Investigating epigenome dynamics the RITE way
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INTRODUCTION AND GENERAL DISCUSSION
INTRODUCTION

The DNA in the eukaryotic cell needs to be packaged to fit in the limited dimensions of the nucleus in an organized manner. The packaged form of DNA is referred to as chromatin. The first level of DNA packaging is the nucleosome, which consists of 146 bp of DNA wrapped around an octamer of histone proteins. There are four canonical histone proteins: H2A, H2B, H3, and H4. In the cell, the arrays of polynucleosomes are further organized into higher orders of packaging.

Chromatin is a very dynamic structure. Histones can be post-translationally modified, histone variants can replace the canonical histones, and histones can be exchanged. These dynamic changes can influence chromatin structure and function. Changes in chromatin structure are called epigenetic changes: they can lead to stable changes in gene expression and thereby stable changes in the phenotype of a cell without a mutation in the DNA sequence. In order for epigenetic memory to work in replicating cells, it has been suggested that the pattern of post-translational modifications (PTMs) is quickly re-established after DNA replication to maintain cell identity. However, so far, evidence for epigenetic memory based on histone modifications is still sparse. The recent observations that histones are quite dynamic also challenge some of the models of epigenetic memory. Histone exchange, the replacement of histones by the same type of histone, provides the cell with a means to renew the chromatin. When cells are growing and dividing, DNA replication and cell division result in a dilution of existing histones with new (mostly) unmodified ones (reviewed in (Alabert and Groth, 2012; Annunziato and Papers, 2005)). When cells are not dividing, replication-independent histone exchange enables replacement of histones (Dion et al., 2007; Jamai et al., 2007; Ray-Gallet et al., 2011; Skene and Henikoff, 2013; Verzijlbergen et al., 2010). The cell could use replication-independent exchange to replace damaged histones, or to erase PTMs in a specific region of the DNA, thereby providing epigenome plasticity. By altering the structure of chromatin by histone exchange, transcription factor binding sites in promoters can be exposed or occluded. Nucleosomes and transcription factors might dynamically compete for DNA binding. This opens up the possibility that histone exchange influences the transcriptional output of a transcription factor binding event (Lickwar et al., 2012). Suppression of histone exchange in coding regions might be a mechanism to suppress transcription from cryptic promoters (Venkatesh et al., 2012). The histone exchange observed in promoters and boundary regions could serve as a mechanism to prevent spreading of chromatin types (Dion et al., 2007).

METHODS TO MEASURE HISTONE EXCHANGE

To measure histone exchange, different strategies have been developed. Several studies make use of an ectopic expression assay where an ectopic copy of the gene of interest is introduced in the cell (Cheng and Gartenberg, 2000; Jamai et al., 2007;
Linger and Tyler, 2006; Rufiange et al., 2007; Schermer et al., 2005). The ectopic copy is tagged and under control of an inducible promoter. Upon induction of the ectopic gene, new proteins (that are tagged) can be followed. A disadvantage of this method is that normal gene regulation is perturbed and that genes may be overexpressed. Another method that has recently been developed is Covalent Attachment of Tags to Capture Histones and Identify Turnover (CATCH-IT) (Deal et al., 2010). This method uses a methionine analog that can bind biotin. After switching to media containing the methionine analog, all new proteins will be labeled. A disadvantage of this method is that specificity is obtained by stringent washes that remove all proteins bound to the DNA, except histones H3 and H4. This restricts the application to these core histone proteins. A method where both old and new proteins can be tracked simultaneously is Recombination-Induced Tag Exchange (RITE) (Chapter 3). This method makes use of a DNA cassette that is incorporated behind the gene of interest in the DNA. The cassette contains two epitope tags and two LoxP recombination sites, the first tag is located between the LoxP sites, the second tag is located downstream of the second LoxP site. When the whole cassette is present, synthesized proteins will contain the first tag. A tag-switch is performed when Cre recombinase is induced. After this the newly synthesized proteins will contain the second tag. A major advantage of RITE is that the gene of interest is expressed from its endogenous location, under control of its endogenous promoter. There are no effects of overexpression caused by ectopic extra copies of the gene. There is no need for media swaps; to induce Cre recombinase only an otherwise inert hormone needs to be added to the growth media. However, because of the induction kinetics of Cre recombinase, the assay is less suitable for proteins that have a very high exchange level, as the tag-switch takes several hours. Another limitation is that current versions of the RITE assay are only suitable for C-terminal tagging of proteins. We occasionally observe that C-terminal tagging can affect protein function, but this can be different for different tags (Chapter 3). A C-terminal tag could also have an effect on mRNA or protein stability, resulting in less protein in the cell. The RITE cassette has an ADH1 terminator behind the first tag, but the second tag is followed by the endogenous terminator. The different terminators could have a different effect on the protein. To prevent such differences, specialized RITE cassettes can be developed in which the terminator before the switch is the same as the terminator after the switch. We have already developed RITE cassettes that have ADH1 terminators after both tags. However, to disrupt the endogenous gene expression as little as possible, it may be preferable to make gene-specific cassettes with endogenous terminators.

**H2A.Z EXCHANGE**

The incorporation of histone variants may serve a similar role as PTMs but can also influence the stability of the nucleosome and thereby promote histone exchange.
H2A.Z, a major variant of H2A, has been shown structurally to destabilize the nucleosome in vitro (Suto et al., 2000). In vivo, H2A.Z is located in regions of the yeast genome where exchange of H3 is high (Jamai et al., 2007; Verzijlbergen et al., 2010). The SWR1 complex incorporates H2A.Z into the nucleosome by replacing H2A. It has recently been shown that the SWR1 complex can also perform this action in reverse, replacing H2A.Z in the nucleosome by H2A (Watanabe et al., 2013). The specificity of the SWR1 complex was shown to depend on acetylation of lysine 56 on histone H3 (H3K56ac). H3K56ac, a hallmark of newly incorporated H3 and positively correlated with H3 exchange, overlaps with H2A.Z localization (Kaplan et al., 2008; Rufiange et al., 2007). Interestingly, H3K56ac stimulates the SWR1 complex to replace H2A.Z in the nucleosome by H2A, whereas H3K56 that is not acetylated stimulates the SWR1 complex to replace H2A by H2A.Z (Watanabe et al., 2013). Watanabe et al. propose that this will lead to multiple rounds of H2A.Z/H2A replacement; first unacetylated H3 allows incorporation of H2A.Z, which in turn destabilizes the nucleosome making it easier to exchange additional H3 proteins. Newly incorporated H3 is H3K56ac, which stimulates the SWR1 complex to incorporate H2A. A SWR1-related complex that is involved in the eviction of H2A.Z from the chromatin is the INO80 complex. This complex can replace H2A.Z in the nucleosome by H2A (Papamichos-Chronakis et al., 2011). H2A.Z can be acetylated on its N-terminal tail by the NuA4 and SAGA complexes in yeast (Babiarz et al., 2006; Ishibashi et al., 2009; Keogh et al., 2006; Mehta et al., 2010; Millar et al., 2006). This acetylation occurs mostly on promoter-located H2A.Z. It has been shown that the INO80 complex is mostly found in coding regions and it preferentially removes unacetylated H2A.Z (Papamichos-Chronakis et al., 2011). All these findings suggest that H2A.Z is very dynamic. To test this idea, we compared global replication-independent exchange of H2A.Z and H3 by immunoblot in Chapter 4. Surprisingly, our results show a global replication-independent exchange of H2A.Z that is similar as, or even lower than H3 exchange. Our result is in contrast with studies that have shown that H2B exchange is higher than H3 exchange (Jamai et al., 2007; Kimura and Cook, 2001). It is possible that H2A.Z is more stable than H2B. However, if we assume that H3 cannot be exchanged without the removal of the H2A.Z-H2B dimers, based on the fact that H2A-H2B (or H2A.Z-H2B) are removed from the DNA before H3-H4 under increasing salt concentrations (Park and Luger, 2006) and by virtue of their assembly pathways (Chapter 1), we do not expect that H2A.Z remains on the chromatin when H3 is evicted. Therefore, our results might suggest that H2A.Z is being recycled and reassembled after a transient eviction. Histones that are evicted and recycled would go undetected with RITE and most other assays that measure histone dynamics. Although recycling of a histone protein may not affect the epigenetic code if it is reassembled at the position from which it originated, (which may not always be the case) dynamic behavior of histones is likely to influence the accessibility of the underlying DNA or
the functional consequences of a transcription factor binding event, as has been shown for Rap1 (Lickwar et al., 2012). Therefore, it will be important to develop methods to detect and measure histone exchange as well as histone recycling. To be able to detect recycling, the histones from different genomic locations could be tagged with different tags, to investigate if histones from location A go to location B. However, if recycled histones stay close to their previous location, this approach might not have enough resolution. To gain resolution, a histone would need to be first labeled as ‘old’, and second, it would have to be labeled with a different label when it is evicted from the chromatin. When histones labeled ‘old’ and ‘evicted’ are found bound to the DNA, it would mean there is recycling.

To investigate H2A.Z recycling, we combined RITE with ectopic overexpression of H2A.Z. If H2A.Z is stably bound to DNA, the resident (old) H2A.Z would not be replaced by the extra new H2A.Z, whereas if H2A.Z is going in and out of the chromatin, the extra H2A.Z could compete for assembly and replace at least part of the old H2A.Z. The ectopic copy of H2A.Z was under control of the inducible GAL1 promoter and contained the same tag as the ‘new’ tag in the RITE assay. After the tag switch, the ectopic copy was induced by releasing the cells in media containing galactose. Our results show that new H2A.Z is incorporated into chromatin to a higher level than cells that do not have H2A.Z overexpression. However, even when H2A.Z new levels were higher in chromatin, not all old H2A.Z disappeared. This suggests that there was more de novo incorporation of H2A.Z, but not all old H2A.Z was competed out of the chromatin. This also suggests that chromatin opens and closes, because H2A.Z was incorporated.

In general, results on histone exchange should be interpreted with caution. For example, previous studies using inducible promoter strategies to label old and new histones in yeast have observed that there is more exchange in promoter regions than coding regions of the DNA. In these studies it cannot be excluded that this is caused by histone recycling in coding regions. The fact that less exchange is observed in coding regions does not mean that the chromatin is less disrupted. Evicted histones in transcribed regions could stay in close proximity of the disrupted chromatin, if they bind to chaperones or complexes that are bound to the DNA or transcription or replication machineries (Chapter 1). It has been shown that chromatin needs to be re-established quickly in the wake of RNA polymerase to prevent undesirable events like transcription from cryptic promoters (Venkatesh et al., 2012). Indeed, H3K36me (histone H3 lysine 36 methylation) has been implicated in suppressing histone exchange (Venkatesh et al., 2012). However, H3K36me might not suppress histone exchange by preventing the exchange itself, but it may act by promoting recycling of histones. Recycling might also be a consequence of suppression of incorporation of newly synthesized histones. Histone chaperones that are implicated in suppressing histone exchange might be involved in recycling mechanisms. Some chaperones have been found associated with histones harboring chromatin-PTMs.
The HIR complex in yeast has been suggested to reassemble H3 in cis (Kim et al., 2007), and the histone chaperone Asf1 has been shown to bind to H3-H4 dimers harboring chromatin-associated PTMs during DNA replication (Groth et al., 2007). Determining whether other chaperones and deposition complexes also associate with histones derived from chromatin might reveal if recycling could be a more widespread mechanism of the cell. We have investigated two H2A.Z chaperones, Nap1 and Chz1, but have found no effect on global H2A.Z exchange. This suggests that there are redundant mechanisms to deliver H2A.Z to the Swr1 complex. It might be more effective to investigate incorporation complexes, since at least two complexes have been shown to be able to perform disassembly and assembly of nucleosomes. The FACT complex has been implicated in removing H2A-H2B during transcription, but it has also been implicated in assembly of H2A-H2B (Formosa, 2011). The Swr1 complex has been shown to be able to swap H2A in the nucleosome for H2A.Z, or the other way around, depending on the stimulus of PTMs (Watanabe et al., 2013).

METYLATION AS MOLECULAR TIMER

As described above, histone exchange seems not to be occurring at random places in the genome. In Drosophila, replacement of histone H3 by the variant H3.3 has been detected mainly in promoter regions, transcribed regions of the genome, and regulatory regions (Mito et al., 2005; Teves et al., 2012). This replacement has been connected with chromatin disruption caused by transcription. In yeast cells, the combination of replication-independent and –dependent histone exchange results in an accumulation of ancestral histones at the 5’ ends of coding regions of long genes that have low to intermediate transcription levels in dividing cells (Radman-Livaja et al., 2011). Interestingly, methylation of H3K79 by Dot1 has been linked to these ancestral histones (Chapter 5). Dot1 methylates H3K79 by a distributive manner, which results in changing ratios of mono-, di-, and tri-methylation over time and throughout the cell cycle. Since so far there have been no demethylases found for H3K79me, it has been suggested that this PTM can only be removed by dilution of histones by replication-coupled or –independent mechanisms. Therefore, and supported by a mathematical model, we predicted that the residence time of a histone in the chromatin would be a determinant of H3K79me level. Using RITE to purify old histone H3 from dividing cells over multiple generations, we were indeed able to show that H3K79me3 increases on aging H3 (Chapter 5). This result shows that methylation continues not only throughout the cell cycle, but also in subsequent generations. In agreement with this, we found that the position of old histones correlates with the position of high levels of H3K79me3 (Radman-Livaja et al., 2011). Ongoing methylation would suggest that none of the daughter cells precisely recapitulates the parental epigenome. This dynamic behavior seems incompatible with the idea that methylated H3K79 functions as an
epigenetic memory mark at single-nucleosome precision. Instead, the cell might use H3K79me as mark to couple cell cycle progression to changes in the epigenome. The connection between histone age and H3K79 methylation suggests that there is a possibility that the epigenome can not only be influenced by modifying and demodifying enzymes but also by factors that affect histone age and localization in the cell. A mathematical model that has been proposed to explain the observed distribution of old histones in replicating cells suggests three possible parameters: 1) replication-independent histone turnover, 2) passback of histones from the 3’ to the 5’ end of coding regions by passing RNA polymerase II, and 3) some diffusion of nucleosomes during DNA replication (Radman-Livaja et al., 2011). Whether the net outcome of these events fine-tunes the H3K79me landscape laid down by Dot1 or whether H3K79me3 functions as a mark to prevent exchange of particular nucleosomes, remains to be determined.

OUTLOOK

Recent studies indicate that histone H3.3 deposition is involved in key cellular processes such as epigenetic reprogramming and cancer development (Filipescu et al., 2013). A large fraction of human pancreatic neuroendocrine tumors (PanNETs) has been found to harbor mutations that inactivate ATRX and Daxx, two H3.3 chaperones implicated in incorporation of H3.3. Loss of ATRX/Daxx might lead to telomere destabilization in these tumor cells (Heaphy et al., 2011). In human pediatric glioblastomas, driver mutations in ATRX/Daxx have been found as well as mutations in the H3.3 gene itself (Schwartzentruber et al., 2012; Sturm et al., 2012). Interestingly, some of the mutations in H3.3 in these glioblastomas reduce global H3 methylation levels in cells by inhibition of SET-domain enzymes, suggesting that the mutant H3.3 proteins act in a dominant manner to also influence canonical H3 (Lewis et al., 2013). These findings stress that histone exchange plays a major role in epigenome control and cell function. Therefore, it will be important to unravel the mechanisms underlying histone exchange to be able to fully understand the significance of this unexplored level of epigenetic regulation and its role in normal development and cancer.
REFERENCES


Introduction and general discussion


