Writing assignment

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Keeping a Genomic Jungle in Check;
How Eukaryotic Cells Regulate the Positive Feedback of Heterochromatin Expansion

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LAYMAN'S SUMMARY

In living organisms, cells form the repeatable building blocks through which tissues like the heart, skin, and lungs are formed. They consist of lipid barriers that contain the cell's DNA, RNA and proteins, thereby separating and protecting these molecules from the outside environment. Of these molecules, proteins are known as the main 'work-horses' of the cell. Different proteins may have different roles in the cell, like speeding-up chemical reactions, forming larger structures together or providing structural support. The information for these proteins is encoded in the cell's collection of DNA or 'genome', where each protein has its own corresponding DNA region or 'gene'. Cells can copy the information from DNA onto RNA, which forms the template that is used to make new proteins. If the DNA of a single human cell were to be stretched out in a line, it would be over 2 meters long. Whilst human cells have around 20 000 genes, most of the cell's DNA does not encode for proteins. Instead, it consists of repetitive sequences, remnants of DNA viruses and sequences used for regulation. To still fit all this DNA into a compact shape, cells tightly wind the DNA around spherical proteins named histones. By modifying these histones with specific molecular groups (i.e., methyl-, acetyl- or phosphor-groups), cells can change how tightly the DNA is compacted.

One of the most studied histone modifications is the addition of one or more methyl-groups to position 9 of Histone 3 (referred to as H3K9me1/2/3). Especially, H3K9me3 has been shown to efficiently con-dense DNA into what is called 'constitutive heterochromatin', thereby making it difficult for proteins to access these regions. Heterochromatin regions simultaneously stabilize and prevent the creation of proteins from the underlying DNA. Due to these properties, cells use constitutive heterochromatin to prevent the non-coding DNA from becoming unstable or creating harmful proteins. These mechanisms have remained very similar from yeast to humans throughout evolution, which is why scientists often use fission yeasts to study the formation of heterochromatin. Using this organism, researchers have found that heterochromatin spreads using proteins that create positive-feedback loops. Meaning that each H3K9me2/3 modification may help create the same modification on a neighboring histone. If this process is left unregulated, however, this may lead to the entire genome of the cell becoming condensed, thereby preventing the creation of new proteins and leading to cell death. How cells prevent this from happening is not entirely known.

In this review, I summarize what has in recent times become known about the formation, spreading and regulation of heterochromatin. For example, how recent research has shown that larger, heterochromatin-covered DNA regions are grouped together and are actively kept separate from the DNA regions encoding for proteins. We then use this information to create up-to-date schematic overviews that explain the overall regulation of heterochromatin, which allows us to speculate on topics for future research and how to treat human diseases where mistakes in these processes are involved, like Hutchinson–Gilford Progeria Syndrome.

Keeping a Genomic Jungle in Check; How Eukaryotic Cells Regulate the Positive Feedback of Constitutive Heterochromatin

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ABSTRACT: Eukaryotic genomes contain many repetitive sequences that, without constant repression, can induce genomic instability via rogue transcription, recombination, and transposition. Repression of these sequences occurs through constitutive heterochromatin, a condensed phase consisting of H3K9me2/3-marked nucleosomes bound to DNA. Interestingly, whilst heterochromatin nucleation in sequence-dependent, its propagation is both sequence-independent and self-reinforced via positive-feedback loops. As the abnormal repression of genes can have large implications for cellular fitness, it is important that these mechanisms be spatiotemporally regulated. How this is mediated is, however, not fully known. In this review, we examine recent findings on the nucleation, spreading and regulation of constitutive heterochromatin. We then use this information to form models explaining the regulation of constitutive heterochromatin, to speculate on topics for future research.

In eukaryotic organisms, the genome is tightly packaged around histones. By interacting with DNA, histones stabilize the genome and facilitate the recruitment of proteins required for DNA-specific processes like transcription, replication, and DNA repair (Ehrenhofer-Murray, 2004). Histones are simultaneously themselves targets for various post-translational modifications. Arguably the best studied histone modification is the trimethylation of lysine on position 9 of Histone H3 (H3K9me3). It has long been known that this modification correlates with a tighter condensation of nearby Histone-DNA nucleoproteins, which reduces the accessibility of the DNA to nuclear proteins (Soufi *et al.*, 2012). These inaccessible regions are generally considered transcriptionally inactive due to their ability to bar transcription factors from interacting with the DNA (Bancaud *et al.*, 2009).

Characteristically, stretches of H3K9me3 have been shown to be long-lasting, epigenetically inheritable, and prevalent among the sex chromosomes or "heterochromosomes" (Ragunathan *et al.*, 2015; Grewal and Klar, 1996; Hsu and Arrighi, 1971). This has led them to be termed as constitutive heterochromatin over time (Heitz, 1928; Berger, 2019). So far, there are at least six main histone methyltransferases (HMTs) that have been shown to catalyze either mono-, di- or trimethylation of H3K9 in mammals, namely: SUV39H1, SUV39H2, SETDB1, SETDB2, G9A and G9A-like protein (Montavon *et al.*, 2021). The individual functions of which have been extensively reviewed in Padeken *et al.* (2022). Interestingly, these six HMT forms are both semi-redundant in function and are able to participate within the same multimeric complex together (Fritsch *et al.*, 2010; Montavon *et al.*, 2021). As a result, whilst the knockout of individual HMT families does not abrogate heterochromatin organization in most cell types, a combined knockout of all six HMTs fully eliminates H3K9me3-containing constitutive heterochromatin within mouse cell lines (Montavon *et al.*, 2021). Due to the large redundancy and overlap in function between HMTs, studying these processes in mammalian cell lines is rather difficult. This has led many in the field to instead utilize certain species of yeasts, due to their simplistic, yet highly conserved heterochromatin systems. Especially the fission yeast (*S. pombe)*, with its singular HMT, is nowadays a commonly used model organism to study heterochromatin and epigenetic modifications (Vyas *et al.*, 2021). Interestingly, not all yeasts have similarly conserved heterochromatin mechanisms to humans. The often used budding yeast (*S. cerevisiae*) model organism, for example, lacks H3K9me1/2/3 modifications and instead utilizes a yeast-specific mechanism that employs SIR proteins to facilitate transcriptional silencing (Brothers and Rine, 2022; Oh *et al.*, 2022).

Using these systems, heterochromatin has over time been implicated in regulating regions of the DNA that contain self-replicating sequences. Due to their self-replicating and repetitive nature, these sequences require repression to prevent rogue transcription, recombination, and transposition events from disrupting the genome (Sassaman *et al.*, 1997; Okita *et al.*, 2019). Some well-known genomic elements utilizing heterochromatin include 'Short and Long Interspersed Retrotransposable Elements' (~34% of the genome), endogenous retroviruses (~8% of the genome) and satellite repeats (~10% of the genome) (Lander *et al.*, 2001; Nurk *et al.*, 2022). While the initial heterochromatin nucleation of these regions follows sequence-specific mechanisms, the propagation of the domain is both sequence-independent and self-reinforcing (Obersriebnig *et al.*, 2016). As a consequence, various, conserved mechanisms have been shown to antagonize heterochromatin. Yet, how these mechanisms can prevent the self-reinforced spreading of heterochromatin into regions associated with active transcription is not well known.

From this description, most if not all regions marked with heterochromatin may seem as transcriptionally unimportant for the functioning of the cell. In reality, this does not have to be the case. For instance, many developmental genes have, often in response to spatial-temporal cues, the ability to switch between a transcriptionally inactive, heterochromatic state and a transcriptionally active, euchromatic state during development (Gorkin *et al.*, 2020). Thereby allowing cells to successfully progress towards a specific cell fate utilizing the same genome as every other cell. This type of heterochromatin is better known as 'facultative heterochromatin' and characteristically contains stretches of H3K27me2/3 histone modifications (Chadwick *et al.*, 2004). Whilst present in humans, facultative heterochromatin is curiously absent from both *S. pombe* and *S.*

cerevisiae. Additionally, since facultative heterochromatin differs extensively to constitutive heterochromatin in its underlying principles, it is, for the sake of clarity, kept outside the scope of this review.

As illustrated, heterochromatin requires an incredible diversity in the sequences it targets, the pathways that facilitate its formation and the way cells maintain it over time and cellular divisions. Thereby begging the question: How do eukaryotic cells prevent constitutive heterochromatin nucleation and propagation from overtaking the chromatin landscape? To answer this, we will in this review be discussing what is currently known about the nucleation, spreading, and maintenance of constitutive heterochromatin in eukaryotic cells. Following this, we will transition into discussing the cellular factors which limit these mechanisms and thereby prevent aberrant H3K9me3 heterochromatin propagation.

Heterochromatin Nucleation in Repetitive Elements

Heterochromatin nucleation can be defined as the *de novo* formation of heterochromatin independently of pre-existing heterochromatin marks. After establishment, the original signals that induced heterochromatin nucleation are often unnecessary for its spreading (and maintenance) (Hall *et al.*, 2002). Instead, as will be discussed later, a self-reinforcing mechanism of propagation takes over (Obersriebnig *et al.*, 2016). Yet, both these independent processes are keenly required for a sustained epigenetic inheritance. Specifically, during meiosis and the early embryonic stages, cells undergo a process of epigenetic reprogramming wherein they exhibit a large loss of epigenetic marks (Wang *et al.*, 2018; Zheng *et al.*, 2016). Generally, this process has been proposed to guide these cells towards an undifferentiated state, thereby broadening which cell fates they can achieve. Following this drop in epigenetic marks, embryonic cells are reprogrammed over several cell cycles, wherein they regain heterochromatin marks through nucleation mechanisms (Fabry *et al.*, 2021). Paradoxically, however, due to the large diversity of heterochromatin-targeted sites, nucleation needs to be both sequence-specific and broadly applicable.

In this context, it is fascinating that these cells can accurately recognize and target the genomic regions that require silencing over those that do not. In this pursuit, cells have been shown to use at least three distinct methods of nucleation, namely: Co-transcriptional RNAi-dependent, Co-transcriptional RNAi-independent, and DNA motif-based nucleation (fig. 1 and 2). We will discuss and summarize some of these mechanisms shortly to showcase the differences in how these mechanisms may target genomic regions for heterochromatin-mediated silencing.

Co-transcriptional, RNAi-dependent nucleation

In cells, transposons, repetitive elements and protein-coding DNA exist side-by-side within the genome. Yet of these, only transposons and repetitive elements require constitutive silencing. So, how do cells make the distinction between these? To explore this question, we will focus in on *S. pombe*, where semi-conserved, RNAi-dependent mechanisms exist. Here, these mechanisms are mainly used to target and condense repetitive elements found within pericentromeric DNA (Hall *et al.*, 2002).

The first mechanism is dependent on so-called primal RNA's (priRNA), which are small RNA fragments produced from the constant degradation of abundant cellular transcripts (Halic and Moazed, 2010). Whilst protein-encoding transcripts are mostly enriched for sense mRNA, however, repetitive elements can transcribe either sense- and antisense RNA (Iida *et al.*, 2008). The resulting priRNA's can be bound by Argonaute 1 (Ago1), which forms part of the RITS complex (Halic and Moazed, 2010). Since priRNA's from repetitive elements have base-pairing capacity, they can direct the RITS complex to matching, nascent-produced RNA transcripts (Verdel *et al.*, 2004). Subsequently, the RITS complex facilitates silencing by recruiting and activating the Clr4 methyltransferase complex (ClrC) that consists of Clr4 (SUV39h1/2 in humans) together with the proteins Cul4, Rik1, Raf1 and Raf2 (Zhang *et al.*, 2008). Together, this complex nucleates H3K9me2/3 in its vicinity and thereby provides the initial signal that is required for heterochromatin propagation. Simultaneously, these transcripts can be digested to prevent potential translation and further mediate silencing (Marasovic *et al.*, 2013; Sugiyama *et al.*, 2005).

This mechanism by itself, however, only produces a small fraction of the siRNA's that cells require to nucleate these repetitive elements (Marasovic *et al.*, 2013). Instead, it is thought to be the initiator of a second, more-prominent mechanism facilitated by the RNA-directed RNA polymerase complex (RdRc), Dicer (Dcr1) and the RITS complex (Motamedi *et al.*, 2004). Here, priRNA- or siRNA-mediated binding of the RITS complex to mRNA recruits RdRc (Colmenares *et al.*, 2007). The RdRc consists of RNA polymerase Rdp1, the RNA helicase Hrr1 and the poly-A polymerase Cid12 (Motamedi *et al.*, 2004). This complex then facilitates the formation of double-stranded RNA (dsRNA) from the transcript. After formation of dsRNA, Dcr1 digests the dsRNA into immature siRNAs that can be bound by the ARC complex, consisting of Ago1, Arb1, and Arb2 (Motamedi *et al.*, 2004; Buker *et al.*, 2007). This complex shuttles the dsRNA to the RITS complex, wherein the dsRNA is digested by either Ago1 or Triman to form a mature siRNA, thereby completing the cycle (Colmenares *et al.*, 2007; Buker *et al.*, 2007; Marasovic *et al.*, 2013).

Interestingly, since new dsRNA is produced following the targeting of the RITS and RdRc complex to nascent transcripts, this process therefore only requires few initial dsRNA to promote siRNA amplification and heterochromatin nucleation (Iida *et al.*, 2008). Similarly, H3K9me2/3 modifications have been shown to reinforce the recruitment of the RITS and RdRc complexes (Petrie *et al.*, 2005; Noma *et al.*, 2004). Thereby introducing an additional positive-feedback loop into this mechanism.

Co-transcriptional, RNAi-independent nucleation

While RNAi-mediated nucleation forms the dominant pathway for *de novo* heterochromatin formation in *S. pombe*, alternative conserved pathways for heterochromatin nucleation exist which bypass the need for RNAi processing. Whilst less is known about these mechanisms, they have become active topics of research in recent years. As, in mammals, the role of RNAi-mediated heterochromatin nucleation has remained somewhat of a mystery. Especially, since mammals do not have RdRp/RITS complexes and so seem

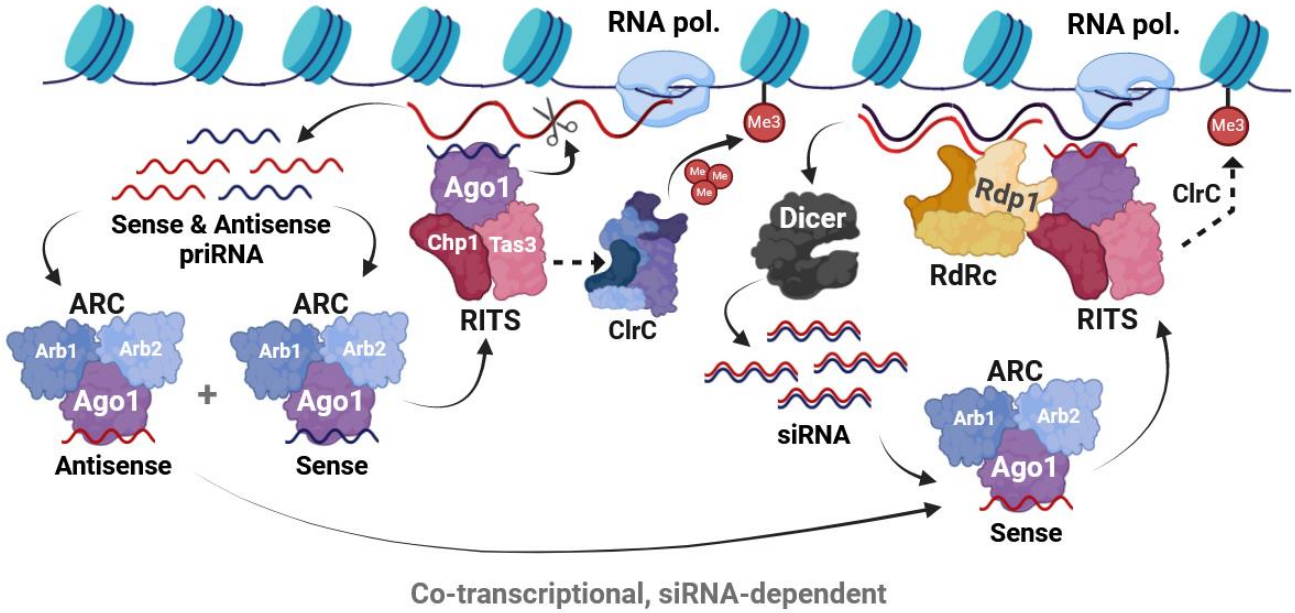

Figure 1. Schematic overview of Co-transcriptional, siRNA-dependent nucleation of heterochromatin in *S. pombe*. *In S. pombe*, degradation of abundant transcripts lead to priRNAs, which can be taken up by the ARC complex. Subsequently, the ARC complex can shuttle these priRNAs to the RITS complex. Since repetitive elements produce both antisense & sense priRNA, the RITS complex can base-pair with matching transcripts from these regions. When this occurs, RITS cleaves the transcript, recruits the ClrC complex and the RdRc complex. The RdRc complex produces dsRNA from the transcript, which is cleaved into new siRNAs through Dicer. Thereby, allowing for the cycle to repeat. Figure was made using BioRender.

to largely restrict utilization of dsRNA-dependent mechanisms to germline cells (Aravin *et al.*, 2007). Thereby, indicating that RNAi-independent methods may be of great importance there. In *S. pombe*, one such RNAi-independent mechanism has been shown to utilize RNA polymerase II stalling on repetitive sequences to induce the nucleation of heterochromatin (Parsa *et al.*, 2018). In this research by Parsa *et al.* (2018), the protein Seb1 was shown to localize to pericentromeric ncRNA transcripts, where it induced stalling of the RNA pol. II. This stalling resultantly led to H3K9me3 formation independent of siRNA. Similarly, follow-up experiments inducing the ectopic stalling of RNA pol. II also induced the formation of heterochromatin domains independent of siRNA, thereby further supporting that nucleation can be RNA pol. II stalling-dependent. What underlying mechanisms induce this nucleation of H3K9me3-stretches is, however, still not entirely known. Although, mechanism likely involve Seb1's ability to recruit the Clr3-containing deacetylase complex SHREC (NuRD in humans) or its potential interaction with the RNA exonuclease Dhp1 (Marina *et al.*, 2013; Chalamcharla *et al.*, 2015). Previously, Dhp1 has been shown to recruit and active the Clr4-containing ClrC complex, thereby inducing H3K9 methylation in the vicinity of stalled RNA pol. II (Chalamcharla *et al.*, 2015). Interestingly, it has been shown that euchromatic H3K4me3 modifications locally recruit proteins (like INTS11) that combat RNA pol II stalling in *S. pombe* (Wang *et al.*, 2023). Therefore, potentially forming a way in which cells can counteract this stalling-dependent formation of heterochromatin near euchromatin regions.

While not pointed out before, it is interesting to note that both the RNAi-dependent and RNAi-independent methods of heterochromatin nucleation that have been discussed so far use transcription to facilitate gene silencing, even though this is in itself semi-paradoxical. Since a heterochromatin domain will suppress the transcription that is required to maintain itself. One solution to this problem is to have a 'leaky' gene silencing, wherein enough ncRNA is transcribed to facilitate RNAi-dependent and -independent forms of heterochromatin nucleation. Recently, in *S. pombe*, it was shown that the anti-silencing factor Epe1 may facilitate this 'leaky' gene silencing in regions containing repetitive elements by partially derepressing these regions (Asanuma *et al.*, 2022). Thereby provides the RNA required for the RITS pathway to work effectively without disrupting heterochromatin functioning.

Nucleation through DNA-motif recognition

When it comes to silencing retrotransposable elements, animals have also evolved RNAi-independent, transcription-independent methods of heterochromatin nucleation. So far, much less is known about these mechanisms and how they contribute to heterochromatin nucleation as a whole.

In humans, one of the more well known DNA-motif based mechanisms is facilitated by a family of proteins named the 'Krüppel-associated box zinc finger proteins' (KZFPs). KZFPs are known to bind to retrotransposable elements and the primer binding sites of integrated retroviruses in a sequence-specific manner using zinc finger domains (Yang *et al.*, 2022; Tribolet-Hardy *et al.*, 2023). With the large sequence complexity found within retrotransposable elements, a DNA-motif based approach would at first seem inefficient. However, over time, KZFP genes have adapted to target highly conserved sequences within Retrotransposable elements, thereby limiting the ability of these Retrotransposable elements to escape detection and

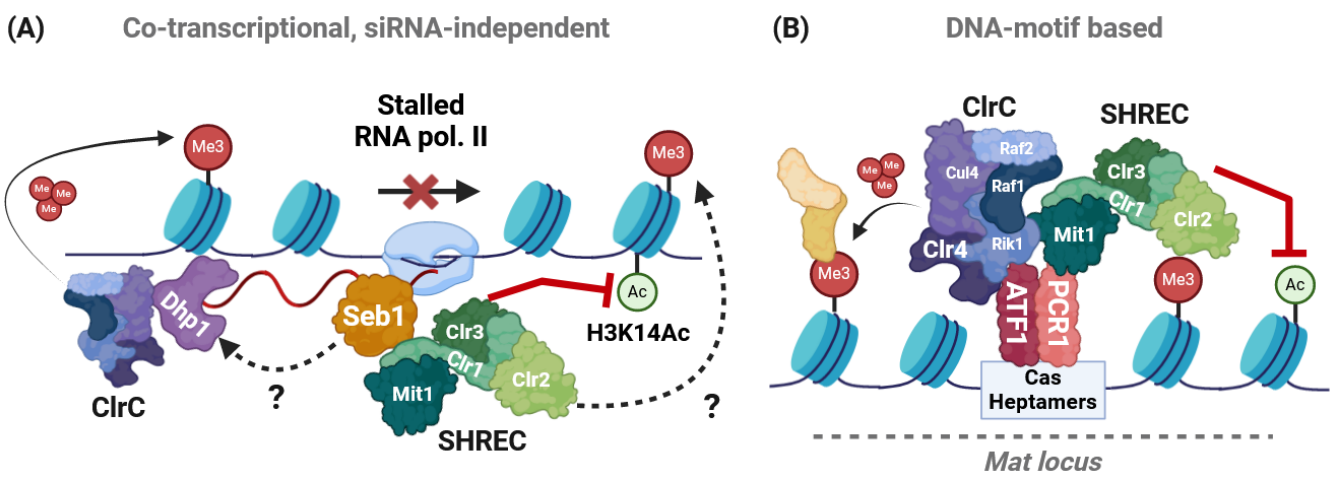

Figure 2. Schematic overview of (A) Co-transcriptional, siRNA-independent and (B) DNA-motif based nucleation of heterochromatin in *S. pombe*. (A) *In S. pombe*, association of Seb1 with RNA pol II in pericentromeric regions promotes the stalling of RNA pol. II. This stalling, through yet unknown mechanisms, induces nucleation of constitutive heterochromatin. Possible mechanisms of action for this process could include the involvement of the SHREC complex or Dhp1 exonuclease. (B) Cas heptamers within the *mat* locus of S. pombe recruit the transcription factor heterodimer ATF1/PCR1. This dimer has been shown to recruit SHREC, ClrC, and Swi6, which subsequently promote nucleation. Figure was made using BioRender.

silencing (Wolf *et al.*, 2020). The nucleation of heterochromatin through KZFPs proceeds through a rather elegant stepwise activation. After a KZFP has bound its target DNA, a conformational change causes its KRAB domain to interact with nearby KAP-1, which forms a hub for the recruitment of several proteins including the Nucleosome Remodeling and Deacetylase (NuRD) complex, HP1, DNA methyltransferases (DNMTs), UBE2i, ATRX/DAXX and SETDB1 (Schultz *et al.*, 2002; Schultz *et al.*, 2001). Looking closer, KAP-1 contains both a PHD- and Bromo-domain in close proximity. Cooperative interactions between the PHD-domain of KAP-1 and a UBE21i protein facilitates the SUMOylation of KAP-1's Bromo-domain, which is required for the recruitment of both the NuRD complex and SETDB1 (Ivanov *et al.*, 2007). The recruited SETDB1 can then catalyze the formation of H3K9me3-stretches surrounding the binding site of KZFP. Additionally, the recruited NuRD complex can remove euchromatin-specific histone acetylation marks surrounding its binding site, thereby further assisting in the formation of heterochromatin domains.

Lastly, an alternative DNA-motif way of targeting heterochromatin nucleation may also occur through the use of transcription factors. Aside from RNAi-mediated nucleation, ATF1 and Pcr1 have been shown to form a secondary important alternative pathway of heterochromatin nucleation in the mating-type region (Jia *et al.*, 2004). In these regions, heterochromatin was abrogated only after both ATF1/Pcr1 and RNAi-mediated nucleation methods were mutated. Binding of ATF1/Pcr1 depended on specific CAS heptamer sequences within the *mat* locus, deletion of which resulted in abrogated heterochromatin nucleation (Yamada *et al.*, 2005). Following its binding of the *mat* locus, the ATF1-Pcr1 heterodimer first binds to the Clr3 histone deacetylase, which is subsequently involved in recruiting both Clr4/Suv39h1 and Swi6/HP1 (Yamada *et al.*, 2005). These proteins then function in facilitating heterochromatin nucleation in the surrounding region and supporting establishment of the domain. Interestingly, similar ATF/Pcr1 pathways have been shown to be present in Drosophila and higher mammals, where they are linked to changes in epigenetic inheritance as a response to (heat) stress (Seong *et al.*, 2011; Sun *et al.*, 2023).

Together, indicating that these mechanisms may complement RNAi-mediated nucleation to provide redundancy, to help synergistically nucleate heterochromatin and to allow for a more tuned response to external factors.

Heterochromatin Spreading, Condensation and Maintenance

Heterochromatin spreading

Nucleation of heterochromatin domain-sized silencing. In actuality, effective repression of genomic sequences requires that H3K9me3 stretches are propagated further than the initial sites of nucleation (Shan *et al.*, 2016). Eukaryotic cells solve this issue by combining nucleation with heterochromatin spreading: a sequence-indifferent, positive-feedback mechanism by which existing H3K9me3 modifications recruit and activate HMT's to modify nearby Histone H3 (fig. 3; Al-Sady *et al.*, 2013). In *S. pombe*, Clr4 (Suv39H1/2 in humans) can 'read' existing H3K9me3 by binding to it using its chromodomain (Akoury *et al.*, 2019). Subsequently, Clr4 is activated and can 'write' on nearby H3K9 using *de novo* mono-, di- and trimethylation. For this reason, this conserved pathway is often referred to as the 'Read-and-write' mechanism of self-propagation. In human cells, the same pathway is largely conserved through Suv39h1/2 (Müller *et al.*, 2016). Specifically, due to Suv39h1/2's preference for catalyzing the H3K9me to H3k9me2/3 transition, cooperation with other region-specific HMTs that facilitate monomethylation is likely required for heterochromatin spreading in higher eukaryotes. At the same time, since monomethylation of soluble H3K9 can also occur immediately following translation, H3K9me may itself be integrated into DNA following replication (Loyola *et al.*, 2009; Rivera *et al.*, 2015). Thereby, providing a separate mechanism by which H3K9me is supplied for heterochromatin

spreading.

Whilst this 'read-and-write' mechanism can recognize H3K9me-modified nucleosomes, a problem becomes clear when considering the fact that the nucleus of eukaryotes is filled to the brim with off-target chromatin. Simultaneously, factors including histone demethylation, histone turnover and histone acetylation may together outcompete the synthesis of new H3K9me3, effectively negating heterochromatin spreading altogether (DiPiazza *et al.*, 2021). As a result, sufficiently enriching HMTs and their supporting factors to newly nucleated heterochromatin is no easy task. The real question, therefore, is how can cells guide these factors towards sites of nucleated H3K9me to facilitate heterochromatin spreading.

Heterochromatin condensation

To approach this dilemma, it is important to not make the mistake of limiting the discussion of these chromatin domains to 2-dimensional, beads-on-string systems. In cells, large DNA sections can fold into complex 3-dimensional loops and organize themselves in topologically associating domains (TADs) (Dixon *et al.*, 2012). Similar, but differently regulated domains also exist near the nuclear periphery as lamin-associated domains (LADs; Guelen *et al.*, 2008). The involvement of which in facilitating heterochromatin will be discussed in detail later on. The formation of independent TADs is still an area of active research, but is known to depend on the processes of cohesion-mediated loop extrusion within TADs and CTCF-mediated isolation between TADs (Chang *et al.*, 2023). These TADs have been shown to organize into cliques with similar heterochromatin landscapes, which could serve to locally enrich and retain heterochromatin factors (Paulsen *et al.*, 2019). Additionally, by grouping together regions with heterochromatin in 3-dimensional space, spreading of heterochromatin may be facilitated in these domains through long-range 'jumping' mechanisms. Evidence for these long-range interactions has recently come from combined experimental & computational approaches to modelling methylation dynamics in *S. pombe*. Recently, for example, Nickels *et al.* (2021) made a case for the existence of long-range methylation of H3K9 by Clr4. In this study, they measured & modeled transcriptional silencing dynamics in varying lengths of integrated DNA constructs. Whilst the measured repression dynamics did not fit that of a system with only short-range methylation mechanics, it could be represented through a combination of both short- & long-range mechanisms. As TADs are grouped in close 3-dimensional space, such a mechanism would allow the H3K9me3-signal to bypass the limitations that come with the linear structure of DNA by producing multiple heterochromatin fronts. Each of these fronts could propagate the H3K9me3-signal forwards and thus effectively help multiply the rate of propagation.

Aside from potentially utilizing the pre-existing TAD structure of DNA, heterochromatin domains are also known to actively form higher density chromatin condensates, which helps facilitate heterochromatin spreading (Hiragami-Hamada *et al.*, 2016). Heterochromatin protein 1 (HP1 in humans or Swi6 in fission yeast) plays an important role in this process. In their monomeric or dimeric state, HP1 / Swi6 bound to H3K9me2/3 forms a hub which recruits many important factors that are required for heterochromatin formation & -spreading, like Suv39h1/2, HDACS, DNMTs and more (Machida *et al.*, 2018; Yamamoto and Sonoda, 2003; Smallwood *et al.*, 2007; Fischer *et al.*, 2009). Additionally, both histone demethylases (HDMs) and HMTs are majorly dependent on HP1-binding for their protein stability, as without it, they become targeted for degradation (Maeda and Tachibana, 2022). The members of the HP1 protein family (HP1α, HP1β, and HP1γ) are generally known as chromatin architectural proteins and are theorized to help condensate heterochromatin in at least three possible ways (Keenen *et al.*, 2021).

First, in its canonical mechanism-of-action the homodimer state can bind two neighboring H3K9me2/3-modified nucleosomes and form a bridge structure between them, thereby enforcing the heterochromatin conformation of the domain (Machida *et al.*, 2018). Since each HP1 protein can recruit heterochromatin factors, long chains of these bridge structures help retain these factors near heterochromatin. Secondly, these proteins have also been shown to cross-link H3K9me2/3-modified nucleosomes on different DNA fibers, thereby creating higher-order condensation and possibly linking different TAD loops together (Hiragami-Hamada *et al.*, 2016). Lastly, aside from HP1 dimer cross-links, oligomerization of HP1 is also considered a possible way through which these higher-order condensations may occur (Kilic *et al.*, 2015).

Recently, a single molecule dynamics study of Swi6 has revealed that, in its dimerized state, Swi6 only has a low binding affinity for H3K9me2/3 (Biswas *et al.*, 2022). A property that would majorly limit Swi6's ability to form condensates. Furthermore, this interaction was consistently antagonized by a competing, more promiscuous affinity of Swi6's hinge region to nucleic acids. If Swi6 only functioned in a mono-/dimer state, non-specific nucleic acid binding would prevent heterochromatin-specific condensation altogether. In their experiments, however, they found that oligomerization of at least two or more Swi6 dimers solved this problem. Specifically, as higher-order oligomers of Swi6 showed an increased affinity for H3K9me2/3 over nucleic acids, a change that was sufficient to induce heterochromatin-specific condensation. Thus, short dimeric bridging, long dimeric bridging and oligomerization of the architectural protein HP1 can help concentrate heterochromatin domains, which enriches individual methylation factors (i.e., HMTs) and thereby creates a unique heterochromatin-focused environment to facilitate H3K9me3 spreading.

Simultaneously, the reasoning described above is unlikely to be the be-all and end-all to explaining the complexity of the system, as it cannot fully encapsulate all the heterochromatin properties that have been found *in vivo*, namely: its propensity to exclude non-interacting proteins, its sensitivity to the loss of hydrophobic interactions and its liquid-like properties (Strom *et al.*, 2017). As a result, the field has in recent times been extensively investigating other possibilities that may fill these gaps. The biggest of which pertains to whether extensive oligomerization of HP1 could lead to a phenomenon known as Liquid-liquid phase separation (LLPS).

LLPS is characterized by the energetic favorability of forming a dense phase (i.e., an oligomer mesh) within a more diluted phase (i.e., the nucleoplasm) (Tang *et al.*, 2021). Within the cell they often, but not always, have the following properties: they are made up of repeating

biomolecules, can form / expand on their own, are highly dependent on concentration and environmental conditions, limit diffusion into- / out of the condensate, and lastly selectively concentrate or exclude certain biomolecules based on affinity (Yuan *et al.*, 2023; Ditlev *et al.*, 2018). As described, LLPS has been shown to exist within the nucleus in a variety of contexts *in vivo* (Gibson *et al.*, 2019). Similarly, HP1α and Swi6 have for some time been known to form phase-phase liquid separations at normal cellular concentrations *in vitro* (Larson *et al.*, 2017; Strom *et al.*, 2017; Hiragami-Hamada *et al.*, 2016). These LLPS condensates were later shown to be depended on HP1/Swi6's ability to reshape histone octamers (Sanulli *et al.*, 2019). Following its binding, HP1/Swi6 was shown to loosen the histone core and expose buried residues that then promote weak, multi-valent interactions between nearby histones. These interactions were subsequently shown to be essential for LLPS, as cross-links preventing this conformational change largely abrogated LLPS formation *in vitro*.

Around the same time, some of the first evidence for HP1α, but not HP1β or HP1γ, inducing LLPS *in vivo* was found (Keenen *et al.*, 2021). Here, condensation was dependent on the phosphorylation of HP1α's N-terminal extension region, which allows it to assume a stretched-out conformation (Larson *et al.*, 2017). Interestingly, whilst neither HP1β or HP1γ could induce LLPS *in vivo*, they have been shown to compete with the oligomerization of HP1α. The relative concentrations of HP1β/γ to HP1α could therefore be a potential way to regulate LLPS size (Keenen *et al.*, 2021). Even so, the role of LLPS in heterochromatin spreading is still a highly disputed topic. Specifically, because some publications suggest that HP1 only has a weak capacity *in vivo* to produce LLPS and that loss of HP1 does not affect the condensation of heterochromatin (Erdel *et al.*, 2020). Overall, from these publications, it becomes evident that HP1-mediated LLPS formation is still an area that benefits greatly from additional research and clarification.

Heterochromatin maintenance

Following heterochromatin nucleation and -spreading, it is of utmost importance that these domains remain actively maintained. In *S. pombe*, ectopically induced heterochromatin cannot be maintained without either Clr4, RNAi-mediated or sequence-specific pathways (Ragunathan *et al.*, 2015). Instead, silencing is abrogated over time through demethylation by the (putative) HDM Epe1, histone turnover or replication-dependent dilution. Therefore, in this process, we pivot away from mechanisms that promote a growing heterochromatin front towards those that prevent the degradation of H3K9me3 over time and cellular divisions.

This change in focus is best embodied by the change of Swi6/HP1's role in these respective processes. In *S. pombe*, heterochromatin spreading can still occur in the absence of HP1 if factors that oppose heterochromatin formation are eliminated (Seman *et al.*, 2023). Namely, the proteins Mst2 and Set1, two enzymes that respectively catalyze the euchromatin marks H3K14Ac and H3K4me in fission yeast. These 'roadblocks' normally hinder the propagation of heterochromatin by increasing histone turnover, actively inhibiting Suv39h1/2 activity and recruiting euchromatin factors (Alper *et al.*, 2013; Greenstein *et al.*, 2020). As a result, Swi6/HP1-mediated recruitment of HDACs and HMTs is an essential prerequisite that 'paves the way' before a growing H3K9me3-front (Zofall *et al.*, 2022). Mutational studies further support this reasoning by showing that a loss of these HDACs prevents the establishment of heterochromatin domains all together (Buscaino *et al.*, 2013). Therefore, HP1-mediated enrichment of heterochromatin factors may not necessarily be required for heterochromatin propagation, as long as counter-acting euchromatin forces are disrupted. In comparison, heterochromatin maintenance is much more dependent on this Swi6/HP1-mediated recruitment of factors. As heterochromatin domains were unable to be maintained over multiple cell cycles without the presence of Swi6 / HP1, even in cases where euchromatin factors were absent (Seman *et al.*, 2023).

This begs the question of why, following the full establishment of heterochromatin, factors like Swi6/HP1 and HMTs are still required for the continued existence of the domain. One major reason can be found in the process of DNA replication. Through continuing cycles of replication, nucleosomes modified with epigenetic marks become diluted in favor of naïve, newly integrated nucleosomes (Alabert *et al.*, 2015). Subsequently, these marks may drop below the critical density that is required to maintain epigenetic inheritance (DiPiazza *et al.*, 2021). As a result, this leads to a slippage in repression and the loss of repressive domains as a whole. As an additional cause, active demethylation of H3K9me3 can similarly result in a decay of heterochromatin marks. This is often actively pursued by cellular HDMs to prevent ectopic heterochromatin formation in euchromatin regions (Audergon *et al.*, 2015). As a result, ectopic heterochromatin can only be maintained in *S. pombe* when the (putative) histone demethylase Epe1 is abrogated. This necessitates mechanisms by which heterochromatin regions are delineated from their euchromatin counterparts.

In humans (but not fission yeast), aside from HP1, long-lasting H3K9me3 stretches are often found to be demarcated with methylated DNA, especially in CpG dinucleotide stretches (Lehnertz *et al.*, 2003). These CpG islands are normally highly enriched in promotors and are associated with active transcription (Vavouri and Lehner, 2012). However, DNA methylation has been shown to reverse this dynamic, leading regions to become transcriptionally silent (Ma *et al.*, 2023). In cells, DNA methylation is propagated through DNMT3a/b and can be maintained in a read-and-write fashion through DNMT1 following replication (Okano *et al.*, 1999; Qin *et al.*, 2015). Interestingly, in mammals, this system of DNA methylation has been shown to interchangeably cross-talk with methylated H3K9me2/3 (Liu *et al.*, 2013). Here, the DNMT1-targeting factor UHRF1 is recruited by both H3K9me2/3 and methylated CpG. UHRF1 subsequently targets DNMT1 to facilitate the methylation of nearby DNA. Crosstalk from DNA methylation to H3K9 methylation is less direct and likely involves its role in genome compartmentalization (Spracklin *et al.*, 2022). Namely, loss of DNA methyltransferases and inhibition of DNA methylation both leads to decreased levels of constitutive heterochromatin, as measured through the amount of interacting H3K9me3-HP1 and their individual abundance.

Lastly, in *S. pombe*, certain DNA sequences have themselves been shown to be capable of maintaining but not establishing heterochromatin domains (Wang *et al.*, 2021). These so-called 'maintainer binding sequences' were shown

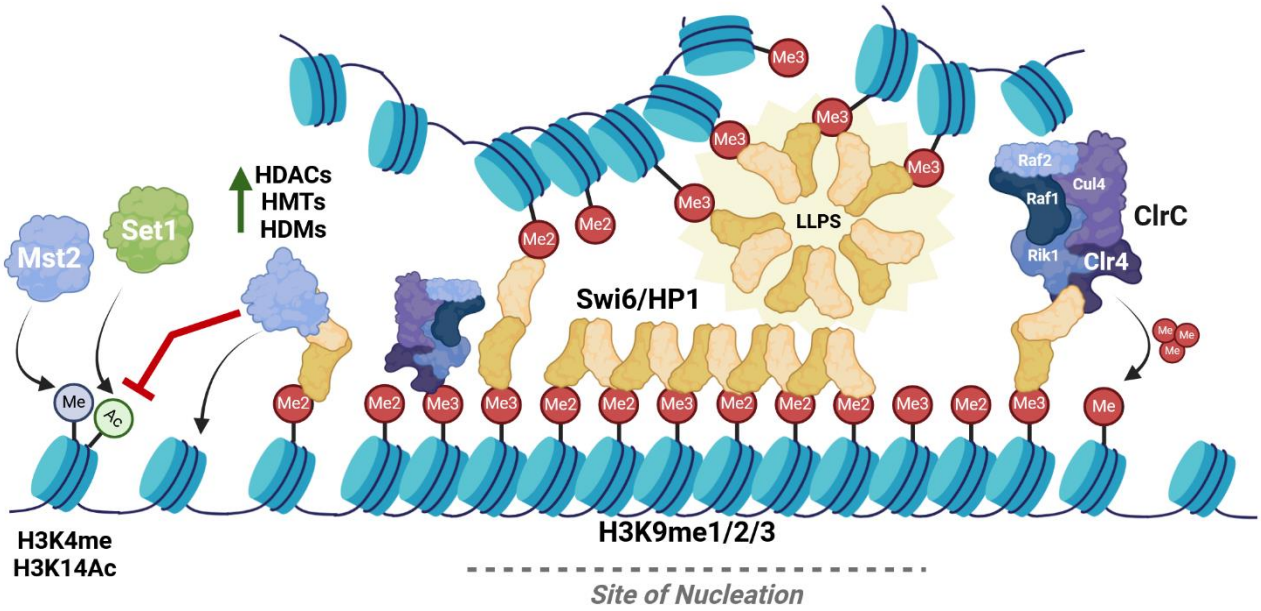

Figure 3. Schematic overview of heterochromatin spreading & condensation mechanisms found in *S. pombe*. *In S. pombe*, positive feedback loops through Clr4 and Swi6/HP1 facilitate the propagation of constitutive heterochromatin domains. Simultaneously, Swi6-mediated recruitment of HDACs, HDMs and heterochromatin factors pave the way for the growing heterochromatin front. Furthermore, Swi6 can, through di-/oligomerization, form DNA condensates that display Liquid-Liquid phase separation properties and help concentrate heterochromatin factors. Figure was made using BioRender.

to contain binding sites for ORC, Atf1, Pcr1 and Deb1 proteins, which accordingly recruited Clr4/Suv39h1 to further catalyze the trimethylation of H3K9. Overall, these resourceful mechanisms form the basis by which heterochromatin domains can concentrate their required factors and stably cover large regions of the genome. Simultaneously, as is the case for many other forms of positive-feedback, applying these principles indiscriminately will inevitably result in genomes consisting of nothing but heterochromatin. Therefore, moderation is required.

■ Factors restricting H3K9me3 Heterochromatin Propagation

As described so far, heterochromatin propagation can be grossly summarized as a positive-feedback process dependent on the interactions between H3K9me2/3, HP1 and HMTs (like Suv39h1/2). Wherein site-specific HMTs may nucleate short stretches of trimethylated H3K9, which as a result recruit both HP1 and Suv39h1/2 to the vicinity. As a recruitment hub for heterochromatin factors, HP1 by itself facilitates additional enrichment of HMTs, HDACs, DNMTs and heterochromatin factors. Thereby, HP1 condensates pave the way for new histone H3 methylation ahead of the heterochromatin front. Once started, this process seemingly becomes self-enforcing. So, how do cells prevent heterochromatin from overtaking regions in which cell expression is essential? In actuality, there is likely not a singular mechanism responsible. In its stead, the regulation of heterochromatin may become feasible through the combination of multiple mechanisms that each exist to functionally separate eu- and heterochromatin (fig. 4). Some of which we will now discuss in further detail.

Histone turnover mechanisms

To start off, we will look at how regulating histone turnover can limit or promote H3K9me3 propagation. Histone turnover is defined as the rate by which integrated histones are replaced with naïve histones. This dynamic process may occur spontaneously, as a result of active regulation or as a side effect of polymerase passaging (during transcription and replication). Both transcription and replication significantly promote histone turnover, as the underlying complexes need to either evict or bypass histones to be able to access the tightly wound DNA (Jamai *et al.*, 2009; Gruszka *et al.*, 2020). Interestingly, in both replication- and transcription-dependent mechanisms, involvement of a conserved nucleosome chaperone complex at least partially regulates these processes through discrete mechanisms. Therefore, we will be discussing these processes independently, starting with active regulation of histone turnover.

Histone turnover can be actively promoted by cells through the modification of heterochromatin marks. This dynamic plays an important role in *S. pombe*, as ectopic heterochromatin located in or near euchromatic regions is prevented from spreading through the local recruitment of the conserved Set1 HMT (Greenstein *et al.*, 2020). Set1, as part of the COMPASS complex, catalyzes H3K4me1/2/3. The presence of these modifications has been shown to both actively inhibit Clr4/Suv39h1 activity and promote the catalysis of histone acetylation by HATs. As discussed previously in the context of HDACs, most forms of histone acetylation increase histone turnover (Zee *et al.*, 2010). Whilst the underlying reasoning is not entirely known, it is thought that acetylation of lysine residues neutralizes the positive charge that normally interacts with negatively charged

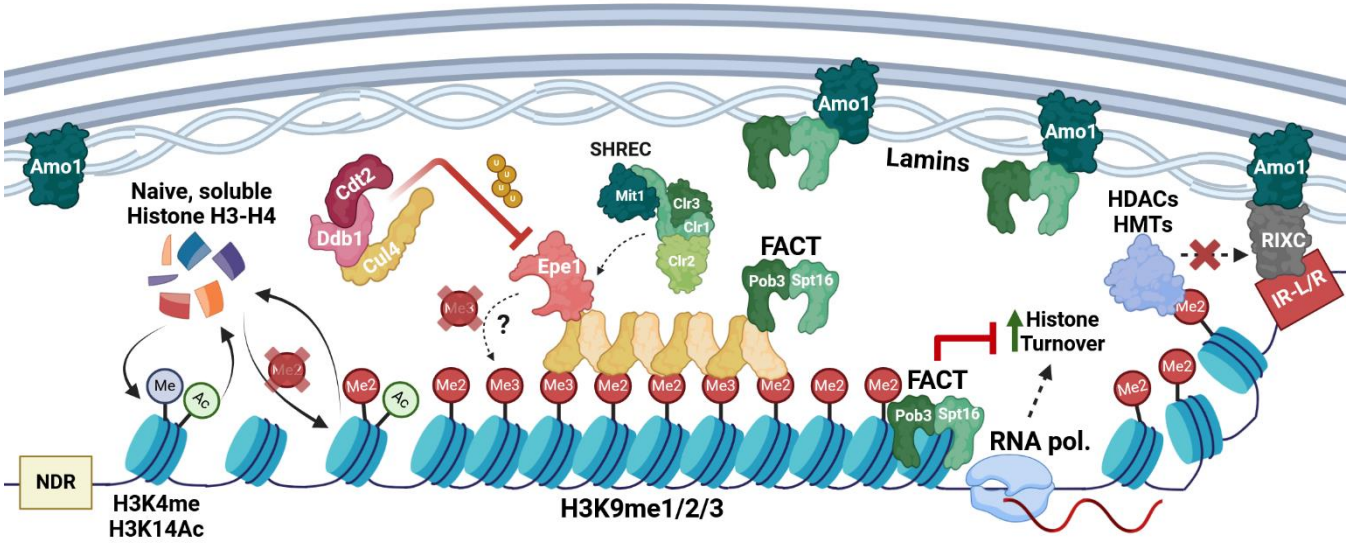

Figure 4. Schematic overview of factors restricting heterochromatin propagation.
Heterochromatin is prevented from spreading through mechanisms including histone turnover, Epe1-mediated degradation, boundary elements, and through spatial segregation to the nuclear periphery. To reduce histone turnover, the FACT complex is actively recruited by both Swi6 and Amo1 to LADs. Here, it reduces turnover in a transcription- and replication-dependent manner. Figure was made using BioRender.

DNA. This would reduce the energetic favorability of the nucleosome-DNA interaction, making it less stable. Thereby, active recruitment of Set1 in euchromatin can promote histone turnover, which restricts the formation of stable H3K9me3 stretches in the vicinity of euchromatin. At least in *S. pombe*, H3K9me3 is catalyzed at much slower rates than H3K9me1/2. As a consequence, comparatively small pools of H3K9me3 can be found at a heterochromatin front (Al-Sady *et al.*, 2013). Simultaneously, H3K9me3 modifications are highly important for the positive-feedback loop of heterochromatin propagation, as the central HMT (Clr4/Suv39h1) for this process preferably binds to H3k9me3 instead of H3K9me2. Active replacement of H3k9me3 with naïve, unmarked histones can therefore significantly antagonize heterochromatin propagation.

In *S. pombe*, stretches of H3K9me2 do not inhibit transcription, a process that promotes histone turnover (Jih *et al.*, 2017). Recent experiments by Takahata *et al.* (2021) have shown that the histone chaperone FACT is essential for keeping H2A/H2B occupancy within heterochromatin stable, through a transcription-dependent mode of action. This in itself is not very surprising. In fact, the FACT complex has for quite some time been known to facilitate non-disruptive passaging of the RNA polymerase by transiently displacing H2A/H2B dimers (Chen *et al.*, 2018). In accordance, the inhibition of the Spt16 FACT subunit has been shown to make transcription more disruptive to nucleosomes, as chromatin-bound H2B and H3 is lost in a transcription-dependent manner. A surprise, however, was that Takahata *et al.* showed that heterochromatin also actively recruits and enriches the FACT complex through dimerized Swi6/HP1 (Takahata *et al.*, 2021). Here, they theorized that FACT is involved in actively maintaining the octamer conformation of histones. Through the loss of FACT, DNA-bound histone octamers are reduced to histone tetramers and hexamers, which induce derepression of heterochromatin regions and thereby could worsen histone turnover. In the past, the FACT complex has generally been considered a mediator of transcriptionally active euchromatin, even in the context of its interaction with Swi6/HP1 (Kwon *et al.*, 2010; Klein *et al.*, 2023). Therefore, this newly discovered role of FACT greatly illustrates how identical protein complexes may be utilized in opposing cellular contexts.

In contrast to transcription, during replication, nucleosomes are mainly evicted instead of bypassed, thereby leading to an approximate two-fold dilution of heterochromatin marks following each replication cycle (Alabert *et al.*, 2015; Gruszka *et al.*, 2020). Hence, for heterochromatin domains to remain stable following replication, parental H3K9me2/3-modified histones require faithful recycling into the nascent-replicated DNA after their eviction upstream. How cells efficiently mediate this process is not well known, however, recruitment of the FACT complex to replisomes has also been shown to be an important factor here (Takahata *et al.*, 2021). During replication, the FACT complex shuttles parental H3/H4 tetramers from DNA upstream of the replication fork to nascent DNA downstream by interacting with Mcm2 and Mcl1 (Wang *et al.*, 2024; Nathanailidou *et al.*, 2024). Interestingly, if Mcl1 (or to a lower extent Mcm2) becomes mutated in a way that prevents it from shuttling the FACT complex, then cells become unable to locally retain parental histones following replication (Nathanailidou *et al.*, 2024). The loss of FACT activity in this process has been shown to have a large impact on heterochromatin domains, as due to the dramatic increase in histone turnover, they became unable to propagate successfully and instead became limited to sites of their nucleation. Additionally, the concentration of soluble, naïve histones around replisomes has also been linked to histone recycling, with lower concentrations making parental recycling more likely (Gruszka *et al.*, 2020). Whilst not established yet, it would be interesting to investigate whether cells change the rate at which histones are recycled, potentially through

FACT or soluble histone concentration, as a way to regulate heterochromatin domains.

Spatial segregation within the nucleus

Overall, the regulation of histone turnover shows that heterochromatin factors (like the FACT complex) can be essential for the continued existence of heterochromatin domains. Subsequently, this makes their spatial enrichment near heterochromatin instead of euchromatin a matter of great importance. How this is spatially regulated is, however, not entirely known.

Interestingly, microscopy studies have long reported that constitutive heterochromatin domains are enriched near the nuclear periphery (Pickersgill *et al.*, 2006). There, H3K9me2/3-marked heterochromatin interacts with lamina- and nuclear transmembrane proteins, like Amo1, Npp106, Lamin B receptors, Lamins and cell-type specific anchors (Iglesias *et al.*, 2020; Polioudaki *et al.*, 2001; Solovei *et al.*, 2013; Biferali *et al.*, 2021). As a consequence, these repressed compartments have generally become known as lamina-associated domains or LADs instead of TADs. Aside from their location, LADs also differ from TADs in other properties. For example, they have been shown to contain fewer cohesion-mediated loops and to consist of mostly heterochromatin (Guelen *et al.*, 2008; Handoko *et al.*, 2011). As LADs make up approximately a third of the genome and are highly enriched in heterochromatin, they represent a significant fraction of the repressed chromatin within cells (Guelen *et al.*, 2008). Interestingly, cells that lack the expression of the fiber-like lamin proteins covering the nuclear membrane, have greatly impacted gene expression profiles (Zheng *et al.*, 2018). Follow-up experiments have shown that losing these proteins is enough to cause LADs to detach from the nuclear membrane, undergo heterochromatin decondensation and experience changes in inter-LAD interactions. Overall, indicating that, by sequestering- and anchoring these domains to the nuclear periphery, their organization is kept in a repressed, heterochromatin state. This repressive state is somewhat lost once LADs move away from the nuclear membrane, therefore spatial separation within the nucleus may be a key process by which genomic sequences are kept in either an euchromatin or heterochromatin state. Other recent findings from combined transcriptomics and scDamID experiments have also backed this theory, as they showed that closer association of TADs with the nuclear lamina (NL) reduced their expression compared to when the same TADs were found away from the NL (Rooijers *et al.*, 2019). Curiously, TADs that were normally located farthest away from the NL were shown to be most impacted by this change in localization.

So, why does the nuclear periphery induce a transcriptionally repressive state? Whilst this is not entirely known, one explanation in *S. pombe* can be found in the nuclear rim protein Amo1. This protein has been shown to: 1. Tether heterochromatin to the nuclear periphery via boundary regions and 2. Locally recruit and enrich the FACT complex at the nuclear membrane (Holla *et al.*, 2020). Thus, by limiting the suppression of histone turnover to the nuclear periphery, formation, and propagation of heterochromatin may also become spatially limited to the nuclear membrane. Simultaneously, the coordinated anchoring of heterochromatin at the nuclear periphery may itself function to create a microenvironment that promotes transcriptional repression. Lastly, by enriching eu- and heterochromatin factors at distinct, far-away locations, cells can minimize the crosstalk between their respective factors. Thereby helping to prevent improper repression or activation of transcription.

Boundary elements and nucleosome free regions

As discussed so far, spatial separation is crucial for preventing crosstalk between domains. Yet, due to the linear and variegated nature of chromosomes, it's a given that eu- and heterochromatin domains will cross paths at some points. From what we've seen, most forms of heterochromatin propagation and suppression are highly dynamic. Nevertheless, in cells, boundaries between eu- and heterochromatin regions are often sharply defined (Yasuhara and Wakimoto, 2008). This begs the question, how do cells stably demarcate the border between active- and repressive regions?

In general, eukaryotic cells have been shown to utilize a large variety of so-called 'boundary elements'. These genomic elements can highly vary, consisting among others of nucleosome-depleted regions (NDRs), tRNA genes, CTCF-binding sites, IR-L/R sequences, and many more (Charlton *et al.*, 2020; Chen *et al.*, 2012; Li *et al.*, 2011; Scott *et al.*, 2006). Therefore, we will focus on some of the mechanisms that may act on these elements to facilitate the suppression of heterochromatin spreading.

In the nuclear periphery, the tethering of chromatin to the nuclear membrane has been implicated in isolating heterochromatin (Charlton *et al.*, 2020). In *S. pombe*, many boundary elements, like tRNA genes and IR-L/R sequences, contain B-box motifs that have been shown to bind the RNA-processing complex RIXC (Holla *et al.*, 2020). RIXC simultaneously can interact with Amo1, thereby tethering the DNA to the nuclear membrane. Interestingly, in this scenario, loss of Amo1, the IR-L/R sequences or the sequences surrounding the B-box motif results in a derepression instead of an overspreading of heterochromatin (Charlton *et al.*, 2020). Indicating that, contrary to how heterochromatin spreading is often viewed, some boundary elements may instead serve to protect heterochromatin from encroaching euchromatin factors, like Epe1, Set1 and HATs.

As heterochromatin spreading requires a critical density of H3K9me3-modified nucleosomes, regions with decreased nucleosome density were theorized to function as heterochromatin isolators (DiPiazza *et al.*, 2021). Specifically, sites of transcriptional initiation were often found to contain NDRs, characterized by lower histone occupancies (Yadon *et al.*, 2010). Recently, in an *in vitro S. cerevisiae* system, NDRs were shown to be able to isolate chromatin domains from one another (Oberbeckmann *et al.*, 2024). In this system, the strength of the isolation similarly depended on the width of the NDR, thereby indicating that sufficiently large NDRs could facilitate the separation of chromatin signals from active- and repressive domains. Even so, the functioning of NDRs in isolating heterochromatin spreading *in vivo*, has yet to be seen. At the same time, there is some evidence that NDRs may actually be actively eliminated through the recruitment of heterochromatin silencing factors (Garcia *et al.*, 2010). A feedback mechanism that would actively negate their isolation function.

Lastly, heterochromatin boundaries are often further enhanced through the anti-silencing factor Epe1, a (putative) HDM in *S. pombe*. Unexpectedly, even though Epe1 is

dependent on Swi6/HP1 for its recruitment, its localization is specific to the heterochromatin boundaries and not heterochromatin as a whole (Braun *et al.*, 2011). This is thought to mainly occur due to two forms of negative feedback, namely degradation via the Cul4-Ddb1 ubiquitin ligase and Swi6/HP1-binding competition with the SHREC complex (Braun *et al.*, 2011; Shimada *et al.*, 2009). Whilst demethylation of H3K9 has long been considered Epe1's canonical mechanism of action, it has yet to be proven *in vivo*. As such, Epe1's anti-silencing activity could also come from its ability to compete with HDACs or HMTs for Swi6/HP1 binding. Thereby, promoting nucleosome turnover over active removal of methyl marks (Aygün *et al.*, 2013).

DISCUSSION

In this review, we set out to tackle the question of how eukaryotic cells mediate the nucleation, self-reinforced spreading and maintenance of constitutive heterochromatin. Ultimately, using this information, we investigate the mechanisms that cells use to prevent constitutive heterochromatin from overtaking the chromatin landscape. Here, we find that the self-reinforced spreading of heterochromatin is highly concentration-dependent, with the formation of a recruitment hub consisting of H3K9me2/3, Swi6/HP1 and SHREC/NuRD being essential in enriching heterochromatin factors for this purpose. Condensation of di-/oligomerized Swi6/HP1, which in recent years has been linked to producing liquid-liquid phase separations *in vivo*, is particularly important here. Simultaneously, the presence of antagonizing euchromatin factors, like Epe1, Set1 and Mst2, provides a large pushback against heterochromatin spreading. Thereby, making the recruitment of these heterochromatin factors also a necessity for the preservation of heterochromatin near euchromatin-heterochromatin boundaries. Similarly, a lack of heterochromatin factors at ectopic sites results in high histone turnover, active demethylation, and HMT inhibition in these locations. Consequently, leading to the rapid loss of these ectopic H3K9me2/3 stretches. All the while, spatial segregation to the nuclear periphery and isolating boundary elements prevent cross-over from heterochromatin to euchromatin regions. Combined, these findings showcase a novel, alternative view wherein the maintenance of heterochromatin domains may require isolation from euchromatin factors as much as euchromatin domains require isolation from heterochromatin. Supporting this reasoning, in *S. pombe*, loss of boundary elements between eu- and heterochromatin often leads to the loss of hetero- instead of euchromatin domains (Charlton et al., 2020).

So far, the field of heterochromatin has been largely limited by the difficulties associated with investigating the highly redundant heterochromatin nucleation, spreading and maintenance processes in higher eukaryotes (like humans). Whilst many of the associated factors have remained conserved throughout evolution, their regulation may still generally change through the emergence of isoforms, additional functions or changes in spatiotemporal control. Therefore, whilst much has become known of heterochromatin nucleation, spreading and maintenance in the eukaryote *S. pombe,* these same processes often remain clouded in humans. Particularly, due to higher eukaryotes displaying many different cell types and levels of cellular potency, we have mostly limited the scope of this discussion to *S. pombe*. In this context, it would be interesting to investigate to what extent human cells of different types and potencies differently approach the regulation of these processes.

In any case, many questions have yet to be answered. Whilst RNAi-dependent nucleation of heterochromatin has been highly studied, the RNAi-independent mechanisms of nucleation often lack the same amount of depth. This is surprising, as in humans, RNAi is only essential in germline cells (Aravin *et al.*, 2007). Future research should, therefore, look into the mechanisms by which RNAi-independent nucleation is facilitated. For example, in the context of Seb1, it is still unknown what signal directly induces the nucleation of heterochromatin following polymerase-stalling and whether similar mechanisms exist in humans.

Simultaneously, it is important to realize that in humans, methylation of H3K9 only forms one of many different types of histone modifications, the majority of which have so far remained unexplored (Zhao and Garcia, 2015). Additionally, histone monomers can consist of different variants in humans, like H2A.Z, H3.Y.2, H4.7 and H1.2 (reviewed in Talbert and Henikoff, 2021). Of these, H2A.Z has recently been implicated in regulating HP1 at pericentromeric sites in humans (González *et al.*, 2023). Therefore, indicating that histone variants may themselves also play a role in regulating these processes. As such, future research should similarly focus on whether other histone modifications or variants are involved in the regulation of constitutive heterochromatin.

To close, it's important to reiterate that the regulation of constitutive heterochromatin is of crucial importance for an organisms genomic stability. To illustrate, a mutation in Lamin A, a factor which facilitates the attachment of LADs to the nuclear periphery, has been shown to lead to Hutchinson–Gilford Progeria Syndrome (HGPS). In this disease, patients experience rapid premature aging and have on average a life-expectancy of only 13,4 years (Shumaker *et al.*, 2006; Pachajoa *et al.*, 2020). Recently, experiments have shown that cells expressing mutated Lamin A undergo demethylation, derepression and nuclear remodeling of lamina-associated heterochromatin (Chojnowski *et al.*, 2020). In this process, loss of heterochromatin specifically corresponded to higher amounts of DNA damage. Further research into the formation, spreading and maintenance of heterochromatin may in the future hopefully help elucidate the cause of- and treatment for these conditions.

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Notes

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ABBREVIATIONS

Ago1, argonaute RISC component 1; ARC, Argonaute siRNA chaperone; CPF, cleavage and polyadenylation factor; Dcr1, Dicer 1; DNMT, DNA methyltransferases; HDAC, histone deacetylase; HDM, histone demethylases; HGPS, Hutchinson–Gilford Progeria Syndrome; HMT, histone methyltransferase; HP1, Heterochromatin protein 1; H3K9me3, histone H3 lysine 9 trimethylation; KZFP, Krüppel-associated box zinc finger proteins; LAD, lamina-associated domain; LLPS, liquid-liquid phase separation; NDR, nucleosome-depleted region; NL, nuclear lamina; NuRD, Nucleosome Remodeling and Deacetylase; priRNA, primal RNA; RITS, RNA-induced initiation of transcriptional silencing; RdRc, RNA-directed RNA polymerase complex; *S. cerevisiae*, *Schizosaccharomyces cerevisiae*; *S. pombe*, *Schizosaccharomyces pombe*; TAD, topologically associating domain;

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