The role of epigenetic inheritance in the maintenance or alteration of cellular identity

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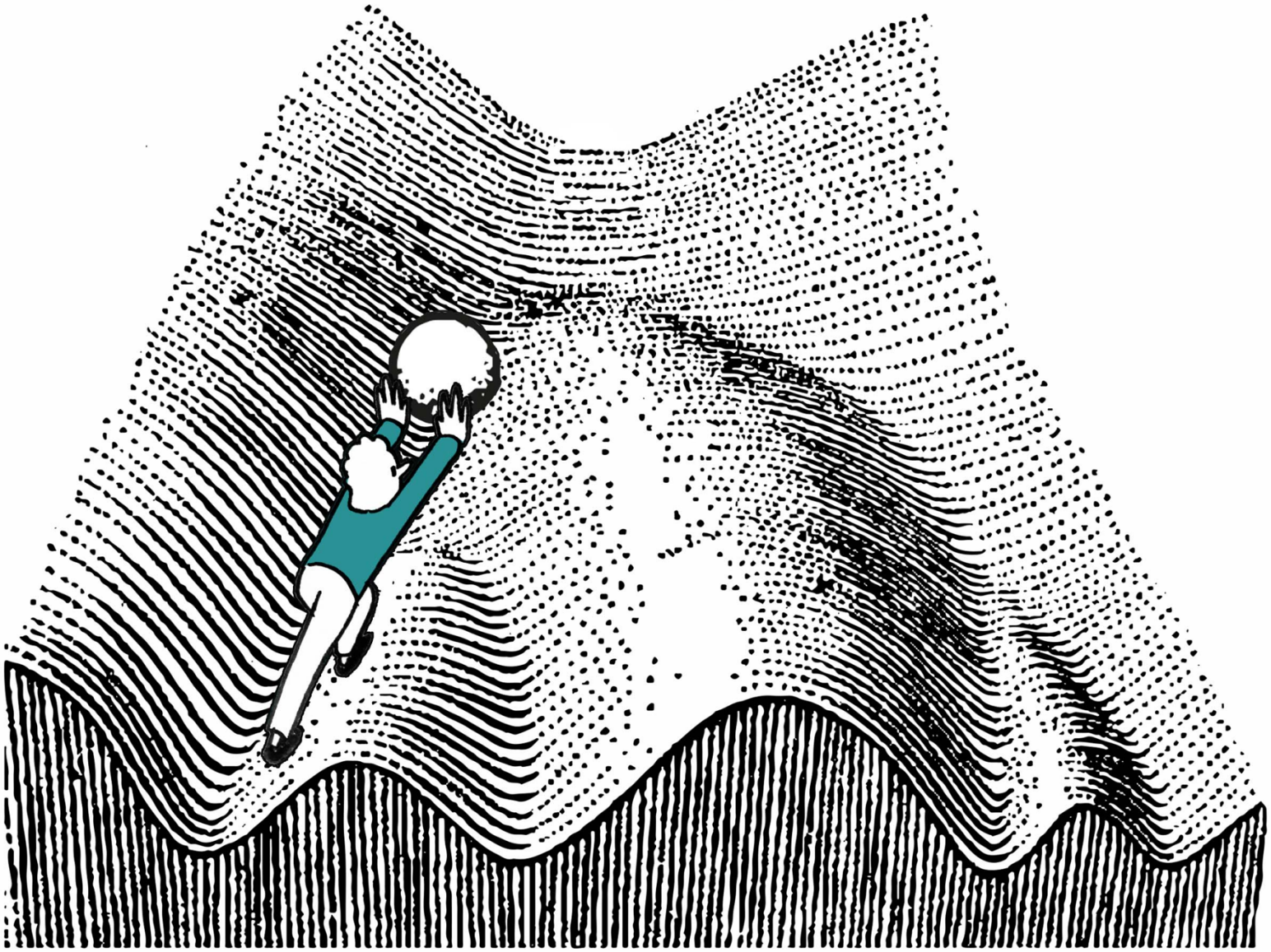

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Layman's Summary
Each cell of the human body contains almost the same DNA code. However, each cell has their own properties and functions due to cell-specific transcriptional programs and expression patterns. These patterns arise by packaging of DNA around nucleosomes into so-called chromatin. "Open" loosely packed euchromatin allows transcriptional initiation while tightly packaged heterochromatin repels this. The chromatin state is tightly regulated by many different epigenetic factors. These epigenetic factors cause heritable changes to gene expression that occur without changing the DNA sequence of a cell. Important epigenetic factors are posttranslational modifications (PTMs) which are attached to histones, which are the subunits of a nucleosome. These PTMs correlate with the chromatin state, so repressive PTMs cause a closed environment while activating PTMs make chromatin accessible. When a cell divides, chromatin needs to be disrupted from its nucleosomes and PTMs for accurate DNA replication fork passage. This means that the epigenome or cell memory is gone for a while. Still, a daughter cell knows exactly which genes to activate or silence after cell division. This means that there must be tightly regulated mechanisms that ensures the epigenetic inheritance. In this review, an overview is given of what is known so far about this research field. It has been established now that repressing PTMs stay attached to histones, which are recycled during DNA replication. Afterwards, these recycled PTMs act as a blueprint for the surrounding naïve histones, which receive the same repressive mark. However, activating PTMs are dispersed during DNA replication. The transcription machinery seems to play a role in the re-establishment of active DNA. But the exact mechanisms remain unsolved. It is important to increase our knowledge about epigenetic inheritance, as PTMs have been found to be a barrier for cell-reprogramming. Efficiently changing of cell fate attracts many research fields, for example the field of regenerative medicine and cancer, as it can have many therapeutic benefits. However, precent techniques that enable cell reprogramming have a low success rate. Increasing our knowledge about epigenetic inheritance could benefit the research to develop more efficient cell-reprogramming techniques.

Abstract
Cell identity is tightly regulated by cell-specific expression patterns. This is caused by epigenetic factors that pack the DNA into chromatin, making it accessible or inaccessible to transcription machinery. The regulation of these epigenetic factors is poorly understood, especially how this epigenetic memory is transmitted to daughter cells after mitosis. Accurate transmission of expression patterns after cell division is essential for preserving proper cell lineages. As DNA replication disrupts the chromatin structure, there must be highly regulated mechanisms involved in the restoration of chromatin. However, these mechanisms remain unclear. In this review, an overview is given of the current finding and models present in the research field of epigenetic inheritance. The focus will be on histone posttranslational modifications (PTMs) as these epigenetic factors are key determinants that correlate with the chromatin state and transcriptional status. Recent studies showed that repressive PTMs are inherited by staying attached to recycled histones during DNA replication. Afterwards, positive read-write loops restore these PTMs on neighboring naïve histones. For activating PTMs more remains unclear as their attached histones are dispersed during DNA replication. Transcription initiation seems an important factor for the appropriate restoration of accessible chromatin, but furthermore the mechanisms involved remain hypothetical. PTMs are a barrier for reprogramming of cell fate as they are important factors in maintaining the cellular epigenetic memory. There are techniques that enable reprogramming of cell fate, but they have low success rates with limited possibilities. Understanding the mechanisms involved in PTM inheritance could be used to develop more efficient reprogramming techniques in the future, which will have many therapeutic benefits.

Keywords
Epigenetic; epigenome; posttranslational modifications; heterochromatin; euchromatin; DNA replication; cell identity; epigenetic memory; reprogramming

Introduction

The identity of a cell is tightly regulated by cell-specific transcription programs. Still, the DNA sequence is almost identical in each cell of the body. The epigenome plays an important role in this, which involves binding of proteins and chemical modifications on the DNA that do not affect the sequence but are inherited to daughter cells. DNA is packed by histones into nucleosomes, which causes structures to form called chromatin. These chromatin structures protect the genome from the environment and organize gene expression[1,2]. Epigenetic factors like posttranslational modifications (PTMs) are attached to histones, which further stabilizes or loosens the chromatin. This makes PTMs important regulators of gene expression.

DNA replication is a well understood and heavily studied phenomenon. However, DNA needs to be unwound and detached from its chromatin for the replication machinery to proceed. Still, daughter cells know exactly which genes it needs to express to maintain cellular identity. How cells know how to establish the correct expression pattern after DNA replication and mitosis, and how chromatin states are inherited is still unclear. Researchers do not agree on certain mechanisms, especially on how posttranslational modifications on histones are transmitted. In this review, an overview is given of the current state of knowledge about epigenetic inheritance. A special focus will be on PTMs, as they are key determinants in the establishment of the chromatin state, but their mechanisms of inheritance are still largely unknown. Learning more about cell memory maintenance is also useful to increase our understanding about how cell fate can be altered. Therefore, it will be discussed how knowledge about PTM inheritance can be used to potentially alter cell identity at the end of this review

How epigenetics regulates cell identity

The identity of a cell arises from tightly regulated cell-specific transcription programs, which are closely related to the organization of chromatin. This means that transcription is regulated by both transcription factors activity and by epigenetic factors such as DNA methylation and histone PTMs. Chromatin can be either transcriptionally active or inactive, which is mostly determined by how tightly neighboring nucleosomes are packed to each other. At transcriptionally active places nucleosomes are not tightly packed together or ‘open’ which is called euchromatin[3]. Because the nucleosomes in euchromatin are not tightly packed, DNA is accessible for proteins like transcription factors and RNA polymerase which makes it a supportive environment for initiation of transcription[4,5]. The packaging of DNA around one single nucleosome is also important for gene expression. Weak binding around a nucleosome is present for example at active promotors which allows binding of the transcriptional machinery. In euchromatin, loosely packed nucleosomes at specific spots like promotors are alternated with normally packed nucleosomes in for example gene bodies, termination sites and in between euchromatic genes. Opposite to this, regions with nucleosomes tightly packed to each other, are called heterochromatin. DNA in these regions is not or much less accessible for the transcription machinery which makes it transcriptionally inactive. The same chromosome can consist of both euchromatin and heterochromatin which cause chromosomes to have certain transcription patterns. This pattern can change in the cell, but this is strictly regulated by signals or transcription factors that promote a dynamic transition between the chromatin states[6]. Expression patterns are highly cell-specific and determine the fate of a cell, the so called cell identity[1].

The basic unit of chromatin is a nucleosome which consist of 147 base pairs of DNA wrapped around a histone octamer. This octamer consists of two H2A-H2B histone dimers and

two H3-H4 histone dimers bridged together as a tetramer[7]. The H3 and H4 histones have amino-terminal domains or also called histone 'tails' which protrude from the nucleosome. These tails are the main platforms for PTMs which are small, chemical modifications that are added and removed post-translationally by highly specific enzymes[8]. The process in which PTMs are attached to histones is called histone maturation. There are many proteins involved in the regulation of PTMs. Besides this, a nucleosome can also vary in its histones, as there exist multiple histone variants like H3.1, H3.2 and H3.3. There are many proteins involved in the regulation of these histone variants and their attached PTMs. For example histone chaperones, a diverse group of proteins with distinct structural and functional properties, play an important role in this[9,10]. They regulate histone availability and fate, which also affects gene expression genome replication and repair. Besides this there are writer proteins which add PTMs to a histone, and erasers which remove these PTMs. Readers are proteins which can bind to PTMs to recruit other components. The presence of certain PTMs or combinations of them correlate with a given transcriptional status and facilitate the formation of chromatin structures which impacts gene expression[11,12]. Activating PTMs include for example acetylation (H4K16ac), monoubiquitinylation at a specific residue, lysine 120 of H2B and methylation of specific histone residues (H3K4me3, H3K36me3)[13]. Trimethylation of H3K9 (H3K9me3) and of H3K27 (H3K27me3) is associated with repressed chromatin. These PTMs provide binding sites for readers that recruit other components to compact chromatin, forming constitutive (H3K9me3) or facultative (H3K27me3) heterochromatin.

Cell identity is determined by the transcriptional programs of a cell in which the interplay between transcription factors, histone chaperones, DNA methylation, histones and its PTMs play a major role. How do these factors co-operate with each other to come to a certain active or silenced expression? The state of chromatinized DNA results in a conformation that allows or inhibits binding of certain regulators, either repressors or activators. The exact order of events remains debated, but it is generally accepted that if the chromatin allows it, based on its already established PTMs and chromatin state, transcription factors bind first at regulatory elements on the DNA, after which they recruit other chromatin remodelers, RNA polymerase and other co-activators to enhancers and promotors[14]. So, euchromatin has an open environment that requires the association of a sequence-specific transcription activator which recruits many factors that associate with RNA polymerase II to facilitate transcription[15,16]. In contrast, heterochromatin is usually rich in repetitive sequences, DNA methylation and 'silencing' PTMs which causes a compacted environment and repels transcription factors. Constitutive heterochromatin remains in the closed state while facultative heterochromatin is more dynamic and can be regulated to become more allowing to TFs. To make it even more complex, all these factors influence each other as well. For example, the histone mark H3K9me3 and CpG methylation frequently co-occur and reinforce each other, while DNA methylation and the H3K4me3 mark exclude each other[17].

How cell identity is maintained after DNA replication

So, many factors work together to establish expression in a cell, but how are these factors passed to the daughter cell? To maintain cell identity, both the DNA sequence and the transcriptional program must be inherited to the daughter cell, rather than established anew. When a cell divides, it undergoes dramatic changes as DNA replication disrupts the chromatin to permit passage of the replication fork. This makes DNA replication both a productive and disruptive mechanism. Despite all of this, daughter cells manage to re-establish the parental chromatin and gene expression patterns. This means that in addition to DNA replication, cells

contain mechanisms that enable the accurate reassembly or segregation of parental nucleosomes and its PTMs to the same location of the daughter cell's DNA. We are only just beginning to understand how this works. It is recognized that mitotic bookmarking, which is the process in which transcription factors remain bound to chromatin during mitosis is an important factor for the re-establishment of expression patterns in daughter cells[15]. Besides this, it is also well known that DNA methyltransferase 1 (DNMT1) recognizes hemimethylated DNA after DNA replication, after which it methylates the newly synthesized DNA strand while copying the methylation pattern of the already existing DNA strand[18,19]. However, understanding the transfer of parental nucleosomes with their associated PTMs during DNA replication remains challenging.

Inheritance of histones

To understand PTM inheritance, it is important to understand the maintenance of histones during DNA replication first. This is because an important feature in the inheritance of repressive PTMs is that parental histones maintain their PTMs during DNA replication[20]. For DNA replication, DNA needs to be free of chromatin, so nucleosomes are disrupted ahead of the replication machinery. This leads to the disassembly of nucleosomes into one $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers[10,21]. The first studies into histone inheritance found that parental histones are recycled and deposited behind the replication fork onto the newly synthesized DNA relatively quickly (Figure 1)[22–24]. Besides this, the daughter strand receives parental and newly synthesized histones in equal ratios[25–27]. So, when DNA is duplicated, parental histones are diluted two-fold. This process of disassembly of parental nucleosomes ahead of the replication fork and placement behind the fork is called parental nucleosome segregation. Ahead of the replication fork, DNA is unwound by the replicative helicase CMG complex which includes CDC45, MCM2−7 and GINS. The force of this unwinding may be sufficient to trigger nucleosome disruption with help of the FACT complex[28–30]. The reassembly starts with the $(H3\text{-}H4)_2$ tetramers[13,29,31]. Besides this, it was found that the H2A-H2B dimers are not tightly associated with the replication fork and not recycled but established anew[32,33]. This means that the $(H3\text{-}H4)_2$ tetramers are likely to be carrying the epigenetic information to the daughter DNA.

It has been shown that $(H3\text{-}H4)_2$ tetramers are deposited behind the replication fork on both leading and lagging strand in a random fashion. However, there are mechanisms that ensure symmetrical partitioning of the tetramers to the leading and lagging strand. Recycling to the lagging strand involves MCM2, a part of the CMG complex which exhibits histone chaperone activity (Figure 1A)[24]. MCM2 has a flexible N-terminal tail which contains a histone-binding domain (HBD) which can chaperone histones. It can only exhibit this function when MCM2 is bound to chromatin, MCM2-7 is inactive and CMG helicase is active[28,34–36]. MCM2 mimics DNA interaction with histone H3-H4 in the nucleosome which allows it to handle all H3 variants, irrespective of their bound PTMs[34]. The HBD protrudes towards the unwinding parental DNA strand which places it in proximity to the histones that are released from the parental DNA strand during chromatin disassembly[37]. This means that the CMG complex causes the evicted histones before the replication fork to be retained. In research in which they disrupted the histone binding domain of MCM2 in mouse embryonic stem cells, parental histones showed a strong preference to the leading strand. In yeast they tried to mimic this situation and they found leading strands preference in two scenarios: 1) a mutation of MCM2 to prevent histone binding and 2) a mutation to disrupt the interaction between DNA polymerase-α (Polα) and chromosome transmission fidelity factor 4 (CTF4)(Figure1B)[24,38]. This

implies that MCM2 ensures deposition of $(H3\text{-}H4)_2$ to the lagging strand in association with Polα and CTF4[26,38]. For deposition to the leading strand another mechanism was described. Polε is the core enzyme involved in leading strand replication[39]. Within this complex, two subunits, POLE3 and POLE4 (Dpb3 and Dpb4 in yeast), have been found to mediate chaperone activity. In yeast, it was found that a deletion of Dpb3 or Dpb4 impaired recycling of parental histones to the leading strand, which could also be the case for eukaryotic POLE3 and POLE4 (Figure 1C)[24,27,40]. This shows that these subunits play a role in chaperoning the parental histones to the leading strand. These findings imply that it is important for histones, as carries of epigenetic information, to stay tightly associated with their genomic location through DNA replication, mediated by specific histone chaperones.

Although parental histones are recycled during DNA replication, the number of histones is diluted two-fold. To counter for this loss of histone density many new histones are synthesized during S-phase. The newly synthesized histones are chaperoned by Antisilencing Factor 1 (ASF1), which leads them to the downstream chaperone complex Chromatin Assembly Factor 1 (CAF-1) (Figure 1A)[24,41]. CAF-1 interacts with proliferating cell nuclear antigen (PCNA) to deposit histones on the replicating DNA[42].

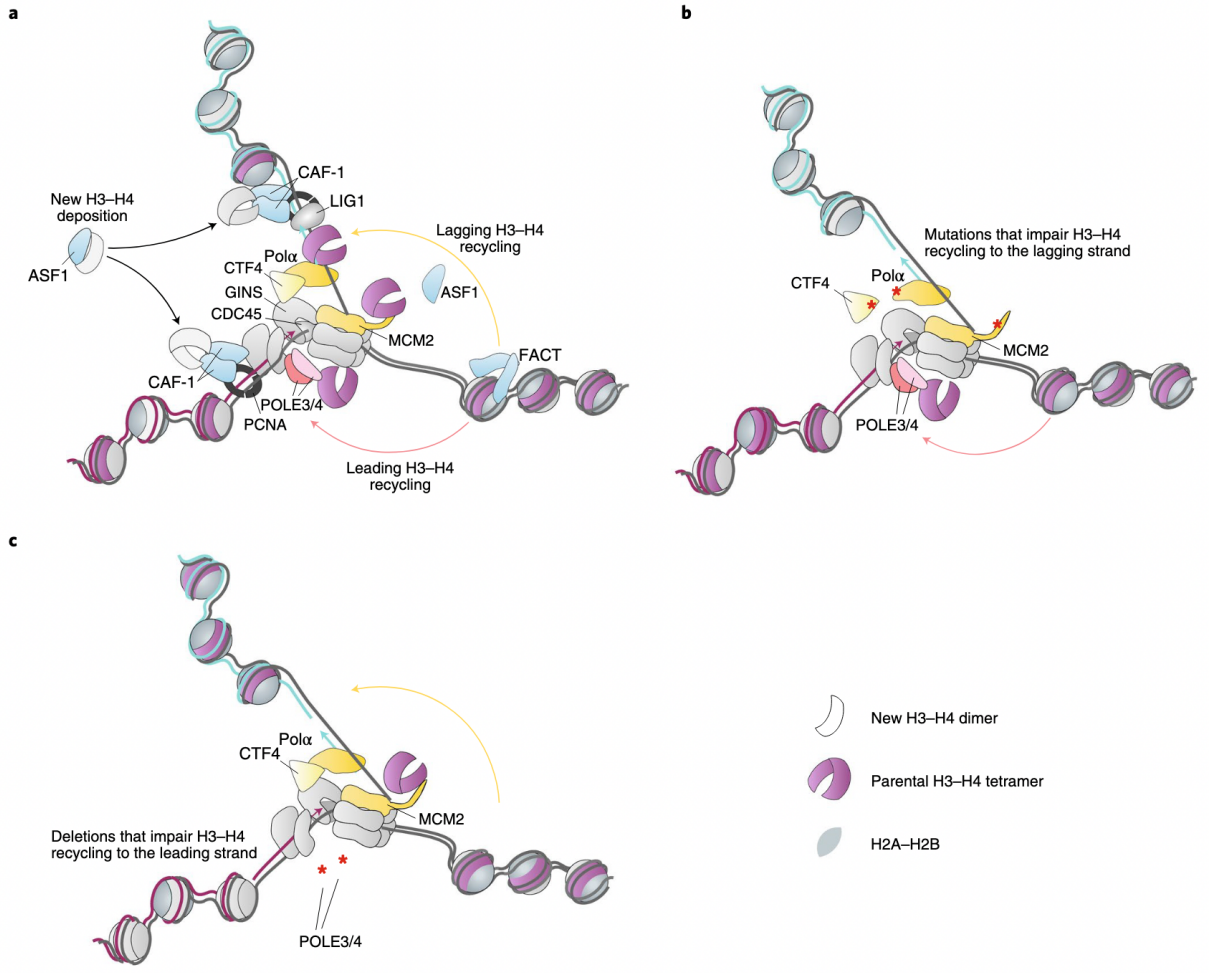

Figure 1: Histone H3-H4 recycling during DNA replication in eukaryotes[24]. A) A simplified scheme of histone H3-H4 recycling and nucleosome assembly at the replisome. Yellow and lilac factors are involved in histone recycling. Light-blue factors are chaperones involved in deposition of newly synthesized histones. B, C) Mutations or deletions (red asterisks) that impair recycling of histone H3-H4 to the lagging strand (B) and to the leading strand (C) are shown.

Inheritance of Post Translational Modifications

After DNA replication, PTMs are believed to play an important role in the transmission of expressed or silenced genes to the daughter cell. An early on established hypothesis was that PTMs remain attached to recycled parental histones during DNA replication. The first clear evidence for this came from studies into the two repressive methylation marks H3K9me3 and H3K27me2/me3[43–45]. Multiple papers showed that the H3K9me3-chromatin state was inherited across multiple generations without presence of the initiating signal[46,47]. Previous research also implied that active mechanisms could be recycled at the replisome. One of them, published by Reverón-Gómez *et al*[48], used an assay which is used to measure chromatin occupancy after DNA replication called ChOR-seq. In this assay, chromatin immunoprecipitation of specific PTMs is combined with EdU pulse labeling[48]. Through this assay, densities of specific PTMs before and after DNA replication were assessed. It was found that both activating (H3K4me3), and repressive marks (H3K27me3) were enriched before and immediately after replication. This suggested that both activating and repressive PTM marks were recycled during DNA replication by staying attached to histones. However, this technique measured presence of PTMs before and after replication, but this alone cannot prove that PTMS are indeed recycled at a specific locus or established quickly anew. The same accounts for the research showing that repressive marks were contained on the histones without the presence of the initial signal[46,47]. Although this research provided insides in the 'bulk' re-deposition or inheritance of epigenetics, it cannot determine the exact mechanism of PTM recycling at the replisome. To prove the local segregation theory of PTMs direct testing of local re-disposition of parental histones at a particular locus was needed. Escobar *et al*[49,50] were the first ones to break this research-gap by developing a proximity-dependent labeling system which irreversibly marked replication-dependent H3.1 and H3.2 histone containing nucleosomes in mouse embryonic stem cells (Figure 2). Their findings showed that only repressed chromatin domains were recycled at the replisome to sister chromatids whereas parental histones from actively transcribed genes were dispersed[49,50]. This shows that different mechanisms are involved in inheritance of activating and repressing PTMS, and that repressive PTMs probably have a more significant role in epigenetic inheritance then active PTMs.

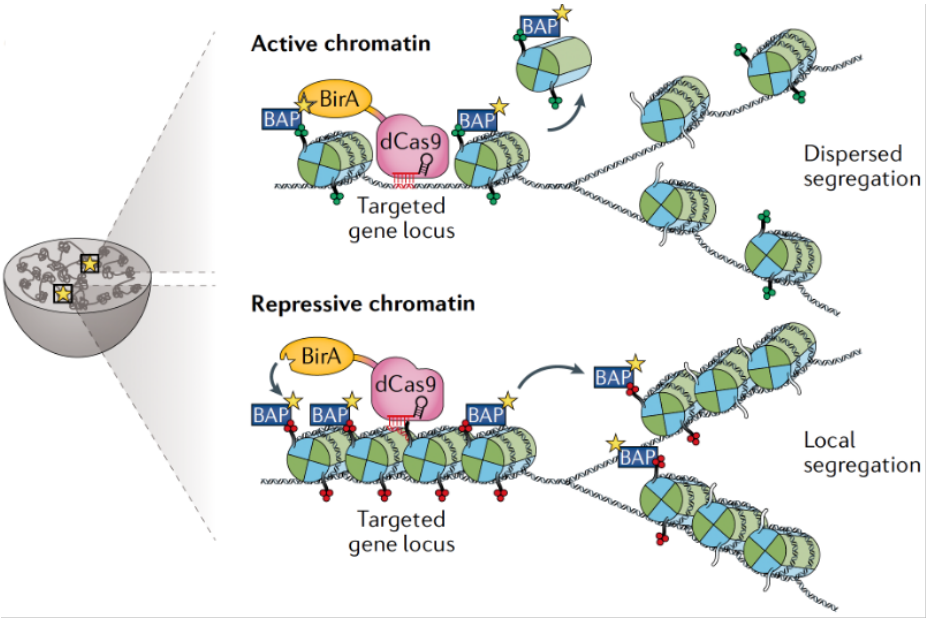

Figure 2: Schematical overview of active and repressive chromatin inheritance[49,50].
Nuclease-deactivated Cas9 (dCas9) is fused to *Escherichia coli* biotin ligase (BirA). With use of a guide RNA, dCas9 with its attached BirA is targeted to transcriptionally active or repressed loci. BirA puts a biotin on a biotin acceptor peptide (BAP) that binds to PTMs before replication. This BAP can be any protein that allows biotin attachment and binds to the PTMs interest. This irreversibly marks histones and their associated PTMs which allows tracing of their fate during DNA replication. Histones of active chromatin are dispersed while repressive chromatin shows local segregation of histones with their associated PTMs.

Inheritance of repressive PTMs

In the yeast *S. pombe*, the *Clr4* gene encodes for the H3K9me3 methyltransferase Clr4. H3K9me3 is a repressing mark and recruitment of Clr4 to euchromatin leads to transcriptional repression and change to heterochromatin. Ragunathan *et al*[47] found that repression of the targeted locus was sustained through ten yeast generations, even when Clr4 was no longer present. Combined with the previously described research of Escobar *et al*[49], this shows that H3K9me3 is a true epigenetic feature, where the histone acts as the carrier of epigenetic information. An important factor in the inheritance of H3K9me3 is the 'read-write' mechanism of Clr4, which is also found in its mammalian homolog SUV39H1/SUV39H2[51,52]. This read-write mechanism exhibits a self-sustaining positive feedback loop which is established with so-called two-state activation[51]. The CD reader domain of the enzyme recognizes nucleosomal H3K9me3, its product of catalysis, and anchors to the chromatin with the N-terminus (Figure 3)[11,51]. This results in allosteric stimulation of the SET writing domain which promotes catalysis of H3K9me3 on neighboring naïve nucleosomes[51]. A similar mechanism is also described for H3K27me, which is a hallmark of facultative heterochromatin. This mark is catalyzed by Polycomb repressive complex 2 (PRC2) which is also involved in the restoration of these domains after DNA-replication[43,44,53]. In this case the EED reader subunit recognizes H3K27me3 which stimulates the reorganization of the PRC2 structure which again stimulates the activity of the EZH2 writer domain (Figure 3)[11,54,55]. These mechanisms seem to maintain the presence of repressive marks after DNA replication and are important for histone maturation after DNA replication. However, there is also research that shows that these mechanisms are probably only a small part of a much bigger network. For example, PRC2 can only maintain presence of H3K27me3 in the presence of Polycomb response elements (PREs) in *Drosophila melanogaster*[56].

To conclude, the best accepted model for the inheritance of repressive marks is that they are recycled at the replisome by staying attached to recycled histones. As this will lead to PTMs being diluted two-fold, recycled PTMs form a platform for methyltransferases which recognize them and further restore the repressive marks on the histones exhibiting a positive feedback loop. However, these positive feedback loops are probably accompanied by other elements which are also involved in the insurance of epigenetic inheritance of the silenced state.

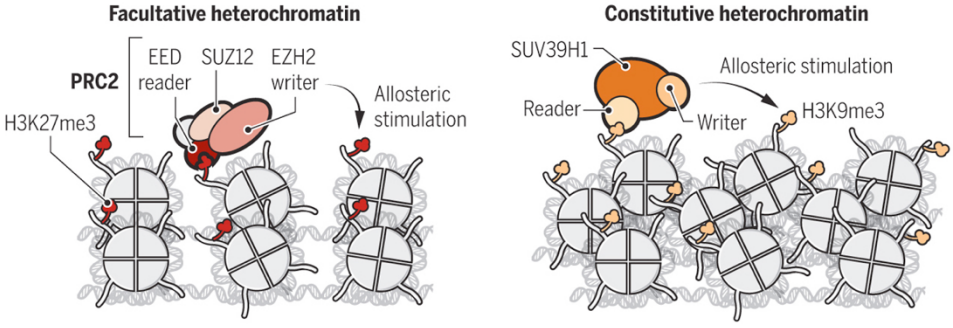

Figure 3: Schematic summary of the heterochromatic repressive state[11]. Left) In facultative heterochromatin, the EED reader subunit of the PRC2 complex recognizes the H3K27me3 mark on nucleosomes and binds to it. This stimulates writer activity of the EZH2 subunit which trimethylates H3K27 on naïve neighboring histones. Right) In constitutive heterochromatin, the reader domain or also called chromodomain (CD) of SUV39H1 recognizes nucleosomal H3K9me3 and subsequently anchors to the chromatin using its N-terminus (not shown). This stimulates methyltransferase activity of the SET or writer domain, which trimethylates H3K9 of naïve neighboring histones.

Inheritance of activating PTMs

In contrast to repressive marks, activating PTMs do not seem to be inherited by staying attached to recycled histones (Figure 2). There must be other mechanisms that restore these PTMs in daughter cells. However, propagation of the active chromatin state is much less understood then repressed regions. Research did confirm that these activating marks contribute to the transcription process. High levels of histone acetyltransferases (HATs) put activating acetyl groups on histones during transcription initiation and elongation. This is counteracted by high levels of histone deacetylases (HDACs) that in their turn remove the acetyl groups. This is a dynamic process in which chromatin is reset which is needed for the next round of transcription[57]. Although this might not have anything to do with replication, this does show that activating marks are short-lived modifications that are not maintained but that are highly dynamic.

Nevertheless, it was found that H3K4me3, a mark of active chromatin, formed an epigenetic barrier to nuclear reprogramming in *Xenopus* and human nuclear transfer embryos (Figure 4)[58]. In this research, donor nuclei were transferred to unfertilized eggs. The first wild-type donor nuclei contained the activating H3K4me3 mark. In this case, cells were resistant to reprogramming and after nuclear transfer a healthy embryo did not develop. When the H3K4me3 mark was removed from the donor nucleus, the DNA became permissive to reprogramming and nuclear transfer led to an efficient cell-type switch and successful development of an embryo. This suggests that H3K4me3 encodes some form of cell memory and acts as a safeguard for cellular identity.

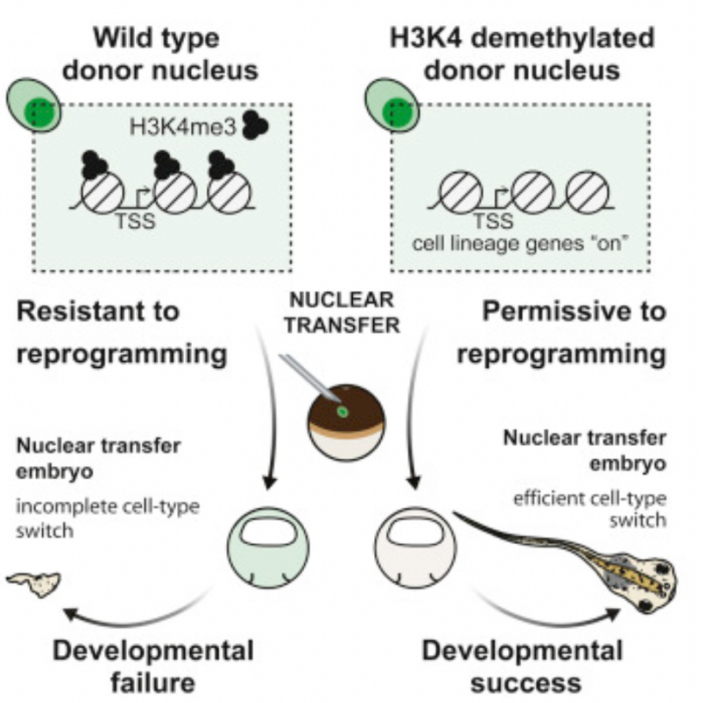

Figure 4: H3K4me3 is an epigenetic barrier to nuclear reprogramming[58]. Left) When H3K4me3 is present in the nucleus of the donor cell, this cell shows resistance to reprogramming which leads to developmental failure. Right) When H3K4me3 is removed from the donor nucleus, the cell becomes permissive to reprogramming which leads to successful development of an embryo. TSS: transcription start site.

The role of transcription in the re-establishment of gene expression

In yeast, active regions are re-established within minutes after replication[59]. In contrast, active regions remain incompletely restored one hour after replication in *Drosophila*[60]. To investigate this in mammals, Stewart-Morgan *et al*[16] developed a replication-coupled assay for transposase-accessible chromatin with next-generation sequencing (repli-ATAC-seq). Using this technique and ChOR-seq they found that nascent chromatin was inaccessible for RNA polymerase and transcriptionally silenced in mouse embryonic stem cells (mESCs). After replication, regions which were accessible before were now inhibited genome wide, except at CpG dense regions. Thirty minutes after replication, RNA polymerase and DNA accessibility began to re-appear. It took two hours for the steady state to be completely restored, and RNA polymerase occupancy and accessible chromatin states were re-established. These findings

were in line with research done in *Drosophila* which showed that replication-coupled nucleosome assembly indeed temporarily ablates nucleosome depleted regions at promotors and enhancers[60]. The restoration of transcription seemed to be locus specific as it was found that super enhancers (highly active enhancers) were faster restoring in contrast to normal enhancers, and that CpG content also positively correlated with restoration speed. This suggests that the genetic nucleotide code, rather than transcription itself influences the switch back to accessible chromatin and that there is hierarchy involved in this, in which some enhancers become active earlier than others based on their DNA sequence. This could hypothetically be used by a cell to regulate cell cycle-specific expression patterns.

Stewart-Morgan *et al*[16] also found that transcription was required to restore active and accessible chromatin after DNA replication (Figure 5). They inhibited transcription by adding two transcription inhibitors. First they used triptolide which blocks XPB helicase activity to prevent formation of the transcription machinery resulting in inhibition of transcriptional initiation[61]. The second drug they used was DRB, which inhibits CDK9 whose phosphorylation activity is required to release paused RNA polymerase II complexes and permit transcriptional elongation[61]. When these drugs were added and transcription was inhibited, active chromatin failed to mature, and transcription factor occupation was not restored. Because of this it is reasonable that transcription is a driving force in active chromatin maturation. However, this should be tightly regulated as collisions between RNA polymerase and the replisome must be avoided, as this can increase genome instability[62]. Besides this, in contrast to normal enhancers and promotors, restoration of active chromatin at super enhancers was only minimally affected by inhibition of transcription. This could be due to the fact that super enhancers are highly active and harbor multiple transcription factor binding sites[63]. This may be a special case in which transcription factor activity is sufficient to re-establish active chromatin in the absence of RNA polymerase.

The previously described research argues that the euchromatic state remains inaccessible for approximately two hours after replication and depends on transcription initiation and elongation for its re-establishment. However, the exact mechanisms behind the restoration of active chromatin after DNA replication still needs to be unraveled.

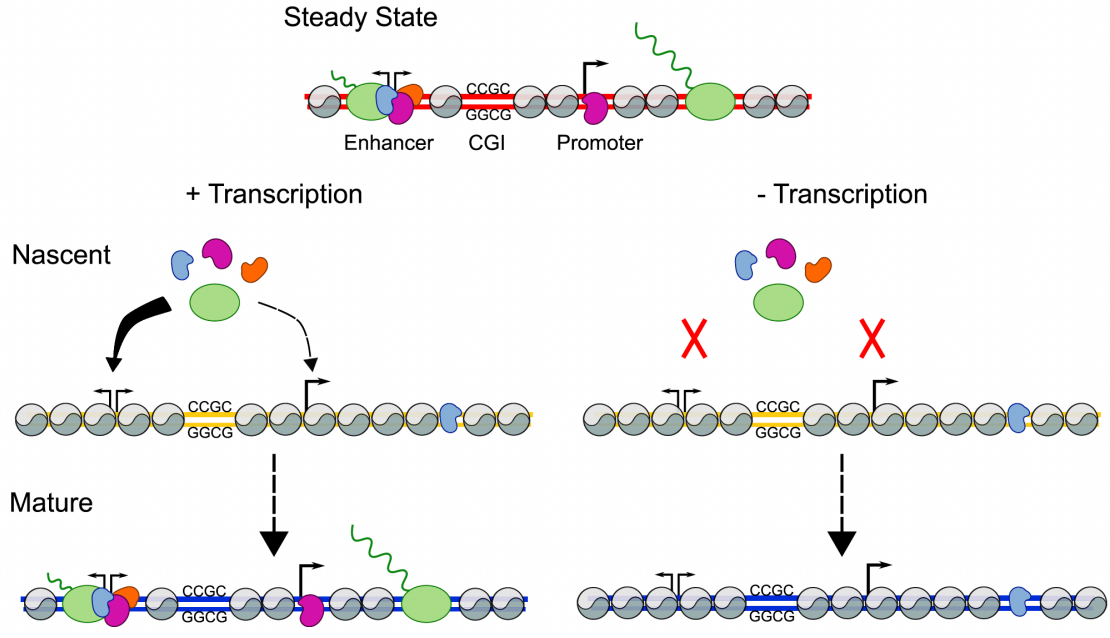

Figure 5: Transcription re-establishes active and accessible chromatin after DNA replication[16]. After DNA replication, nascent chromatin is inaccessible for RNA polymerase and transcription factors genome wide, except at CpG dense regions.

Left) In the presence of active transcription, accessibility is restored approximately 2 hours after replication. Right) In the absence of active transcription, accessible chromatin fails to restore itself.

Hypothetical mechanisms for the re-establishment of gene expression

Although progress has been made in research into the inheritance of different chromatin states, many questions remain unanswered. It has been established now that with the help of histone chaperones, histones are recycled behind the replisome with repressive PTMs still attached to it. This shows that repressive PTMs are true epigenetic carriers. In contrast, activating PTMs are not maintained on histones during DNA replication. Transcription seems to play a role in the re-establishment of open chromatin, but how transcription factors and RNA polymerase recognize the regions that used to be active prior to replication is still under debate.

Stewart-Morgan *et al*[16] hypothesized that transcription restart could rely on inherited modifications that were maintained during replication by staying attached to recycled histones. They hypothesized their experiments implied that the H3K4me3 mark for active chromatin was maintained during replication[48]. As H3K4me3 and other activating marks can stimulate pre-initiation complex formation and recruitment of other transcription factors needed for transcription, it could be that recycling of these marks may enable re-establishment of transcription after DNA replication involving positive feedback loops[64]. This could further influence restoration of the active chromatin state by promoting transcription-coupled modifications on new histones. A similar hypothesis was made by Reinberg and Vales[11]. The H4K16ac mark functions to establish open chromatin and attracts other transcription factors[65]. To sustain transcription, constant presence of an initiator is needed. Initiators subsequently attract co-activators like histone acetyltransferases that further spread the activating PTMs. Also, phosphorylation of the carboxyl terminal domain of RNA polymerase II (Ser^5-P and Ser^2-P) recruit methyltransferases that further methylate H3K4 and H3K36 which facilitates transcription (Figure 6)[11,66]. So, if activating marks could be maintained on histones they could be responsible for the re-establishment of transcription[11]. However, Escobar *et al*[49] showed that activating PTMs are actually not maintained on histones during replication. So, although these kinds of mechanisms seemed well argued there must be other ways to initiate them.

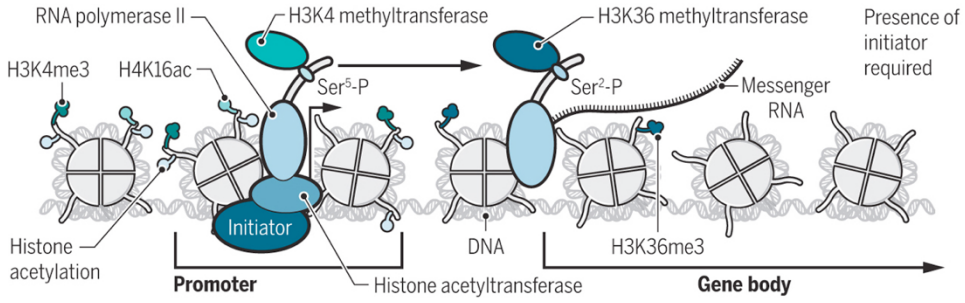

Figure 6: Schematic summary of the euchromatic active state[11]. To sustain active transcription the constant presence of an initiator is required. This initiator recruits RNA polymerase II and histone acetyl or methyltransferases that further spread the presence of activating PTMs like H3K4me3 and H4K16ac. Also, phosphorylation of the carboxyl terminal domain of RNA polymerase II (Ser^5-P and Ser^2-P) recruits H3K36 methyltransferases, increasing presence of the H3K36me3 mark. This leads to a chromatin state that stimulates transcription.

As activating PTMs are not inherited and histones containing activating PTMs are dispersed, it could be that regions that were euchromatic before only receive newly synthesized histones after DNA replication. A hallmark of these new histones it that they only contain acetyl groups[67–70]. Histone tails with acetyl groups are negatively charged which repels the negatively charged DNA, making the DNA more accessible (Figure 7)[71]. This means that chromatin containing newly synthesized histones is potentially accessible by default. So, a hypothesis is that open chromatin is not actually inherited and not a real epigenetic event. It could be that only repressive PTMs are inherited and that the "rest" of the chromatin becomes accessible again for RNA polymerase and transcription factors, due to acetyl-groups being present.

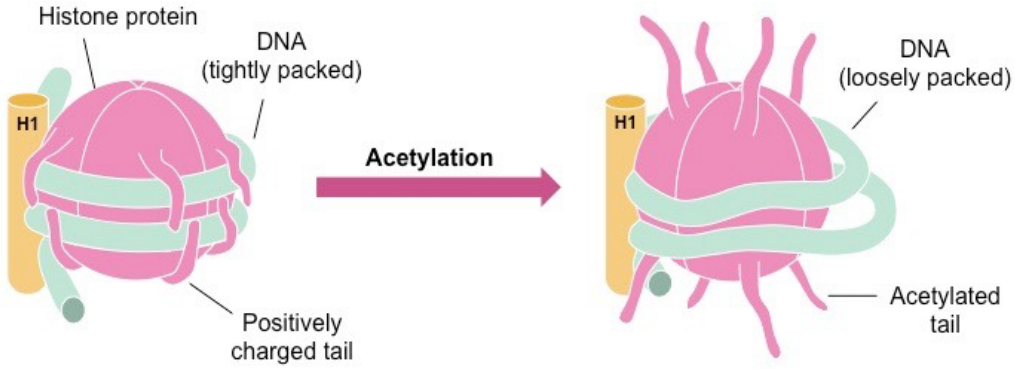

Figure 7: Schematic figure showing the effect of histone acetylation on DNA packaging around nucleosomes[71]. Left) Positively charged histone tails bind to negatively charged DNA, packaging the DNA tightly around the nucleosome. Right) When histone tails become acetylated, they become more negatively charged which repels them from the DNA. This causes the DNA to be more loosely packed around nucleosomes then before.

As mentioned before in this review, mitotic bookmarking is also involved in inheritance of gene expression[15]. Bookmarking transcription factors remain on chromosomes during mitosis and have been shown to reactivate certain genes afterwards. Mitotic transcription has been overlooked for a long time mostly due to the assumption that mitotic chromatin was to compact for transcription to take place. But with the development of better and more sensitive techniques this turned out not to be true[72]. Cells that expressed approximately 28,000 transcripts during interphase, contained measurable expression of up to 8,000 transcripts though mitosis[72]. The maintenance of basal promotor activity together with active transcription activity, although at a low level, allows robust expression of genes at exit of mitosis. Expression is at low level because enhancer-promotor loops are lost due to the absence of the majority of transcription factors and DNA being more tightly packed (Figure 8)[15]. Hereby, mitotic bookmarking functions as an epigenetic mechanism that is involved in the propagation of transcriptional memory. A hypothesis is that some transcription factors remain bound to DNA during replication and that they recruit other transcription factors which leads to the re-establishment of transcription. Because there is only a low level of transcription present at first, it takes some time to restore the previous level after replication, which would be in line with the findings of Stewart-Morgan *et al*[16]. Maybe bookmarking factors influence addition of activating PTMs like H4K16ac and H3K4me3 to histones, which further spreads the re-establishment of transcription as described before (Figure 6). However, this is still a hypothetic situation, and more research is needed into the inheritance of activating PTMs and the restoration of transcription after mitosis.

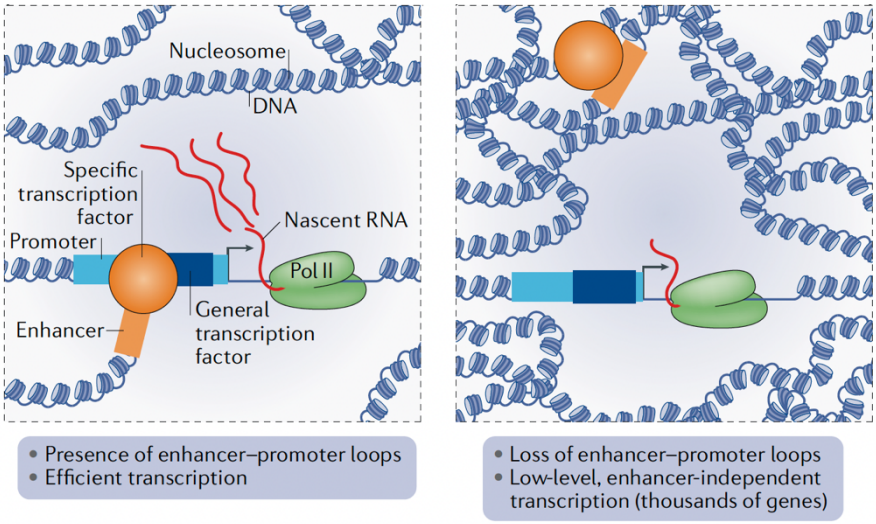

Figure 8: Mitotic bookmarking causes low levels of transcription during mitosis[15]. Left) During interphase, many gene specific transcription factors are present which leads to the presence of enhancer-promotor loops and efficient transcription. Right) During mitosis, specific transcription factors are not present and enhancer-promotor loops cannot be formed. However, some general transcription factors are still present. This leads to low levels of transcription for thousands of genes.

A relatively unknown form of epigenetics involves prion proteins. Prions are misfolded proteins which were first described as infectious particles that cause infectious spongiform encephalopathies[73]. Later they were found to be involved in different physiological functions as well. For example in translation termination in yeast, antiviral immune response[74] and in the function of p53 as a tumor suppressor prions seem to play an important role[75]. Prion conformers are proteins which cause the misfolding of certain proteins into prions, which has further downstream effects. These prion conformers seem to be transmitted and passed through to daughter cells after mitosis and meiosis, which means that they could function as epigenetic factors[76]. Harvey *et al*[77] decided to investigate if prions could be involved in the inheritance of active gene expression. In the yeast *S. cerevisiae*, a prion-like conformational change of the Snt1 subunit of the Set3C histone deacetylase complex interferes with DNA binding of a transcriptional repressor which leads to active transcription. After induction of Snt1 by phosphorylation in response to prolonged cell cycle arrest, the [ESI+] prion is formed from misfolded Snt1. This prion can bind to DNA and activates transcription of otherwise repressed genes. It does this by reshaping the Snt1 and Set3C complex activity, which causes recruitment of RNA polymerase II and inhibits the repressor-activator site binding protein (Rap1). Harvey *et al*[77] found that this mechanism was stably transmitted across multiple cell divisions. Which suggest that prions could be involved in the re-establishment of active gene expression.

To conclude, these hypothetical mechanisms suggest that activating PTMs are not maintained on histones because other mechanisms are responsible for the restoration of open chromatin after DNA replication. Acetyl groups on newly synthesized histones, mitotic bookmarking and prions could play a role in this.

How epigenetics can be used to alter cell identity

It is important to unravel the mechanisms behind cell identity propagation, as this knowledge can be possibly used to reprogram cell fate. Especially for the research field of regenerative medicine this could be important. For example, mastering cell fate change could allow generation of any cell type which is needed for replacement therapies. But it could also lead to better cancer therapies, as change in cell identity is common at different stages of tumor progression. Besides, by inducing cell reprogramming and looking further into the effect on chromatin, we can learn more about epigenetics. Cell fate was long considered as irreversible

in differentiated cells. However, there are now artificial methods that enable the reprogramming of somatic sells into embryonic cells, although the efficiency rates are very low[78,79]. These techniques are somatic cell nuclear transfer (SCNT), induced pluripotent stem cells (iPSCs), cell fusion and trans-differentiation[80]. In these techniques, cell fate is altered without touching the DNA sequence of a cell, which implies that epigenetics is involved. When Yamanaka established the iPSC technique, he introduced four re-programming factors to somatic cells which seemed to be sufficient to reverse the cell back into an embryonic state. The factors he introduced were Oct3/4, Sox2, Klf4 and c-Myc, afterwards called Yamanaka factors[79]. These factors only bind to a limited number of target genes, which suggests the presence of other mechanisms that further spread the change in chromatin needed for reprogramming of the cells[81]. Upon binding of Yamanaka factors, increased chromatin accessibility was found on reprogramming factors binding sites[82,83]. Besides this, chromatin was also found to shift from an open to a closed and from closed to open state during reprogramming[82]. This implies that binding of Yamanaka factors stimulates chromatin remodeling which could subsequently regulate binding of other transcription factors resulting in reprogramming.

Multiple studies showed that the epigenetic memory of a cell forms a barrier to reprogramming. SCNT-embryos display aberrant gene expression patterns then the donor cell type[84,85]. However, a lot of genes that are stably silenced in the donor cell fail to be fully reactivated after SCNT[58,86]. Besides this, SCNT-embryos also express donor cell-type specific genes in the wrong cell-lineages, due to epigenetic memory of their active state[58,87]. This epigenetic memory is hypothesized to lead to severe disruption of cell differentiation in SCNT-embryos. In iPSCs and trans-differentiation similar findings were published[88–90]. It is important to investigate how genes remain resistant to reprogramming, and epigenetic factors like PTMs are hypothesized to play an important role in this. This is a logical assumption, as it is likely that the epigenetic factors that stabilize cell fate memory in the donor cell also are the factors that prevent efficient cell fate reprogramming.

As repressive PTMs are considered to be true epigenetic factors while this is still under debate for activating PTMs, the focus of epigenetics involved in cell-reprogramming was also mainly on repressive PTMs. As expected, genes that are not efficiently reprogrammed from inactive to an active state, are rich in the repressive marks H3K9me3, H3K27me3 and DNA methylation[91]. So, repressive marks seem to be a shield for reprogramming and removal of these marks led to an improvement of reprogramming efficiency[92]. A study on iPSCs showed that the removal of histone H3K9me3 increased successful iPSCs generation[93]. It was also found that the H3K9me3 mark was enriched at binding loci of the four Yamanaka factors[93,94]. The same thing was found to happen in SCNT[86]. H3K27me3 has also been reported as a barrier to efficient reprogramming in many species and removal of this mark is needed for successful SCNT[92,95–98]. However, additional deposition of H3K27me3 to silence donor cell-type specific somatic genes is also important for efficient reprogramming. This suggests that the effect of removal or addition of a certain marks is gene specific.

As the part of activating PTMs in cellular memory is still unknown, this also counts for their role in cell-reprogramming. However, as mentioned before, the H3K4me3 mark seems to be a barrier for efficient cell fate reprogramming (Figure 4)[58]. In contrast, demethylation of the same mark was found to be a barrier for iPSC generation[99]. The activating histone mark H3K79me3 has also been described as an important barrier to cell reprogramming[100]. Inhibition of the H3K79me3 methyltransferase Dot1l reduced expression of genes specific for

the original somatic cell and improved iPSC generation. The role of activating PTMs in cellular memory is still under debate, but these studies show that they play an important role.

While most studies looked at the global effect of PTM removal, it seems that the same mark can have different effects. This suggests that the effect of PTM interference is gene specific depending on its original state and more targeted approaches are needed. However, these studies show the importance of PTMs as epigenetic barriers to cell fate changes. This could be caused partly by PTMs stabilizing the expression patterns in the original cell, but also by reprogramming factors being too weak to counteract this. This results in the "reprogrammed" cell still containing a form of epigenetical memory. This could be beneficial when you want to reprogram a cell to a closely related cell type, but otherwise it hinders reprogramming. However, in iPSCs it has also been observed that donor cell-type specific gene expression is reduced after additional cycles of cell division. This suggests that while PTMs are propagated at first, multiple cycles of DNA replication eventually lead to a dilution of the epigenetic barriers and loss of cell memory[90]. However, successful reprogramming techniques require efficient programming of the epigenome and removal of the epigenetic memory. This underscores again the importance of understanding the mechanisms behind this.

DNA replication and cell fate reprogramming

Waddington, who presented his view on the epigenetic landscape a long time ago, stated that at some point cells are in a state of instability, in which induction by some signal pushes the ball, here symbolizing a cell, into one developmental pathway (Figure 9)[101]. In this model the ball or cell can never return to its original place, as a ball would never roll against gravity itself, which means that cell fate is irreversible. However, using the Yamanaka factors it seemed that the ball could be pushed up the hill, taking it back to a state before differentiation. The highest point of the ball or cell can be seen as a stem cell, which is in a state of instability and not differentiated or rolled of the hill yet. However, this highest point can also be seen as a cell in mitosis, as DNA replication brings a cell in a state of instability as well. This means that DNA replication may be an opportunity for cell reprogramming. This means that we can try to look at the mechanisms involved in epigenetic inheritance during DNA replication described before in this review and see if they can possibly be used to alter cell fate.

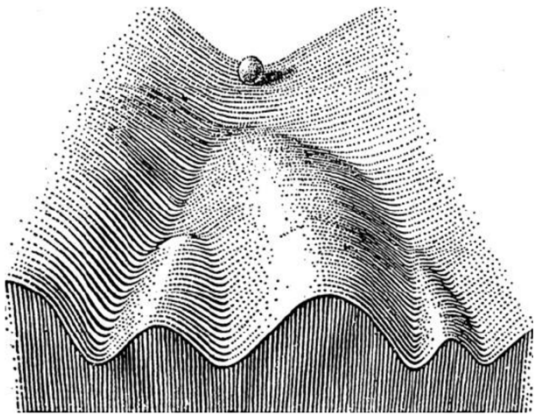

Figure 9: The epigenetic landscape as proposed by C.H. Waddington[101].

A hypothetic mechanism in which DNA replication can possible alter gene expression is asymmetrical parental nucleosome segregation[50]. Although it has been discussed before in this review that during DNA replication histones are equally recycled, mechanisms have been described in which histones were asymmetrically distributed among sister-chromatids[102]. There are some examples in biology in which this is used to generate two different daughter cells after mitosis. For example, in *Drosophila* this is used to produce a new germline stem cell and a differentiated gonialblast during germline development[103,104]. A similar phenomenon was found in embryonic mouse muscle stem cells, in which parental H3 and H4 histones have a leading strand bias which leads to asymmetrical cell division[105]. This could cause one sister chromatid to receive all repressive marks while the other one receives none at all (Figure

10a)[50]. POLE3/POLE4 is responsible for histone recycling to the leading strand, while CTF4, Polα and MCM2 chaperone histones to the lagging strand. Mutations interfering with these chaperones led to strand bias of histone recycling (Figure 1b, c). If we can find ways to modulate these factors so that certain genes become active or not, we could possibly alter cell fate. However, this is still far-fetched as the mechanisms involved in strand specific recycling are not yet completely understood. Besides this, it remains a question if asymmetrical nucleosome segregation also happens in mammals so, this theory needs further investigation.

Another hypothetical mechanism that could be used to alter cell fate is tetramer splitting. Sometimes, in the same nucleosome, both repressive (H3K27me3) and activating (H3K4me3) PTMs are present. Promotors for which this accounts are called bivalent promotors[2,106]. This bivalency is tissue specific and tightly regulated. For example, PRC2 plays a role in maintaining bivalent genes in a repressed state[107]. Modulation of these bivalent promotors may be important for cell fate reprogramming. Although most tetramers stay attached to each other during replication, a small fraction of them show splitting events[31,108,109]. These splitting events could modulate epigenetic inheritance as it leads to unequal distribution of the activating and repressing PTMs (Figure 10b)[50]. However, for asymmetrical histone segregation we know possible histone chaperones that could interfere with this process, but for tetramer splitting this is not the case yet. Xu *et al*[31] found splitting events only in H3.3-containing tetramers. This leads to the question if tetramer splitting is histone-variant or chromatin-region specific. As H3.3 histones are enriched in euchromatin[110,111], a possible model is that splitting events differ for euchromatin or heterochromatin and happens more frequently in euchromatin. Detecting possible 'splitting hot spots' is interesting for future research into the inheritance of activating modifications and cell-reprogramming.

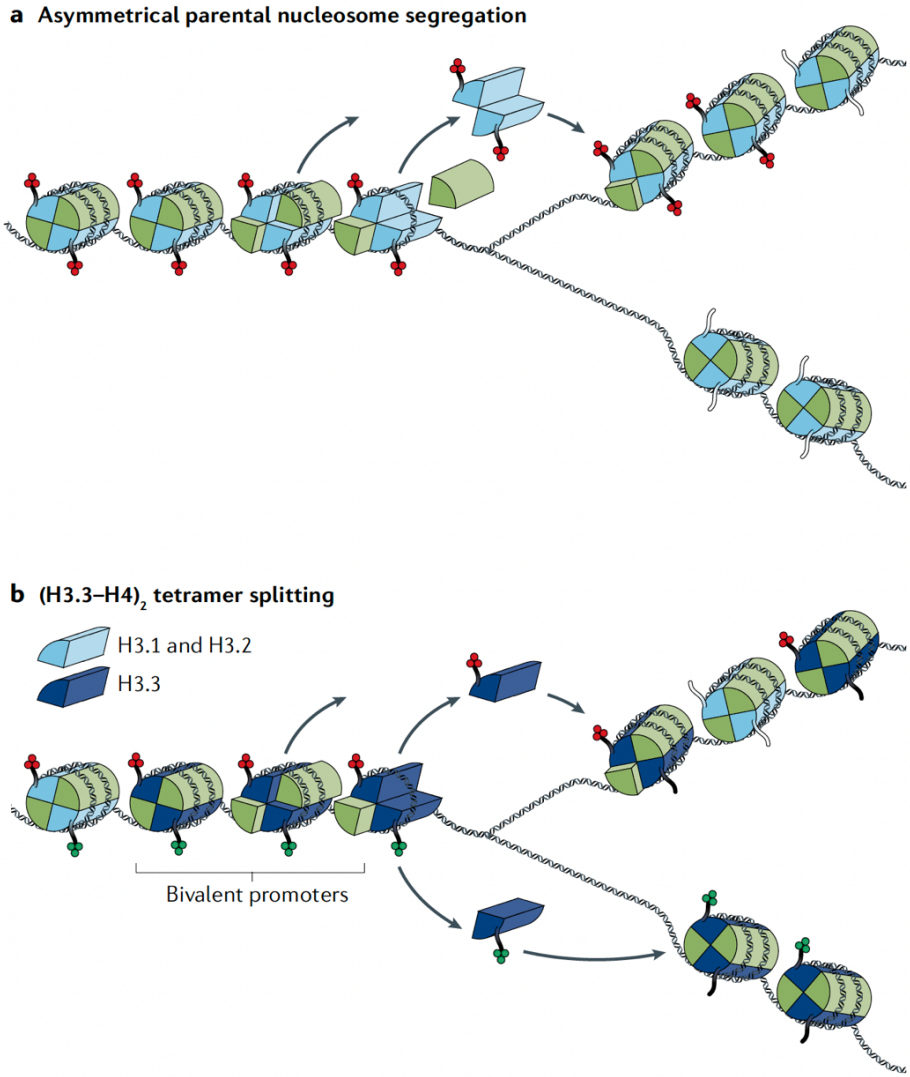

Figure 10: Schematical figure showing possible mechanisms involved in altering cell fate[50]. A) In asymmetrical nucleosome segregation, histones are not distributed equally to each sister-chromatid. This could lead to one sister-chromatid receiving all repressive PTMs, while the other one does not receive any. B) In the tetramer splitting model, H3.3 containing histone tetramers split into two dimers. If this happens at bivalent promotors, this could lead to one sister-chromatid receiving activating PTMs while the other chromatid receives only repressive PTMs.

Conclusions and future perspective

During the last decade it has become clear that PTMs play an important role in the inheritance of cell identity. Especially for repressive PTMs progress have been made and recent studies proved them to be true epigenetic factors, as they stay attached to recycled histones during DNA replication. The read-write positive feedback loops mediated by PRC2 and Suv39H1 also were a breakthrough, as they cause the attachment of repressive marks on newly synthesized histones. For activating PTMs more questions remain unanswered, and it is still debated if they are true epigenetic factors. Some studies showed epigenetic involvement of activating PTMs as a barrier to reprogramming. However, histones with activating PTMs are dispersed during DNA replication. Transcription seems to play a role in the re-establishment of accessible chromatin and other mechanisms like mitotic bookmarking, prions and acetyl groups on newly synthesized histones could be involved in DNA becoming accessible after DNA replication. This suggest that not activating PTMs on histones, but other mechanisms are important for the restoration of euchromatic regions. However, the exact mechanisms behind the restoration of active chromatin after DNA replication remain to be discovered. A hypothesis is that activating PTMs are not epigenetically inherited because being careful with gene activation is an evolutionary benefit. Positive feedback loops for activating PTMs could be a big risk as they could lead to permanent mistakes. However, this hypothesis could also count for repressive PTMs, as the silencing of for example a repressor can have the same effect as an activator.

PTMs are also important in the artificial change of cell fate which could be used for therapeutic use, as they establish cell memory and form a barrier to reprogramming. Now that we better understand epigenetic inheritance, we can speculate on mechanisms that could possibly be used to alter cell identity. Asymmetrical nucleosome segregation and tetramer splitting are examples of this. Reprogramming studies show that epigenetic cell memory is difficult to reset which hinders efficient reprogramming. To improve efficiency, it is important that epigenetic inheritance is better understood. Until now, most studies looked at the global effect of PTM interference in cell identity change. But as some PTMs show different effects in different situations, more gene-specific research is needed. The rapidly advancing techniques on sequencing, CRISPR-Cas9 technology, single molecule and single cell analysis could be used for this. Epigenetics inheritance is complex as many different components including PTMs, DNA methylation, histone variants, mitotic bookmarking factors and histone chaperones are all working together, resulting in a chromatin state which is inherited to the daughter cell. In this review we mainly focused on histone PTMs inheritance, but all other epigenetic factors must be re-established as well. Future experiments should address the interplay of these factors. Taken together, despite the great advances in this research field, many questions remain unanswered.

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