleosomes, except that they can bind SIR more strongly than nucleosomes (by a reduced SIR off-rate) and, since silencer proteins are not histones, we assume they are not subject to inhibitory histone modifications. SIR dissociation from a silencer, as from nucleosomes, is inhibited by SIR-mediated interactions with other SIR-bound nucleosomes or the other SIR-bound silencer. A SIR-bound silencer has the same Sir2 activity as a SIR-bound nucleosome.

SIR dosage and spreading. The current model does not invoke any differences between the DNA inside or outside the *HM* locus. The only distinct DNA elements available for containing SIR spreading are the silencers themselves. The model also takes some account of the limiting abundance of Sir3 protein for silencing.[19,45] With 1,400 Sir3 proteins per haploid cell,[46] there is only enough for ~20 Sir3 dimers for each of the 34 Sir3 silenced regions (32 telomeres + 2 *HM* loci). In our standard model, we make a larger pool of 50 SIR complexes available for binding to the *HM* locus, reflecting some ability for the locus to compete SIR away from telomeres.

Simulations

The system comprises a string of $L = 300$ nucleosomes, equivalent to a genomic region of ~60 kb. Each nucleosome can be in one of 10 different states (**Fig. 1**). Nine states are generated by alternative acetylation states at the 2 H4K16 residues of each nucleosome (AA, AU, UU), combined with alternative methylation states at the 2 H3K79 residues of each nucleosome (mm, mu, uu). All these 9 states are assumed to be associated with

transcriptional activity. The tenth nucleosome type (S) is a UU/uu nucleosome with the SIR complex bound; this is assumed to be associated with transcriptional silencing.

Two 'nucleosome' positions are used to represent the silencers and are treated specially. These are located at positions 139 and 160 (i.e., with 20 nucleosomes between), reflecting the spacing of the *E* and *I* silencers at *HML*.[47] Silencers have only 2 states, SIR bound (S) or unbound (U).

Between DNA replications, nucleosomes and silencers are interconverted between the different states by 7 processes depending, in some cases, on the states of other nucleosomes in the system. The simulation uses a Gillespie algorithm, with each process selected randomly with a rate defined by a specific parameter:

- *sas2* - **Histone acetylation**. A random nucleosome is chosen. If it is in the UU or UA state, the nucleosome is moved one step toward the fully acetylated state, AA (i.e., UU→AU or AU→AA). The methylation status is not changed. We assume that SIR-bound nucleosomes are not subject to acetylation, as the close contact between Sir3 and the H4 tail in the Sir3-BAH-nucleosome complex[39,40] seems likely to make the tail inaccessible.
- *dot* – **Histone methylation**. A random nucleosome is chosen. If it is in the uu or um state, the nucleosome is moved one step toward the fully methylated state, mm (i.e., uu→mu or mu→mm). The acetylation status is not changed.
- *hdac* – **Histone deacetylation**. A random nucleosome is chosen. If it is in the AA or AU state, it is moved one step toward the fully deacetylated state, UU (i.e., AA→AU or AU→UU). The methylation status is not changed.
- *sir2* – **Sir2-mediated histone deacetylation**. A random position is chosen. If it is in the S state (whether a nucleosome or the silencer), a second position is chosen a random distance away from the first. To reflect the effect of DNA separation on contact efficiency,[48] this distance step x is chosen with a probability $1/x$ normalized by the sum $1/1 + 1/2 + \ldots + 1/300$. The second position is selected to be x steps to the left or right (with equal probability) of the first position. If the second position is within the system (1,300) and is in the AA or AU state, it is moved one step toward the fully deacetylated state, UU (i.e., AA→AU or AU→UU). The methylation status is not changed.
- *exch* – **Nucleosome exchange**. A random nucleosome is chosen. If it is not in the S state, it is converted to the AA/uu state.
- *SIRon* – **SIR binding**. (*totalSIR*). A random position is chosen. If it is in the UU/uu state or is a silencer in the U state, it is converted to the S state with a probability *freeSIR*/*totalSIR*, where *freeSIR* is *totalSIR* minus the number of S nucleosomes or silencers. That is, there is a pool of *totalSIR* complexes available for binding to this genomic region.
- *SIRoff* – **SIR dissociation**. (*SIRcoop*, *siloff*). A random position is chosen.
    - If the position is a nucleosome in the S state, the dissociation step is attempted. SIR dissociation occurs only if the nucleosome is not engaged with another S nucleosome or S silencer, which we determine by an engagement test, as follows. An S nucleosome is assumed to have 2 interfaces able to contact other S nucleosomes. We want to allow long-range (beyond nearest-neighbor) interactions, but this generates a very large number of potential ways in which multiple S nucleosomes could engage with each other in 3 dimensions, and the chromatin folding rules for such structures are unknown. We thus make a computationally feasible estimate of the probability of an S nucleosome being unengaged, by determining the probability that the nucleosome is both free from binding to the nearest S nucleosome or S silencer to its left and free from binding to similar sites to its right. The relative probability of it being bound on the left side is $l = SIRcoop/x\_left$, where *SIRcoop* is a cooperativity factor and *x_left* is the distance to the nearest S nucleosome or S silencer on the left. The relative probability of it being bound on the right side is $r = SIRcoop/x\_right$. The probability of being bound to neither is calculated as $(1+h)/(1+l+r+l.r+h)$, where $h = SIRcoop/(x\_left + x\_right)$ is the relative probability that the 2 flanking S positions are engaged to each other but not the central position. (That is, we compare the 2 states where the S nucleosome is unbound to all 5 possible engagement states of the 3 S nucleosomes). This calculation produces a roughly proportional decrease in the probability of an S nucleosome being unengaged as the number of S nucleosomes in the system increases. Dissociation results in the S state nucleosome being converted to the UU/uu state.
    - If the position chosen is a silencer, then the SIR dissociation step is aborted with a probability 1–*siloff*. Thus, when $siloff < 1$, the silencer binds SIR more strongly than a nucleosome. Dissociation from the silencer is subject to the engagement test above, and results in the S state silencer being converted to the U state

Each of the reaction rates is scaled by the number of nucleosomes in the system and the generation time. Thus, $hdac = 1$ means that on average 1 deacetylation attempt is made per nucleosome per generation, $SIRoff = 3{,}000$ means that on average 3,000 SIR dissociation attempts are made per nucleosome per generation. Note that the number of completed reactions is substantially fewer than the number of attempts because the chosen nucleosomes are often unsuitable for the chosen reaction.

Once a generation time is reached, DNA replication is simulated by converting the nucleosome string to that which would form on one of the daughter DNA strands, according to random distribution of parental nucleosomes[14,15] and filling of gaps with acetylated, unmethylated nucleosomes, as new histones are rapidly acetylated in *S. cerevisiae*.[49,50] Effectively, each nucleosome has a 1/2 probability to be replaced by an AA/uu nucleosome. In

the standard model, we remove all SIR complexes from parental nucleosomes and silencers.

SIR binding can be contained near the silencers without the need for barriers

The model was able to reproduce a stable, replication-robust high SIR occupancy of the *HM* region in the presence of active silencers, the SIR proteins, H4 acetylation and H3K79 methylation (**Fig. 2A**). SIR binding was dynamic, generally extending over a ~10 kb region that changed position over time but retained reasonably dense SIR binding between the silencers.

No specific barriers were needed to restrict SIR to the *HM* region. Essentially the limited availability of SIR proteins and the strong SIR-SIR interactions, both direct and via histone modifications, combine to create a patch of SIR binding that prefers to overlap the silencers.

Specific barrier elements, which are not included in our model, are likely to constrain spreading of silencing-associated features at real *HM* loci.[51] However, considerable spreading of these features is evident in ChIP-chip data deposited in the SGD database,[52] showing a ~7–10 kb region of elevated Sir2 occupancy at *HMR* and *HML*,[53] a ~7 kb region of low H4ac at *HMR*,[54] and a ~6 kb region of low H3K79me at *HMR*.[55] ChIP-seq mapping of H4K16ac also showed low acetylation extending 1–3 kb beyond the silencers.[56]

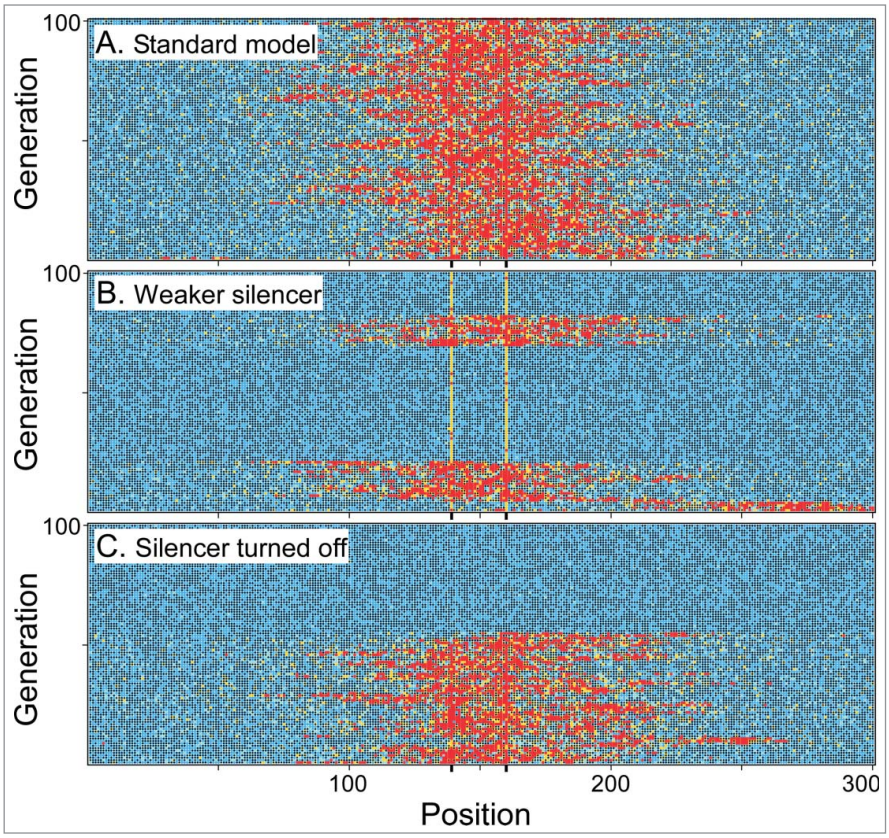

Figure 2. Containment of silencing and its control by the silencers. (**A**) A space-time plot, showing the evolution of the 300 position nucleosome string (horizontal) over 100 generations (vertical) with the standard model. The silencer positions are 139 and 160. Nucleosome acetylation and SIR binding status at the end of each generation (before DNA replication) are displayed: AA/** – blue; AU/** - cyan; UU/** or U silencer– orange; S nucleosome or silencer – red. * indicates that the acetylation/methylation status is ignored. Methylated nucleosomes are marked with a black dot. The standard parameter values are: *sas2* = 100, *dot* = 2, *hdac* = 1, *sir2* = 500 , *exch* = 1, *SIRon* = 100, *totalSIR* = 50, *SIRoff* = 3,000, *SIRcoop* = 500, *siloff* = 0.2. (**B**) Weakening the silencer produces bistable behavior. Parameters were as for B, except that the silencers were weakened by making the strength of SIR binding equal to that of a UU/uu nucleosome by setting *siloff* = 1 (the silencers remained immune to acetylation or deacetylation). (**C**) SIR binding is silencer dependent. At generation 50, the silencers were turned off by making positions 139 and 160 act as simple nucleosomes (having normal SIR binding and being subject to histone modifications).

The model also suggests that the regions flanking *HM* loci could experience substantial fluctuations in SIR occupation, with high and low SIR states stable for a few generations. This is consistent with observations that reporter genes inserted adjacent to *HM* silencers can show the variegated expression resulting from heritable bistability.[57] Spreading of silencing-associated features and variegated reporter expression is also seen for SIR silencing at telomeres.[24]

By weakening the activity of the silencers in the model it was possible to make the system bistable, with stable silenced and active states persisting for ~20 generations (**Fig. 2B**). This is consistent with experimental observations for *sir1* and other mutants that decrease SIR complex recruitment to the silencers.[22,23,58,59] The model predicts that genes lying adjacent to the *HM* region would also be silenced or active in a bistable fashion when the silencers are weakened.

The model also reproduces the experimental observation that association of SIR with the silencers is more efficient under conditions when the surrounding chromatin is silenced[43] (**Fig. 2B**), supporting the idea that recruitment of the SIR complex to silencers is stabilized by interactions with deacetylated nucleosomes.[20]

Importantly, high level SIR binding and the H4K16 hypoacetylation and H3K79 hypomethylation in the model are completely dependent on the silencers; silencer inactivation results in a uniform high level of H4 acetylation and H3K79 methylation across the whole region (**Fig 2C**).

The effect of SIR dosage

Alterations in the levels of SIR proteins are known to affect the degree of silencing at the *HM* loci and telomeres.[19,45] Increasing Sir3 levels leads to a greater strength and extent of Sir3 ChIP signals at telomeres.[60,61]

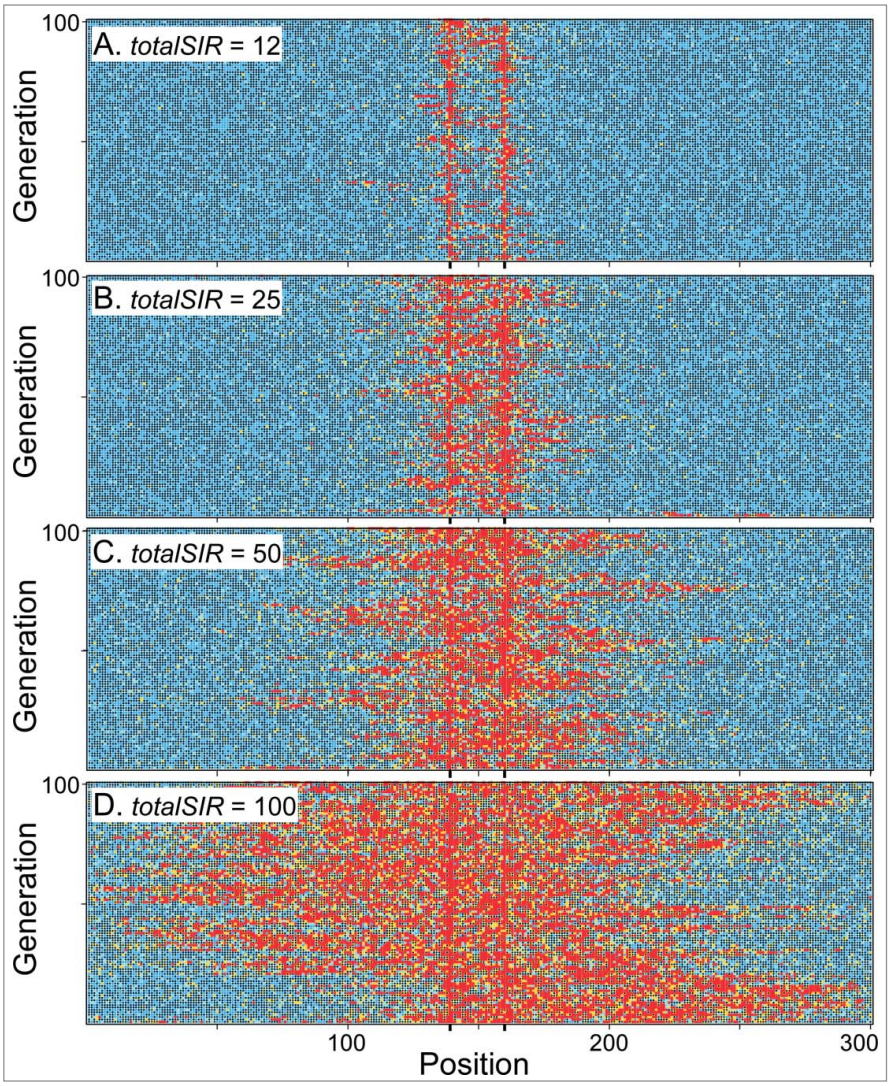

Figure 3. Effect of Sir dosage on silencing. The *totalSIR* parameter was varied from 12 (**A**), 25 (**B**), 50 (**C**, the standard value), and 100 (**D**).

The model reproduces this sensitivity to SIR dosage (**Fig. 3**). Reducing the amount of available SIR complex by 4-fold resulted in an almost complete loss of SIR occupation of the *HM* locus, with remaining SIR binding closely associated with the silencers (**Fig. 3A**). Halving the SIR availability produced moderate bistability of SIR occupation and H4 deacetylation within the *HM* locus (**Fig. 3B**). Doubling available SIR levels increased the extent of the SIR-bound region, while maintaining the mobility of this region. A strong effect of available SIR concentration on containment of silencing is also seen in other models.[25-27]

The role of antisilencing modification by Dot1

Deciphering the *in vivo* role of Dot1 methylation of H3K79 in SIR silencing has been complicated by artifactual effects of Dot1 removal on a widely used telomeric silencing reporter construct.[62] However, most experiments not involving this reporter are consistent with mild effects of Dot1 removal on silencing. *URA3* and *GFP* reporters placed within *HML* or *HMR* have shown either slightly increased expression[63] or no change in expression[33,64] in *dot1*Δ strains. Expression of *ADE2* or *GFP* reporters placed adjacent to *HMR* increased slightly in *dot1*Δ strains.[57] The lack of Dot1 did not derepress endogenous *MAT* genes,[55,64] and expression of natural telomeric genes was not strongly affected.[55,62]

Complete removal of Dot1 activity in the model caused a moderate increase in spreading of SIR binding, as shown by time-averaged profiles (**Fig. 4A, B**) and space-time plots (**Fig. 4C, D**), in accordance with the proposal that methylation of H3K79 by Dot1 helps restrict the spreading of silenced chromatin.[29] There was a consequent slightly lower overall density of SIR occupation in and adjacent to the *HM* locus, and a slightly higher density of SIR occupation at sites further from the silencers. The lack of large changes in SIR density upon removal of *Dot1* methylation is consistent with the observed mild effects of the *dot1*Δ mutation.

We also used the model to simulate increased Dot1 activity. Overexpression of Dot1 restored growth on uracil-deficient media when URA3 genes were inserted at *HML* or *HMR*,[63] and inhibited establishment of silencing of a *GFP* reporter at *HMR*.[33] We found that very high Dot1 activity could eliminate SIR binding in the model (not shown). Interestingly, a moderate increase in Dot1 activity produced weak bistability (**Fig. 4E**), with stabilities of low-SIR and high-SIR states possibly sufficient to be detected by single-cell assays or even colony variegation assays.

Dot1 methylation sharpens the contrast between SIR-bound and SIR-unbound regions because it injects additional positive feedback into the system. As pointed out by Ng et al.,[61] Dot1 provides a double-negative feedback loop in which methylation of H3K79 inhibits SIR binding, and SIR binding inhibits methylation of H3K79. This results in methylation at one nucleosome making methylation more likely at other nucleosomes. Cooperative stabilization due to SIR-SIR inter-nucleosomal contacts provides a more direct way to complete the Dot1 feedback loop, because reduced SIR binding at one nucleosome due to H3K79me can directly destabilize SIR binding to other nucleosomes, increasing their availability for methylation. The same is true for any modification that both inhibits SIR binding and is inhibited by SIR binding.

It has also been proposed that H3K79me is involved in an additional positive feedback loop, where H3K79me stimulates transcriptional activity (through its inhibition of SIR binding)

and transcriptional activity in turn stimulates H3K79 methylation via Dot1 association with transcription.[65] We explored this Dot1 recruitment feedback mechanism by making Dot1 action dependent on pre-existing H3K79me in the simulations. That is, when the *dot* action is chosen, 2 adjacent nucleosomes are chosen at random and if both of these are non-S (active) then one of the neighbors (to left or right) is methylated one step toward the mm state. The effect of this implementation is that a patch of non-S nucleosomes, where transcriptional activity should be higher, has a chance to maintain itself and even spread. Addition of this Dot1 feedback loop sharpened the contrast between the *HM* locus and outside regions and significantly improved the stability of the SIR-ON and SIR-OFF states when the silencers were weakened (**Fig. 4F**, compare with **Fig. 2B**). Thus, in our model, this additional positive feedback makes the system more switch-like but is not essential. In contrast, a Dot1 recruitment mechanism was necessary for bistability in the SIR model of Mukhopadhyay and Sengupta.[27]

The contribution of SIR cooperative stability to silencing and its containment

Cooperation between SIR complexes has a strong impact on the behavior of the *HM* locus in our model, contributing strongly to stable SIR binding and localization. Removing cooperative stabilization without compensation abolished SIR binding. SIR binding could be restored in the model by increasing the strength of SIR binding to individual nucleosomes. However, SIR binding became very dispersed, with SIR density between the silencers only slightly higher than in the outside region (**Fig. 5A**). In the absence of cooperative stability, we were unable to find any parameters that gave a high contrast between SIR binding within and outside the *HM* locus.

Cooperative stabilization provides positive feedback in SIR occupation, which stabilizes both low and high densities of SIR binding, allowing an increased contrast between the *HM* locus and the outside region. A direct positive feedback loop results because the presence of a SIR complex bound to one nucleosome makes it more likely that a SIR complex will remain

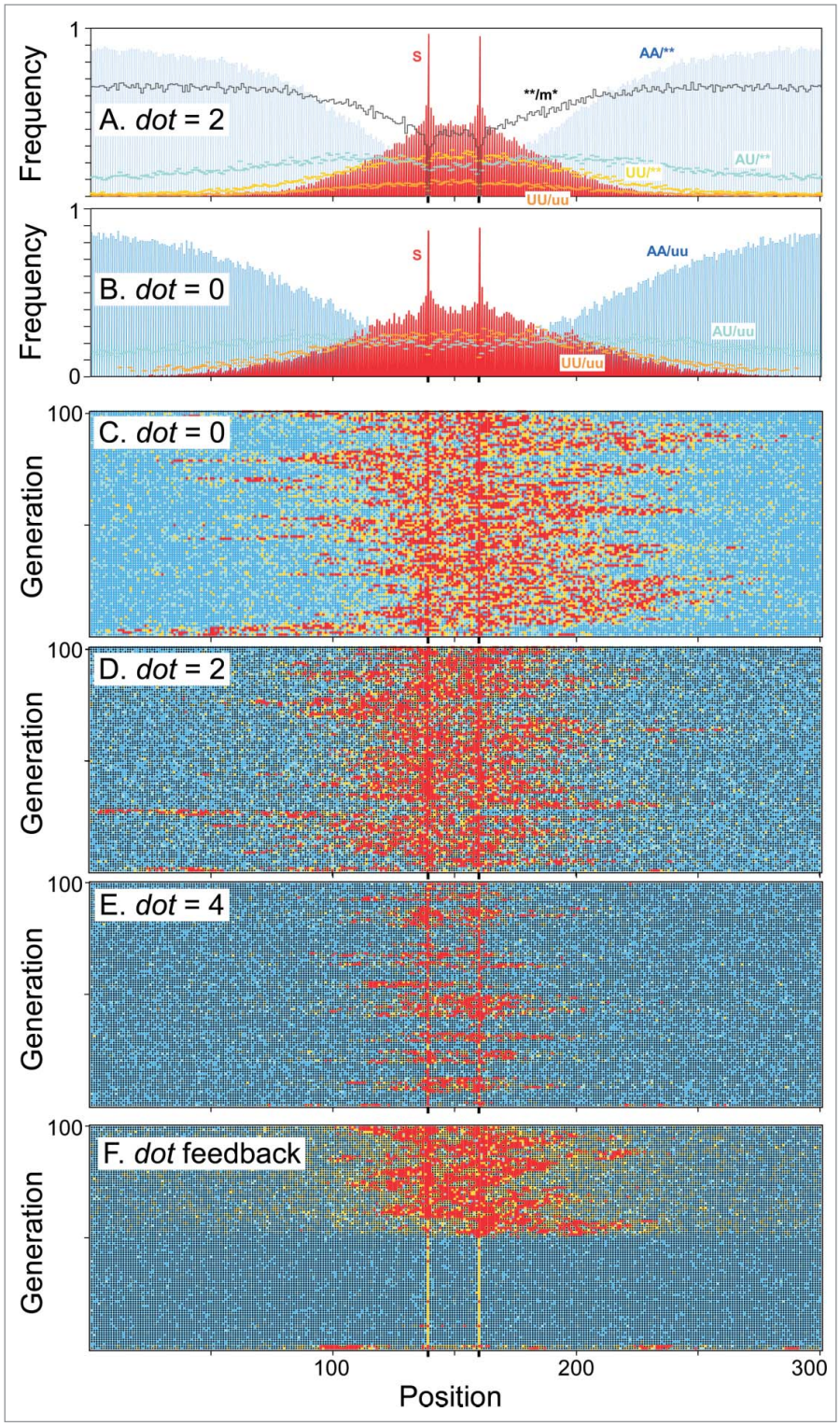

Figure 4. Effect of Dot1 methylation on silencing. (**A**) Histograms of the time-averaged fraction of the status of each position over 200 generations for the standard model (dot = 2). AA/**- blue bars; AU/** - cyan dots; UU/** - yellow dots; UU/uu – orange dots; **/m* - black line; S - red bars. * indicates that the acetylation/methylation status is ignored. (**B**) As (A) except *dot* = 0 (*dot1*Δ). AA - blue bars; AU - cyan dots; UU - orange dots; S - red bars. C-E. Space-time plot (as **Fig. 2A**) with (**C**) *dot* = 0 (*dot1*Δ), (**D**) *dot* = 2 (standard), and (**E**) *dot* = 4. F. A model that includes recruitment of Dot1 by H3K79me nucleosomes can increase bistability. *dot* = 10, *sas2* = 50, *siloff* = 0.8.

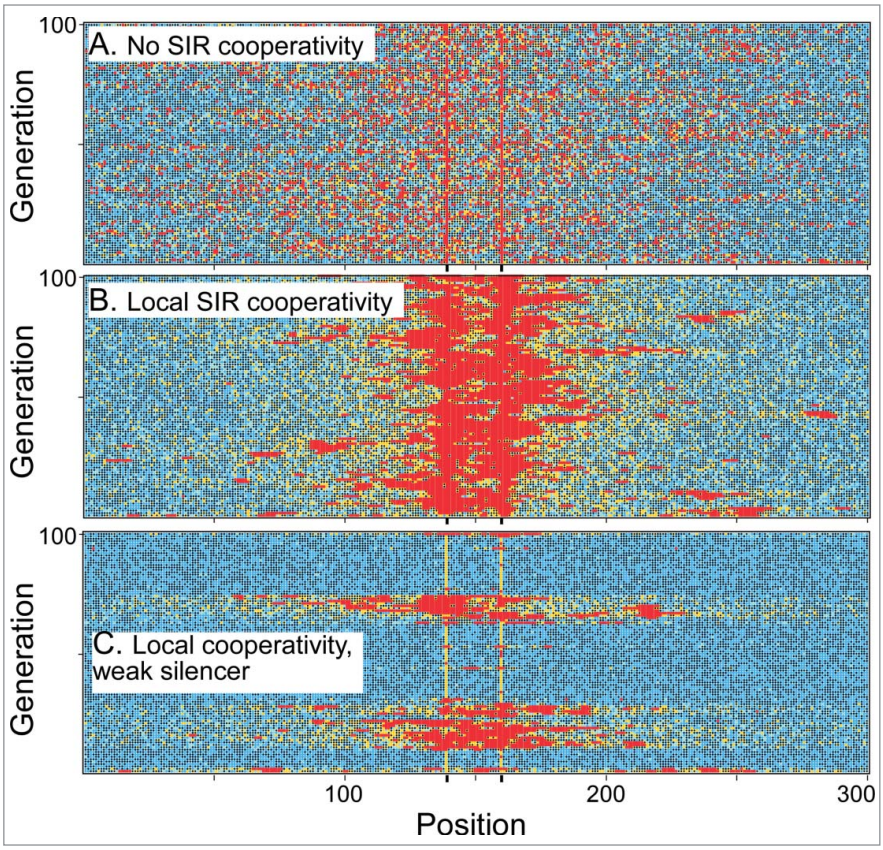

Figure 5. Cooperative stabilization helps localize SIR. (**A**) Loss of localized SIR binding in the absence of SIR-SIR inter-nucleosome interactions. *SIRcoop* = 0, *SIRon* = 100, *SIRoff* =10, *siloff*= 0.2. Figure elements as in **Figure 2B**. (**B**) Localization of SIR binding when SIR-SIR cooperativity is local, that is, limited to adjacent nucleosomes. *SIRoff* = 2,000, *SIRcoop* = 3,000, *Sir2* = 800, *sas2* = 90, *siloff* = 0.2 (strong silencer). (**C**) Local SIR-SIR cooperativity can give bistability when the silencers are weakened. As B, except *siloff* = 1.

bound to another nucleosome in the vicinity. Indirect positive feedback loops also result because cooperative stabilization connects the effects of SIR-favoring or SIR-inhibiting histone modifications on one nucleosome to increased probabilities of those modifications on nearby nucleosomes.

Our standard scheme for cooperative stability allows long-range SIR-stabilizing contacts between SIR-bound nucleosomes, which is consistent with the 3-dimensional clumping of SIR-complexed nucleosome arrays *in vitro*.[16] In the model, the probability of contact decreases with increasing separation of the bound nucleosomes along the DNA, consistent with *in vivo* measurements.[48] This decreasing cooperativity with distance allows different regions of the system in the model to behave somewhat independently, enabling a stable high-SIR state in the *HM* locus to coexist with a low-SIR state outside.

We also tested the effect of a more restricted cooperativity scheme, where a SIR-nucleosome complex is stabilized only if one or other of its *adjacent* nucleosomes is also SIR-bound. We found that dense SIR binding could be reasonably well confined over the *HM* locus with such local inter-nucleosomal SIR-SIR interactions (**Fig. 5B**), and such a system also displayed bistable behavior when the silencer was weakened (**Fig. 5C**). Thus, cooperative stabilization can be effective even if inter-nucleosomal SIR-SIR interactions are restricted to adjacent SIR-bound nucleosomes. In this situation, the long-range positive feedback requirement for a bistable system[7,26] is provided by recruited Sir2 acting to deacetylate distant nucleosomes.

Conclusions

Key system behaviors of a SIR-silenced *HM* locus can be reproduced by a discrete stochastic model that incorporates: (1) a 2-step SIR-mediated deacetylation reaction capable of acting beyond adjacent nucleosomes; (2) cooperative binding of the SIR complex, also with interactions beyond adjacent nucleosomes; (3) ubiquitous modifications that inhibit, and are inhibited by, SIR binding; (4) limited availability of the SIR complex; and (5) silencer elements that act solely by recruiting the SIR complex. The model displayed localization of SIR binding around the *HM* region and a lack of binding to non-silenced regions, without invoking barrier elements or different properties of DNA inside and outside the locus. The system was sensitive to SIR dosage and relatively insensitive to loss of Dot1 methylation. Critically, the model reproduced the dependence on the silencer elements, with weakening of the silencers producing stable and heritable SIR-bound and SIR-free states. The model predicts that other perturbations, such as reduced SIR dosage or increased Dot1 activity, can expose the bistability inherent in the system. This could be tested by experiments in which the expression of Sir or Dot1 genes were modulated by controlled induction, for example using the TET system.[66] Mukhopadhyay and Sengupta[27] incorporated similar processes in a model of SIR binding at telomeres, and came to similar conclusions, although the stability of alternative states through DNA replication was not examined in their model.

We have made the assumption that SIR binding is completely inhibited by H4K16 acetylation or H3K79 methylation. However, it is unlikely that this inhibition is absolute. SIR complexes retain some ability to bind enzymatically modified nucleosomes *in vitro*[16,31,67] (though the modifications may have been incomplete in these experiments). Any reduction in the SIR binding differential due to histone modifications that are removed by or inhibited by SIR, reduces positive feedback in the system and hinders bistability, especially if the bound SIR is active in deacetylation. Further work is needed to explore the binding differential required for proper system behavior.

Simple cooperative interactions between SIR complexes bound to different nucleosomes are critical in our model for localization of SIR binding to the *HM* locus and for bistable SIR binding. Although other researchers have treated SIR binding in their models,[25,27] an explicit protein-bound state was not included in our previous models. We found that addition of this state made it easier to find parameter values capable of generating heritable bistability, often the most stringent requirement for such models. SIR-SIR interactions provide positive feedback interactions that link the state transition probabilities of a nucleosome to the state of other nucleosomes in the system. This positive feedback provides for strong contrast between SIR-bound and SIR-unbound states. The decay of interaction probability with distance, coupled with the same distance dependence in Sir2 deacetylation, promotes the formation of clusters of SIR-bound and SIR-unbound nucleosomes that can stably coexist in reasonable proximity.

Cooperative interactions between SIR-bound nucleosomes have long been invoked in SIR spreading.[44] 'Lateral interactions' between bound SIR complexes were proposed to explain the decreased occupation of the silencers by SIR under conditions where SIR binding to nearby nucleosomes is reduced,[20,43] behavior that is also reproduced in the model. However, definitive evidence for cooperativity between SIR-bound nucleosomes has not been reported. Though we favor a model where these cooperative interactions can occur beyond nearest neighbors, we show that reasonable SIR localization, as well as heritable bistability, can be obtained when SIR-SIR interactions are limited to adjacent nucleosomes, and Sir2 deacetylation provides the sole long-range reaction in the system. Nucleosome-interacting protein complexes with the potential for such cooperative interactions are involved in other histone modification-based epigenetic memory systems, such as the HP1/SWI6 and CLRC complexes in Schizosaccharomyces pombe[18,37,68] and the PRC1, PRC2, and TrxG complexes in *Drosophila*.[17] Better understanding of the intra- and inter-nucleosome interactions mediated by these complexes will be critical for understanding how they confer epigenetic memory.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Danesh Moazed, Chenning Lu, Mohammed Altaf, and Kevin Struhl for discussions.

Funding

Kim Sneppen was supported by the Danish National Research Foundation through the Center for Models of Life. Ian Dodd was supported by Australian NHMRC grant GNT1025549.

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