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Heterochromatin maintenance and establishment: lessons from the mouse pericentromere

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Abstract

Defined as a chromatin structure that remains condensed throughout the cell cycle heterochromatin is generally transcriptionally silent and is characterized by a specific molecular signature. Constitutive heterochromatin at the pericentromere is a conserved feature throughout evolution, which impacts genome stability. Here, we will summarize recent advances in our understanding of the dynamics of mouse pericentric heterochromatin during the cell cycle and development. Comparison with heterochromatin maintenance in fission yeast will enable discussions of the common basic principles and various mechanisms exploited in the distinct organisms.

Words: 83

Introduction

Initially discovered based on differential staining with DNA dyes, heterochromatin is defined as chromosomal domains that, in contrast to euchromatin, remain condensed throughout the cell cycle [1]. Studies in different model organisms have identified heterochromatin regions close to centromeres and telomeres, which make up constitutive heterochromatin [2]. These forms of heterochromatin are critical for genome maintenance and are considered to be vital for repression of transposable elements. Especially, heterochromatin that forms at repetitive

elements at the pericentromeres has been characterized extensively and emerged as the paradigm for constitutive heterochromatin. Here, we will summarize our knowledge on pericentric heterochromatin establishment and dynamics during murine development. We will also compare the features of pericentric heterochromatin in fission yeast and mice to illustrate possible common principles of heterochromatin maintenance.

While formation of a repressive chromatin environment at pericentric repeats is evolutionarily conserved, in different organisms distinct combinations of chromatin marks characterize pericentric heterochromatin at the molecular level. These chromatin marks include DNA methylation, specific histone variants, hypoacetylation and repressive histone modifications, in particular H3K9 methylation, which constitutes a binding site for the Heterochromatin protein 1 (HP1) family. A shared element, whose exact importance remains to be elucidated, is the clustering of pericentric heterochromatin into chromocenters in interphase nuclei (Figure 1). This spatial arrangement, which can compartmentalize heterochromatin away from the rest of the genome, might serve to create a local environment where silencing factors could possibly concentrate and help maintain the heterochromatin status. In fission yeast, the three chromosomes bring their centromeres together in a single chromocenter [3] while in mouse as well as in certain plants such as *Arabidopsis*, the arrangement of the chromosomes into subgroups in interphase gives rise to several chromocenters [4, 5]. Interestingly, chromocenter organization is globally maintained even through S-phase [6] and dissociation in individual chromosomes is only observed during mitosis. However, this organization can differ in distinct cell types and developmental stages as has been documented in mouse [7-11]. This demonstrates the fact that chromocenter organization is dynamic during development; it is established early during development, and depending on the cell type and the state of differentiation different numbers of chromocenters are formed.

Maintenance of heterochromatin marks throughout the cell cycle

Studies in *S. pombe* have been instrumental in gaining insight into the mechanisms in place in order to ensure the maintenance of heterochromatic features during the cell cycle. At fission yeast centromeres, heterochromatin forms at repetitive DNA elements, referred to as pericentric *dg* and *dh* repeats (Figure 1) and is essential for proper loading of the centromere specific H3-variant Cenp-A and kinetochore assembly [12]. Nucleosomes at the pericentric repeats are hypoacetylated and enriched in H3K9me2, a histone mark catalyzed by the histone methyltransferase Clr4 and bound by chromodomain-containing proteins Chp1 and

the HP1 homologues Swi6 and Chp2, which have the capacity to multimerize. Clr4 itself can bind H3K9me2 via its proper chromodomain and methylate neighboring nucleosomes thereby creating additional binding sites for HP1 proteins. Hypoacetylation of histones is ensured by histone de-acetylases recruited by the HP1 homologs [13]. Together these chromatin marks limit Pol II occupancy and ensure the transcriptionally repressed state at pericentric heterochromatin. Interestingly, expression of Forward and Reverse pericentric transcripts is differentially regulated: Forward transcripts accumulate both in strains deficient in Swi6 and RNAi pathway components, while Reverse transcripts accumulate only in RNAi mutants. This suggests that expression of the Forward strand is under transcriptional and the Reverse strand under post-transcriptional control [14]. However, also in wild type cells, an exquisite equilibrium exists between transcriptional silencing and activating activity. Indeed, low-level, bidirectional transcription of the pericentric repeats is actually required to ensure the stable maintenance of molecular heterochromatin marks during the cell cycle. In wild type cells, transcription of pericentric repeats peaks in S-phase. Transcription during this specific time window of the cell cycle could possibly occur as a consequence of the phospho-methyl switch in mitosis which leads to Swi6 delocalization and the dilution of repressive histone marks at the daughter strands [15, 16], Figure 2A. Pericentric transcripts are thought to form double stranded RNA or hairpin structures [17], which are further processed by the RNAi machinery [14]. The resulting small RNAs (siRNAs) that accumulate transiently in S-phase then target the RNA-induced transcriptional silencing (RITS) complex [18] and Clr4 to re-establish heterochromatin features. The RNA-directed RNA polymerase complex (RDRC) ensures amplification of the siRNA signal. However, in the absence of the RDRC, small degradation products of pericentric transcripts can mediate H3K9 methylation [19]. Taken together, these data suggest that the continuous presence of heterochromatin at pericentric repeats actually requires re-establishment of heterochromatin features after each round of replication. Collectively, work in fission yeast has revealed a critical role for pericentric transcripts and the RNAi-dependent pathway in cell cycle maintenance of heterochromatin in locations where heterochromatin was already established.

In mice, the AT-rich major satellites are arranged in arrays up to several megabases in length [20]. These highly methylated repeats that are organized into hypoacetylated chromatin show high H3K9 methylation that depends on the Suv39h1/2 methyltransferases and are bound by the three isoforms of HP1. Spreading of heterochromatin features is thought to exploit a self-sustaining loop mechanism through HP1 self-association and interaction with the

H3K9HMTase, Suv39h1/2, which can add more H3K9me [21, 22], and the capacity of HP1 to recruit *de novo* methyltransferase activity [23]. Observations of reduced DNA methylation at pericentric repeats observed in Suv39h mutant cells supports this model [23]. In both *S. pombe* and mouse pericentromeres, H3K9HMTase, working as the writer, together with the reader, HP1/Swi6, propagate the heterochromatin state. However, a role for RNA-based mechanisms in the maintenance of heterochromatin features has remained difficult to establish in mammals [24-26]. In mammals, pericentric repeats are transcribed [23, 27] and regulated in a cell-cycle dependent manner [28], yet, during the cell cycle, transcripts accumulate in mitosis and in G1/S. In contrast, in fission yeast, transcription ceases in mid S-phase when pericentric heterochromatin replicates, and therefore, the analogy to fission yeast cannot be extended to include the exact timing during the cell cycle. Furthermore, if RNAi-based mechanisms play a role, as reported for the transposon-control in oocytes [29] the mechanism possibly operates without amplification of the siRNA signal, since a properly defined RNA-directed RNA polymerase still remains to be characterized. It is also possible that maintenance of molecular heterochromatin marks through chromosome duplication may be ensured by the mutual reinforcement of mechanisms that maintain DNA methylation and inheritance of modified histones as discussed [30]. In this context, the parental histone mark would be sufficient to re-initiate the whole process of heterochromatinization by recruiting HP1 and in turn the H3K9me methyltransferase. Furthermore, the maintenance methyltransferase Dnmt1 together with the SRA-domain protein Np95 [31] could also ensure methylation maintenance and recruit histone deacetylase activity necessary to remove acetylation marks on the newly incorporated histones [32, 33]. Np95 shows affinity to hemi-methylated DNA [34, 35] and interacts with H3 tails methylated at position K9 [36], thereby linking DNA and histone modifications to ensure Np95's function during replication of pericentric domains.

Notably, the chromatin assembly machinery at the replication fork contributes in a decisive manner to heterochromatin maintenance. The Chromatin Assembly Factor 1 (CAF-1) deposits new H3-H4 in a DNA-synthesis coupled manner [37]. New histone H3 bound to CAF-1 mono-methylated by SetDB1 [38] is a favoured substrate for Suv39h, which can then establish H3K9me3 and HP1 binding [39]. How CAF-1 possibly contributes to HP1 inheritance at the replication fork by transferring parental HP1 to the replicated daughter chromatin remains to be established [6, 40], Figure 2B. The recently identified chromatin-remodelling factor, SMARCAD1, which interacts with PCNA, is likely to participate in these dynamic events connecting histone methylation and HP1 recruitment [41]. In summary, in mammalian

pericentric regions, a combination of factors act in concert to ensure maintenance of heterochromatin: DNA methylation maintenance and histone de-acetylation are coupled at the replication fork, as well as histone deposition with further post-translational modifications. Thus, given the robustness of these multiple layers involved in the cell cycle maintenance of heterochromatin in mammals, an RNA-dependent mechanism may either: not be necessary at all, or may not be possible to reveal under normal conditions.

Interestingly, plant heterochromatin, similar to mammalian heterochromatin, is enriched in both DNA methylation and H3K9methylation. These two chromatin marks are tightly interconnected; histone methylation depends on DNA methylation [42, 43] and vice versa [44]. DNA methylation is critical to silence transposable elements and repeated sequences [45] and its maintenance is ensured by active mechanisms operating at the replication fork. However, plants lack HP1-like proteins at heterochromatin that could allow spreading of heterochromatin marks in a self-reinforcing loop as is proposed to occur in fission yeast and mammals. Instead, small RNA molecules processed from satellite transcripts or transposable elements do reinforce heterochromatin at pericentromeres in *Arabidopsis* [46]. While small RNA molecules do not target histone methylation activity as in yeast, they direct *de novo* DNA methylation activity to homologous sequences through a mechanism termed RNA-directed DNA methylation (RdDM, [47]. In plants, it remains to be established whether transcription of pericentric repeats is particularly increased following passage of the replication fork in order to reinforce DNA methylation maintenance. These examples illustrate that while most of the heterochromatin features are shared among different species, certain heterochromatin marks are specific to a given organism, as is the way their heterochromatin maintenance is coordinated with other chromatin modifications.

Pericentric heterochromatin dynamics during reproductive development

During mammalian germ cell development and gametogenesis genome organization undergoes important changes, and this is clearly visible at pericentric heterochromatin domains. While in mature oocytes, pericentric satellite repeats retain most of the somatic heterochromatin marks with the exception of HP1α, in sperm, the majority of histones are replaced by protamines or sperm-specific histone variants [48], therefore requiring *de novo* establishment of most heterochromatic features. Upon fertilization, protamines and the

sperm-specific histone variants are removed [49] and a nucleosomal organization is restored. As histone assembly occurs in a DNA-synthesis independent manner, only H3.3 and not the replicative H3 variants are incorporated [50]. This accentuates the chromatin differences at pericentric satellite repeats between the two parental genomes as paternal pericentric domains also lack H3K9me3, and H4K20me3 [51](Figure 3). Instead, during the first cleavage stages, alternative repressive chromatin factors, the Polycomb group binding proteins, are enriched at paternal pericentric satellites [52]. In the zygote, the paternal genome undergoes active global DNA demethylation [53], however some residual DNA methylation is retained at pericentric repeats [54] where it could play a role in the establishment of other heterochromatin features.

During the early cleavage stages, the spatial organization of pericentric satellite repeats is particularly dynamic; the pericentric domains reorganize from the gamete-specific organization to form rings around the nucleolar precursor bodies [11]. These ring-structures progressively reorganize into chromocenters during the 2-cell stage. During this particular developmental time window, transcripts from pericentric major repeats undergo a burst in transcription reaching levels far above the amount observed in cycling embryonic fibroblasts [55]. While the repeats were known to be transcribed bi-directionally [27], unexpectedly, their transcription and spatial localization is controlled in a strand-specific manner during early embryonic development [55]. The Forward strand is transcribed during the S-phase of the second cell cycle and peaks in late S/G2 followed by upregulation of the Reverse strand. Transcription from both strands is then strongly downregulated at the 4-cell stage, when chromocenters have formed, which are faithfully propagated during the following cleavage stages (Figure 3). Interference with transcripts during the 2-cell stage results in embryos that fail to form chromocenters and arrest their development in the G2 phase of the second cell cycle [55]. These experiments illustrate a critical necessity for pericentric transcription during heterochromatin establishment in early embryonic development. How transcription of the two strands is regulated in time and space and which promoters and transcription factors are necessary to drive satellite transcription specifically during this developmental transition are still unresolved questions. Differences in timing of transcription and differential regulation of subsets of repeats by distinct silencing pathways have been observed in fission yeast [14, 15] and plants [46]. Investigating the strand-specific expression of major satellite sequences in mutant backgrounds for chromatin modifiers may help to better understand their mode of regulation.

The exact mechanism of how pericentric transcripts affect chromocenter formation and development as well as the role of the two respective transcripts remain to be dissected, however, recent data have allowed speculation about their mode of action. Fertilized eggs expressing a mutated form of H3.3 (K27R) arrest at the 2-cell stage and show HP1β mis-localization. This arrest, in addition to proper HP1β localization can be rescued by injection of double-stranded, but not single stranded major RNA, implying processing of double stranded RNA molecules and a role for small RNA based mechanisms [56]. Alternatively, pericentric transcripts could function in recruiting heterochromatin components or modifiers, similar to the long non-coding transcript Air, involved in imprinting that interacts with the histone methyltransferase G9a [57]. An intriguing candidate to interact with pericentric transcripts is HP1, which has RNA-binding capacity [58, 59]. HP1 indeed binds major satellite RNA, however, it does so only when sumoylated and it also shows specificity for Forward transcripts. Interestingly, sumoylation of HP1 enhances its capacity to be recruited *de novo* to pericentric satellites lacking H3K9me3 and HP1 [60].

In short, the specific organization of DNA in the sperm and the loss of somatic heterochromatin marks entail a challenge for the early embryo to cope with two distinct states of pericentric heterochromatin in the same nucleus. Early development therefore necessitates setting most somatic heterochromatin marks at paternal and requires consolidation of maternal heterochromatin domains; it may also be one of the rare situations in which *de novo* formation of constitutive heterochromatin can be studied in mammals. The demonstration of a critical role of pericentric satellite transcripts may be one of the first steps towards a better understanding of the mechanisms implicated in mammalian heterochromatin establishment.

Figure legends:

Figure 1:
Pericentric heterochromatin clustering

A. 4′,6-diamidino-2-phenylindole (DAPI)-stained images and schematic representations of mouse nuclei in interphase (top) or chromosomes in mitosis (bottom). Individual pericentric heterochromatin domains (red) from distinct chromosomes cluster together in chromocenters

during interphase. Scale bar, 10μm. Image is reproduced, with permission, from Maison et al, Nat Rev Mol Cell Biol, vol. 5 (4) pp. 296-304 © (2004) Macmillan Publishers Ltd. All rights reserved. B. Clustering of pericentric heterochromatin is a recurrent feature of nuclear organization in a number of organisms independent of the size of their genomes and the number of chromosomes. While in *S. pombe* the three centromeres gather to a single cluster [3], in both mouse and *Arabidopsis*, heterochromatin organizes in several chromocenters [4, 5]. Pericentric heterochromatin nucleates at repetitive elements. In *S. pombe* centromeres, these consist of the outer *dg* and *dh* repeats present on each chromosome. In Arabidopsis, heterochromatin forms on 180bp satellite repeats interspersed with Athila retrotransposons and their remnants as well as various types of transposons that flank these repeats [46, 61]. In mouse, the pericentric domains consist of large uninterrupted arrays of AT-rich 234bp-long major satellite repeats [20], which can extend to more than 2 Mb per chromosome.

Figure 2:

Pericentric heterochromatin maintenance during the cell cycle in S. pombe and mouse

A. Fission yeast spends most of its lifetime in G2 phase, during which time, H3K9me2, at pericentric repeats constitutes a binding site for the chromodomain protein Swi6, the HP1 homolog. Phosphorylation of serine 10 of H3 (phospho-methyl switch) during mitosis (M) induces loss of Swi6 binding. This, together with the subsequent dilution of repressive histone modifications upon replication of pericentric repeats in early S-phase creates a small time window during which Pol II gains access and pericentric transcripts accumulate. These transcripts are processed by the RNAi machinery into small RNAs (siRNAs) that target the methyltransferase Clr4 (the homolog of mammalian SUV39H) to pericentric repeats. After deacetylation of the newly incorporated histones, Clr4 then remethylates K9, Swi6 is recruited, and transcriptional silencing is reestablished.

B. In G2, pericentric heterochromatin is enriched in DNA methylation and H3K9me3 that is bound by HP1 in mice. While HP1 binding is affected by the phospho-methyl switch in mitosis [62, 63], the extent of HP1 disruption and the mechanisms of its restoration in G1 phase are as yet unknown. Transcripts from pericentric satellites have been observed in mitosis and in G1/early S-phase, however major satellite transcription is downregulated during mid S-phase when pericentric domains replicate [28]. Coupled to DNA replication, histone de-

acetylase activity is recruited by the DNA clamp PCNA and the DNA methyltransfease Dnmt1 [32] to deacetylate the newly incorporated histones. Dnmt1 restores DNA methylation. PCNA further recruits the Chromatin Assembly Factor 1 (CAF1). CAF1 can assemble H3 already monomethylated at K9 by SetDB1 [38] into nucleosomes, but it also plays an important role in HP1 dynamics by transferring parental HP1 to daughter strands [40].

Figure 3.

Heterochromatin organization and transcription during early cleavage stages in mouse

Mouse sperm DNA tightly compacts around a core of pericentric repeats (blue) in the centre of the sperm [10]. After fertilization, the egg completes meiosis II and extrudes one haploid genome in form of the polar body (PB). Two independent pronuclei form, in which pericentric domains reorganize into ring-structures around the nucleolus-like bodies [11]. Maternal (pink) and paternal (blue) pericentric domains are epigenetically distinct in the zygote [11, 51, 52] and retain asymmetry up to the 8-cell stage [52, 64]. Paternal pericentric repeats lack H3K9me3 and H4K20me3 and either do not, or only weakly bind HP1β [51, 52]. During the 2-cell stage chromocenters progressively form and are then propagated during further cleavage stages. The first transcription of the zygotic genome (minor zygotic gene activation, ZGA) takes place at the end of first cell cycle, followed by the major ZGA in the 2-cell stage. The formation of chromocenters during the 2-cell stage coincides with a transcriptional burst of pericentric satellites ([55] see text).

Acknowledgments:

We apologize for not quoting all of our colleagues for their contributions due to space limitations. We thank C. Maison for Figure 1A and Alysia vandenBerg for critical reading of the manuscript. G.A. is supported by la Ligue Nationale contre le Cancer (Equipe labellisée Ligue 2010), PIC Programs, the European Commission Network of Excellence EpiGeneSys (HEALTH-F4-2010-257082), the European Commission ITN FP7-PEOPLE-2007-215148 "Image DDR" and FP7-PEOPLE-2008-238176 "Nucleosome 4D", ERC Advanced Grant 2009-AdG_20090506 "Eccentric", the European Commission large-scale integrating project FP7_HEALTH-2010-259743 "MODHEP", ANR "ECenS" ANR-09-BLAN-0257-01, ANR "ChromaTin" ANR-10-BLAN-1326-03 and INCa "GepiG".

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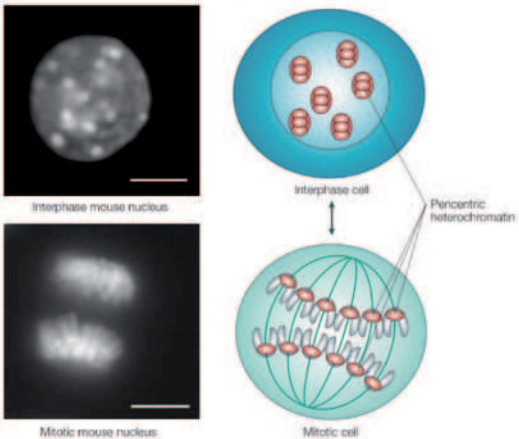
FIGURE 1

A.



B.

organism	# of chr	chromocenters	repetitive elements
S. pombe	3	1	dg, dh repeats
A. thaliana	5	~8	180bp satellites, Athila retrotransposons
M. musculus	20	~8-30	234bp major satellites

FIGURE 2

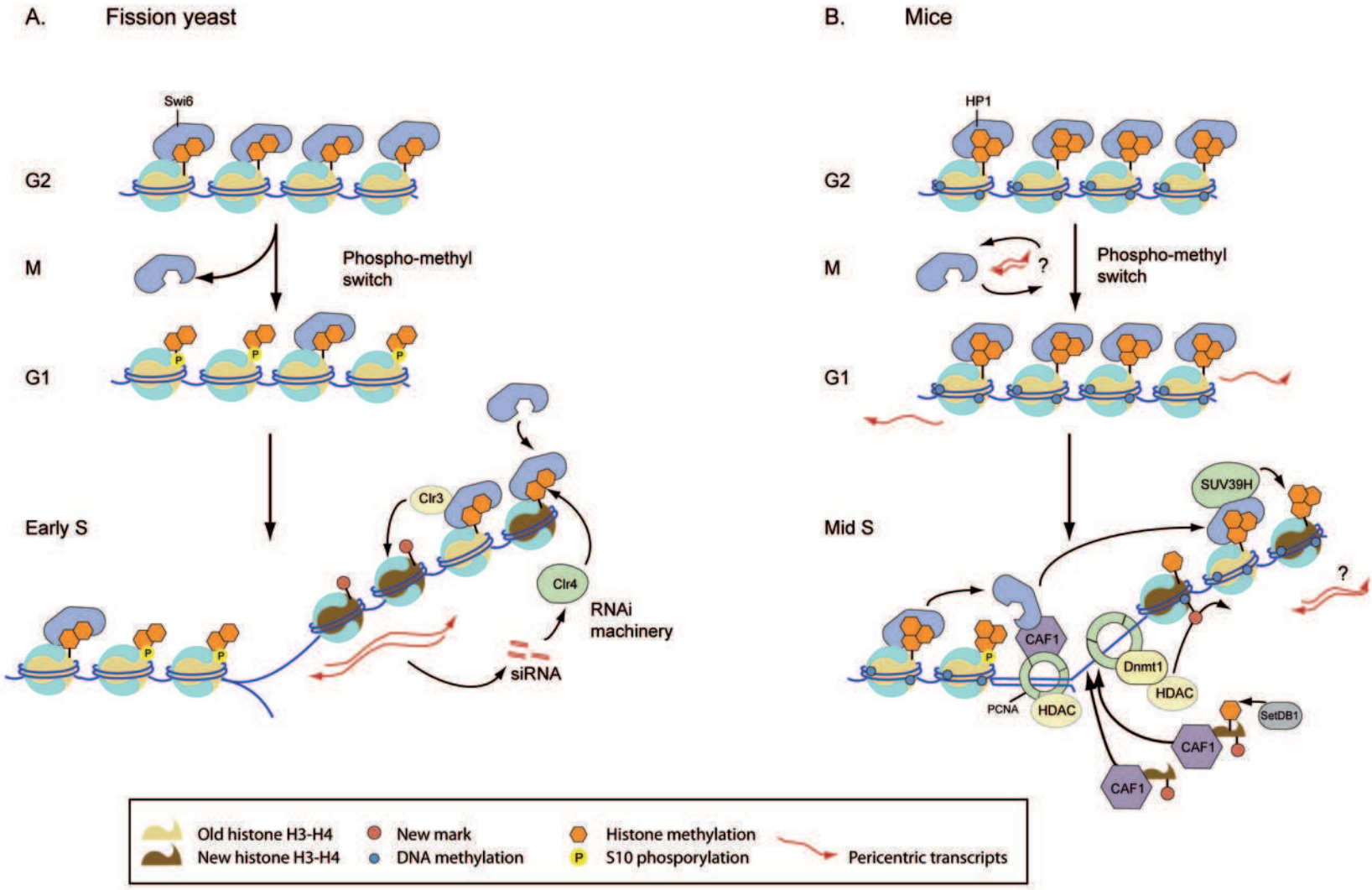
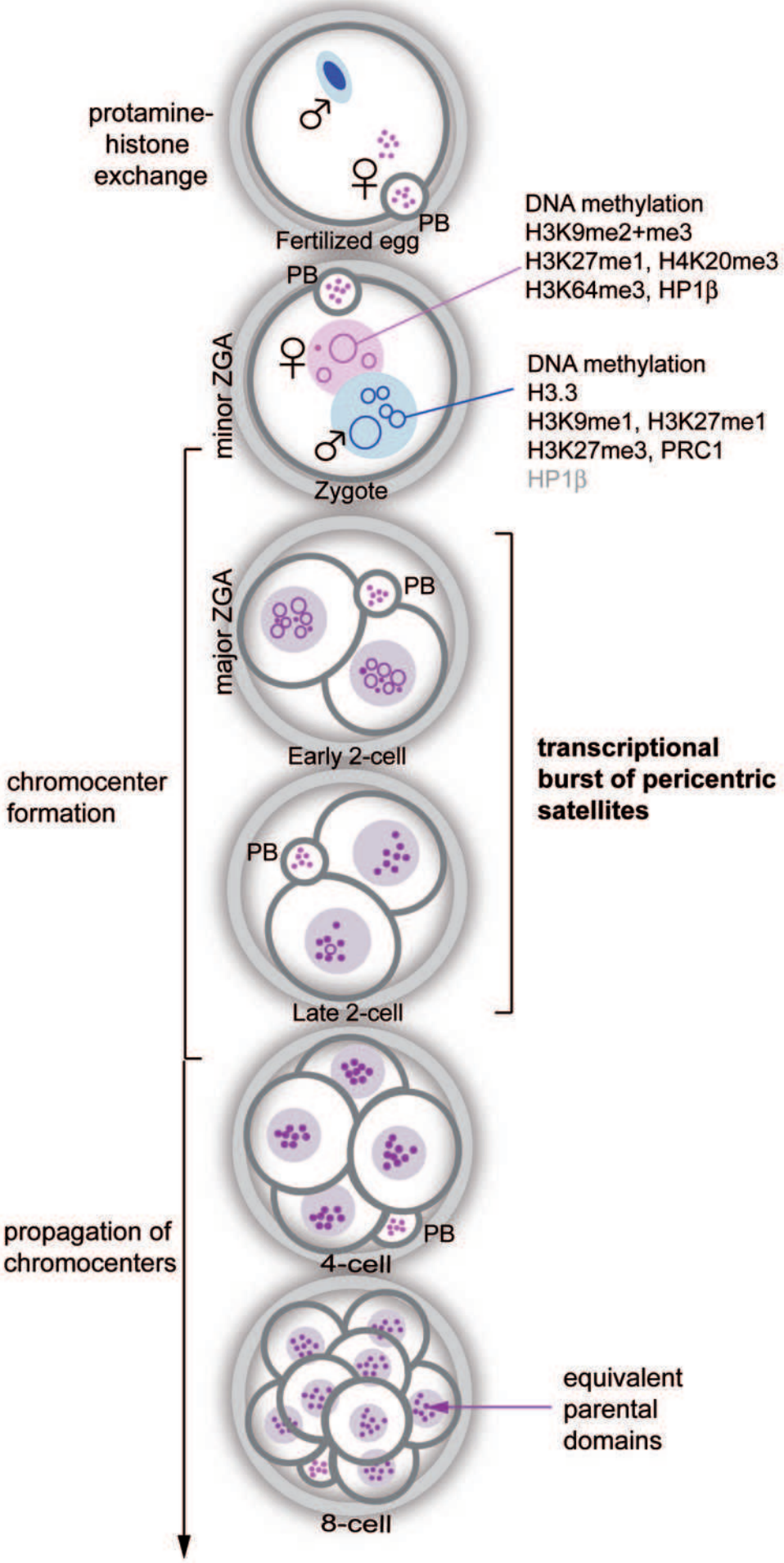

A.
Fission yeast
Swi6
G2
M
Phospho-methyl switch
G1
P
Early S
Clr3
Clr4
RNAi machinery
siRNA
B.
Mice
HP1
G2
M
?
Phospho-methyl switch
G1
Mid S
SUV39H
?
CAF1
Dnmt1
HDAC
PCNA
HDAC
SetDB1
CAF1
CAF1
Old histone H3-H4
New histone H3-H4
New mark
DNA methylation
Histone methylation
S10 phosporylation
Pericentric transcripts

FIGURE 3


protamine-
histone
exchange
♂
♀
PB
Fertilized egg
DNA methylation
H3K9me2+me3
H3K27me1, H4K20me3
H3K64me3, HP1β
PB
minor ZGA
♀
♂
DNA methylation
H3.3
H3K9me1, H3K27me1
H3K27me3, PRC1
HP1β
Zygote
major ZGA
PB
Early 2-cell
transcriptional
burst of pericentric
satellites
chromocenter
formation
PB
Late 2-cell
propagation of
chromocenters
PB
4-cell
equivalent
parental
domains
8-cell