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Declaro que esta dissertação de candidatura ao grau de Doutor é da minha autoria e que os dados aqui incluídos são o resultado de trabalho original por mim desenvolvido entre Fevereiro de 2009 e Abril de 2013 no laboratório do Dr Lars Jansen, Instituto Gulbenkian de Ciência em Oeiras, Portugal. Este doutoramento foi realizado no âmbito do Programa Doutoral do Instituto Gulbenkian de Ciência PGD 2008. Todas as colaborações estão indicadas em cada capítulo, na secção de Acknowledgements. Esta dissertação teve o apoio financeiro da FCT BD nº SFRH/BD/33567/2008 e dos projectos BIA-BCM/100557/2008, BIAPRO/100537/2008 e EMBO instalação.

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Abstract

Cell proliferation and differentiation into distinct cell types during development requires the preservation of cellular phenotypes during cell divisions. How the cell maintains its identity and how cells with the same genetic information display different heritable phenotypes or gene expression profiles are fundamental questions in biology. The mitotic and/or meiotic inheritance of changes in gene expression that are not due to changes in DNA sequences is known as epigenetic inheritance. The transmission of the epigenetic state is dependent on molecular markers that are predicted to be transmissible across cell divisions, have the capacity to template their own duplication and propagate under control of the cell cycle. There are a range of markers that control gene expression in cis involved in epigenetic phenomena, including DNA or histone modifications, DNA or histone binding proteins and histone variants. Although histone modifications are widely studied, their inheritance and potential underlying mechanism of propagation are controversial. A key aspect to elucidate the role of histone proteins and their modification as an epigenetic mark is the dynamic equilibrium between histone turnover and the dynamics of the modifications of those histones. The basic unit of chromatin is the nucleosome. The nucleosome is formed by an octamer of four core histones (H2A, H2B, H3 and H4) wrapped by 147bp of DNA. With the exception of H4 all histones have variants and their preferential localization serves different roles in preserving epigenetic identity.

The major H3 variants are H3.3 and centromere protein A (CENP-A). Unlike canonical H3.1, H3.3 is deposited at promoters, sites of active transcription, enhancers and subtelomeric regions throughout the cell cycle; whereas CENP-A is deposited into centromeric chromatin during G1 phase. The dynamics of histones has
been analyzed using powerful methods that include metabolic pulse labeling of nascent proteins, fluorescence recovery after photobleaching, conditional expression of tagged histones and the use of self-labeling tags. Nevertheless, methods for quantitative histone turnover at specific loci have been lacking. In chapter 2, I describe the development of TimeChIP, a pulse labeling strategy coupled to chromatin precipitation that provides temporal information of histone occupancy at high resolution. We show that using the self-labeling SNAP-tag, we can isolate pulse labeled histones at different times following their labeling for the analysis of their dynamics. This method provides temporal resolution, distinction between old and new pools of proteins and high spatial resolution in living cells. We employ this method for the analysis of CENP-A in proof of principle experiments and show that CENP-A is stably maintained in centromeric chromatin and assembled in nucleosomes following its targeting in G1 phase.

In chapter 3, we used the TimeChIP strategy to determine to what extend histones H3 variants H3.1 and H3.3 can be maintained at different loci of mitotically dividing cells. We find that canonical H3.1 is locally retained at genes and non-gene loci, whereas H3.3 exhibits a faster turnover. Genome-wide analysis demonstrates that H3.3 turnover at active genes correlates with the rate of transcription. However, there is a strikingly high degree of histone retention of both H3.1 and H3.3 for the duration of the cell cycle, even at gene bodies of highly transcribed genes. Moreover, we find that transcription activation at an inducible gene locus does not lead to faster histone turnover. Conversely, inhibition of RNA polymerase II leads to an increased retention of H3.1 and H3.3 during the cell cycle.

This work describes the development of a versatile strategy for the analysis of histone dynamics: turnover and assembly. We used this method to demonstrate for the first time that while subsets of H3.1 and
H3.3 nucleosomes are dynamic at active gene loci, a significant pool is retained in cis during the extent of a cell cycle. Our findings are consistent with a model in which nucleosomes contribute to the inheritance of epigenetic information and make predictions about the dynamics and retention of histone modifications.
A proliferação e diferenciação das células durante o desenvolvimento requerem a preservação dos fenótipos celulares durante a divisão celular. O modo como uma célula mantém a sua identidade e o modo como células com a mesma informação genética conseguem exibir diferentes fenótipos hereditários ou diferentes perfis de expressão génica são questões fundamentais em biologia. A herança epigenética é definida como a hereditariedade mitótica e meiótica de alterações na expressão dos genes que são independentes da sequência de DNA. A transmissão do estado epigenético é dependente de marcadores moleculares que se esperam ser transmissíveis através das divisões celulares, que têm a capacidade de servir de modelo para a sua própria duplicação e cuja propagação está dependente do controlo do ciclo celular. Existem uma série de marcadores que controlam a expressão génica em cis envolvidos em fenómenos epigenéticos tais como modificações de DNA ou de histonas, proteínas de ligação ao DNA ou a histonas e variantes de histonas. Apesar das modificações em histonas serem amplamente estudadas, a sua herança e o possível modelo subjacente à sua transmissão hereditária são ainda muito controversos. De modo a elucidar o papel das histonas e suas modificações enquanto marca epigenética é fundamental perceber o equilíbrio entre o seu turnover e a dinâmica das suas modificações. A unidade básica da cromatina é o nucleossoma que consiste em 147 pares de bases de ADN enrolados em redor de um octámero de quatro histonas centrais (H2A, H2B, H3 e H4). Com a excepção da H4, todas as histonas têm variantes e as suas diferentes localizações determinam papéis distintos na preservação da identidade epigenética.
As principais variantes da histona H3 são as H3.3 e a proteína A do centrómero (CENP-A). Ao contrário da proteína canónica H3.1, a H3.3 é depositada em promotores, em locais de transcrição activa e em regiões intensificadoras e subteloméricas durante todo o ciclo celular. Por sua vez, a proteína CENP-A é depositada na cromatina centromérica durante a fase G1. Neste estudo, a dinâmica das histonas foi analisada utilizando métodos poderosos que incluem pulse labeling (marcação com recurso a compostos especiais) metabólico de proteínas nascentes, recuperação de fluorescência após a fotodegradação, expressão condicional de histonas com tags e a utilização de self-labeling tags. No entanto, não foram possíveis métodos para quantificar o turnover de histonas em loci específicos.

No capítulo 2, descrevemos o desenvolvimento do método TimeChIP, uma estratégia de pulse labeling associada à precipitação da cromatina, que fornece informação temporal em alta resolução da localização de histonas. Mostramos que o uso da self-labeling SNAP-tag permite isolar histonas marcadas em diferentes tempos, seguindo a sua marcação de modo a analisar a sua dinâmica. Este método permite distinguir entre pools novos e antigos de proteínas, oferecendo alta resolução temporal e espacial em células vivas. Este método foi aplicado para a análise da CENP-A, onde mostramos que esta proteína se encontra estável na cromatina centromérica e é incorporada em nucleossomas após a sua focalização na fase G1.

No capítulo 3, descrevemos a utilização do método TimeChIP para determinar até que ponto as variantes de histonas H3, H3.1 e H3.3, podem ser mantidas em diferentes loci de células em mitose. Os nossos resultados mostram que a proteína canónica H3.1 é mantida localmente em loci gênicos e não-gênicos, enquanto que a H3.3 apresenta um turnover mais rápido. Uma análise genómica em larga escala mostra que o turnover da H3.3 em genes activos se
correlaciona com a taxa de transcrição. No entanto, existe um elevado grau de retenção de ambas variantes (H3.1 e H3.3) durante todo o ciclo celular, mesmo em conjuntos de genes altamente transcritos. Adicionalmente, descobrimos que a activação da transcrição de um locus de um gene indutível não resulta num turnover mais rápido das histonas. Por outro lado, a inibição da RNA polimerase II leva a um aumento da retenção das variantes H3.1 e H3.3 durante o ciclo celular.

Este trabalho descreve o desenvolvimento de uma estratégia versátil para a análise da dinâmica de histonas: seu turnover e montagem (assembly). A utilização deste método permitiu mostrar pela primeira vez que pequenos conjuntos de nucleossomas compostos por H3.1 e H3.3 são dinâmicos em loci de genes activos mas que uma parte significativa é retida em cis durante a extensão de um ciclo celular. Os nossos resultados estão de acordo com um modelo em que os nucleossomas contribuem para a herança de informação epigenética e permitem fazer previsões sobre a dinâmica e a retenção de modificações das histonas.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTB</td>
<td>β-Actin</td>
</tr>
<tr>
<td>AHA</td>
<td>Azidohomoalanine</td>
</tr>
<tr>
<td>ATRX</td>
<td>α-thalassemia X linked mental retardation protein</td>
</tr>
<tr>
<td>BG</td>
<td>O(^6)-benzylguanine</td>
</tr>
<tr>
<td>CAF1</td>
<td>Chromatin Association Factor-1</td>
</tr>
<tr>
<td>CATCH-IT</td>
<td>Covalent Attachment of Tags to Capture Histone and Identify Turnover</td>
</tr>
<tr>
<td>CATD</td>
<td>CENP-A Targeting Domain</td>
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<tr>
<td>CCAN</td>
<td>Constitutive Centromere Associated Network</td>
</tr>
<tr>
<td>CCNA</td>
<td>Cyclin A</td>
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<tr>
<td>CDK</td>
<td>Cyclin Dependent Kinase</td>
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<tr>
<td>CENP-A</td>
<td>Centromeric Protein A</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>CiA</td>
<td>Chromatin in vivo Assay</td>
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<tr>
<td>DAXX</td>
<td>Death Associated Protein</td>
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<tr>
<td>FPALM</td>
<td>Fluorescence Photoactivation Localization Microscopy</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence Recovery After Photobleaching</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>hAGT</td>
<td>human O(^6)-Alkylguanine-DNA Alkyltransferase</td>
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<tr>
<td>HFD</td>
<td>Histone Fold Domain</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>HIRA</td>
<td>Histone Regulator A complex</td>
</tr>
<tr>
<td>HJURP</td>
<td>Holliday Junction Recognizing Protein</td>
</tr>
<tr>
<td>LacO</td>
<td>Lac Operon</td>
</tr>
<tr>
<td>IncRNAs</td>
<td>long non coding RNAs</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>MYC</td>
<td>Myc proto–oncogen protein</td>
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<tr>
<td>MyoD</td>
<td>Myoblast Determination protein</td>
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<tr>
<td>ncRNA</td>
<td>non-coding RNAs</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear Transcription factor-κβ</td>
</tr>
<tr>
<td>PAFPs</td>
<td>Photoactivatable Fluorescent Proteins</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PRC</td>
<td>Polycomb Repressive Complex</td>
</tr>
<tr>
<td>PRE</td>
<td>Polycomb Response Elements</td>
</tr>
<tr>
<td>RITE</td>
<td>Recombination-Induced Tag Exchange</td>
</tr>
<tr>
<td>RPKM</td>
<td>Reads per kilobase per million</td>
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<tr>
<td>RPL13A</td>
<td>Ribosomal protein L13a</td>
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<tr>
<td>RPLP0</td>
<td>Ribosomal protein P0</td>
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<tr>
<td>Sat2</td>
<td>Pericentromeric Satellite 2 repeats</td>
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<tr>
<td>SILAC</td>
<td>Stable Isotope Labeling of Amino Acids in Cell Culture</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
</tbody>
</table>
TrxG/PcG Trithorax and Polycomb protein
TSS Transcription Start Site
TTS Transcription Termination Site
XCi X Chromosome Inactivation
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Chapter 1 – General Introduction
1. Epigenetic inheritance

The term epigenetics has had different meanings throughout the last half century. Generally speaking the concept of epigenetics refers to heritable changes that occur independent of genetic changes. Conrad H. Waddington coined the term (1942) to refer to the study of “causal mechanisms” by which the genes of the genotype bring about phenotypic effects during development. Later, D.L. Nanney introduced the term epigenetic control systems as “auxiliary mechanisms with different principles of operation that are involved in determining which specificities are to be expressed in any particular cell”, and that such systems were “presumably limited by the information contained in the genetic library…” “An epigenetic change should not result in a permanent loss of information and a return to a previous condition of expression is always theoretically possible” (Haig, 2004). Currently epigenetics is more strictly defined as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequences”

Maintenance of epigenetic information relies on the ability to persist through cell divisions in the absence of the initial inducing signals. The ability of an epigenetic mechanism to sustain itself is characterized by: 1) stability, where the agent responsible for the transmission of the information is expected to self maintain across the cell cycle; 2) self-duplication, the ability to copy information into novel structures so as to maintain cellular information through cell division and growth; and 3) cell cycle control, a temporal regulation of the propagation of the epigenetic system (Gómez-Rodríguez and Jansen, 2013). Mechanistically, an epigenetic state can in principle be self-sustainable by trans-acting or cis-acting molecular signatures.
1.1.1 Epigenetic Inheritance in *trans*

Trans-acting epigenetic signals are maintained by positive feedback mechanisms involving transcription factors and non-coding RNAs (ncRNAs). In the case of transcription factors, there are many examples; classic cases include the phage lambda and the cell type specific master regulator MyoD in eukaryotes. The phage lambda codes for two transcription factors, Cro and lambda repressor. Although the two proteins are not homologous at the amino acid sequence level, they recognize the same operator sites. Binding of one or the other, leads to two physiologically distinct states, lysogeny and lytic growth. Cro autoregulates its own production positively while the lambda repressor does so positively and negatively. The bi-regulation of the repressor is given by the cooperative binding of its dimers to adjacent sites generating a state poised to respond to a transient signal and switch state. The epigenetic nature of the binary phage lambda states comes from the positive feedback loop of either the repressor or the Cro transcription factor that activates expression of its own gene and is stable for many cell divisions as the respective transcription factor is distributed to daughter cells. (Johnson et al., 1978, 1981; Ptashne et al., 1976). Similarly, the basic-helix-loop-helix (bHLH) transcription factor MyoD binds to private E boxes of target genes and induces myogenesis in a wide variety of cell types in mammals. Importantly, in addition to its role as an activator of downstream genes, it functions in a positive autoregulatory loop increasing the levels of its own expression thereby stabilizing myogenic commitment (Chanoine et al., 2004; Fong et al., 2012; Lassar et al., 1989; Thayer et al., 1989).

A growing number of ncRNAs are found to be involved in epigenetic phenomena but the underlying mechanisms are largely unknown. For instance, in plants, Dicer like protein 3 (DCL-3)
generates mobile 24–nucleotide (24-nt) sRNAs (Molnar et al., 2010). 24-nt sRNA moves between cells directing RNA-dependent DNA methylation in the recipient cell that control transcriptional gene silencing of transposon elements from three separate genomic loci (Melnyk et al., 2011; Molnar et al., 2010).

1.1.2 Epigenetic Inheritance in cis

Epigenetic states that are maintained in cis are typically chromatin based. Chromatin is a dynamic DNA-protein complex in which the genetic information is packaged inside eukaryotes cells. Its organization directly impacts on control of gene expression. Chromatin as a high order structure shows different levels of organization: 1) the basic unit of chromatin is the nucleosome which is formed by an octamer of four core histones (H3, H4, H2A and H2B) wrapped with 147 bp of DNA and separated by a 10-80 bp linker DNA associated with linker histone H1. Together, this complex forms a 10 nm diameter fiber, 2) in vitro, a helical fiber of 30 nm in diameter has been observed containing 6-11 nucleosomes per turn. 3) chromatin fibers that interact within or between larger fibers (Higher order in vivo chromatin has been described as a “polymer melt” state where nucleosomes interact across chromatin regions and not in a linear-fashion between neighbors), 4) chromosome territories and 5) chromosomes (de Graaf and van Steensel, 2013; Hübner et al., 2013) (Figure 1.1)

Figure 1.1 Chromatin organization in the mammalian nucleus. a) Chromosomes are organized in chromosome territories. b) Chromosome
territories are comprised of so-called fractal globules that interact in cis or can interdigitate with adjacent chromosome territories. c) Chromatin fibers interact (i) within a fractal globule, (ii) between fractal globules of the same chromosome territory, or (iii) between adjacent chromosome territories. d) Chromatin may form a 30nm fiber. e) Chromatin is resolved as a 10nm beads on a string fiber consisting of nucleosomes. Adapted from Hübner et al., 2013.

The accessibility to the DNA is, at its most basic level, determined by the nucleosome. The octamer is arranged along a twofold dyad symmetry axis, the intersection point with the middle of DNA fragment. The typical histone protein features three α-helices (α1, α2 and α3) separated by two loops (L1 and L2) that constitute the histone fold domain (HFD) and an amino-tail. The HFDs fold together in antiparallel pairs: H3 with H4 and H2A with H2B. The assembly of the eukaryotic nucleosome begins with an (H3-H4)₂ tetramer, held together by a strong four-helix bundle between the two H3 molecules, which is followed by the addition of two H2A-H2B dimers that form weak four-helix bundles between H4 and H2B (Luger et al., 1997; Talbert and Henikoff, 2010).

**Figure 1.2 Architecture of nucleosome core particle.** The four histone dimers H3, H4, H2A and H2B are colored in blue, green red and yellow,
respectively. The four histone dimers are arranged about a twofold dyad symmetry axis, which also intersects the middle of the DNA fragment. Adapted from Biswas et al., 2011.

The canonical histones, that are among the slowest evolving proteins known, are encoded by gene clusters whose expression is tightly coupled to DNA replication. DNA replication is highly regulated and occurs in two steps: licensing and initiation. During the licensing step, the prereplicative complex (pre-RC) is formed at multiple origins of replication in G1 phase, where it loads ORC, Cdc6, Cdt1 and the inactive replicative helicase Mcm2-7 complex (Remus and Diffley, 2009). In the initiation step, active helicase containing the CMG (Cdc45-Mcm2-7-GINS) complex unwinds the double-stranded DNA for the loading of DNA polymerases. The initiation step is regulated by the S phase specific cyclin-dependent kinase (S-CDK) and Cdc-7-Dbf4-dependent kinase (DDK) (Tanaka and Araki, 2010). Assembly of newly synthesized genomic DNA requires large amounts of histones produced during S phase and ancestral histones that are transferred from the parental strand to the daughter strands (Groth et al., 2007; Osley, 1991). Assembly of \((\text{H3-H4})_2\) tetramers is mediated by the histone chaperone chromatin assembly factor-1 (CAF-1) that binds to the proliferating cell nuclear antigen (PCNA) (Shibahara and Stillman, 1999) and followed by deposition of two H2A-H2B dimers to complete the nucleosome.

*Cis* epigenetic states that are propagated in chromatin are maintained by so-called epigenetic markers that include DNA methylation, histone variants and their modifications, DNA or histone binding proteins and ncRNAs.
1.1.2.1 DNA methylation

Cytosine DNA methylation is a stable and heritable long term silencing mark important for processes such as gene and transposon silencing, imprinting and X chromosome inactivation in most of eukaryotes. There are three different nucleotide sequence context where DNA methylation has found to occur: symmetrically in CG and CHG, and asymmetrically in CHH (where H= C, T or A). In mammals DNA methylation occurs symmetrically in the CG context. The majority of CG dinucleotides across the genome are methylated except for high density CG regions called CpG islands at active promoters that are typically unmethylated. The unmethylated state is important for gene activation. The acquisition of methylation during development results in the establishment of long term repression (Bird, 2002; Lee et al., 2010). The establishment is driven by the DNA methyltransferases DNMT3A and DNMT3B during the blastocyst stage and re-establishment in gametogenesis of embryonic development (Law and Jacobsen, 2010).

DNA methylation is semiconservatively transmitted during the cell cycle because of the nature of DNA replication. A DNA sequence carrying symmetrical methylation marks on both strands gives rise to two hemi-methylated double strands. The DNA methyl transferase DNMT1 methylates new CpGs during DNA replication at sites where the parental strand already carries a methyl group. DNMT1 is recruited by a ubiquitin-like plant homeodomain and RING finger domain 1 (UHRF1) to hemimethylated DNA and through a transient interaction with proliferating cell nuclear antigen (PCNA) associates with replication machinery, where PCNA enhances the efficiency of the catalytic activity of DNMT1 (Bird, 2002; Law and Jacobsen, 2010; Schermelleh et al., 2007; Spada et al., 2007). Consequently, DNMT1 is responsible for the heritable maintenance of DNA methylation in a
reading and writing manner. This occurs during S phase through the UHRF1 interaction as well as in G2 and M phases (Easwaran et al., 2004). DNMT1 is positively regulated along the cell cycle by CDK1, 2 and 5 which phosphorylate Ser154 in humans (Lavoie and St-Pierre, 2011).

1.1.2.2 Polycomb proteins

Another important class of factors implicated in carrying epigenetic states are non-histone chromatin-binding proteins. Among the most extensively studied are the Trithorax and Polycomb (TrxG/PcG) protein family. TrxG and PcG were originally identified in D. melanogaster due to their roles in tuning HOX genes expression by promoting histone modifications (Simon and Kingston, 2009). In flies, TrxG and PcG associate to chromatin by complex DNA elements named Trithorax and Polycomb response elements (TRE/PRE), which are located kilobases from the promoters they control. These sites are depleted of nucleosomes and form looping interactions with their target repressed genes in the case of PcG and non repressed genes in Trx in Drosophila (Petruk et al., 2012; Simon and Kingston, 2013). Although, TrxG and PcG were initially found on HOX genes, approaches like genome wide chromatin immunoprecipitation (ChIP) studies have identified target genes besides HOX in Drosophila and mammals (Simon and Kingston, 2009).

PcG proteins are involved in silencing of genes in a non-constitutive conditional manner. In development, PcG is implicated in holding developmentally controlled genes in a poised but inactive state in mouse and human embryonic stem cells (ES). The system is composed of many complexes, Polycomb repressive complex 1 (PRC1), PRC2, PRC1 variant, PHO-RC and PR-RUB, of which the best characterized are PRC1 and PRC2. PRC1 silences target genes, in part, by monoubiquitylation of histone H2AK119u1 (Wang et al.,
that inhibits the recruitment of FACT, blocking RNA polymerase II release at an early stage of elongation (Zhou et al., 2008) and creating a compacting state in chromatin. Experiments using the mammalian SV40 replication system show that PRC1 is maintained on the template after replication fork passage and binds tightly to single stranded DNA (Francis et al., 2009; Lo et al., 2012) which may contribute to the heritable nature of PRC1 mediated silencing.

PRC2 carries the major methyltransferase for H3K27 methylation, a modification critical for maintaining repressed gene expression programs throughout development (Pengelly et al., 2013).

The mechanism for PRC2 targeting has not been elucidated, except the presence of Polycomb response elements (PRE) located many kilobases from the promoters they control in Drosophila. In mammals, recruitment is even more poorly defined. In addition to DNA elements similar to PREs, unmethylated CpG islands and lncRNAs are believed to target PcG functions in mammals (Simon and Kingston, 2013). Once, PRC2 is recruited to chromatin, the presence of pre-existing H3K27me3 marks on neighboring nucleosomes activates the methyltransferase activity of PRC2 contained in the E(z)/Ezh2 subunit, to carry out further methylation on unmethylated H3K27 (Hansen et al., 2008; Margueron et al., 2009; Xu et al., 2010a). This suggest that PRC2 self-replicates by acting as adaptor that reads (recognize) and writes (methylates) its own signal to be sustained (Gómez-Rodríguez and Jansen, 2013).

*In vivo* FRAP experiments in Drosophila show that Pc and Ph, members of the PRC2 complex, have a relative short residence time of 2-6 minutes, suggesting that PcG complexes are highly dynamic where the chromatin state is inherited in a dynamic fashion rather than a stable fashion (Ficz et al., 2005). *In vivo* experiments in Drosophila
embryos show that Trx, PRC1 and PRC2 associate transiently with PCNA, indicating that these PcG proteins are maintained during S phase (Petruk et al., 2012). Regarding maintenance of PcG proteins during mitosis, evidence comes from FRAP studies in Drosophila stem and differentiated cells. PcG proteins binds mitotic chromatin with up to 300 fold longer residence times than in interphase potentially contributing to the mitotic propagation of the silent state. This retention is governed by phosphorylation of H3 at Ser 38 (H3K27me3Ser38) (Fonseca et al., 2012).

The PRC2 complex is cell cycle regulated at its catalytic subunit. Ezh2 contains an evolutionally conserved consensus CDK phosphorylation motif. Phosphorylation at this motif correlates with the oscillatory activity of CDK1 and 2 during the cell cycle, without affecting assembly and histone methyltransferase (HMT) activity of PRC2 (Chen et al., 2010), thereby CDK1 and 2 act as positive factors for the maintenance of PRC2.

1.1.2.3 Histone modifications

In addition to the above mentioned histone methylation events, other histone modifications contribute to chromatin dynamics and epigenetic states. Histone modifications, either on the surface of the DNA wrapped core of the nucleosome or on the amino-terminal tail that extends from the nucleosome surface, have been described (Table 2). They can be functionally categorized in: 1) modifications that disrupt chromatin structure. These include negatively charged acetylation and phosphorylation that neutralize the positively charged histones thereby generating repulsion between histones and DNA. This results in the relaxation of histone-DNA contacts for processes like transcription, replication and DNA repair. In contrast, deacetylases have the ability to induce condensation of chromatin important for chromosome segregation. 2) Regulation of chromatin binding factors
that act as effector proteins of the modification. Such factors are characterized by Chromo, Tudor, MBT, PhD finger domains that bind to methyl-lysine modifications, Bromo domains bind to acetyl-lysine modifications and 14-3-3 domains bind phosphorylated H3S10. Numerous domains can recognize the same histone modification and create binding platforms for various factors (Bannister and Kouzarides, 2011; Goutte-Gattat et al., 2013; Kouzarides, 2007; Zentner and Henikoff, 2013).

<table>
<thead>
<tr>
<th>Chromatin Modifications</th>
<th>Modified Residues</th>
<th>Regulated Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation</td>
<td>K-ac</td>
<td>Transcription, repair, replication, condensation</td>
</tr>
<tr>
<td>Methylation (lysines)</td>
<td>K-me1,K-me2,K-me3</td>
<td>Transcription, repair</td>
</tr>
<tr>
<td>Methylation (arginines)</td>
<td>R-me1, R-me2a, R-me2s</td>
<td>Transcription</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>S-ph, T-ph</td>
<td>Transcription, repair, condensation</td>
</tr>
<tr>
<td>Ubiquitinylation</td>
<td>K-ub</td>
<td>Transcription, repair</td>
</tr>
<tr>
<td>Sumoylation</td>
<td>K-su</td>
<td>Transcription</td>
</tr>
<tr>
<td>ADP ribosylation</td>
<td>E-ar</td>
<td>Transcription</td>
</tr>
<tr>
<td>Deimination</td>
<td>R &gt; Cit</td>
<td>Transcription</td>
</tr>
<tr>
<td>Proline Isomerization</td>
<td>P-cis &gt; P-trans</td>
<td>Transcription</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>T-OGlcNAc S-OGlcNAc</td>
<td>Transcription</td>
</tr>
</tbody>
</table>

Modified from Kouzarides, 2007.

A large number of histone modifications are associated with epigenetic phenomena; nevertheless, few cases have been defined as heritable epigenetic markers. The methyl marks H3K9, H3K27 and H4K20 are involved in constitutive heterochromatin formation, gene silencing, DNA damage repair and mitotic chromosome condensation. All three marks come in three different flavors (mono, di or tri-methylation) that have different functional consequences, the trimethylated state being the most strongly associated with the silent chromatin state. These marks have a slow turnover rate compared
with acetylation and phosphorylation and are marks postulated to be epigenetic in the sense that they drive an *in cis* heritable chromatin state (Barth and Imhof, 2010; Zee et al., 2010).

### Table 2. Turnover rates for histones carrying different post translational modifications

<table>
<thead>
<tr>
<th>Histone Modification</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation</td>
<td></td>
</tr>
<tr>
<td>All histones</td>
<td>&lt;15min</td>
</tr>
<tr>
<td>H2A</td>
<td>80% 2-3 min</td>
</tr>
<tr>
<td>H2B</td>
<td>75% 3 min, 20% 40 min</td>
</tr>
<tr>
<td>H3</td>
<td>60-70% 3 min, 25% 30 min</td>
</tr>
<tr>
<td>H4</td>
<td>50% 2-3 min, 45% 40 min</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>Short pulse: 3h</td>
</tr>
<tr>
<td>H2A</td>
<td>60% 40 min; fast, 30 min; slow</td>
</tr>
<tr>
<td>H2B</td>
<td>Not determined</td>
</tr>
<tr>
<td>H3</td>
<td>Fast, 30 min; slow, 2-3h</td>
</tr>
<tr>
<td>H4</td>
<td>Fast, 30 min; slow, 2-3h</td>
</tr>
<tr>
<td>Methylation</td>
<td>Half-life (days)</td>
</tr>
<tr>
<td>Overall H3</td>
<td>1.298 +/- 0.007</td>
</tr>
<tr>
<td>H3K4me1</td>
<td>0.959 +/- 0.129</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>0.342 +/- 0.001</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>1.031 +/- 0.060</td>
</tr>
<tr>
<td>H3K18me1</td>
<td>1.207 +/- 0.116</td>
</tr>
<tr>
<td>H3K27me1</td>
<td>0.470 +/- 0.005</td>
</tr>
<tr>
<td>H3K27me2</td>
<td>1.145 +/- 0.001</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>3.128 +/- 0.032</td>
</tr>
<tr>
<td>H3K36me1</td>
<td>0.751 +/- 0.085</td>
</tr>
<tr>
<td>H3K36me2</td>
<td>0.571 +/- 0.000</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>1.105 +/- 0.070</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>3.609 +/- 0.283</td>
</tr>
<tr>
<td>Overall H4</td>
<td>1.385</td>
</tr>
<tr>
<td>H4K20me1</td>
<td>0.297 +/- 0.005</td>
</tr>
<tr>
<td>H4K20me2</td>
<td>1.467 +/- 0.001</td>
</tr>
<tr>
<td>H4K20me3</td>
<td>4.809 +/- 1.483</td>
</tr>
<tr>
<td>H4R3me1</td>
<td>2.788 +/-. 1.806</td>
</tr>
<tr>
<td>Overall H1.4</td>
<td>0.976 +/- 0.065</td>
</tr>
<tr>
<td>H1.4K25me1</td>
<td>1.294 +/- 0.166</td>
</tr>
</tbody>
</table>

From Barth and Imhof, 2010.

H4K20 methylation, an evolutionarily conserved chromatin modification, is linked with repression of transcription, DNA damage repair and X inactivation in mammals. Initial methylation of H4K20 is mediated by SET8/PRSet7, whereas further H4K20 methylation to H4K20me2 and H4K20me3 is performed by SUV4-20H1 and SUV4-
20H2 enzymes (Jørgensen et al., 2013). The methyltransferase Set8 has been found to be associated with chromatin and/or the PCNA replication clamp during replication (Huen et al., 2008). H4K20me1 is present throughout the cell cycle but PRSet7, which is regulated during the cell cycle, has been detected only during G2 and early M phase. This indicates that the presence of the methyl mark does not need the continued presence of the methyl transferase. Consistently, persistence of H4K20me has been observed in Drosophila embryos lacking PRSet7 (Trojer and Reinberg, 2006), suggesting that the marker is stable retained. Moreover, H4k20me1 is required for Suv4-20 dependent establishment of H4K20me2 that is recognized by the ORC1 component of the origin of replication complex in vitro, and might cooperate in marking replication origins (Kuo et al., 2012).

Stable isotope labeling of amino acids in cell culture (SILAC) in combination with mass spectrometry (MS) in HeLa cells has shown that H4K20me3 and H3K27me3 have the slowest rates of formation (Zee et al., 2010). Studies of the turnover of histone modifications indicates that H3K27me3 has a half life of 3 days (Zee et al., 2010). The steady-state of H3K27 is achieved by the action of demethylases and the methyltransferase PRC2. Once established, H3K27me3 recruits PRC2 to sites of DNA replication, facilitating the maintenance of H3K27me3 via the action of EZH2 containing PRC2. Thus H3K27me3 is replicated onto nascent deposited histones (Hansen et al., 2008). Despite the reported association of PRC2 and PCNA during replication, a recent report by Petruk et al., showed that at least in Drosophila embryos, S phase nuclei are depleted of H3K27me3 as well as H3K4me3 marks which appear to be replenished only later, during G2 phase. They therefore suggest that these marks are unlikely to be the only epigenetic mark that propagates the transcriptional state of TrxG and PcG target genes, at least in Drosophila.
Another hallmark of silenced chromatin is H3K9 methylation. Di and trimethylation of H3 lysine 9 like H3K27me3 is propagated by a positive feedback loop, in which Heterochromatin Protein 1 (HP1) binds specifically to methylated H3K9, forms oligomers to bridge neighboring nucleosomes and recruit Suv39h1/2, KMT1F and SETDB1 histone methyltransferases to methylate neighboring nucleosomes thereby spreading the silent state (Bannister and Kouzarides, 2011; Bannister et al., 2001; Lachner et al., 2001). Moreover, the association of SETDB1 with the HP1-CAF1(chromatin association factor) complex and the interaction between DNMT1 and the HMT G9a that associates with PCNA during DNA synthesis, suggest that H3K9me3 propagation is maintained during DNA replication (Estève et al., 2006; Loyola et al., 2009). Hathaway et al., developed a chromatin in vivo assay (CiA) system with the ability to induce heterochromatin formation through the recruitment of HP1 to examine the epigenetic properties of H3K9me3 through cell division of embryonic stem cells. HP1 tethering leads to suppression of gene activity, spreading of heterochromatin over a 10kb domain after 5 days of HP1 recruitment independent of sequence elements. H3K9 methylation is stable in the absence of HP1 with a turnover rate similar to global histone turnover in HeLaS3 cells and rates of histone H3 displacement form chromatin in Drosophila S2 cells (Deal et al., 2010; Dodd et al., 2007; Hathaway et al., 2012; Zee et al., 2010).

The transmission of histone modifications through a closed positive feed-back loop, where reader and writers propagate the histone mark, has been simulated in a mathematical model based on the silent mating type of Schizosaccharomyces pombe. The region that contains the mating type cassettes is normally in a silenced state but mutants which have a portion of the silenced region depleted and replaced by a reporter gene, generate a bistable condition, flipping between silenced...
and active state. This bistability is under the control of activating acetylations and silencing methylations that are dynamically produced by their respective transferases and removed by deacetylases and demethylases, respectively (Dodd et al., 2007). The model showed that 1) positive feedback loops in nucleosome modifications are an effective and robust mechanism for epigenetic memory, 2) histone modification can be highly dynamic without compromising stability, and 3) bistability, requires cooperativity that results from the ability of modified nucleosomes to not only stimulated addition of same modification but also to stimulate removal of competing modifications (Dodd et al., 2007).

The role of histone modifications associated with transcriptional activation to maintain an active chromatin state is less explored. The turnover rates of histone modifications estimated by SILCA-MS show that active modification marks turn over ~2.5 fold faster in comparison with repressive marks, suggesting that cells require more fine temporal control over activated genes than repressed genes (Zee et al., 2010). Analysis of histone modifications at gene promoters and coding regions in mammalian cell lines show that H3K4ac, H3K4me and H3K79me persist upon inhibition of transcription and through mitosis (Kouskouti and Talianidis, 2005).

1.1.2.4. X chromosome inactivation

In mammals, the imbalance in X-linked genes between XX and XY individuals is regulated by silencing of a large percentage of genes on female X chromosomes called X chromosome inactivation (XCI). The initiation and maintenance of the inactive chromosome (Xi) is controlled by the x-inactivation center that produces Xist, a ~17kb ncRNA. Once the inactive X coated by Xist which is transcribed in cis by unknown mechanisms, transcription marks across the chromosome are lost and the Polycomb Repressive Complex 2 (PRC2) is recruited
followed by broad distribution of H3K27me3, and enrichment of the histone H2A variant macroH2A. As a result promoters of X-linked genes eventually undergo DNA methylation at later stages to stably maintain silencing (Calabrese et al., 2012; Heard and Disteche, 2006). X chromosome inactivation is a classic example of in cis chromatin-mediated epigenetic silencing that does not affect the homologous X chromosome that shares the same nucleus.

1.1.2.5. Histone variants

With the exception of H4 all histones have variants that are not linked specifically to DNA replication. These variant histones differ in amino acid sequence from the canonical histones (Figure 1.3) and are encoded by separate genes that are expressed throughout the cell cycle. Different variants are enriched in specific chromatin domains rather than the broad distribution across all chromatin as is the case for canonical replication-coupled histones (Talbert and Henikoff, 2010). An important property of histone variants is that by creating specialized nucleosomes, they generate different functional chromatin domains with distinct functions by influencing nucleosome stability or through downstream effectors that bind to specific variants (Kurumizaka et al., 2013). In humans, eight H3 variants have been identified: H3.1, H3.2, H3.3 H3.4 (H3T), H3.5, H3.X, H3.Y and CENP-A. The major H3 variants are CENP-A and H3.3. Two of these H3 variants, CENP-A and H3.3 that are relevant for the work described in this thesis will be introduced in more detail below.
Figure 1.3 Sequence alignments of human H3 variants. Alignment of amino acid sequences corresponding to human H3 variants. Sequences are compared in relation to the H3.3 variant and the amino acid differences are highlighted. H3.1 and H3.2 differences are highlighted in purple, H3t in gray, H3.X and H3.Y in yellow, and CENP-A in light blue. The position numbers of amino acids that are different between H3.3 and H3.1/2 are indicated. The positions of the N-terminal tail and of the α-helixes of the histone-fold motif are shown (Szenker et al., 2011).

1.1.2.5.1 CENP-A

The histone H3 variant CENP-A uniquely marks centromeric chromatin and is found in virtually all eukaryotes investigated thus far. It shares only about 50% amino acid identity with canonical H3 (Kurumizaka et al., 2013). The centromere is a chromosomal region that functions as a platform for the assembly of the kinetochore, which drives segregation of chromosomes to daughter cell during cell division (Allshire and Karpen, 2008; Cleveland et al., 2003). In most eukaryotes, centromeres are propagated on repetitive satellite DNA which in humans corresponds to α-satellite, a complex family of long
tandemly repeated monomers of 171bp that constitute 5% of total human DNA (Henikoff et al., 2001; Waye and Willard, 1986). However, α-satellites are not sufficient for the function and maintenance of centromeres, as is evident from neocentromeres, ectopic centromeres that are stably transmitted through mitosis and meiosis (Tyler-Smith et al., 1999; Vafa and Sullivan, 1997; Warburton et al., 1997).

While specific DNA sequences are non-essential, centromere function strongly depends on CENP-A. Depletion of CENP-A\textsuperscript{CID} in Drosophila, leads to mislocalization of most kinetochore proteins while the depletion of kinetochore proteins does not affect the localization of CENP-A\textsuperscript{CID}. Indeed, CENP-A\textsuperscript{CID} mislocalization and formation of ectopic kinetochores is observed when CENP-A\textsuperscript{CID} is overexpressed (Blower and Karpen, 2001; Heun et al., 2006). These findings strongly suggest that CENP-A provides identity and position of the centromere in dividing cells.

CENP-A is also structurally divergent of canonical H3 nucleosomes by inducing negative supercoil, left-handed wrapping and by containing a specific domain at the HFD (Black et al., 2004; Sekulic et al., 2010). The CENP-A targeting domain (CATD), that encompasses the L1 and α2 helix of CENP-A, is pivotal for the definition of centromeric chromatin. It has been shown that when the CATD domain is placed within the HFD of canonical H3, the chimeric histone generated is sufficient to target to centromeres (Black et al., 2004; Panchenko et al., 2011). Moreover, it has been shown that the CATD is the target sequence for Holliday junction recognizing protein (HJURP), the chaperone for prenucleosomal CENP-A. HJURP contributes to the efficient incorporation into centromeres (Foltz et al., 2009) and transmit stability throughout the histone fold domains of CENP-A and H4 (Panchenko et al., 2011).
Throughout the entire cell cycle, CENP-A nucleosomes are present at centromeres. Experiments using fluorescent pulse labeling techniques based on SNAP-tagging and fluorescence recovery after photobleaching (FRAP), whose details will be introduced in section 2, have shown that in human cells, Xenopus extracts, Chicken cells and Drosophila, CENP-A is targeted to centromeres during late telophase/early G1 phase of the cell cycle (Bernad et al., 2011; Dunleavy et al., 2012; Jansen et al., 2007; Schuh et al., 2007; Silva et al., 2012). In human cells CENP-A has been found to be stably retained throughout multiple cell divisions and segregates in a semi conservative manner during DNA replication (Bodor et al., 2013; Hemmerich et al., 2008; Jansen et al., 2007). Such high stability is consistent with maintaining an epigenetic memory of centromere identity.

Recently, direct evidence for the epigenetic nature of the centromere has been shown by means of the induction of formation of a new centromere. This was achieved by the artificial tethering of CENP-A\textsuperscript{CID} in Drosophila S2 cells and tethering of HJURP in human culture cells to a non-centromeric site (Barnhart et al., 2011; Mendiburo et al., 2011). Barnhart et al. forced the CENP-A chaperone HJURP onto a LacO (Lac operon) domain at a non-centromeric locus. This was found to be sufficient to nucleate CENP-A chromatin and form a functional centromere. Mendiburo et al. used a similar approach to show that the ectopic targeting of CENP-A\textsuperscript{CID} is sufficient to recruit nascent untagged CENP-A and create an epigenetic feed-back loop that triggers heritable kinetochore formation for several subsequent cell divisions.

As mentioned above, contrary to canonical histones, nascent CENP-A is loaded in late telophase/early G1 phase in human cells (Jansen et al., 2007). Prenucleosomal CENP-A is targeted to
centromeres by HJURP (Foltz et al., 2009) through an interaction either with the constitutive centromere complex (CCAN) or the Mis18 complex. Among the components of CCAN, CENP-N are CENP-C are the factors that recognize the CENP-A nucleosome directly through the CATD domain and the carboxyl terminus of CENP-A respectively, for the recruitment of the CCAN to the CENP-A nucleosome (Carroll et al., 2009, 2010). In fission yeast it has been reported that the recruitment of HJURP requires the Mis18 complex (Pidoux et al., 2009; Williams et al., 2009), but there is no evidence of a direct interaction between Mis18 and HJURP. A possible link between the Mis18 complex and HJURP are the RbAp46/48 proteins that interact with both the Mis18 complex and HJURP (Dunleavy et al., 2009). Alternatively, Mis18 may change centromeric chromatin structure to facilitate the recruitment of HJURP. In support of this view, the Mis18 complex has been suggested to affect the acetylation state of chromatin (Fujita et al., 2007; Ohzeki et al., 2012), and recruitment of HJURP to a human artificial chromosome depends on the transcriptionally active chromatin mark with H3K4me2 (Bergmann et al., 2011). Furthermore, the requirement for Mis18 in the initiation of de novo centromere assembly on α-satellite DNA can be bypassed by targeting histone acetyl transferases to the satellite array (Ohzeki et al., 2012). Combined, this evidence points to an elaborated protein network that possibly involves changes in chromatin structure that constitutes the feed-back loop for the self templating of CENP-A (Figure 1.4).
The assembly of nascent CENP-A is tightly coupled to the cell cycle. HJURP binds to prenucleosomal CENP-A in late S/G2 phases of the cell cycle and chaperones new CENP-A–H4 until its assembly in early G1 phase (Dunleavy et al., 2009; Foltz et al., 2009; Jansen et al., 2007); preventing misincorporation of CENP-A. The cell cycle is controlled by Cdk2/cyclin A and Cdk1/cyclin B complexes during S, G2 and mitotic phases. By blocking CDK activity and visualizing newly synthesized CENP-A in human culture cells, Silva et al., proposed that CDKs hold the CENP-A assembly machinery in an inactive state until mitotic exit for deposition of CENP-A (Silva et al., 2012). This model was substantiated by the finding that CDK inhibition leads to premature recruitment of Mis18α and Mis18BP1\(^{\text{HsKNL2}}\) to centromeres in G2 phase.
and concomitant CENP-A assembly. Depletion of Mis18α, Mis18BP1HsKNL2 or HJURP in combination with CDK inhibition prevents assembly of CENP-A in G1 and G2 phases. Thus, cell cycle restricted loading of nascent CENP-A provides a direct coupling between the S-phase turnover, cell division and subsequent assembly after mitosis, potentially ensuring a proper homeostasis of centromere size across cell division cycles (Gómez-Rodríguez and Jansen, 2013).

1.1.2.5.2. H3.3

In contrast to CENP-A, histone H3.3 differs from canonical H3 by just four amino acids which render the incorporation of H3.3 independent of DNA replication. Genome wide analysis of H3.3 distribution showed that H3.3 is deposited at chromatin regions that are actively transcribed and in promoter regions coinciding with enrichment of H3K4me1 and RNA polymerase II (Mito et al., 2005).

H3.3 assembly is not cell cycle regulated, but can occur throughout the cell cycle, thereby replacing a fraction of the nucleosome pool assembled during S phase (Gómez-Rodríguez and Jansen, 2013; Wu et al., 1982). Recently, Deal et al., suggested that H3.3 turnover is fast at active genes and regulatory elements (Deal et al., 2010) but H3.3 in bulk is stable indicating that not the entire pool is turned over. H3.3 histones are deposited by the Histone regulator A (HIRA) complex in a replication-independent manner (Tagami et al., 2004). HIRA forms a complex with Cabin1 and Ubinuclein (UBN1), which copurifies with H3.3 (Tagami et al., 2004).

In addition to HIRA, studies in ES cells have described the α-thalassemia X linked mental retardation protein - death associated protein (ATRX-DAXX) complex, and DEK to be involved in the H3.3 deposition at telomeres, pericentromeres and centromeres (Drané et al., 2010; Goldberg et al., 2010; Sawatsubashi et al., 2010). The
function of H3.3 in the organization of centromeres and telomeres chromatin is unknown (Goldberg et al., 2010). Whether it is associated to transcription or needed for cell division and genome stability needs to be established.

A direct role of H3.3 in maintaining memory of a chromatin state is, as of yet, poorly defined. One study has implicated H3.3 in memory. Nuclear transfer experiments in Xenopus laevis showed that misexpression of the donor-originated MyoD gene occurs upon initiation of zygotic transcription in nuclear transfer embryos. Memory of MyoD target gene expression is maintained in the absence of transcription during 24 mitotic divisions and this epigenetic memory is dependent upon H3.3 lysine 4 methylation (Ng and Gurdon, 2008).
2. Measuring protein dynamics

Key to our understanding of the contribution of chromatin to epigenetic memory is a determination of the dynamics of chromatin components. Below I will review different strategies that have been used to determine different aspects of chromatin dynamics, stability and turnover.

2.1 Fluorescence recovery after photobleaching (FRAP)

The green fluorescence protein (GFP) technology has made it possible to apply methods such as Fluorescence recovery after photobleaching (FRAP) and Fluorescence correlation spectroscopy (FCS) to probe dynamic behavior of proteins in vivo. Fluorescence recovery after photobleaching (FRAP) was developed by Axelrod and colleagues as a quantitative technique to study dynamics of protein mobility in living cells by measuring the rate of fluorescence recovery at a previously bleached site (Ishikawa-Ankerhold et al., 2012). In a FRAP experiment, fluorescent molecules in a small region of the cell are irreversible photobleached using a high-powered laser beam. Subsequently, movement of the surrounding non-bleached fluorescent molecules into the photobleached area leads to recovery of fluorescence with a particular velocity, which is recorded at low laser power (Figure 2.1.) (Ishikawa-Ankerhold et al., 2012).
Figure 2.1 Fluorescence recovery after photobleaching. Typical plot of fluorescence intensity in a region of interest versus time after photobleaching a fluorescent protein. The prebleach is compared with the asymptote of the recovery curve to calculate the mobile and immobile fractions. The diffusion rate of the fluorescent protein is determined from the recovery curve. Adapted from Lippincott-Schwartz et al., 2001.

Recovery of fluorescence into the bleached area occurs as a result of the diffusional exchange between bleached and unbleached molecules. Quantitative FRAP studies define two kinetic parameters: the mobile fraction (M), which is the fraction of fluorescent proteins that can diffuse into the bleached region during the time course of the experiment; and the immobile fraction, which is the fraction of molecules that cannot exchange between bleached and nonbleached regions (Lippincott-Schwartz et al., 2001). Knowledge of the rate of molecular exchange can provide important insights into the properties and interactions of molecules within the cellular environment. FRAP, has recently been applied to 3D environments like the cytoplasm or
inside the nucleus to determine the binding and diffusion parameters of proteins in these environments. FRAP has been used to measure the residence time of canonical histones, which exhibit a longer residence time that most chromatin-associated proteins (see chapter 2 and Kimura and Cook, 2001; Thiriet and Hayes, 2005).

### 2.2 Inducible fluorescent proteins

Photoactivatable fluorescent proteins (PAFPs) are capable of displaying pronounced changes in their spectral properties in response to irradiation with light of a specific wavelength and intensity (Lukyanov et al., 2005). Rather than observing the recovery of fluorescence into the bleached area (as in FRAP), the movement of photoactivated molecules away from the activation spot is assessed (Sparkes et al., 2011). The main advantage of PAFPs is the capability for the performance of pulse-chase labeling, which contributes to elucidate protein transport pathways, intricate connections between compartments, protein turnover and super-resolution imaging (Lippincott-Schwartz and Patterson, 2009).

More than 20 different PAFPs have been described, which can be categorized in three classes: 1) irreversibly switches from a dark to bright fluorescent state, 2) irreversible photoconversion from one fluorescent color to another or 3) reversibly photoconversion, enabling on/off switching capability. The first group includes PA-GFP a photoactivatable variant of GFP and PA-mRFP1 and PA-mCherry1 that is derived from DsRed that are less phototoxic due to their low energy red light emission. The irreversible photoconverters class has the advantage that molecules are visible prior to photoconversion which enables tracking and selection of specific regions to photoactivate. Among these are EosFP and Dendra2 that convert from green to red and PS-CFP2 a photoswitchable cyan FP that photoconverts from cyan to green. The class of reversible highlighters with on/off switching
is characterized by their ability to be repeatedly photoconverted between two states using light of two distinct wavelengths. They include FP595, Dronpa PA-Cherry and DsRed timer among others (Lippincott-Schwartz and Patterson, 2009). Different applications in diffraction limited and super-resolution imaging, can be achieved by the combination of optical and biochemical characteristics of the PAFPs. These include their brightness level, oligomeric state, contrast ratio, rate of spontaneous conversion into an activated state and rate of photobleaching (Lippincott-Schwartz and Patterson, 2009).

2.3 Self labeling tags

Self-labeling proteins are those that are capable to bind irreversibly or with high affinity and specificity to a synthetic probe in vitro or in vivo (Keppler et al., 2004). Self labeling protein tags are used as a fusion to a protein of interest. Self labeling proteins include SNAP, SNAPf, CLIP, CLIPf, Halo and TMP. The most well established system is the SNAP-tag (Gautier et al., 2008; Keppler et al., 2004). SNAP is a stable suicide-enzyme protein-fusion tag that catalyzes its own covalent binding to cell permeable O6-benzylguanine (BG) derivatives and thereby permits the labeling of SNAP-tag fusion proteins with a variety of different synthetic probes. It was generated in a stepwise manner from human O6-alkylguanine-DNA alkyltransferase (hAGT) by introduction of 19 point mutations and deletion of 25 C-terminal residues. Relative to hAGT, SNAP-tag possess a 52-fold higher reactivity toward BG derivatives, does not bind to DNA and is expressed as well in cells as on cell surfaces (Mollwitz et al., 2012).

The main advantages of self labeling tagging are: an alternative for detection of proteins in organisms that are not suitable for the expression of autofluorescent proteins, the ability to distinguish young and old copies of a protein by labeling at different time points with different fluorophores, simultaneous and specific labeling of self-
labeling fusion proteins in vitro and in living cells (Bodor et al., 2012; Gaietta et al., 2002; Gautier et al., 2008; Jansen et al., 2007). Although, this system is not suited for the analysis of proteins with high turnover, it has been used for in vivo analysis of chromatin dynamic assembly of H3 histones. SNAP-tag quench-chase-pulse and pulse-chase experiments (see chapter 2) have contributed to the elucidation of the stability of CENP-A chromatin and the cell cycle timing of CENP-A assembly that is restricted to G1 phase (Jansen et al., 2007). In addition, it has facilitated the characterization of factors involved in H3.1 and H3.3 deposition (Ray-Gallet et al., 2002).

2.4 Recombination-induced tag exchange (RITE)

Epitope tags are used to study protein expression and localization using specific antibodies raised against peptides that are used as tags. Detection of tagged proteins provides a steady state snapshot of protein levels. Recently, genetically encoded epitope tags have been used to develop a versatile strategy to study protein dynamics called Recombination-induced tag exchange (RITE) (Verzijlbergen et al., 2010). RITE is a genetic method that induces a permanent epitope-tag switch in the coding sequence of a protein of interest after a transient induction of a site-specific recombinase. RITE is composed of two parts: a tandem-tag cassette that can be integrated in frame of the gene of interest for conditional C-terminal tagging, and a stably integrated and constitutively expressed hormone-dependent Cre recombinase for the control of epitope switching. RITE cassettes encode for one epitope tag between two LoxP recombination sites and a second, epitope tag downstream of the second LoxP site. The tagged proteins are encoded by a single gene and under the control of the endogenous promoter (Terweij et al., 2013; Verzijlbergen et al., 2010). Cre induced recombination leads to the switch in expression of the protein of interest with one tag to being
expressed with another tag. In effect, this allows for the detection of lifetime and fate of both the old pre-switch pool of protein and the new post-switch pool of protein. Advantages of RITE are selective tagging and following of one protein of interest, monitoring of old and newly synthesized proteins and identification of proteins controlling protein turnover (Verzijlbergen et al., 2010). The method is not suited for the analysis of proteins with high turnover. The epitope tags used in RITE also allows for application of proteomic methods, DNA based high-throughput screens, protein dynamics in single cells by live imaging (Radman-Livaja et al., 2011; Terweij et al., 2013; Verzijlbergen et al., 2010). The use of RITE has allowed to determine directly the dynamics of histones in yeast. This analysis has shown that nucleosomes are dynamically retained for multiple cell divisions, that the rate of turnover is dependent of transcription and that ancestral histones accumulate towards the 5’ end of long and less transcribed genes (Radman-Livaja et al., 2011; Verzijlbergen et al., 2010).

### 2.5 Metabolic labeling of proteins

#### 2.5.1 Stable isotope labeling by amino acids in cell culture (SILAC)

Mass spectrometry (MS) is an analytical strategy to identify known and novel PTMs on proteins, as well as determine the relative quantity of proteins (Sanz-Medel et al., 2008). Mass spectrometric measurements are carried out in the gas phase on ionized analytes.

A mass spectrometer measures the mass to charge ratio (m/z) of freely moving gas-phase ions in electric and/or magnetic fields (Soldi et al., 2013). A mass spectrometry based proteomics experiment consists of five stages: 1) protein isolation that might include one dimensional gel electrophoresis, 2) enzymatic degradation of proteins to peptides, 3) separation of peptides by high-pressure
liquid chromatography and elution into an electrospray ion source, where they are nebulized in small, highly charged droplets. After evaporation, multiply protonated peptides enter the mass spectrometer 4) a mass spectrum of the peptides eluting at this time point is taken, and 5) generation of a prioritized list of these peptides for fragmentation (Aebersold and Mann, 2003).

Recently, a stable isotope labeling strategy called stable isotope labeling by amino acids in cell culture (SILAC) has been described (Ong et al., 2002). In SILAC, a growth medium is prepared where natural (light) amino acids are replaced by heavy SILAC amino acids. Cells grown in this medium incorporate the heavy amino acids. When light and heavy cell populations are mixed, they remain distinguishable by MS (Ong et al., 2002; Soldi et al., 2013). This allows for quantitative measurements of changes in protein levels or post translational modifications between experimental and control conditions. It also allows heavy labeling to be used as a pulse label to determine protein turnover by mass spectrometry. The advantages of SILAC are: 1) there is no need of peptide labeling step which minimize the loss of starting material, 2) no differences in labeling efficiency between samples because incorporation is virtually 100%, 3) peptides from the same protein can be compare due to the uniform labeling, 4) labeling of peptides is specific to its sequence and the mass differential between two states can be specified more directly and 5) quantification of small proteins is possible (Ong et al., 2002). SILAC has been used to profile protein levels, profile PTM dynamics during the cell cycle, in spike in strategies and in pulse experiments to measure the turnover of PTMs and histone variants (Soldi et al., 2013). SILAC has been applied to profile the bulk dynamics of histone modifications by labeling newly synthesized histones with a heavy isotope. In this way the presence and quantity of new and old pool histones can be determined.
by MS (See chapter 2 and Ong et al., 2002). This strategy revealed that H3.1 nucleosomes remain largely octameric throughout the cell cycle whereas a proportion of H3.3 nucleosomes split and old H3.3 histones form hybrid nucleosomes with nascent H3.3 (Xu et al., 2010b).

2.5.2. Covalent attachment of tags to capture histone and identify turnover (CATCH-IT)

CATCH-IT is a modified pulse labeling method specifically designed for estimating nucleosome turnover rates. It is based on metabolically labeling newly synthesized histones with an amino acid analog that can be subsequently coupled to an affinity tag (Deal and Henikoff, 2010). In CATCH-IT native histones are pulse labeled with the methionine (Met) surrogate azidohomoalanine (AHA). AHA is a small bioorthogonal functional group used to tag proteins, glycans and lipids in cells. It is stable at physiological temperatures and incorporated by the biosynthetic machinery of the cell. After pulse labeling AFA labeled proteins are subsequently ligated to biotin which allows their detection (Dieterich et al., 2006). In the case of analysis of chromatin dynamics, isolated nucleosomes containing newly synthesized histones are affinity purified with streptavidin, and washed stringently to remove non-histone proteins as well as H2A-H2B dimers. Finally, the affinity purified DNA from chromatin that is pulse labeled or allowed to turn over during a chase period is hybridized to high density tiling microarrays to create a map of nucleosome occupancy that is dependent on its rate of turnover following the initial pulse (Deal et al., 2010). The advantage of CATCH-IT is that no transgenes or tags are needed. However, the method does not allow for the detection of a specific protein nor is it suitable to measure fast dynamics. The use of CATCH-IT showed that newly synthesized H3/H4 is incorporated at
active genes and enhancers in Drosophila and is turned over with a half-life ~1-1.5 hours (Deal and Henikoff, 2010).

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<tr>
<th>Table 3 Methods to Analyze Protein Turnover</th>
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<td>FRAP</td>
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<td>Measures local protein turnover after photobleaching</td>
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<td>Floxed protein tag that is replaced by a different tag upon Cre-expression</td>
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<td>Metabolic labeling of protein (SILAC,CATCH-IT)</td>
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Modified from Bodor et al., 2012.
## 3. Aims of this thesis

Chromatin states can potentially be propagated through multiple cell divisions thereby contributing to epigenetic inheritance. However, the mechanistic basis for chromatin inheritance is not clear, nor is the dynamics and the rate of turnover of histones at specific loci well established. Histones and their post translational modifications are candidates to mark epigenetic states. Gaining insight into the dynamic state of chromatin is important for the understanding of the epigenetic inheritance.

Early experiments in bulk chromatin suggest that histones are stable and survive critical processes challenging stability such as DNA replication and transcription, thereby supporting the argument that histones have a role in epigenetic inheritance. However, whether histones are stably maintained at all loci is not known. Indeed stability has been questioned with the finding of residence times of nascent histones on the order of 1.5h at active genes and promoters of Drosophila cells. The determination of the genomic localization and timescales of histone turnover is pivotal to elucidate the molecular basis for the propagation of chromatin.

In chapter 2, I introduce TimeChIP, an assay that allows capturing histone dynamics whose main advantage is that it allows for the distinction of old and new pools of histones and high spatial resolution. In chapter 3, we show for the first time that a population of ancestral H3.1 and H3.3 histones is stably retained across the cell cycle in a human cell line. Finally in chapter 4, I discuss these results and present an outlook to the future based on the results and conclusions of this work.
References


Chapter 1 – General Introduction


Chapter 1 – General Introduction


Chapter 1 – General Introduction


Chapter 1 – General Introduction


Chapter 2 – TimeChIP
AUTHOR CONTRIBUTION

All the experiments were planned by the author and the supervisor Lars E.T. Jansen. All the experiments in this chapter were executed by the author.

SUMMARY

Proteins are dynamic entities. The steady-state of proteins is defined by the combination of synthesis, localization, maintenance and degradation. Protein stability is likely an important parameter in protein-based epigenetic memory. Histones have been proposed to act as markers of epigenetic memory. Bulk analysis of histone dynamics suggests that histones turnover slow. Although, recently a genome wide analysis of newly synthesized histones indicates that histones turnover rapidly, at least at active genes, challenging the role of histones as epigenetic markers. To measure the dynamics of histones, we developed a strategy based on the SNAP system for the performance of pulse-chase experiments, we call TimeChIP. We show that TimeChIP facilitates the study of histone dynamics because of its temporal resolution, analysis in intact cells, differentiation of old and new pools of histone and the ability to determine turnover at high spatial resolution across the genome.
INTRODUCTION

Proteins undergo continuous changes in their interactions, stability and location with consequences for their functions. One striking example of such a consequence is the loss of post-translational modifications and need for re-establishment once the protein is replenished. The spatio-temporal dynamics of proteins involves coordination of synthesis and assembly of nascent proteins. Of special relevance to this thesis is protein inheritance across cell divisions which is directly impacted by protein turnover rates. For the analysis of different features of protein dynamics there are an array of methodologies which differ in the degree of both spatial and temporal resolution. Bulk measures of protein turnover are based on differential chemical labeling. Proteins are \textit{in vivo} labeled e.g. with isotope labeled amino acids and followed for a certain period of time. Then, the distribution of new and old proteins is determined in bulk. Traditionally this was done by measuring density differences after isolation of proteins of interest (Jackson and Chalkley, 1974; Jackson et al., 1975). A modern incarnation of this is the use of non-radioactive isotopes for stable isotope labeling of amino acids in cell culture that can be detected by mass spectrometry. This method is rendered quantitative when internally controlled with stable isotope labeling by amino acid in cell culture (SILAC) (Ong et al., 2002). Although such assays are highly quantitative they do not provide information about the localization of the protein and the temporal resolution is low.

In contrast to such bulk measures, methodologies have been developed that assay turnover at specific loci. These include microscopy-based methods such as photoactivation or photobleaching of fluorescent proteins fused to the protein of interest. In fluorescence recovery after photobleaching (FRAP) an area of a cell expressing a fluorescently tagged protein is rapidly and irreversibly bleached and
the influx of unbleached molecules (recovery of fluorescence) is measured as a function of time (Ishikawa-Ankerhold et al., 2012). FRAP has been used for the analysis of histone dynamics, where after photobleaching a small area of nuclei, H3-GFP and H4-GFP enter the bleached area more slowly than H2B-GFP. Once incorporated into replication foci during S phase, H3 and H4 are stably maintained and can even persist through mitosis (Kimura and Cook, 2001). Photoactivation experiments function reciprocally to FRAP where a population of dark inactive molecules is locally activated. In fluorescence photoactivation localization microscopy (FPALM) activated molecules are switched on by photoactivation and yield a signal over a dark background. Its tracking over time provides spatial and temporal dynamics of the protein of interest (Hess et al., 2006). Albeit, FRAP provides a high temporal resolution and FPALM subcellular specificity, both are phototoxic, and restricted to short timescales. Furthermore, although specific nuclear domains can be analyzed, dynamics cannot be analyzed at genome sequence resolution.

To circumvent these limitations, novel methods have recently been developed specifically to determine dynamics of chromatin that include recombination-induced tag exchange (RITE). This method allows to measure ancestral and newly synthesized proteins pools encoded by the same transgene in budding yeast (Verrijlbergen et al., 2010). In RITE the protein of interest is encoded by a transgene containing an epitope tag and a stop codon flanked by loxP recombination sites followed by a second epitope tag. When Cre recombinase activity is induced the sequence encoding the first tag is removed and expression of second tag commences. This, in effect creates a pulse labeling condition where the differentially tagged proteins represent old and new protein. Accordingly, the two tagged
proteins can be affinity purified and relative protein occupancy can be mapped across the genome by microarrays or sequencing. Besides the simultaneous detection of old and new protein, this method allows for the measurement of dynamics at long time scales at DNA sequence resolution. The ability to measure dynamics at short timescales is limited by the efficiency of Cre-recombination. In addition, histone dynamics is measured in relative terms derived from the ration between old on new protein. RITE experiments have shown the dynamics of replication-independent histone H3 in budding yeast, whose rate of exchange vary between cell cycle phases. Particularly in G1 phase half of old histones are replaced and continued histone deposition was maintained during at least three cell divisions (Verzijlbergen et al., 2010). Importantly, when considering retention, ancestral histones accumulate toward 5’ end of long and less transcribed genes as opposed to turning over uniformly across the genome. Consistently, a mathematical model which considers histone turnover, histone spreading dependent of replication and transcription-dependent passback, predicted that maternal histones are reincorporated within two nucleosomes of their original site (Radman-Livaja et al., 2011). A technique with a similar application as that of RITE is a method called covalent attachment of tags to capture histones and identify turnover (CATCH-IT). In CATCH-IT all nascent proteins are metabolically labeled with an amino-acid analog that can subsequently be coupled to a biotin affinity tag (Deal et al., 2010). Following biotinylation, chromatin is extracted and fragmented. Sequences associated with biotinylated chromatin are analyzed. This method provides high spatial information down to nucleotide sequence resolution but is limited to the measurement of nascent protein incorporation and turnover at a short timescale. CATCH-IT has been used to measure the incorporation of nascent histones across the Drosophila genome. Somewhat surprisingly, using CATCH-IT Deal et
al., found that new synthesized H3 has a half-life ~1-1.5 hours at active genes and enhancers (Deal et al., 2010). Similar to what has been observed in yeast by RITE (Verzijlbergen et al., 2010), this study demonstrated that the rate of histone turnover correlates with the rate of gene expression.

A powerful approach for protein dynamics studies is the use of self-labeling tags that can overcome some of the limitations of the methodologies described above. The SNAP-tag is derived from the human DNA repair protein O⁶-alkylguanine-DNA alkyltransferase (hAGT) that catalyzes its own covalent binding to cell permeable O⁶-benzylguanine (BG) derivatives (Keppler et al., 2004) (Figure 2.1). The self-labeling nature of the SNAP-tag makes it ideally suited for pulse labeling studies. The SNAP-tag can be used to label proteins in living cells as well as in vitro. Importantly, SNAP reactive substrates can be conjugated with different molecules that include fluorescent dyes as well as e.g. biotin or Digoxin. This renders this tag applicable to both fluorescence and well as biochemical techniques where e.g. biotinylation allows for the immobilization of a protein of interest. Thus in SNAP pulse-chase experiments, the SNAP tagged protein is pulse labeled with BG bearing a dye molecule, then chase for a period where the pulse labeled protein is turned over and replenished by new unlabeled protein. Alternatively, in SNAP quench-chase-pulse experiments, the SNAP-tagged protein is labeled with unconjugated BG (quench), chased for a certain time where new protein is synthesized and subsequently labeled with a dye-conjugated BG molecule (Figure 2.2). Experiments using the SNAP tag have provided insight in the dynamics of histone H3 variants.

Initially this method was used to characterize the dynamics of the histone H3 variant CENP-A whose localization is restricted to the centromere. This strict localization makes CENP-A an ideal substrate
for fluorescence-based approaches. Pulse-chase experiments demonstrated that CENP-A is a stable protein at centromeres, diluted only by DNA replication and newly synthesized CENP-A is targeted to centromeres in a strict cell cycle regulated manner in early G1 phase (Bodor et al., 2013; Jansen et al., 2007). In contrast to CENP-A, H3.3, another H3 variant, was initially proposed to be deposited throughout the cell cycle. Importantly, assembly does not dependent on DNA synthesis in S phase while canonical H3 is loaded (Ahmad and Henikoff, 2002). In contrast to CENP-A, the histone H3 variants H3.1 and H3.3 display very different dynamics. While assembly of H3.1 is restricted to S phase by direct coupling to the DNA replication machinery, (Shibahara and Stillman, 1999; Smith and Stillman, 1989), H3.3 is dynamically assembly at active genes, promoters and subtelomeric regions and is also detected at centromeres (Deal et al., 2010; Goldberg et al., 2010; Ray-Gallet et al., 2011). A recent study used fluorescence SNAP-technology to characterize H3.1 and H3.3 dynamics across the cell cycle based on the quench-chase-pulse strategy described above. This confirmed the strict S-phase dependency of H3.1 assembly and the dynamic assembly of H3.3 (Ray-Gallet et al., 2011). However, such microscopy-based analysis does not have the resolution to study the stability of histone H3.1 or H3.3 at specific loci.
Figure 2.1 SNAP-tag. SNAP-tag (brown) is fused to the protein of interest (green). Self-labeling with benzylguanine results in a covalent irreversible bond between the benzyl moiety and a reactive cysteine in SNAP with the consequent releases of guanine.

To gain insight into locus-specific assembly and turnover of specific histone variants, we sought to develop a strategy based on pulse labeling of SNAP-tagged histones, followed by chromatin isolation and fragmentation, immobilization of nucleosomes dependent on the SNAP pulse label and DNA isolation to determine occupancy at specific loci. We call this method TimeChIP as it resembles a Chromatin immunoprecipitation (ChIP) protocol but provides temporal information of histone occupancy. We aimed to generate a system to determine the site specific dynamics of histone H3 variants across the cell cycle in human somatic cells.
Figure 2.2 SNAP labeling strategies. **A)** Pulse-chase: cells that produce and turnover SNAP-tagged protein (light green spheres, represent protein population) are labeled with SNAP substrate at time 0 (dark green spheres). Then, the protein is chased by allowing cells to continue to synthesize SNAP protein that is not labeled, while the pulse labeled pool turns over. **B)** Quench-chase-pulse: cells that produce and turnover SNAP-tagged protein are quenched with an unconjugated SNAP substrate (no dye, grey spheres) at time 0, blocking subsequent labeling. Then, cells continue to synthesize SNAP protein that is not labeled for a certain period and new SNAP protein is specifically labeled with SNAP substrate bearing a dye.
MATERIALS AND METHODS

Cell lines and constructs

HeLa cell lines stably expressing H3.1-SNAP-3XHA, H3.3- SNAP-3XHA or CENP-A-SNAP-3XHA (Jansen et al., 2007; Ray-Gallet et al., 2011) were cultured in DMEM medium supplemented with 10% newborn calf serum, 2mM L/Glutamine, 100/mL Penicillin and 100µg/mL Streptomycin (all from Gibco), at 37°C and 5% CO₂. The constructs 3xHA-SNAPf-2xPreScission-CENP-A, 3xHA-SNAP-2xPreScission-CENP-A, 3xHA-CLIPf-2xPreScission-CENP-A and 3xHA-CLIP-2xPreScission-CENP-A named pLJ471, pLJ509, pLJ473 and pLJ492 respectively, were constructed by a PCR-generated fragment carrying a triple HA-tag, SNAPf, SNAP, CLIPf or CLIP-tag and a double PreScission protease site cloned into the NdeI and BamHI sites of pSS26m. The resulting ORF was subcloned into the EcoRV and BamHI sites of pCEMS1-CLIP-CENP-A (pLJ310), replacing the CLIP fragment.

The SNAP-3XHA fragment from pSS26m-SNAP-3XHA (pLJ222) was subcloned into EcoRV and NotI sites of pCEMS1-CLIP (pLJ299), replacing CLIP, resulting in pLJ447. T98G SNAP-3XHA cells were cultured in DMEM medium supplemented with 10% newborn calf serum, 2mM L/Glutamine, 100/mL Penicillin and 100µg/mL Streptomycin (all from Gibco), at 37°C and 5% CO₂ and selected with 0.5mg/mL G418.

DNA transfections

T98G cells were transfected with 200ng of DNA, 1µl Plus Reagent and 1.25µl of Lipofectamine (Invitrogen) in Optimem reduced serum media (Gibco) according to manufactures instructions.
Cell synchronization

HeLa CENP-A-SNAP-3XHA cells were synchronized by double Thymidine block. Cells were treated with 2mM of Thymidine (Sigma) for 17 hours, washed twice in medium and released in medium containing 24µM of Deoxycytidine (Sigma) for 9 hours. Subsequently, cells were treated again with Thymidine for 16 hours and released into medium containing Deoxycytidine.

SNAP quench-chase-pulse labeling

Synchronized HeLa cells expressing CENP-A-SNAP were pulse labeled by addition of 5µM BTP (SNAP-Cell Block New England Biolabs) in presence of Thymidine in growth medium for 30 minutes at 37°C for irreversible non-fluorescent labeling of the preexisting CENP-A-SNAP pool. 3 hours after the quench, cells were treated with 9µM RO-3306 (Calbiochem) for 8 hours and followed for 3 hours treatment with 24µM MG132 (Sigma).

Pulse labeling of intact cells was performed with 10µM BG-Biotin or 2µM TMR Star (SNAP-Cell TMR-Star New England Biolabs), in growth medium for 1 hour or 15 minutes, respectively at 37°C. After each labeling step (fluorescent and non-fluorescent) cells were washed twice with medium and reincubated at 37°C to allow excess SNAP substrate to be release from cells. After 30 minutes, cells were washed again once in medium.

Pulse labeling of permeabilized cells: 2-3 x 10^7 cells were collected, resuspended in chromosome isolation buffer (CIB buffer) and pulse labeled with 10µM BG-Biotin (New England Biolabs) for 1 hour at room temperature.
Soluble Nucleosome Preparation

Chromatin was prepared by a modification of the method of Yoda and Ando, 2004. The following volumes and numbers are given for one purification (~5 x 10⁷ cell equivalent): cells were equilibrated in 15mL CIB buffer (3.75mM Tris pH7.5, 20mM KCl, 0.5mM EDTA, 0.5mM DTT, 0.05mM Spermidine (Sigma), 0.125mM Spermine (Sigma), 0.1% recrystallized Digitonin (Sigma), 1mM PMSF, 1:1000 Protease inhibitor cocktail (Sigma), aprotinin (Sigma)), dounced 10x with tight pestle and centrifuged at 300g for 5 min at 4ºC. Dounce homogenization and centrifugation was repeated once more. Pelleted nuclei were washed in 15mL washing buffer (20mM Hepes sodium pH7.7, 20mM KCl, 0.5mM EDTA, 0.5mM DTT, 0.5mM PMSF, 1:1000 Protease inhibitor cocktail (Sigma) and aprotinin (Sigma)), and centrifuged at 300g for 5 min at 4ºC. Pelleted nuclei were washed again in 7.5mL washing buffer with 0.3M NaCl and centrifuged at 500g for 10 min at 4ºC. Chromatin resuspended in 500µL washing buffer was digested with 800U/mL Micrococcal nuclease (Roche) in the presence of 0.3M NaCl and 3mM CaCl₂ while rotating for 1 hour at room temperature. The reaction was quenched with 5mM EGTA, 0.05% NP40 and centrifuged at 10000g for 15 min at 4ºC. Soluble nucleosomes were recovered.

SNAP-Nucleosomes Purification

The soluble nucleosomes concentration was adjusted to 1 A₂₇₆ units in 340µL washing buffer A with 0.3M NaCl. 0.2mg Streptavidin Magnetic Beads (Thermo Scientific) were washed twice with washing buffer B (20mM Hepes sodium pH7.7, 20mM KCl, 0.5mM EDTA, 0.5mM DTT, 0.5mM PMSF, 1:1000 Protease inhibitor cocktail (Sigma), aprotinin (Sigma), 0.5%NP40 and 0.5M NaCl), while rotating for 5 min at room temperature. Beads were blocked with washing buffer B supplemented with 50mg BSA and 200µg/mL yeast tRNA (Invitrogen)
while rotating for 1 hour at 4°C. SNAP biotinylated nucleosomes were recovered by adding 300µL of the adjusted fraction to the pretreated beads, and rotated for 1 hour at 4°C, while the 40µL of nucleosomes were saved as input fraction. After 3 washes with washing buffer B with rotation for 5 min at room temperature each, 250µL TNES buffer with 100µg/mL RNaseA (Invitrogen) was added directly to the beads and incubate for 10 minutes at room temperature. The resulting RNase treated samples were subjected to a phenol-chloroform extraction followed by purification on a Qiagen column. For protein analysis, beads were directly eluted in 2X Sample Buffer, incubated for 10 minutes at 100°C and centrifuged.

**Quantification of bound nucleosomes**

The recovered DNA was quantified with a Quant-iT PicoGreen dsDNA assay kit (Invitrogen). Quantification of nucleosome occupancy at specific loci was performed by real-time PCR. The recovered DNA was quantified in triplicate by real-time PCR using iTaq Universal SYBR Green supermix (Bio-Rad) and the 7900HT Fast Real-time PCR System (Applied Biosystems). Quantification of DNA was achieved by comparing qPCR signals to those obtained from a standard curve derived from 10 fold serial dilutions of the input DNA which also served as a measure of the linear dose response of the qPCR reaction.

In addition to the qPCR standard curve, a TimeChIP standard curve was generated for each pulse-chase experiment to determine the dose-response of the qPCR to the fraction of biotinylated nucleosomes. The TimeChIP standard curve was generated by mixing of equal amounts of *in vivo* pulse labeled cells expressing SNAP-tagged histones mixed with unlabeled cells in a 2 fold ratio series. The quantitative value of TimeChIPed DNA of the chase samples at each locus was interpolated on the TimeChIP standard curve. The
TimeChIP signal was defined as the fraction of biotin retained relative to the standard curve.

**Immunofluorescence**

HeLa cells were grown on glass coverslips coated with poly-L-Lysine (Sigma) and fixed with 4% formaldehyde (Thermo Scientific) for 10 minutes. Cells were extracted after fixation and processed for immunofluorescence using standard procedures. Cells were stained with 1µg/mL anti-HA (clone HA11, Covance) and FITC-conjugated anti-mouse (Jackson Immunoresearch Laboratories). Samples were stained with DAPI (4',6-diamidino-2-phenylindole; Sigma) before mounting in Mowiol (Calbiochem).

**Microscopy**

Widefield fluorescence microscopy was performed using a DeltaVision Core System (Applied Precision) that controls an inverted microscope (Olympus, IX-71), coupled to a Cascade 2 EMCCD camera (Photometrics). 512 by 512 pixel images were collected at 1x binning using a 100x, 1.4 NA oil immersion objective (UPlansApo) at 0.2µm axial sections spanning the entire nucleus. Quantified images were acquired using the same exposure conditions for each fluorescent channel. TRITC and FITC images of a uniformly slide were automatically flatfield- and camera-noise-corrected using softWoRx (Applied Precision). All images presented are maximum intensity projections of deconvolved pictures.

Centromeric TMR-Star fluorescence intensity was quantified using CRaQ, a macro specifically developed for ImageJ (NIH). For specific details about methods and parameters of CRaQ see Bodor et al., 2012.
Immunoblotting

Proteins were separated in 12% SDS/PAGE gel and transferred to Hybond PVDF membranes (GE Healthcare) using standard procedures. Blots were probed with anti-HA (Covance) at 1:2000 dilution. Anti-mouse HRP conjugated secondary antibody was purchased from Jackson Immunoresearch Laboratories.

Flow cytometry

HeLa CENP-A-SNAP cells ($10^6$) were harvested and fixed 1 hour at 4°C with 70% ethanol. Cells were washed twice in PBS containing 3% BSA (Sigma) and incubated for 30 minutes at room temperature with 5μg/mL propidium iodide (Sigma) and 200μg/mL of RNaseA in PBS containing 3% BSA. Subsequently flow cytometry analysis was performed on FACScan (Becton Dickinson) using CellQuest software.
RESULTS

TimeChIP strategy

To determine aspects of protein dynamics in living cells, common approaches include fluorescence recovery after photo bleaching (FRAP), photoactivation, among other novel techniques (see introduction above) that assess turnover at specific loci at a relatively short time frame. Alternatively, high resolution localization to a specific genomic region is achieved through chromatin immunoprecipitation (ChIP), but this approach cannot assess protein dynamics. A strategy that combines both protein dynamics and localization at high genomic resolution is currently lacking. We sought to develop a biochemical approach to determine histone dynamics in living cells at specific loci using the SNAP-tag system (TimeChIP, see figure 2.3)
Figure 2.3 Outline of the TimeChIP assay. A) Cells stably expressing H3.1- or H3.3-SNAP-HA are in vivo pulse labeled with BG-biotin at time 0 (blue). Cells continue to synthesize H3-SNAP protein that is not labeled, while the H3-SNAP biotinylated pool turns over. Cells are harvested and nuclei are isolated by mechanical forces using a Dounce homogenizer in the presence of 0.1% Digitonin. Salt extracted chromatin is fragmented to mononucleosomes with Micrococcal nuclease and soluble biotinylated H3-SNAP nucleosomes are affinity purified with Streptavidin magnetic beads. B) Detection of pulse labeled histone. Soluble H3.3-SNAP nucleosomes either pulse labeled or unlabeled were purified and separated by SDS-PAGE, blotted and detected using an anti-HA antibody. C) DNA associated with purified pulse labeled H3.3-SNAP nucleosomes can be identified by
quantitative (q) PCR. Biotinylation dependent enrichment for the ACTB gene is shown.

HeLa cells stably expressing histone-SNAP-3XHA cells were *in vivo* pulse labeled with benzylguanine (BG) conjugated with a biotin moiety. The specific and tight binding of biotin-streptavidin makes it a widely used system for labeling, purification and immobilization of protein complexes. Once cells were collected, chromatin was extracted and fragmented into mononucleosomes by MNase treatment. Histone-SNAP-biotinylated nucleosomes were captured on Streptavidin magnetic beads. Recovery of SNAP-tagged protein as well as the presence of co-purified DNA by western blot and spectrophotometry respectively was assessed followed by the determination of the localization of tagged histones at a particular locus. The resulting protocol is outlined in figure 2.3. Unlike strategies based upon antibody-antigen specificity, TimeChIP allows to purify the protein of interest depending on self-labeling of the SNAP-tag with high affinity given by biotin-streptavidin interaction. To minimize non-specific background binding of DNA, we used yeast tRNA to block the Streptavidin solid phase. Finally, the isolation of chromatin in a native manner provides single nucleosome resolution to the assay.

To make TimeChIP a suitable assay for the study of nucleosome dynamics, the following parameters were assessed: labeling efficiency, mononucleosome isolation and purification. In contrast to fluorescent BG substrates, nonfluorescent BG or BTP enters cells efficiently (Bodor et al., 2012). We tested *in vivo* and *in vitro* labeling of CENP-A-SNAP cells using the BG-biotin substrate to determine the efficiency of labeling. CENP-A-SNAP cells were labeled in cell culture media at 37°C for 1 hour either as intact cells or as permeabilized cells in buffer containing nonionic detergent at room temperature for 1 hour as well. After purification of biotinylated
proteins, the protein levels were detected by western blot. We found that labeling of CENP-A-SNAP in conditions where the cell membrane is permeabilized with nonionic detergents is improved (Figure 2.4 A). This suggests that cell permeability is a rate limiting step in SNAP labeling with BG-biotin. Furthermore, we tested a novel variant of BG-biotin, CP-biotin, whose structure is modified to facilitate crossing cell membrane (synthesized by Ivan Correa, New England Biolabs, Ipswich, MA). We found that the amount of H3.3 purified increases ~1.5 fold when H3.3-SNAP is labeled with CP-biotin in comparison with BG-biotin (Figure 2.4 B).

Recently, new variants of both the SNAP and CLIP tags have been developed (named SNAPf and CLIPf, respectively) that show an improved reaction kinetics (Pellett et al., 2011; Sun et al., 2011). We fused these novel tags to CENP-A. Cells transiently transfected with CENP-A-SNAP, CENP-A-CLIP, CENP-A-SNAPf or CENP-A-CLIPf fusion proteins were in vivo fluorescence pulse labeled with BG carrying TMR-STAR at different concentrations and incubation times. The activity of the tags was determined by measuring the TMR-Star fluorescence relative to HA fluorescence intensity and normalized to the standard conditions. HA is encoded by the SNAP transgene and therefore provides a measure of the protein level independent of labeling activity. While CLIPf showed only a modest improvement over CLIP, SNAPf performed ~3 to 5 fold better across different concentrations of substrates and incubation times (Figure 2.5). Next, we sought to create stable S3 HeLa and HeLa cell lines expressing HA-SNAPf-PreScission-CENP-A but efforts to isolate cell lines bearing properly localized tagged-CENP-A failed suggesting that the tag interfered with CENP-localization.

Obtaining a homogeneous preparation of soluble mononucleosomes is an important factor in the successful application
of this assay. It is known that the histone octamer can be extracted from DNA at 2M NaCl, while lower concentrations result in the dissociation of H2A-H2B dimers leaving behind the H3-H4 tetramer (Eickbush and Moudrianakis, 1978). We used 300mM NaCl to extract chromatin which leaves the full nucleosome intact but removes non-chromatin proteins and low-affinity chromatin interacting proteins. Histone octamers protect ~147bp of DNA from Micrococcal nuclease (MNase) digestion (Axel, 1975; Luger et al., 1997). We used MNase protection as parameter for the definition of nucleosomes isolated at 0.3M NaCl as well as a method to isolate mono or oligo nucleosomes (Figure 2.4 C). By titrating the MNase concentration and incubation time we optimized conditions to maximize the yield of mononucleosomes. Finally, although the biotin-streptavidin purification system is based on the tight and essentially irreversible complex that biotin forms with streptavidin; we tested the efficiency of binding of the biotinylated SNAP-tag to streptavidin magnetic beads. T98G cells transiently expressing SNAP-HA tags were in vivo biotinylated and soluble labeled SNAP-HA (non histone fused) was immobilized on streptavidin magnetic beads. In order to test whether all the biotin-labeled protein binds to streptavidin, the unbound fraction was re-incubated with fresh streptavidin beads. Recovery of any SNAP-HA from this unbound fraction would indicate the initial binding reaction did not deplete all protein during the first binding step. We found that all biotinylated SNAP-HA protein binds efficiently to streptavidin, as no additional protein could be recovered using fresh streptavidin beads (Figure 2.4 D). In addition we tested whether all biotinylated proteins are eluted from the beads by re-eluting beads a second time (Figure 2.4 D).
Figure 2.4 TimeChIP optimization. A) Western blot analysis of CENP-A-SNAP labeling of intact cells vs. permeabilized cells. Cells were either unlabeled or labeled with BG-biotin for 1 hour. Soluble nucleosomes were prepared and bound to streptavidin beads. Bound material was eluted, separated by SDS-PAGE, blotted and detected using an anti-HA antibody. Alternatively (right), 0.1% recrystallized Digitonin was included during the biotinylation reaction to permeabilized cells. Percentage indicates the % of sample loaded. B) Experiment to compare the efficiency of biotinylation using BG-biotin or CP-biotin. Intact H3.3-SNAP expressing cells were pulse labeled under identical conditions with either CP-biotin, BG-biotin or were left unlabeled. Chromatin was extracted and solubilized by MNase. Nucleosomes
were bound to streptavidin beads and the quantification of co-bound DNA was determined by qPCR for the ACTB gene. **C) Generation of soluble mononucleosomes.** Salt extracted chromatin was digested with Micrococcal Nuclease in a 2 fold dilution series. Following digestion, DNA was isolated and analyzed by agarose gel electrophoresis and run on a Bioanalyzer for quantification. Size of fragments is indicted as well as concentration of mononucleosome peak. **D) Determination of binding efficiency.** Left: T98G cells expressing SNAP-HA were either unlabeled or BG-biotin pulse labeled. Soluble extract was bound to streptavidin beads. Input, unbound and bound fractions were separated on SDS-PAGE and analyzed by Western blot to detect *in vivo* biotinylated SNAP-HA using an anti-HA antibody. Both primary and secondary elutions were analyzed (compare lanes 6 & 7). The unbound fraction (lane 5 and 8) was tested for the presence of residual biotinylated SNAP-HA by reincubation with streptavidin to detect biotinylated SNAP-HA not purified during the first purification. Lane 9 shows that very little SNAP-HA binds to streptavidin in the second purification (indicating that unbound SNAP-HA is unlabeled). Right: Pulse labeling of T98G cells expressing SNAP-HA cells with TMR-Star show SNAP-HA is diffusely localized.
Figure 2.5 Evaluation of SNAPf-tag performance. A) HeLa cells transiently transfected with either CENP-A-SNAP, CENP-A-SNAPf, CENP-A-CLIP or CENP-A-CLIPf fusion proteins were labeled with TMR-Star at different concentrations and incubation times and processed for immunofluorescence with anti-HA. Representative images of cells are shown with TMR-Star signals in green and DAPI (DNA) in blue. B) TMR-Star and HA fluorescence intensity were determined using CRaQ. TMR/HA ratios are a measure of SNAPf and CLIPf activity. Results are plotted as fold difference normalized against signals obtained after incubation with 2µM TMR-Star for 15 minutes.

Proof of Principle

Following the initial pilot experiments to determine the feasibility of purifying biotin-pulse labeled SNAP from cells, we adopted the method to apply to the purification of histone H3 variants. Histone variants are implicated in epigenetic memory, however their dynamics at specific loci is poorly defined (Gómez-Rodríguez and Jansen, 2013). The reported dynamics of H3 variants varies from less than an hour to days (Bodor et al., 2013; Deal et al., 2010; Jansen et al., 2007; Kimura and Cook, 2001; Radman-Livaja et al., 2011; Xu et al., 2010). Using
our TimeChIP strategy, histone dynamics is measured by pulse biotinylation of SNAP-tagged histones in chromatin. The fraction of biotinylated histone retained in nucleosomes diminished over time as a consequence of turnover. First, we tested the dynamic range of the assay indicating the sensitivity at which we can detect retention of histones. Equal amounts of \textit{in vivo} labeled HeLa H3.1-SNAP cells were mixed at different ratios with unbiotinylated HeLa H3.1-SNAP cells. These mixtures were then assayed by the TimeChIP protocol and the genomic DNA recovered was quantified as a measure of the amount of histone retained. We found a linear response corresponding to the 2 fold serial dilutions of the biotinylated H3.1 cells indicating there is good agreement between the degree of biotinylation and the amount of DNA recovered. Moreover, we found that with the TimeChIP assay we can detect a minimum of 6.25% of histone retained (Figure 2.6).

\begin{figure}[h]
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\caption{Proof of principle. \textit{In vivo} pulse labeled H3.1-SNAP cells are mixed with unlabeled H3.1-SNAP cells in a 2 fold serial dilution, subsequently native chromatin is isolated from the 6 different pools of cells. Nucleosomes are purified by immobilization on Streptavidin. Total DNA purified was}
\end{figure}
quantified with a PicoGreen Assay. The graph indicates average of five reads of the amount of genomic DNA that is recovered and indicates the dose-response of DNA recovery as a function of biotinylation.

**Pulse-chase with TimeChIP**

SNAP fluorescence-based experiments have shown that CENP-A is stably localized at centromeres. To test the TimeChIP procedure in a pulse-chase labeling setting we used CENP-A-SNAP as a trail, since its *in vivo* stability has been previously established (Bodor et al., 2013). We performed pulse-chase experiments in asynchronous CENP-A-SNAP cells. The primary locus for CENP-A assembly is on α-satellite repeats, the major DNA component of the centromeres (Vafa and Sullivan, 1997). We measured the enrichment of CENP-A nucleosomes at α-satellite I, pericentromeric domain (Sat2) and 5SrDNA (Figure 2.7 A). As expected, CENP-A is most enriched at α-satellite, while levels drop to approximately half of that at the 5SrDNA and are reduced to one fourth at the pericentromeric domain. When normalizing to the initial enrichment for each locus, after 12 hours of chase, approximately 50% retention of CENP-A was seen at all loci tested. While CENP-A-SNAP levels were eventually reduced to background levels at all loci, levels remained high (~40%) at α-satellite I and Sat2 repeat. At 5SrDNA turnover appears to be faster (Figure 2.7 B). These results suggest that CENP-A is stably retained at centromeres and pericentromeric domains during the cell cycle compared with the retention at ribosomal 5SrDNA repeats.
Figure 2.7 CENP-A-SNAP Pulse-chase with TimeChIP. A) Quantification by qPCR of total enrichment of CENP-A at α-satellite I, pericentromeric domains (Sat2) and 5SrDNA. Average %IP is plotted and SEM is shown for two independent experiments. B) Time course of CENP-A-SNAP pulse-chase shows quantification by qPCR of CENP-A retained at α-satellite I, pericentromeric domains (Sat2) and 5SrDNA. CENP-A is more stably retained at α-satellite I and Sat2 in comparison to 5SrDNA where the turnover is faster.

Quench-chase-pulse with TimeChIP

The use of the SNAP system combining fluorescent and nonfluorescent SNAP substrates in a quench-chase-pulse experiment, allows for determining the dynamics and fate of the nascent protein pool. Cells are quenched using the unconjugated SNAP substrate BTP (no dye). Following the quench of the ancestral pool cells are chased during which a nascent unlabeled pool is synthesized. This pool can subsequently be labeled using a labeled substrate. This quench-
chase-pulse strategy has been used to show that in contrast to canonical H3 and H3.3, CENP-A is loaded during G1 phase (Jansen et al., 2007). To determine the utility of TimeChIP for the performance of quench-chase-pulse experiments, we tested whether the assembly of nascent CENP-A into chromatin also occurs in early G1 phase which is the cell cycle window during which new CENP-A is visibly targeted to the centromere. Alternatively, incorporation into chromatin occurs at a later stage in the cell cycle. First, we aimed at detecting newly synthesized nucleosomal CENP-A by western blot. Asynchronous CENP-A-SNAP cells were quenched with BTP. After 16 hours of chase, CENP-A-SNAP cells were pulse labeled with BG-biotin, followed by another 24 hour chase to allow newly synthesized pulse labeled histone to be assembled into centromeric chromatin. Following 24 hours of chase, nuclei were isolated, mononucleosomes were liberated by MNase digestion and biotinylated CENP-A-SNAP was immobile on streptavidin beads. We show that using this Quench-Chase-Pulse-Chase TimeChIP procedure, nascent CENP-A assembly into chromatin can be detected, indicating that CENP-A-SNAP is incorporated into salt-stable chromatin. As expected, the levels of nascent nucleosomal CENP-A detected are significantly lower to the total CENP-A-SNAP levels (Compare lanes 3 with 9 in Figure 2.8). Nevertheless, biotinylated CENP-A-SNAP protein can be readily detected. As described before, the in vitro labeling is higher in comparison with in vivo labeling of SNAP with the BG-biotin substrate (Figure 2.4A). To test whether newly synthesized CENP-A is assembled into chromatin at centromeres or elsewhere during early G1 phase we used TimeChIP with in vitro pulse labeling conditions. A synchronous population of CENP-A-SNAP cells arrested at the G1-S boundary by double thymidine block, were quenched with BTP in the presence of thymidine. The cells were then released into S phase for 3 hours. In order to keep the cells tightly synchronized in G2 phase, the
CDK1 inhibitor RO3306 was added for 8 hours (Vassilev et al., 2006). This treatment arrest cells in late G2 phase preventing mitotic entry. To ensure mitotic arrest, cells were released from RO3306 into MG132 for 3 hours which will arrest cells in metaphase of mitosis. Cells were then release by washout of MG132. Nascent CENP-A-SNAP assembly was analyzed in cells that remained arrested at the G1/S phase boundary, in mitosis, 3 and 11 hours after mitotic exit (Figure 2.9 A). Microscopy analysis showed that nascent CENP-A localizes at centromeres after 3 and 10 hours of mitotic exit (Figure 2.9 C). Strikingly, new CENP-A was detected in chromatin already in mitosis as well as early and late G1 by western blot (Figure 2.9 D). This suggests that while CENP-A is targeted to centromeres only in G1 phase; it may already be incorporated into general chromatin prior to mitotic exit. To determine where nucleosomal CENP-A accumulates we analyzed the TimeChIP precipitates for three families of DNA repeats, α-satellite I, pericentromeric domains (Sat2) and 5SrDNA genes. New CENP-A is detected in early G1 phase at centromeres, however, new CENP-A incorporated at α-satellite I chromatin is almost exclusively detected in late G1, suggesting that CENP-A loads into centromeric chromatin in late G1. However, after 3h chase (early G1) there is a significant proportion of cells still in mitosis making it difficult to determine whether no assembly into α-satellite I occurs at this early stage. Interestingly, new CENP-A is not only assembled at α-satellite I but also to a lesser extent at repeats of pericentromeric domains and 5SrDNA gene (as observed by pulse labeling experiments above, Figure 2.7). The accumulation of this non-centromeric signal may indicate a low level of promiscuity in the CENP-A assembly machinery.
Figure 2.8 Detection of nascent nucleosomal CENP-A. A) *In vivo* pulse labeling scheme of newly synthesized nucleosomal CENP-A-SNAP by quench-chase-pulse-chase TimeChIP. CENP-A-SNAP-HA cells are quenched using 5µM BTP (unconjugated SNAP substrate), then ancestral CENP-A is chased for 16 hours during which a nascent unlabeled pool is synthesized. Nascent CENP-A is pulse labeled with 10µM BG-biotin and chase for 24 hours where is incorporated onto chromatin. B) Western blot analysis of nascent chromatin incorporated CENP-A. Soluble nucleosomes were prepared and bound to Streptavidin beads from cells that underwent the quench-chase-pulse-chase procedure. Samples were separated by SDS-PAGE, blotted and detected using an anti-HA antibody. Lane 3 shows newly synthesized CENP-A incorporated into chromatin after 24 hours chase. Lane 6 shows that CENP-A is not detected when no time is allowed for new protein synthesis between quench and pulse labeling with a BG-biotin pulse. Lane 9 shows labeling of the total CENP-A pool.
Figure 2.9 Incorporation of newly synthesized CENP-A into chromatin during the cell cycle. A) Schematic of cell synchronization and labeling of chromatin incorporated nascent CENP-A. B) FACS analysis of DNA content to monitor cell cycle position of CENP-A-SNAP cells arrested in mitosis, released and progressed in G1 phase. Estimated fractions of cells in respective cell cycle positions is indicated. C) Visualization of new CENP-A at centromeres by TMR-Star labeling of nascent pool. Microscopy analysis of pulse labeled cells reveals CENP-A targeting to centromeres in early and late G1 phase. D) Newly synthesized CENP-A pull down in a biotin dependent manner in mitosis, early and late G1 phase. Nascent nucleosomal (left) and soluble (right) CENP-A in mitosis, early and late G1 phase were separated by SDS-PAGE and detected with anti-HA antibody. CENP-A-SNAP is detected in the input and unbound blots of the chromatin and soluble fraction in all conditions. Nascent nucleosomal CENP-A is detected in chromatin bound fractions in mitosis, 3h chase, late G1 (lanes 2, 3 and 4). Soluble CENP-A is not detected in mitosis, early and late G1 (lanes 7, 8 and 9). As expected,
CENP-A quenched with unconjugated SNAP substrate (BTP) and subsequently \textit{in vitro} pulsed with BG-biotin without a chase period (quench-pulse; lanes 1 and 5) could not be detected. Membranes were stained with Ponceau to detect histones as a loading control and a measure of purity of soluble fractions. E) Quantification of CENP-A incorporated in chromatin by qPCR. Amount of DNA associated to CENP-A nucleosomes quantified by qPCR (%IP) for $\alpha$-satellite I, pericentromeric domains (Sat2) and ribosomal loci in mitosis, early and late G1 phase. Enrichment of CENP-A is observed in late G1 phase at the three different loci by comparing with quench-pulse CENP-A.
DISCUSSION AND CONCLUSIONS

The comprehension of histone dynamics is important for understanding the contribution of chromatin to epigenetic inheritance. Thus far, chromatin dynamics has been analyzed using bulk pulse labeling of the amino acids, FRAP of GFP-tagged histones, conditional expression of tagged histones and self-labeling tags. Labeling of histones by the use of isotope labeled amino acids or amino acid analogs in SILAC and CATCH-IT allows for the analysis of protein complex turnover and, in the case of the latter, provide spatial resolution during turnover without the need to use of tags (Deal et al., 2010; Jackson, 1990; Xu et al., 2010). RITE which depends on the conditional induction and concurrent loss of differentially tagged histones, allows to measure old and new pools of histones simultaneously at DNA sequence resolution (Radman-Livaja et al., 2011; Verzijlbergen et al., 2010). FRAP and SNAP-tag fluorescent pulse labeling has the power to uncover variation in dynamics within the cell population or during the cell cycle because the analysis is performed on single cells. While FRAP provides a high temporal resolution from seconds to minutes, SNAP-tag based analysis operates on a longer time scale from hours to days (Bodor et al., 2012, 2013; Jansen et al., 2007; Kimura and Cook, 2001). Although, these methods are powerful and have provided insight on histone dynamics, what is lacking is a measure of histone turnover at specific loci. The RITE assay has the capacity to do this but requires highly efficient Cre-mediated recombination of the gene locus expressing tagged-histones which is not feasible in human cells.

Histone H3 variants are of particular interest to study due to their implication in epigenetic processes. Canonical H3 (H3.1) is assembled strictly in S phase, CENP-A assembly is restricted to early G1 phase, while H3.3 assembles throughout the cell cycle (Bodor et
al., 2013; Jansen et al., 2007; Ray-Gallet et al., 2011). Each variant has a particular localization. H3.1 is widely distributed throughout chromatin, H3.3 is ubiquitously localized at promoters and gene body of active genes, enhancers and subtelomeric regions, while CENP-A uniquely loads at centromeres (Goldberg et al., 2010; Vafa and Sullivan, 1997).

In this chapter I describe the development of the TimeChIP strategy for use in analyzing histones dynamics. Critical parameters to be considered are the SNAP labeling, fragmentation of chromatin by Micrococcal nuclease and the efficiency of chromatin purification. Regarding SNAP labeling we refer to two main aspects: the SNAP substrate and the SNAP tag. We conclude that a limiting factor for the assay is the cell permeability of SNAP substrates. We observed that when cell membrane is permeabilized the SNAP substrate significantly improve the reaction with the tag, an approach that should be considered for experiments like quench-chase-pulse or pulse. For labeling of intact cells, CP-biotin provides an improvement of labeling over BG-biotin. In relation to the tag itself, we saw that the faster variant of SNAP (SNAPf) exhibits a faster reaction kinetics which favors the in vivo labeling step as a larger fraction of SNAPf can be labeled within the same timeframe and substrate concentration. The second parameter is fragmentation of chromatin. To obtain high spatial resolution of histone turnover, soluble chromatin should be fragmented to mononucleosomes. By titration of MNase incubation times we optimize conditions such as to maximize the amount of mononucleosomes and limiting the amount of di- tri-nucleosomes or over digestion of chromatin. We find that this should be re-optimized for each batch of MNase. This is also important in the context of deep sequencing of isolated material (see chapter 3) where MNase digestion can create a bias in detection of specific sequences (Rizzo et
al., 2012). The last parameter that we highlight is the use of blocking reagents for the purification of the protein. Although we used one of the strongest non-covalent interactions, biotin-streptavidin for the purification of histones, to find the appropriate buffer conditions for the binding reaction was important. To avoid non-specific binding we found that the use of bovine serum albumin, yeast tRNA and non ionic detergent NP40 were important factors to block unoccupied binding sites and dislodge loosely bound molecules, respectively. Overall, the major limitation of the strategy is the inefficient labeling of intact cells by SNAP substrates that due to cell impermeability. While this may be a limiting factor for the analysis of CENP-A which is low in abundance, highly abundant proteins such as H3.1 and H3.3 can still successfully be analyzed (see chapter 3).

**CENP-A nucleosome dynamics along the cell cycle**

Centromeres are composed of α-satellite repeats that constitute 5% of total human DNA but the number of CENP-A nucleosomes per centromere is likely much smaller than the total number of nucleosomes occupying α-satellite arrays (D. Bodor, personal communication). Because CENP-A is relatively low in abundance compared to other histones and is broadly distributed across large alphoid arrays it served to test the detection limit of the TimeChIP method. We show that CENP-A-SNAP is stably retained as has been shown by FRAP and SNAP fluorescent pulse labeling (Hemmerich et al., 2008; Jansen et al., 2007). We take this observation beyond the general stability of CENP-A by demonstrating CENP-A dynamics at different loci. Specifically, we find that CENP-A is stable at α-satellite I repeats. Furthermore, our TimeChIP analysis suggests that CENP-A turnover is not only slow at centromeres but also at pericentromeric domains, even though CENP-A levels appear to be lower at these loci. In contrast, the enrichment of CENP-A at 5SrDNA repeats that can be
detected during the first 12 hours is rapidly lost in subsequent time points indicating that outside the centromere domain, CENP-A is not only lower in abundance but also turns over faster. Quench-chase-pulse analysis suggests that CENP-A is incorporated into nucleosomes in G1 phase. Interestingly, assembly of CENP-A is not restricted to alphoid DNA but is also observed at major satellites and at the rDNA repeats. In addition, we detect nascent CENP-A-SNAP protein in chromatin before detection of specific DNA loci suggesting that low levels of CENP-A assembly throughout chromatin may occur as early as in mitosis. Taken together, we speculate that CENP-A not only is targeted at centromeres in G1 phase where it is stably maintained but that there is also a subpopulation of CENP-A that assembles distal to centromeres and turns over faster. An explanation is that like the labile histone H3.3-H2A.Z variant nucleosome shown in human cells (Jin et al., 2009), the population of CENP-A with fast turnover constitute a CENP-A-H2A.Z double variant nucleosome where H2A.Z is less stable as has been described in S. cerevisiae (Meneghini et al., 2003). Interestingly, purification of the CENP-A nucleosome complex indicates that CENP-A containing nucleosomes are likely to contain the histone H2A.Z variant (Foltz et al., 2006). Alternatively, the same study identified a proportion of CENP-A-H3 hybrid nucleosomes which may confer a different stability.

Our ability to detect CENP-A by TimeChIP led us to question whether assembly into chromatin of newly synthesis CENP-A-SNAP occurs in early G1 as microscopy analysis has indicated or whether, nucleosome assembly is delayed following initial targeting. Our results indicate that at present we do not have the resolution to determine CENP-A turnover dynamics. Clear enrichment of CENP-A into centromeric chromatin is observed only in samples enriched in late G1 phase. However, the fact that we are unable to obtain a homogenous
population of early G1 cells does not allow us to draw conclusion on
the precise timing of CENP-A assembly into centromeric chromatin. In
addition, although our PCR probes detect an abundant class of alphoid
repeats only a small subset of these are likely occupied by CENP-A at
any time thereby reducing our sensitivity. The development of SNAP
substrates with higher capability to cross the cell membrane will
significantly contribute to the performance of in vivo pulse labeling
experiments and may help the analysis of CENP-A dynamics in
chromatin in future efforts.

A comprehensive understanding of protein function includes the
elucidation of its dynamics. The role of chromatin in epigenetic process
has been assessed through histone modifications and their regulation.
We show here that TimeChIP as a method for the study of protein
dynamics provides: 1) the ability to asses protein dynamics at long
time scales, 2) analysis in living cells, 3) distinction between ancestral
from newly synthesized pools of proteins, 4) high spatial resolution.
Thus, TimeChIP is potentially a versatile method for the analysis of site
specific histone dynamics such as turnover in living cells with which we
may gain insight in the relationship between histone dynamics and
epigenetic memory as well as the molecular mechanisms involved.
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REFERENCES


Chapter 3 – H3.1 and H3.3 Turnover
AUTHOR CONTRIBUTION

All the experiments were planned by the author and the supervisor Lars E.T. Jansen. All experiments were executed by the author, except those depicted in figure 3.6 which were performed in collaboration with Daniel Sobral from the Bioinformatics Unit at IGC.

SUMMARY

Epigenetic forms of inheritance are implicated in the transmission of cellular states independent on the DNA sequence. Chromatin is a pivotal component of carriers of epigenetic information. The steady-state maintenance of chromatin components dependents on the rates of nucleosome assembly and disassembly, as well as the rate of reestablishment of histone modifications which have been implicated in epigenetic memory. There is evidence for rapid histone turnover at specific loci, however the proportion of histone stably retained at specific loci is unknown. By using a TimeChIP strategy, based on the self-labeling SNAP-tag for the isolation of pulse labeled histones of different ages, we show that a significant pool of H3.1 and H3.3 is retained at genes and non-genes for the duration of the cell cycle. Turnover of H3.1 and H3.3 does not increase when transcription is activated, whereas inhibition of RNA polymerase leads to retention of H3.1 and H3.3. Our results support a model where nucleosomes contribute to inheritance of non genetic information.
INTRODUCTION

Inheritance of gene expression profiles and associated phenotypes that cannot be explained by changes in the DNA sequence is known as epigenetic inheritance. The maintenance of epigenetic information depends on the ability to persist through cell divisions (Waddington, 2011). Many factors may contribute to the epigenetic state. These include positive feedback loops that maintain a network of transcription factors that in this way perpetuate the transcriptional program. However, maintenance also requires the existence of molecules that propagate specific chromatin structures to either activate or repress gene expression, especially when identical sequences within the same cell are controlled differently in cis. This implies that chromatin may be a pivotal carrier of epigenetic information. There is a plethora of chromatin regulators involved in epigenetic phenomena, including DNA or histone modifications, DNA or histone binding proteins and histone variants (Gómez-Rodríguez and Jansen, 2013). The most extensively studied among these are histone modifications and the enzymes that regulate them, although whether modifications are heritable is a controversial issue and possible mechanisms are unclear. To consider histone modifications as epigenetic carriers it is necessary to understand the dynamic equilibrium between histone turnover and the dynamics of the modifications of those histones.

The basic unit of chromatin is the nucleosome. The nucleosome is formed by an octamer of four core histones (H2A, H2B, H3 and H4) wrapped by 147bp of DNA (Luger et al., 1997). Nucleosome assembly occurs in two steps: deposition of (H3-H4)$_2$ tetramer followed by the addition of two H2A-H2B dimers (Jackson 1990). With the exception of H4 all histones have variants that are not linked to DNA replication. The specific enrichment of histone variants
at different genomic loci indicate that they serve different roles in preserving epigenetic identity. In mammals, the major H3 variants are H3.3 and centromere protein A (CENP-A) (Talbert and Henikoff 2010). H3.3 differs from canonical H3.1 by only four amino acids and its incorporation into chromatin occurs throughout the cell cycle and is mainly localized at sites of active transcription as well as enhancers and telomeres (Goldberg et al., 2010; Ray-Gallet et al., 2011). CENP-A localization is restricted to the centromere, its deposition occurs in G1 phase after which the protein is stably maintained (Bodor et al., 2013; Jansen et al., 2007; Vafa and Sullivan, 1997).

The inheritance of nucleosomes across cell division cycles cannot be understood intuitively as is in the case of DNA. The semiconservative nature of DNA replication and segregation is given by the complementary base-pairing of its sequences. Propagation of a chromatin state requires an orchestration between nucleosome assembly, positioning, disassembly and stability (Luger et al., 2012; Simon and Kingston, 2013). Moreover, in the case of nucleosomes, there appears to be little opportunity for direct templating new molecules onto older ones indicating that maintenance of a “state” requires self-templating in an indirect manner (Gómez-Rodríguez and Jansen, 2013).

Classical radioactive pulse-chase analysis on bulk chromatin and FRAP experiments on GFP-tagged histones have shown that, in general, H3/H4 and H2A/H2B are stable molecules. However, H2A/H2B dimers exchange into chromatin faster and part of the H2A/H2B dynamics is suppressed when RNA polymerase II is inhibited (Jackson, 1990; Kimura and Cook, 2001). This indicates that transcription is a driver of H2A/H2B exchange. Indeed, genome wide analysis of pulse labeled histones indicates that nucleosomes undergo fast turnover at active genes, epigenetic regulatory elements and
origins of replication in Drosophila cells (Deal et al., 2010). However, in this study a method is used (CATCH-IT) in which histones are labeled during synthesis using a methionine analogue that is subsequently coupled to biotin. Therefore, the analysis is biased towards the dynamics of newly incorporated nucleosomes which may not represent the overall histone pool. By using an inducible recombination tagging system in budding yeast, Radman-Livaja et al., followed the fate of parental histones and compared their distribution with nascent histones genome wide through multiple cell divisions. They found that ancestral nucleosomes distribute to adjacent locations after replication (Radman-Livaja et al., 2011). Nevertheless, there is no evidence of retention of ancestral histones at specific loci in mammals.

To understand the mechanisms of chromatin-based epigenetic inheritance it is necessary to know at what rate histones turnover and at which loci in living cells. We know that CENP-A exhibits a strikingly slow turnover using SNAP-based pulse labeling experiments (Bodor et al., 2013; Jansen et al., 2007). We have developed a methodology based on the SNAP system (which we call TimeChIP) to determine the turnover of histone pools of different ages at specific genomic loci. We find that both H3.1 and H3.3 are turned over faster at active genes versus non-gene loci, with H3.3 displaying faster dynamics than H3.1, particularly at active genes. Moreover, we find that H3.1 and H3.3 turnover is slowed upon inhibition of transcription. For both histones a variable but significant pool is retained for the duration of the cell cycle. These findings are therefore consistent with the idea of nucleosomes as candidate carriers of epigenetic information.
MATERIALS AND METHODS

SNAP labeling and drug treatments

Pulse labeling of intact cells was performed with 10µM CP-Biotin (provided by Ivan Correa, New England Biolabs), in growth medium for 1 hour at 37ºC. After labeling, cells were washed twice with medium and reincubated at 37ºC to allow excess SNAP substrate to be released from cells. After an additional 30 minutes, cells were washed again once with medium and reincubated with growth medium and further treated for analysis as indicated. TNF-α was used at 50ng/mL (R&D Systems), Thymidine at 2mM (Sigma), DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole) at 100µM (Sigma) and α–Amanitin at 10µg/mL (Sigma).

Soluble nucleosome preparation, SNAP nucleosomes purification and quantification of bound nucleosomes and qPCR analysis were performed as described in chapter 2.
Chapter 3 – H3.1 and H3.3 Turnover

Immunofluorescence

HeLa cells were grown on glass coverslips coated with poly-L-Lysine (Sigma) and fixed with 4% formaldehyde (Thermo Scientific) for 10 minutes. Cells were extracted after fixation and processed for immunofluorescence using standard procedures. Cells were stained with anti-NF-κB (1:25; sc8414, Santa Cruz Biotechnology). For BrdU staining, HeLa cells were fixed in Methanol/Acetone (1:1; Sigma) for 3 minutes, acid treated with 2M HCl during 30 minutes at RT followed by 3 x 10 minute washes with 100 mM Borax (Sigma). HeLa cells were extracted with 0.1% Triton X-100 and stained with anti-BrdU (1:100; MoBU-1, Santa Cruz Biotechnology). Secondary antibodies used were FITC-conjugated anti-mouse (Jackson Immunoresearch Laboratories) and Cy3-conjugated anti mouse (Sigma). Cells were stained with DAPI (4’,6-diamidino-2-phenylindole; Sigma) before mounting in Mowiol

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Table 1. List of primers used for TimeChIP

<table>
<thead>
<tr>
<th>Description</th>
<th>Target</th>
<th>Sequence (5’ -3’)</th>
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<tbody>
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<td>exon 3</td>
<td>GCCCGTGCTCAGGGCTTCTT</td>
</tr>
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<td>ACTBe3R</td>
<td>exon 3</td>
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<td>GAPDHHR</td>
<td>exon 1</td>
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<td>exon 1</td>
<td>TTATCAACGCGGCCCAGG</td>
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<td>TBPR</td>
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<td>promoter-exon join</td>
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</tr>
<tr>
<td>RPL13AR</td>
<td>promoter-exon join</td>
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</tr>
<tr>
<td>13/21-3A *</td>
<td>α-satellite I Chr 13 &amp; 21</td>
<td>CTTCTGTCTAGATTTTAGA</td>
</tr>
<tr>
<td>13/21-1B</td>
<td>α-satellite I Chr 13 &amp; 21</td>
<td>CATAGAGATGACATG</td>
</tr>
<tr>
<td>Sat2_F **</td>
<td>pericentromeric satellite 2</td>
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</tr>
<tr>
<td>Sat2_R</td>
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</tr>
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</tr>
<tr>
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<td>promoter</td>
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<tr>
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<td>promoter</td>
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<td>NFkB</td>
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<tr>
<td>NFkB</td>
<td>exon 2</td>
<td>AGAGGCACAGGTAGTCCACCA</td>
</tr>
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*(Warburton et al., 1991), **(Bergmann et al., 2011).
Cells treated with TNF-α were stained with Rhodamine Phalloidin (Invitrogen) before DAPI staining.

**Microscopy**

Digital images were captured using a DeltaVision Core System (Applied Precision) that controls an inverted microscope (Olympus, IX-71), coupled to a Cascade 2 EMCCD camera (Photometrics). Images (512x512) were collected at 1x binning using a 100x oil objective (NA1.40, UPlanSApo) with 0.2µm axial sections spanning the entire nucleus. Images were subsequently deconvolved and maximum signals were projected as 2D images using softWoRx (Applied Precision). Fluorescence quantification was performed on nondeconvolved images. The BrdU fluorescence signal intensity was quantified using ImageJ (NIH). The sum of the mean and two times the standard deviation of cells for which BrdU treatment was omitted was used as background cutoff. BrdU treated cells that displayed a signal higher than this cutoff were considered positive.

**Flow cytometry**

HeLa cells (10^6) were harvested and fixed 1 hour at 4°C with 70% ethanol. Cells were washed twice in PBS containing 3% BSA (Sigma) and incubated for 30 minutes at room temperature with 5µg/mL propidium iodide (Sigma) and 200µg/mL of RNaseA in PBS containing 3% BSA. Subsequently flow cytometry analysis was performed on FACScan (Becton Dickinson) using CellQuest software and for quantification FlowJo software.

**Next-generation sequencing**

10ng of purified TimeChIPed DNA quantified by PicoGreen assay were submitted. Sequencing libraries were generated and barcoded for multiplexing according to Illumina recommendations. Resulting libraries were submitted for Illumina sequencing on a 4x
Illumina HiSeq at the Gene Core Facility at EMBL, Heidelberg Germany.

**RNA extraction and qRT-PCR**

RNA was isolated from $10^6$ HeLa cells with TRizol (Life Sciences), according to manufacturer’s instructions. Prior to cDNA synthesis, RNA was treated with RNase-Free DNase (Promega) following the provided protocol. cDNA synthesis of 1µg of DNase-treated RNA was performed using SuperScript II Reverse Transcriptase (Invitrogen). qRT-PCR analyses were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems), using the iTaq Universal SYBR Green Supermix (BioRad), and $2^{\Delta \Delta Ct}$ method for relative quantification (Livak and Schmittgen, 2001). Expression values were normalized using threshold cycle (CT) values obtained for RPLP0 and GAPDH genes.

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence (5' -3')</th>
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<td>rcIAP2F</td>
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</tr>
<tr>
<td>rcIAP2R</td>
<td>CTGTTCAAGAAGATGAGG</td>
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<tr>
<td>rCCNA2F</td>
<td>TCCTCGTGGACTGGTTAGTTG</td>
</tr>
<tr>
<td>rCCNA2R</td>
<td>ACAGCCAAATGCAGGGTCTC</td>
</tr>
<tr>
<td>rGAPDHF</td>
<td>GGACTCATGACCACAGTCCATGCC</td>
</tr>
<tr>
<td>rGAPDHR</td>
<td>GCGGCCATCAGCAGCCACAGTT</td>
</tr>
<tr>
<td>rH31LF</td>
<td>CGAGAAATCGCCCAAGACTTC</td>
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<tr>
<td>rH31LR</td>
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<tr>
<td>rRPL13AR</td>
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</tr>
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Table 2. List of primers used for qRT-PCR
RESULTS

Quantitative retention of ancestral H3.1 and H3.3

HeLa cells stably expressing H3.1-SNAP-3XHA or H3.3-SNAP-3XHA were \textit{in vivo} pulse labeled with CP-biotin and chased for 3, 6 and 12 hours. Chromatin was extracted and fragmented into mononucleosomes by MNase treatment. H3.1-SNAP or H3.3-SNAP biotinylated nucleosomes were immobilized on Streptavidin magnetic beads. Co-purified DNA from recovered H3.1 or H3.3-SNAP nucleosomes was quantified by spectrophotometry. The occupancy of pulse and pulse-chase H3.1 and H3.3 nucleosomes at a particular locus was quantified by qPCR. The loci analyzed were exon 1 of TATA binding protein (TBP) a lowly transcribed gene as estimated from HeLa RNA-seq data (3.2-3.5 reads per kilobase per million mapped reads RPKM), exon 3 of the β-actin gene (ACTB), the promoter-exon 1 junction of the ribosomal protein L13a (RPL13A) and exon 1 of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene which transcripts levels correspond to 90-93, 110-220 and 600-900 RPKM, respectively (ENCODE Project Consortium, 2011). To measure the turnover rate of H3.1 and H3.3 outside genes, we selected the α-satellite I repeat, pericentromeric satellite 2 repeats (Sat2) and a conserved non-coding element that is not detectably transcribed (gene desert) (Nobrega et al., 2003). Initially, cells expressing SNAP-tagged canonical H3.1 and H3.3 were chased for 3, 6 and 12 hours after pulse labeling. We observed that canonical H3.1 exhibits slow turnover at the α-satellite I and pericentromeric satellite 2 where ~50% of ancestral histone is retained after 12 hours chase. In comparison, H3.1 nucleosomes are turned over faster at the active genes ACTB, GAPDH and RPL13A. Surprisingly, the extent of turnover is limited and after 12 hours ~20-30% of nucleosomes are still retained at these genes. Interestingly, the gene desert locus that is not transcribed,
Chapter 3 – H3.1 and H3.3 Turnover

displays a similar kinetics to active genes (Figure 3.1). H3.3 turnover is slightly faster than canonical H3.1 at all loci analyzed (Figure 3.1 A & B). Statistical analysis indicates that H3.3 turnover is significantly faster after 3 and 6 hours chase at genes and after 6 and 12 hours chase at non-genes (Figure 3.3). Interestingly, nucleosome turnover appears to be biphasic in nature at active genes, particularly for H3.3. Only half of the parental H3.3-SNAP population is retained after 3 hours chase at all loci except Sat2 and the gene desert locus. After this initial rapid turnover, the rate of loss of nucleosomes slows down and significant retention is observed up to 12 hours chase at the four genes. This suggests that nucleosome turnover is not homogenous and that qualitatively different populations of nucleosomes exist at active genes (Figure 3.1 A).
Figure 3.1 TimeChIP analysis of H3.1-SNAP and H3.3-SNAP. A) Cells expressing histones H3.1-SNAP and H3.3-SNAP were pulse-biotinylated and chased for indicated times. Chromatin was isolated and fragment followed by biotin-dependent pull down and qPCR analysis for quantification of the following DNA loci: beta actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA binding protein (TBP) and ribosomal protein L13a (RPL13A) genes at indicated time points. B) Analysis as in A but processed for detection of α-satellite I repeat, pericentromeric satellite 2 repeats (Sat2) and a conserved non-coding element (gene desert) at indicated time points. Fraction of biotin retained values are derived from fitting of the %IP measurements of chased samples on a linear regression of a TimeChIP standard curve that was included in each experiment (see Chapter 2). Data shown represents an average of six independent experiments, error
bars indicate standard error of the mean (S.E.M). Statistically significant difference in the comparison is indicated by asterisk (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ (Kruskall-Wallis test).

Since significant levels of ancestral histones could be detected after 12 hours we next tested whether canonical histone H3.1 and H3.3 are retained at the time scale of the cell cycle of HeLa cells. To this end cells were chased for 12, 24 and 48 hours following pulse labeling. In general, we observed that number of cells was maintained from 0h (5x10$^7$ cells) to 24h (6-7x10$^7$cells) and duplicated from 24 to 48 hours (1-1.2x10$^8$ cells), indicating that during the course of the experiment cells underwent at least one cell cycle. We found that ~20% of ancestral H3.1 is retained after 24 hours chase at ACTB, GAPDH, TBP, RPL13A genes and the gene desert locus. After 48 hours levels are further reduced to ~10% but are still detectable above background levels (Figure 3.2). In contrast, canonical H3.1 shows a slower turnover at the $\alpha$-satellite I repeats and the pericentromeric domain, but is eventually also reduced to 10% after 48 hours of chase at Sat2 but not at $\alpha$-satellite I repeats where retention is maintained at ~35% (Figure 3.2 B). Turnover rates of H3.3 at this timescale are similar to H3.1 although H3.3 appears slightly faster at most loci with the exception of TBP (Figure 3.3). Nevertheless, these differences were found not be significant. We observed that 20% of H3.3 is retained after 24 and 48 hours chase at $\alpha$-satellite and pericentromeric satellite 2 (Figure 3.2 B). In sum, these results suggest both H3.1 and H3.3 are retained stronger at the $\alpha$-satellite and pericentromeric satellite 2 in comparison to genes. However, despite these possible trends we were unable to establish whether these differences between loci are significant, given the current experimental variance in the data (Figure 3.3). Both nucleosome types turnover in a biphasic pattern where most nucleosomes are lost in the first 12 hours followed by a much slower turnover at longer time scales (Figure 3.2).
Figure 3.2 TimeChIP analysis of H3.1-SNAP and H3.3-SNAP. A) TimeChIP analysis as described in Figure 3.1 of H3.1-SNAP and H3.3-SNAP cells at ACTB, GAPDH, TBP and RPL13A genes for indicated time points. B) Histones H3.1-SNAP and H3.3-SNAP were biotinylated and chase at α-satellite I repeat, pericentromeric satellite 2 repeats (Sat2) and a conserved non-coding element (gene desert) at indicated time points. Fraction of biotin retained values are derived from fitting of the %IP measurements of chased samples on a linear regression of a TimeChIP standard curve that was included in each experiment (see Chapter 2). Data shown represent average of five independent experiments, error bars indicate standard error of the mean (SEM). Statistically significant difference in the comparison is indicated by asterisk (*) p<0.05, (**) p<0.01 (Kruskall-Wallis test).
Figure 3.3 Comparison of the rate of H3.1 and H3.3-SNAP retention at genes and non-gene loci. Fraction of H3.1-SNAP and H3.3-SNAP retained at genes (top) and non-genes (bottom) at indicated time points. Fraction of biotin retained values are derived from fitting of the %IP measurements of chased samples on a linear regression of a TimeChIP standard curve that was included in each experiment (see Chapter 2). Data shown represent average of six (left) and five independent experiments (right), error bars indicate standard error of the mean (SEM). Statistically significant difference in the comparison is indicated by asterisk (***) p<0.01, (****) p<0.001, no statistically significant difference in the comparison is indicated by ns (Two-way ANOVA, Bonferroni correction)

Local retention of H3.1

The finding that ancestral H3.1 and H3.3 are retained at the different loci raises the question whether such retention occurs locally. Three different scenarios could be considered: 1) histones are locally retained; 2) histones turnover and 3) alternative to turnover, ancestral histones could be turned over in another location, released into the soluble pool and "recycled" by re-incorporation at the locus under
investigation. In this later case, ancestral histones are detected but they are dynamically retained rather than locally (Figure 3.4). There is however no precedent for histones that are released from chromatin into the soluble pool to be reincorporated since chromatin based histones differ in their post translational modification from the soluble histone pool (Groth et al., 2007). Nevertheless, to address this possibility we took advantage of the assembly dynamics of H3.1. Deposition of nascent H3.1 occurs during S phase by the histone chaperone CAF-1 in strictly DNA replication dependent manner (Ahmad and Henikoff, 2002; Ray-Gallet et al., 2011; Shibahara and Stillman, 1999). In contrast, incorporation of newly synthesized H3.3 occurs throughout the cell cycle including S phase but independent of DNA synthesis by the HIRA complex, ATRX and DAXX chaperones (Ahmad and Henikoff, 2002; Drané et al., 2010; Lewis et al., 2010).

Because H3.1 assembly is S phase dependent there will be no deposition of either nascent or ancestral H3.1 outside of S phase. This creates an opportunity to determine whether the retention of ancestral canonical H3.1 is due to local or dynamic retention. We treated cells with Thymidine for 14 hours to prevent DNA replication and incorporation of both labeled new and labeled old H3.1-SNAP while ancestral H3.1 was chased (Figure 3.5 A). The efficiency of the arrest was assessed with BrdU labeling which was included during the duration of the experiment, and showed that 5% of H3.1-SNAP cells thymidine treated do not incorporated BrdU while non-arrested H3.1-SNAP cells incorporated BrdU in 88.4% (Figure 3.5 C). FACS analysis shows that when H3.1-SNAP cells are arrested with Thymidine there is an enrichment of cells in G1 phase and a concurrent loss of G2/M cells in comparison with untreated cells. This indicates that Thymidine arrest does not allow cells to passage through S phase cells as expected (Figure 3.5 D). TimeChIP analysis shows that following Thymidine
arrest levels of ancestral H3.1 are maintained at GAPDH and Sat2, with a small increase at the actin gene (Figure 3.5 B). If the apparent retention of H3.1 at these loci would be the result of dynamic reincorporation of old H3.1-SNAP than one would expect that upon blocking of assembly histone loss would become apparent and no signal would be retained. The fact that H3.1 levels are maintained, or even increases, strongly indicates that H3.1-SNAP histones are retained locally. Although the levels of H3.3 did not change at the different loci when cells were arrested with Thymidine, we cannot determine conclusively whether reassembly of labeled H3.3 has occurred as this may continue during the S phase arrest (Figure 3.5 B). From these results, we conclude that ancestral canonical H3.1 is locally retained at both active and silent loci.

Figure 3.4 Outline of possible modes of H3.1-SNAP retention. A) Local retention. The amount of labeled H3.1-SNAP in a given time (time n, t₀) is equal to the number of the initially labeled H3.1-SNAP pool (t₀). B) Turnover. The number of labeled H3.1-SNAP at time n is lower than in time 0. C) Dynamic retention. The number of labeled H3.1-SNAP at time 0 is equal than at time n, but there is an intermediate stage during the chase where H3.1 is lost after which labeled H3.1-SNAP from a different locus is incorporated.
Figure 3.5 H3.1-SNAP is locally retained. A) Schematic of H3.1-SNAP and H3.3-SNAP pulse-chase and blocking of DNA replication. B) TimeChIP analysis of H3.1-SNAP and H3.3-SNAP at Actin and GAPDH genes and major satellite 2 (Sat2) during normal cell proliferation and during Thymidine arrest for 12 hours. Mean +/- S.E.M. are shown, n=3. No statistically significant difference in the comparison is indicated by ns (paired, t-test p<0.05). C) Thymidine arrested and asynchronous H3.1-SNAP cell populations were pulse labeled with BrdU in the presence or absence of Thymidine to determine efficiency of arrest. BrdU labeling was visualized by immunofluorescence (Cy5). BrdU staining is absent from Thymidine arrested, contrary untreated to cells. Below: Quantification of the number of BrdU+ cells. Number of cells analyzed (n) is indicated. D) FACS analysis of DNA content (Propidium iodide).
content of H3.1-SNAP cells either pulse labeled (pulse), released (12h chase) and released in Thymidine. H3.1-SNAP cells arrested with Thymidine exhibit enrichment in G1 phase and lack a G2/M population indicating an S phase arrest. Green line represents an estimation of the G0/G1, S and G2/M population sizes.

**Genomic distribution and turnover of H3.3**

We have shown that ~10-20% of parental H3.3 is present after 48 hours chase at different loci (Figure 3.2). However, H3.3 is incorporated at virtually all expressed gene loci as well as at enhancer elements (Goldberg et al., 2010; Jin et al., 2009; Ray-Gallet et al., 2011). Therefore, to determine whether our results for the loci we analyzed are indicative for what happens across the genome we carried out TimeChIP-seq. In TimeChIP-seq, H3.3-SNAP cells were *in vivo* pulse labeled with CP-biotin and chased for 12 and 24 hours. Salt-extracted chromatin was fragmented into mononucleosomes by MNase treatment. H3.3-SNAP biotinylated nucleosomes were captured on Streptavidin magnetic beads. 10ng of co-purified DNA from recovered H3.3-SNAP nucleosomes were submitted for generation of sequencing libraries followed by Illumina sequencing at high coverage. TimeChIP allows for the analysis of a limited number of loci while with TimeChIP-seq the coverage is genome wide. However, we cannot estimate the absolute retention rate of H3.3-SNAP with TimeChIP-seq. H3.3 enrichment is measured as the number of sequence reads for each locus normalized against the total number of reads. Due to the wide distribution of H3.3 across the genome, both local read counts as well as global read counts change significantly during the chase period, making it difficult to make an absolute quantitative statement on local histone retention. However, the approach does allow for relative measurements between loci over time. In agreement with our observations based on qPCR, we find that H3.3-SNAP turnover is faster at the most transcribed genes compared
to unexpressed or lowly expressed genes (Figure 3.6 C). Turnover is more rapid near the transcription termination site (TTS) compared to the transcription start site (TSS) (Figure 3.6 B & C). Furthermore, H3.3-SNAP exhibits to be highly enriched at enhancers and displays a turnover rate exceeding that of active genes (Figure 3.6 C). At loci enriched in H3.3 we find that turnover is more rapid in the first 12 hours compared to the 2nd 12 hours indicating a discontinues turnover rate, similar to what we have observed for specific loci by qPCR (Figure 3.2). Interestingly, in this experiment we find that, overtime, ancestral H3.3 accumulates just downstream of the TSS, reminiscent of the distribution of ancestral H3 observed in budding yeast (Figure 3.6 A) (Radman-Livaja et al., 2011).

Figure 3.6 TimeChIP analysis of H3.3-SNAP at the genome-wide level. A) Coverage of H3.3-SNAP at the top 1000 expressed genes at 0 (dark blue), 12 (blue) and 24 hours (light blue). Enrichment of H3.3-SNAP (y axis) was estimated based on the number of reads per total number of reads relative to the number of read per total number of reads in the input. X axis represents distribution of H3.3-SNAP at 5 kb upstream of TSS, across the gene body and
5kb downstream of the TTS of most expressed genes (GEO code GSM958735) (ENCODE Project Consortium, 2011). **B)** H3.3-SNAP turnover for highly expressed genes. The average turnover index of H3.3-SNAP was calculated for regions described for A) for the top 1000 genes according to RNA-seq data from ENCODE. The turnover rate (y axis) for H3.3 was plotted as the ratio of enrichment at 12h relative to 0h (dark blue) and as the ratio of enrichment at 24h relative to 12h (light purple) at top 1000 expressed genes. 1= no change; <1 = histone loss; >1 = histone enrichment. **C)** Relative H3.3-SNAP turnover at transcription start site (TSS) and transcription terminal site (TTS) of top and bottom 1000 expressed genes, as well as enhancers. Distribution is shown by box plots that show median (black bar), 25th and 75th percentile (box) and Min-Max (whiskers) H3.3-SNAP turnover of the first 12 hours (dark blue) and H3.3-SNAP turnover the following 12 hours (12-24 hours) (light blue).

**Differential histone retention within active gene**

Previous reports have indicated that nucleosomes are unstable just upstream of the transcription start site (Jin et al., 2009). To determine whether this correlates with fast histone turnover we sought to quantify the retention of H3.3-SNAP by TimeChIP at the promoter region and gene body of the basally expressed nuclear transcription factor-κβ (NF-κβ). We find H3.3 turnover to be much slower at exon 2 of NF-κβ gene body compared to the transcription start site where only ~20-30% of H3.3 is retained in the first 3 hours of chase with a further reduction to <10% after 12 hours (Figure 3.7). Interestingly, H3.1 is retained longer at the TSS in comparison to H3.3 while H3.1 turnover in the gene body is comparable to that of H3.3 (Figure 3.7). We can conclude that at least at of NF-κβ, nucleosome turnover is faster at the TSS and that H3.1 and H3.3 are impacted differently at the promoter of this gene.
Figure 3.7 Histones turnover within NF-κB gene. TimeChIP experiment as in Figure 3.1 A) Levels of H3.1 and H3.3-SNAP retained at transcription start site of NF-κB gene. B) Levels of H3.1-SNAP and H3.3-SNAP retained at gene body (exon 2) of NF-κB at indicated time points. Mean +/- S.E.M. are shown, n=3. Statistically significant difference in the comparison is indicated by asterisk (* p<0.05, (*** p<0.001 (One way ANOVA Bonferroni correction). C) Schematic of NF-κB gene indicating regions flanked by PCR probes used to quantify H3.1 and H3.3 occupancy at transcription start site (TSS) and gene body (exon 2).

Dynamics of H3.1 and H3.3 during transcription activation

Our assessment of different gene loci by genome wide analysis indicates that nucleosome retention correlates negatively with transcription. i.e. higher transcription rates drive more histone loss. Next, we sought to directly test whether gene activity correlates with higher rates of histone turnover. We used the activation of the nuclear transcription factor-κB (NF-κB) gene by the cytokine TNF-α as a means of conditionally activating transcription. The rapid transcriptional activation of NF-κB by TNF-α is well characterized (Mahoney et al., 2008). We measured the rate of H3.1 and H3.3 retention following gene activation by TNF-α addition. A 3h treatment with TNF-α resulted
in an increase of NF-κβ mRNA by ~2 fold and a 3 fold increase in the transcript levels of the downstream gene cIAP2 (Figure 3.8 C). To confirm that the activation of NF-κβ pathway occurs in the majority of the cells, we determined the localization of NF-κβ by immunofluorescence (Figure 3.8 D). ~94% of cells exhibited NF-κβ translocation to the nucleus upon TNF-α treatment. Interestingly, despite the NF-κβ activation in the vast majority of cells and the concurrent transcriptional activation of this gene, retention of H3.1 did not change during transcription activation of NF-κβ, both at the gene body and transcription start site (TSS) (Figure 3.8 B). H3.3 turns over faster at the TSS of NF-κβ in comparison with canonical histone H3.1, and this rapid rate is not further accelerated by TNF-α. There is a minor but not significant increase in retention of H3.3 in the gene body of NF-κβ following induction (Figure 3.8 B). These results suggest that activation of a gene does not lead to a measurable increase in the turnover of histones H3.1 and H3.3.
Figure 3.8 Histone turnover during transcription activation. A) Schematic of pulse-chase labeling of cells expressing SNAP-tagged histones and gene induction by TNF-α. B) Levels of ancestral H3.1-SNAP and H3.3-SNAP at the transcription start site and exon 2 of the TNF-α induced NF-κβ gene. Schematic representation of NF-κβ gene, indicating regions flanked by PCR probes. No statistically significant difference in the comparison is indicated by ns (paired, t-test p<0.05). C) Quantification of TNF-α mediated induction of NF-κB mRNA and the downstream cIAP2 gene in H3.3-SNAP cells by qRT-PCR. D) Subcellular localization of NF-κB in untreated and TNF-α treated cells. 94% of cell population displayed nuclear staining following TNF-α addition. Results of TimeChIP and levels of mRNA represent average of three independent experiments, error bars indicate standard error of the mean (SEM).
Dynamics of H3.1 and H3.3 during transcription inhibition

Our analysis of histone turnover at an active gene during transcription induction did not reveal a detectable increase in histone loss from chromatin. We next examined whether the turnover of H3.1 and H3.3 can be slowed down by directly interfering with transcription. We treated the cells with DRB and α-Amanitin. DRB prevents activating phosphorylation of the RNA polymerase II C-terminal domain (CTD), which results in repression of transcription elongation (Yankulov et al., 1995). α-Amanitin binds to the largest subunits of RNA polymerase II and III (Cassé et al., 1999). To assess the inhibitory effect of DRB and α-Amanitin on genes transcribed by RNA polymerase II, we selected H3.1L, cyclin A (CCNA), Myc proto-oncogen protein (MYC) and ribosomal protein P0 (RPLP0) genes. H3.1L, CCNA and MYC genes have in common that the half life of mRNA is relatively short (less than 4 hours) due to their cell cycle regulated expression (Lam et al., 2001). RPLP0 has a half life of 8.5 hours (Jb et al., 2009). These half-lives allows us to detect the inhibitory effect of DRB and α-Amanitin by analysis of steady-state mRNA levels. In a pilot experiment to determine optimal drug concentration and incubation times to be included in the TimeChIP experiment we observed that transcript levels decrease with 100µM DRB for 3 hours and 10µg/mL α-Amanitin for 12 hours (Figure 3.9). Following treatment with these inhibitors we measured histone turnover by TimeChIP. The retention of ancestral H3.1 increased ~2-2.5 fold at ACTB, GAPDH, TBP, the gene desert and Sat2, and 5 fold at RPL13A when cells are treated with α-Amanitin for 12 hours (Figure 3.10 B, C). DRB treatment slowed H3.1 turnover at the Sat2 and gene desert loci. At the active RPL13A locus a trend of slow turnover was observed although not significant given the error in measurement (Figure 3.10 B, C). Similarly, slow turnover of H3.3 during transcription inhibition with DRB is observed at TBP, RPL13A and gene desert,
although again not significant from the experimental variance (Figure 3.10 B, C). When transcription is blocked with \( \alpha \)-Amanitin, retention of H3.3 increases 5 fold at GAPDH and at the conserved non-coding gene desert locus, ~2.5-3 fold at ACTB, TBP, Sat2, \( \alpha \)-satellite I repeats and RPL13A (Figure 3.10 B, C). Although increased retention is not statistically significant for some loci, all loci follow the same trend. Because inhibition of RNA polymerase II decreases the turnover of H3.1 and H3.3 at the conserved non-coding element, we postulated that this locus might, in fact, be transcribed and as a result respond to DRB and \( \alpha \)-Amanitin treatment. However, we could not detect any mature transcripts at this locus by qRT-PCR analysis (data not shown). Moreover, RNA-seq analysis of HeLa cells reports no read counts at this locus (GEO code GSM958735)(ENCODE Project Consortium, 2011). Instead, it has been reported to be a lamina associated domain in Tig3 cells and proximal to an enrichment of H3K27Ac mark (ENCODE Project Consortium, 2011).

Figure 3.9 DRB and \( \alpha \)-Amanitin inhibit transcription. Relative mRNA levels of H3.1L, cyclin A (CCNA), Myc proto–oncogen protein (MYC) and ribosomal protein P0 (RPLP0) normalized to an untreated control following treatment with RNA polymerase II inhibitors DRB and \( \alpha \)-Amanitin. Mean +/- S.E.M. are shown, n=3.
Figure 3.10 Histone turnover during transcription inhibition. A) Schematic of H3.1-SNAP and H3.3-SNAP pulse-chase experiments in combination with transcription inhibitors. B) TimeChIP analysis of H3.1-SNAP and H3.3-SNAP at ACTB, GAPDH, TBP and RPL13A genes after 100µM DRB and 10µg/mL α-Amanitin treatment for 3 hours and 12 hours, respectively. Untreated samples were analyzed for the same time frame. C) TimeChIP analysis of H3.1-SNAP and H3.3-SNAP at α-satellite I repeat, pericentromeric satellite 2 repeats (Sat2) and a conserved non-coding element (gene desert). Mean +/- S.E.M. are shown, n=3. Statistically significant difference in the comparison is indicated by asterisk (*) (paired t-test p<0.05).
The retention of H3.1 and H3.3 increases in most of the loci analyzed when RNA polymerase II transcription is inhibited with DRB and α-Amanitin, including surprisingly, a non-transcribed locus. To examine whether this effect extends to other loci that are not transcribed by RNA polymerase II, we measured the retention of H3.1 and H3.3 at the 5SrDNA locus, which is transcribed by RNA polymerase I and insensitive to α-Amanitin and DRB (Price and Penman, 1972; Yankulov et al., 1995) (Figure 3.11 B). Despite the prediction that RNA polymerase II does not impact transcription of rDNA loci we found that retention of ancestral H3.1 and H3.3 increases by two fold after α-Amanitin treatment, and ~1.5 fold when cells are treated with DRB (Figure 3.11 A).

**Figure 3.11 Histone turnover at 5SrDNA locus.** A) TimeChIP analysis of H3.1-SNAP and H3.3-SNAP at 5SrDNA during treatment with 100µM DRB and 10µg/mL α-Amanitin. B) qRT-PCR results show that levels of 5SrDNA do not decrease by treatment with 10µg/mL α-Amanitin and 100µM DRB. Mean +/- S.E.M. are shown, n=3. Statistically significant difference in the comparison is indicated by asterisk (*) (paired t-test p<0.05).
DISCUSSION

In this chapter we applied our newly developed Time-ChIP methodology to the analysis of histone retention in mitotically dividing HeLa cells in culture. We find that while histones are turned over, a significant fraction of canonical H3.1 is retained across the cell cycle at non-genes and genes loci. Importantly, we show that retention of H3.1 at different loci is maintained in the absence of DNA synthesis during which no deposition of H3.1 occurs. This shows for the first time that H3.1 is retained locally at active loci in mammalian cells. H3.1 is generally more stable at centromeric and pericentromeric repeats in comparison to active genes. It is not clear from our results whether this stability is as high as has been observed for CENP-A at centromeres, although CENP-A appears to be more strongly retained at early time points (Chapter 2, Figure 2.7). Consistently, quantification of histone turnover at centromeres in a cell by cell analysis at optical resolution, indicates that H3.1 and H3.3 turn over faster than CENP-A even when present at the centromere locus (Bodor et al., 2013). Ectopic targeting of CENP-A is sufficient to lead to self-replication of centromeric chromatin and heritable kinetochores formation for several cell divisions (Mendiburo et al., 2011). This indicates that CENP-A has the capacity to induce and sustain a self-maintaining feedback mechanism. The existence of such a mechanism of propagation for H3.1 which is distributed throughout chromatin is unlikely.

We find that a proportion of H3.1 is locally retained even at active genes. Early in vivo experiments in budding yeast have shown that nucleosomes are quickly and efficiently positioned on nascent chains after the passage of the replication fork (Lucchini et al., 2001). Recently, a quantitative model based on measurements of ancestral H3 in budding yeast considering the total histone turnover, lateral movement of histones and dissociation/re-association during
replication; indicates that ancestral histone are reincorporated within 400bp of the original site (Radman-Livaja et al., 2011). We now provide direct evidence for such local retention of nucleosomes also during transcription in human somatic cells. We find that during Thymidine arrest, during which histone H3.1 cannot be assembled, the previously incorporated pool is retained at non genes and genes. Similarly, we see that H3.3 levels are unaffected by Thymidine addition. However, since H3.3 assembly is not cell cycle restricted, we cannot conclude whether the retention is local or whether dynamic reassembly occurs.

We see that both H3.1 and H3.3 turnover in a biphasic manner. Particularly, we find that retention of ancestral H3.3 at active genes decreases by half after the first 3 hours of chase while the levels drop at a slower pace after 6 and 12 hours. Like H3.3, H2A.Z is deposited into chromatin throughout the cell cycle. Unlike H3.3, H2A.Z is enriched at promoters and sites of DNA double strand breaks (Kalocsay et al., 2009). Nucleosomes carrying both the H3.3 and H2A.Z variants have been shown to be highly enriched at transcription start sites (TSS) and CTCF binding sites. In addition, such double variant nucleosomes are highly unstable as inferred by their low salt stability (Jin 2009). In contrast, Nekrasov et al., suggest that the instability at CTCF binding site is given by the heterotypic histones H2A.Z-H2A nucleosome (Nekrasov et al., 2012). High-resolution maps of homotypic and heterotypic Drosophila H2A.Z nucleosomes show that homotypic nucleosomes are enriched downstream of active promoters and intron-exon junctions, while heterotypic nucleosomes are depleted from these regions (Weber et al., 2010). The enrichment of H2A.Z at active genes may provide an explanation for the rapid initial loss of ~50% of H3.3 at genes during the first 3 hours chase. Possibly the ancestral H3.3 that is associated with H2A.Z is lost rapidly
while the pool that is complexed with canonical H2A is more stably retained.

We show that ancestral H3.3 turnover is fast at promoters while is stronger retention is observed at active gene bodies and non-gene loci. In contrast, a previous study in Drosophila cells indicates that H3 has a turnover rate of ~1-1.5 hours at active genes bodies, promoters and regulatory elements suggesting a rapid nucleosome turnover in manner dependent on gene expression (Deal et al., 2010). However, the approach used by Deal et al., where histones are metabolically labeled, measures the turnover rate exclusively of recently assembled newly synthesized histones. A possible explanation to reconcile our results with the Deal et al study is that there are qualitatively different populations of nucleosomes with different turnover rates, a dynamic population that is replaced several times during one cell cycle and a second population that is retained at longer timescales part of which can be retained throughout across the cell cycle. While we have obtained evidence that H3.1 is maintained locally in chromatin, we cannot determine whether H3.3 is retained locally despite the fact that some retention of parental H3.3 is observed after a 48 hours chase at active genes. Deposition of H3.3 is facilitated by HIRA, ATRX and DAXX throughout the cell cycle; which make it difficult to determine whether retention is local or whether ancestral pools of H3.3 are dynamically reassembled overtime (Drané et al., 2010; Goldberg et al., 2010; Lewis et al., 2010; Ray-Gallet et al., 2002). In agreement with previous studies, genome-wide analysis shows that H3.3 is enrichment at highly transcribed genes, promoters and enhancers (Goldberg et al., 2010). Interestingly, we see that turnover rates are different for these different loci with enhancers sporting the highest H3.3 dynamics. Moreover, we found that similar to dynamics of H3 in budding yeast (Radman-Livaja et al., 2011), H3.3 nucleosomes accumulate toward
the 5’ end of genes over time, although this result is preliminary at this stage. An explanation could be that nucleosomes slide during disassembly/reassembly process while RNA polymerase II acts in conjunction with chromatin remodeling (Fazzio and Tsukiyama, 2003; Radman-Livaja et al., 2011).

Nucleosomes form barriers for transcription. By inducing transcription of the stress response gene NF-κB, we found that, contrary to our expectation, turnover of H3.1 and H3.3 was not further increased significantly. There are two alternatives that histones have during transcription, either histones are evicted or transferred behind the polymerase. *In vitro* studies with phage SP6 RNA polymerase and yeast RNA polymerase III proposed the formation of an intranucleosomal DNA loop that leads to an intermediate pause of the RNA polymerase while histones are transferred to the same DNA molecule (Bintu et al., 2011; Felsenfeld et al., 2000; Studitsky et al., 1994, 1997). An increase of transcription activity could lead to an increase of the exposure time of accessible nucleosomal DNA which implies that histones will be prone to eviction during elevated transcription. Possibly in our human cell experiments nucleosome retention at active genes is achieved through similar intranucleosomal DNA transfer mechanisms. An increase in transcription may be achieved either by a faster transcription rate or by a higher density of RNA polymerases. In the latter case the dynamics of transitional intranucleosomal DNA looping during elongation of RNA polymerase may not need to change, thereby maintaining a similar degree of nucleosome turnover as we observe at the NF-κB gene upon activation by TNF-α.

Jin et al., have proposed that an initial group of RNA polymerases during transcription elongation, acting as a pioneer, will evict histones to facilitate transcription by the trailing RNA polymerases
(Jin et al., 2010; Orphanides and Reinberg, 2000). This may be a mechanism that gives rise to differential nucleosome stabilities. Nucleosome retention during transcription requires the participation of histone chaperones and chromatin remodelers. Evidence comes from Jamai et al., who show that inactivation of the nucleosome assembly factor Spt16 leads to loss of H2B and H3 on active and inactive genes. Histone loss is rescued when transcription is inhibited, indicating that Spt16 contributes in the reassembly of histones during transcription (Jamai et al., 2009). A similar role has been proposed for the FACT complex that facilitates chromatin transcription by RNA polymerase II, by reassembly of H2A-H2B dimers after enzyme passage. However, in this study FACT did not impact on H3-H4 tetramers (Belotserkovskaya et al., 2003). In future efforts it will be of interest to determine the role of these histone chaperones in the retention of nucleosomes during transcription.

We find that histone turnover decreases significantly at all loci tested when transcription by RNA polymerase II is inhibited. This is contrary to our expectation of reduced histone loss only at sites of transcription by RNA polymerase II. Panse et al., have described that transcription of 45S RNAs is maintained even if RNA polymerase II transcription is arrested by DRB (Panse et al., 1999). In agreement, we see that 5SrDNA is transcribed under DRB and α-Amanitin arrest but histone turnover is nevertheless significantly diminished.

At this point we have no adequate explanation of this phenomenon. Possible models include: 1) all genomic sequences are transcribed at some rate by RNA polymerase II leading to Pol II driven nucleosome turnover, irrespective of transcription rate (if so, this would mean that transcription occurs below the detection limit of our mRNA measurements). 2) The chromatin response is systemic in nature rather than local. Either the drug treatments induce a global stress...
response that leads to an overall reduction in nucleosome turnover or, more interestingly, transcription inhibition leads to changes in local chromatin domains. In light of this, it has been proposed that the transcriptional program of a cell may be dependent of the spatial organization of the genome. Genes that are actively transcribed share transcription factories, described as sub-nuclear foci with high local concentrations of RNA polymerase II that are sensitive to α-Amanitin. These clusters are specialized in particular transcriptional pathways and driven by discrete transcription factors (Bickmore and van Steensel, 2013; Sexton et al., 2007). Moreover, 3D analysis of chromatin domains identified two structures: ridges and antiridges; that differ in transcriptional activity, the presence of specific proteins and noncoding RNAs and have a distinct chromatin folding. Although evidence for the molecular mechanisms is lacking, it seems that specific sub-nuclear microenvironments are formed that promote high transcription rates (ridges) or allow only low to moderate transcriptional activities (antidridges) (Goetze et al., 2007). One speculative explanation for our results could be that transcription inhibition leads to histone retention in a genomic region much broader than individual genes. This may involve changes in histone modifications, compaction of chromatin, and formation of clusters that drive changes in nuclear organization and dynamics. Hypothetically, the global inhibition of transcription could stimulate the mobilization of chromatin to the nuclear lamina after chromatin has been disengaged of transcription factories. The results of histone turnover during transcription inhibition are intriguing. Further experiments in the context of genome architecture are needed.

In conclusion, our results show that a population of parental H3.1 and H3.3 histones is stably retained at the timescale of the cell
cycle. Consequently such subpopulation could be responsible to carry PTM contributing to epigenetic maintenance of gene expression.
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Chapter 4 – General Discussion
The inheritance of cellular gene expression profiles and associated phenotypes is critical for the generation and stability of different cell types. This epigenetic inheritance is carried by non-DNA sequence based factors across mitotic divisions. Although several factors have been identified that play a role in this process, their mechanism to provide memory is in many cases unknown. We have proposed that mechanisms of epigenetic inheritance should, in the most parsimonious view, fulfill with the following aspects: stability, self-templated duplication and cell cycle coupling (Gómez-Rodríguez and Jansen, 2013). Several epigenetic markers are chromatin based, implicating nucleosomes, that are central to chromatin, as important players in epigenetic inheritance but the mechanistic basis is not clear. Histone H3 variants have been involved in the transmission of active and repressive gene states (Bannister et al., 2001; Hathaway et al., 2012; Lachner et al., 2001; Ng and Gurdon, 2008) which raises an important question: Are histone H3 variants inherited across mitotic cell divisions? The work presented here provides evidence to answer this question.

In chapter 2, we introduced TimeChIP, an assay that allows capturing histone dynamics (Figure 2.3) by covalent pulse labeling of uniquely tagged histone variants. The main advantages of TimeChIP are the distinction of old (ancestral) and new pools of histones, the ability to determine dynamics at a timescale of hours to days, and the high spatial resolution. Importantly, this allows for the determination of histone turnover at specific loci. In chapter 3, we show for the first time that a population of ancestral H3.1 and H3.3 histones is stably retained across the cell cycle in a human cell line. In this chapter, I will discuss several hypotheses and predictions that follow from the results presented in this work.
Can qualitative differences between nucleosomes result in differential stability?

Our initial results in chapter 2 show that a subpopulation of CENP-A assembles outside centromeres that displays a faster turnover in comparison to centromeric pools (Figure 2.7 & 2.9). One explanation for differential turnover of CENP-A at different loci may be differential rates of transcription rates. rDNA loci are transcribed at a much higher rate compared to centromeric loci.

Another hypothesis is that CENP-A nucleosomes are intrinsically different between different loci. Purification of CENP-A nucleosomes from human cells indicated that CENP-A containing nucleosomes are enriched in the histone H2A.Z variant (Foltz 2006). H2A.Z is involved in transcription regulation, DNA repair, heterochromatin formation, chromosome segregation and mitosis, but it does not exhibit a uniform localization within the genome (Guillemette and Gaudreau, 2006; Kalocsay et al., 2009; Meneghini et al., 2003; Zlatanova et al., 2009) A particular feature of H2A.Z is that it is broadly but non-uniformly distributed throughout the chromosomes and can be actively and selectively removed at specific loci (Hardy and Robert, 2010). Bönisch et al., suggest that H2A.Z localization is due to difference of the stability between homo and heterotypic H2A.Z nucleosomes (Bönisch and Hake, 2012).

Based on these findings, a hypothesis that could explain the incorporation of unstable CENP-A nucleosomes outside the centromere locus is the formation of CENP-A/H2A.Z double variant nucleosomes that may be unstable in a manner analogous to the previously reported destabilization of H3.3/H2A.Z double variant nucleosomes (Jin et al., 2009). As an alternative, Foltz et al, also found CENP-A/H3 hybrid nucleosomes. Possibly, these heterotypic
nucleosomes provide a different stability. However it is not known whether the CENP-A/H2A.Z or CENP-A/H3 hybrids represent the non centromeric pool. To explore these hypotheses a TimeChIP experiment could be carried out to isolate CENP-A (chapter 2) followed by a reChIP for H2A.Z and/or H3. This will determine where such hybrid nucleosomes are located and whether they correlate with high CENP-A turnover, e.g. at the 5SrDNA loci.

In addition to CENP-A, our results indicate that H3.1 and H3.3 also turnover at different rates. However, in this case differential turnover occurs at the same locus as we observe turnover in a biphasic manner (Figure 3.1 & 3.3). As discussed above, nucleosomes carrying both H3.3 and H2A.Z variants have been shown to be highly unstable (Jin et al., 2009). The crystal structure of H2A.Z nucleosomes indicates a subtle destabilization of the interaction interface between the H2A.Z docking domain and H3 (Suto et al., 2000).

One possible explanation of nucleosomes with different stabilities at specific loci (e.g. at promoter versus the gene body) may be the presence or absence of the H2A.Z variant histone. To determine this I would propose to conduct a H2A-SNAP and H2A.Z-SNAP TimeChIP Pulse-Chase experiment. If rapid nucleosome turnover is a consequence of H2A.Z presence we would expect H2A.Z turnover to be fast and largely completed during the first 3 hours. In contrast H2A-SNAP would be expected to turnover at a slower but constant rate reflecting the slow phase we observe in our experiments in Figures 3.1 and 3.3. In addition, one could directly determine the presence of H2A.Z or H2A in young (Pulsed) versus old (chased) SNAP-tagged H3.1 and H3.3 by a re-ChIP experiment of H3.1/H3.3-SNAP followed by H2A.Z ChIP or by mass spectrometry in young (Pulse) versus old (chased) SNAP-tagged H3.1 and H3.3.
Transcription drives histone turnover

During transcription elongation RNA polymerase II must overcome nucleosome barriers. Consequently, displacement of nucleosomes is expected to occur to some extend during transcription. Surprisingly however, upon induction of transcription the turnover of H3.1 and H3.3 did not increase (Figure 3.7). Histone chaperones and chromatin remodelers are involved in the disassembly/reassembly of nucleosomes during transcription. FACT is proposed to be involved in exchanging histones during transcription elongation, specifically H2A/H2B dimers (Belotserkovskaya et al., 2003). In addition, deposition of H3.3 by FACT has been reported in Drosophila (Nakayama et al., 2007). Highly transcribed loci show an enrichment of FACT and H3.3 indicating a high degree of histone turnover (Aida et al., 2013).

How can we reconcile transcription dependent histone replacement with the stable nucleosome retention we observe for a subset of nucleosomes upon transcription induction? As outlined above, one model would involve different retention rates of histones depending on intrinsic nucleosome differences. Possibly, chromatin remodelers turn over selected dynamic nucleosomes leaving intact a more stably subset. However, while chromatin remodelers have a clear role in histone dynamics during transcription elongation, it is unknown whether they selectively discriminate between ancestral and newly synthesized histones to be deposited. Using a conditional reporter to induce or repress transcription in cells expressing H3.3-SNAP combined with RNAi, one might be able to determine the contribution of specific chaperones to the retention of recently assembled nucleosomes versus older ones. This may establish whether, for instance, FACT plays a role in disassembly/reassembly of ancestral histones to support maintenance of epigenetic information.
Importantly, the same conditional shut off reporter system in combination with TimeChIP, will allow to test our prediction that histone turnover diminishes when transcription does not occur. Instead of using global inhibitors of transcription (as discussed below) such an experiment would allow for a more subtle manipulation of local transcription.

Our experiment thus far using small molecule inhibitors of transcription have produced puzzling results. While a slowing down of histone turnover can be observed upon transcription inhibition, this increase in retention appears to be global and not correlated with sites of transcription (Figure 3.9 & 3.10). One possibility explanation would be that these inhibitors of RNA polymerase II induce a non-specific cellular stress that indirectly affect chromatin dynamics.

Alternatively, the inhibition of transcription by DRB and α-Amanitin leads to the migration of large scale chromatin loops distal of transcription factories. Chromatin hub structures are formed prior to entering a transcription factory (Mitchell and Fraser, 2008). Likely, those chromatin hubs are compacted or migrate to the nuclear lamina upon transcription inhibition as has been proposed to occur as a mechanism of gene regulation during development (Peric-Hupkes et al., 2010). This in turn may lead to a change in nucleosome dynamics even at sites distal to active genes. An approach to define whether transcription inhibition leads to high retention of histones by formation of chromatin hubs will be to obtain high coverage TimeChIP-seq data to determine whether changes in histone dynamics upon transcription inhibition occur in large genome segments. Another, perhaps more challenging approach would be to combine the use of 3C (chromosome conformation capture) technology (de Wit and de Laat, 2012) in parallel with TimeChIP to dissect this relationship.
Chapter 4 – General Discussion

Figure 4.1 Model for differential turnover of histones at active genes. We propose that during transcription, a population of histones (either H3.1 or H3.3) is locally retained (green) whereas a proportion of histones is lost and replaced by nascent histones that are selectively turned over at higher rates (grey)

The role of histones as epigenetic markers

How does histone turnover affect maintenance of histone modifications? It has been proposed that histone modification patterns of newly synthesized histones are loaded based on the template of histone modifications present on the neighboring parental nucleosome (Annunziato, 2005; Bannister et al., 2001; Felsenfeld and Groudine, 2003; Lachner et al., 2001). This proposal is based, in part, on the studies that have shown global stability of nucleosomes e.g. during DNA replication (Ishimi et al., 1991; Jackson, 1990; Katan-Khaykovich and Struhl, 2011; Xu et al., 2010). Now, we are providing evidence for stable retention of a population H3.1 and H3.3 at specific loci
throughout a cell cycle, and at least in the case of H3.1, the retention is local. In support of histone retention during DNA replication that we described, Groth et al. have shown that the histone chaperone Asf1 in association with MCM2-7 helicase, through the Asf1-(H3-H4)-MCM intermediate, functions as a histone acceptor and donor of both old and new nucleosomes to assemble on daughter DNA strands. They proposed that the Asf1-(H3-H4)-MCM complex transfers parental nucleosomes. Part of this argument is based on the fact that when DNA synthesis is stalled, histone modifications H4K16Ac and H3K9me3, that are not found on nascent histones, become enriched in the complex, suggesting that during normal progression of the DNA replication fork these parental nucleosomes transiently associate with this complex (Groth et al., 2007). Recently, artificial induction of H3K9me3 in vivo has been achieved with the so-called chromatin in vivo assay CiA that involves local tethering of HP1 (Hathaway et al., 2012). Using CiA Hathaway and colleges, have measured the kinetics and maintenance of H3K9me3 through cell division following transient HP1 tethering. They suggest that maintenance of H3K9me3 occurs by symmetric propagation, where a neighboring nucleosome is marked on average every 5-7 hours depending on the cell type and propose that the inheritance of noncentromeric H3K9me3 domains involves a combination of nucleation, local propagation and histone turnover (Hathaway et al., 2012; Hodges and Crabtree, 2012). Similarly, earlier work has proposed the transmission of H3K27me3 using a heterologous reporter system (Hansen et al., 2008). In a cell line expressing GAL4-EED under the control of a tetracycline regulated promoter, EED-mediated inhibition of transcription was induced, leading to the formation and recruitment of the PRC2 (EED-EZH2-SUZ12) complex to the promoter. Upon removal of tetracycline, propagation of H3K27me3 at the TSS and downstream sequences as well as endogenous PRC2 was detected up to four cell divisions.
(Hansen et al., 2008). While transmission of the silent state was observed these studies do not proof that maintenance of the chromatin state is mediated solely through histone stability and methyltransferase activities. Nevertheless, these relatively slow propagation rates are consistent with the multi-hour retention we observe making it possible that slow turnover and slow propagation of marks may be sufficient to heritably maintain the epigenetic state mediated by the mark.

Recent analysis of nucleosome dynamics questioned the role of histones as players to transmit epigenetic information, because half-lives of histones at active genes were measured to be \(~1\text{-}1.5\) hours, (Deal et al., 2010). We describe for the first time a sub-population of H3.1 that is locally retained for many hours at active genes. An intriguing question, to be addressed in future efforts, will be whether the fraction of H3.1 retained carries ancestral histone modifications. Does the fraction of stable H3.1 correspond to the nucleation factor for the subsequent propagation of histone modifications like H3K9me3 and H3K27me3? One approach to define whether parental histones preferentially carry specific histone modifications will be to quantify parental histones by TimeChIP followed by the measurement of histone modifications with stable isotope labeling by amino acid in cell culture (SILAC). Additionally, site specific analysis would allow determining whether histones turn over more slowly at a locus targeted for silencing of gene expression. Using a conditional shut-off reporter e.g. the one developed by Hansen et al., (Hansen et al., 2008) one could determine the enrichment of old histones at the silenced locus followed by re-ChIP for H3K27me3 to determine whether this modification is enriched in old (chased) SNAP-tagged histones H3.1 or H3.3.

In closing, in the future it will be important to integrate histone dynamics (retention/replenishment) with the direct transmission of
histone modifications during transcription and silencing. Understanding the relative contributions of turnover and propagation of both histones and their modification is key to establish the contribution of histone modifications to epigenetic memory. Finally, according to Waddington’s epigenetic landscape, repressed markers are more stable than active ones (Ferrell, 2012). In light of this, one may have to consider how a histone modification that accompanies transcription is propagated and turned over (Clayton et al., 2006). An example is acetylation, where instead of a stable steady state, an equilibrium of acetylation/deacetylation cycles define the chromatin state (Verzijlbergen et al., 2011). This represents a challenge that requires the development of techniques with high temporal and spatial resolution to obtain a more complete understanding of inheritance of active chromatin states.
Chapter 4 – General Discussion

References


