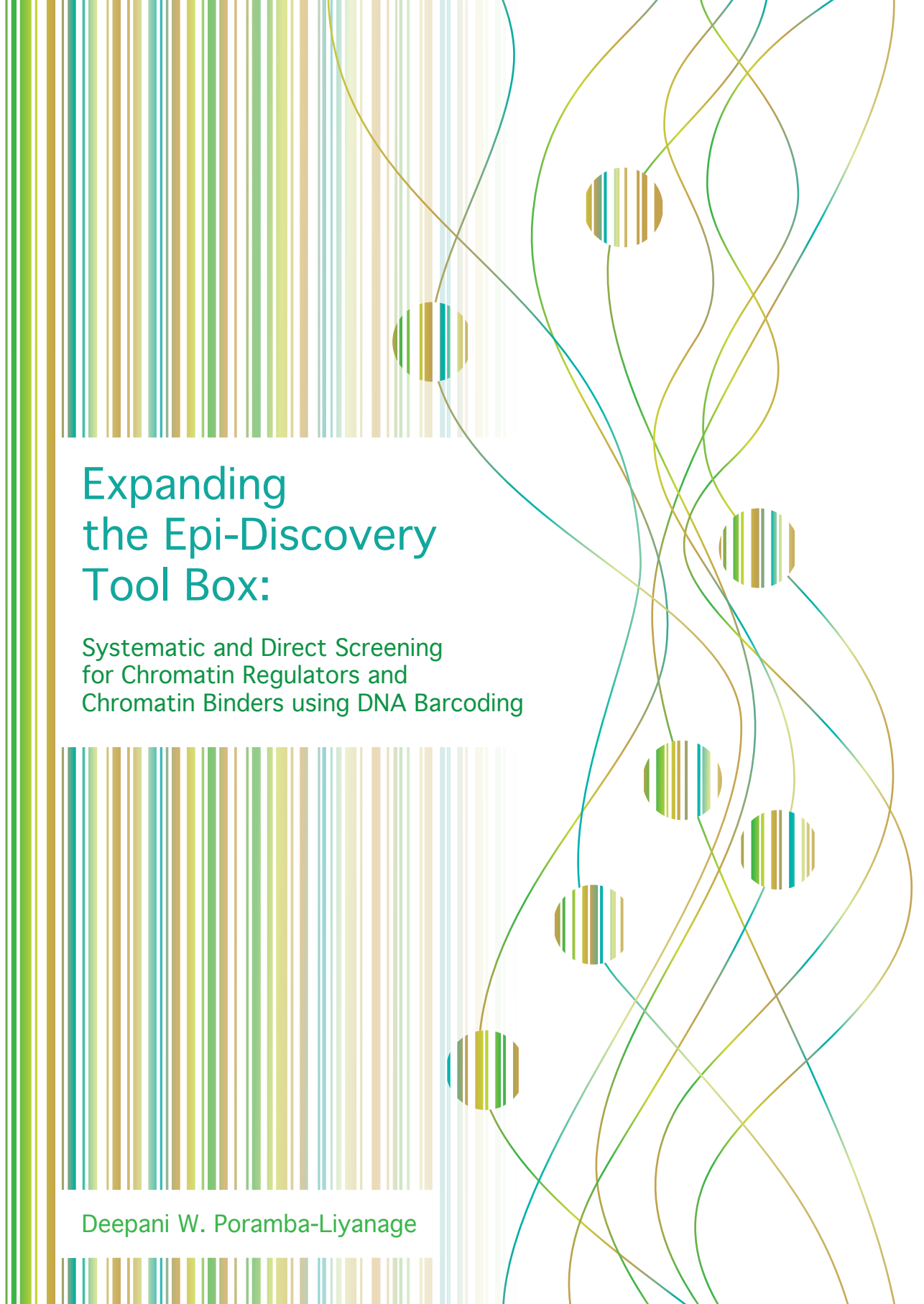


The background features a series of vertical stripes in shades of green, yellow, and blue on the left side. On the right side, there are several wavy, overlapping lines in the same color palette. Scattered along these wavy lines are several circular icons, each containing a unique pattern of vertical stripes.

Expanding the Epi-Discovery Tool Box:

Systematic and Direct Screening
for Chromatin Regulators and
Chromatin Binders using DNA Barcoding

Deepani W. Poramba-Liyanage

Expanding the Epi-Discovery Tool Box:

Systematic and Direct Screening for Chromatin Regulators and Chromatin

Binders using DNA Barcoding

Deepani W. Poramba-Liyanage

Cover design by Eliska Slovakova

About the front cover: A visualization showing how cells travel through different epigenetic landscapes throughout their existence, inspired by Waddington's epigenetic landscape. The thesis describes technology to deconstruct epigenetic loci by introducing DNA barcodes in cells.

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Expanding the Epi-Discovery Tool Box

Systematic and Direct Screening for Chromatin Regulators and Chromatin

Binders using DNA Barcoding

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,

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CHAPTER 1

General introduction:
Understanding the dynamic
nature of chromatin and
insights into technologies
for decoding the epigenome

1

General introduction: Understanding the dynamic nature of chromatin and insights into the technologies and challenges for decoding the epigenome.

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1.1 General introduction: chromatin

Eukaryotic genomes are packaged in chromatin, which consists of DNA, histones, and associated proteins (Figure 1). Structurally, there are 4 core histones, H2A, H2B, H3, and H4 and the linker histone, H1. The core histones form an octameric histone core around which DNA is wrapped to form the basic structural unit of chromatin, the nucleosome. Histone H1 binds to linker regions, connecting adjacent nucleosomes and contributing to the further folding of the chromatin fiber. The core histones contain 'tail' domains that are disorganized regions that further contribute to interactions with DNA and between nucleosomes (Maeshima, Ide, Hibino, & Sasai, 2016). Electrostatic forces between the histones and the DNA allow the chromatin fiber to be compacted, and unwound depending on the environment (Maeshima et al., 2016). Histones can be further classified into two major groups, those that are incorporated into chromatin during replication (canonical histones) and those that are replication independent (histone variants). Canonical histones are often present in gene clusters (Eirín-López, González-Romero, Dryhurst, Méndez, & Ausió, 2009) so that their expression can be coordinated and increased during the S phase of the cell cycle. The expression of histone variants is typically not restricted to S-phase. The presence of histone variants is particularly seen in specific genomic regions. At centromeres the H3 variant, CENP-A, is assembled into specialized nucleosomes that form the foundation for kinetochore assembly while the H3 variant H3.3 is deposited at the 5' and 3' ends of active genes as well as on telomeres and pericentric heterochromatin and loci containing endogenous retroviral elements (Elsasser, Noh, Diaz, Allis, & Banaszynski, 2015; Fang et al., 2018). At genes, both H3.3 and the H2A variant H2A.Z preferentially occupy nucleosomes that flank gene promoters, particularly at the +1 nucleosome that borders the transcription start site (TSS) (Henikoff & Smith, 2015).

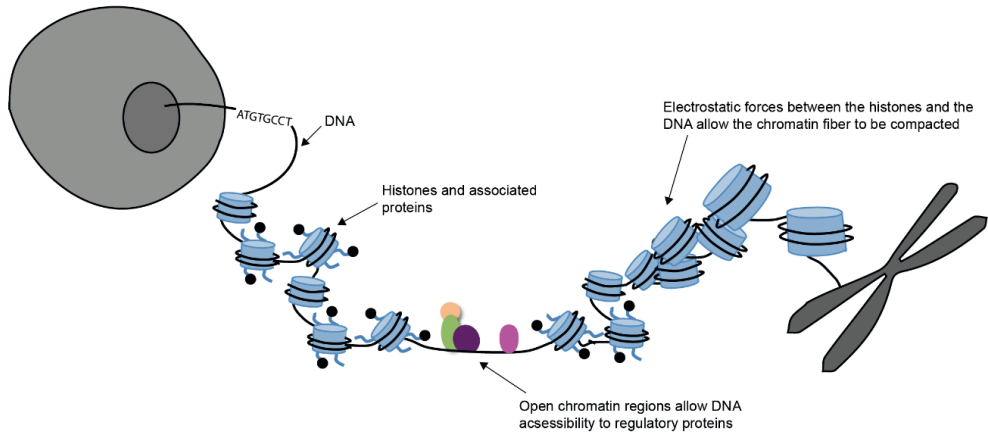


Figure 1. *In the nucleus of a eukaryotic cell, the genome is packaged in chromatin, which consists of DNA, histones, and associated proteins. The core histones contain ‘tail’ domains that are disorganized regions that contribute to interactions with DNA and between nucleosomes. All histones are subject to a large variety of post translational modifications (black dots on the histone tails). Electrostatic forces between the histones and the DNA allow the chromatin fiber to be compacted, and unwound depending on the environment, the interacting proteins, and the modifications. Open chromatin regions allow DNA accessibility to regulatory proteins.*

In addition to the structural variability in the nucleosome caused by histone variants, all histones are subject to a large variety of post translational modifications (PTMs). Many different and physiologically relevant PTMs have been identified and to date, acetylation (Allfrey, Faulkner, & Mirsky, 1964; Marmorstein & Zhou, 2014; Phillips, 1963), methylation (Allfrey et al., 1964; Hyun, Jeon, Park, & Kim, 2017), phosphorylation (Kleinsmith, Allfrey, & Mirsky, 1966; Rossetto, Avvakumov, & Côté, 2012), ubiquitination (Goldknopf, French, Daskal, & Busch, 1978; Krajewski, 2019; Uckelmann & Sixma, 2017), SUMOylating (Garvin & Morris, 2017; Shiio & Eisenman, 2003), glycosylation (Dehennaut, Leprince, & Lefebvre, 2014; S. Zhang, Roche, Nasheuer, & Lowndes,

2011), glycation (Ansari, Chaudhary, & Dash, 2018; Guedes, Vitorino, Domingues, Amado, & Domingues, 2011; Zheng et al., 2019), and crotonylation (Kelly et al., 2018; M. Tan et al., 2011) are some of the best studied. These covalent modifications of the histone subunits are one mechanism by which chromatin is organized in substructures. The modifications are deposited or “written” by several classes of enzymes, while their removal or “erasing” is facilitated by other classes of enzymes. PTMs on histones can directly affect chromatin structure. In addition, these modifications are further interpreted or “read” and the actions they specify are transmitted by specific chromatin-associated proteins leading to several layers where chromatin can be regulated and its organization defined. Even though these histone modifications alone often do not determine whether a gene is “on” or “off” (Helin & Minucci, 2017; Laugesen & Helin, 2014), together with the writers, erasers and readers, they facilitate or introduce barriers to gene expression or binding of transcription factors, thus providing a mechanism for modulating gene expression and for determining cell fate decisions (Krajewski, 2019). This interaction of DNA with the many associated proteins leads to the existence of different types of chromatin (Filion et al., 2010).

1.2 Dynamic states of chromatin

The epigenome of a cell determines its identity and how it will respond to signals. On the other hand, during development and differentiation, the epigenome is rewired to allow for adopting of new cell fates. Therefore, the epigenome supports stability as well as change. At the heart of this dual role is the maintenance and plasticity of the structure of the chromatin template as it undergoes obligate rearrangements during transcription, replication, and repair. For example, the disassembly of nucleosomes in an orderly manner allows RNA polymerase II (Pol II) to access the DNA, while nucleosome re-assembly impedes Pol II access, preventing aberrant or ectopic transcription and mRNA synthesis from within transcribed regions (de Jong & van Noort, 2019; Nagai, Davis, Mattei, Eagen, & Kornberg, 2017; Simon et al., 2011). In extended chromatin fibers,

the core histone tails are able to interact with the DNA in its vicinity, while in a folded structure the tails can also interact with neighboring nucleosomes. Aiding to the fluid state of chromatin is the ongoing unwrapping of parts of the nucleosome (Alabert, Jasencakova, & Groth, 2017; K. Luger, Mader, Richmond, Sargent, & Richmond, 1997) or the removal of an entire histone octamer, followed by replacement with either newly synthesized histones or different components (Chereji & Clark, 2018; Henikoff & Smith, 2015). This exchange or histone turnover can have many implications for the composition, structure and function of different genomic regions. Increased histone exchange could increase the transient accessibility of a particular genomic region to cellular components such as promoter accessibility to Pol II, thereby facilitating transcription. Canonical histones can be replaced by histone variants that facilitate subsequent rounds of exchange, such as the histone variant H2A.Z and H3.3, which could further enhance DNA availability and facilitate transcription (Giaimo, Ferrante, Herchenrother, Hake, & Borggreffe, 2019; Schlesinger et al., 2017). Replacement of existing chromatin-bound, post translationally modified histones by newly synthesized histones will alter the chemical nature and physical properties of the nucleosome and thereby affect gene expression (Henikoff & Smith, 2015; Krajewski, 2019). Chromatin is therefore in a constant dynamic or fluid state of wrapping and unwrapping that facilitates or impedes DNA accessibility for transcription, DNA repair and replication (Maeshima et al., 2016). In summary, in the past decade, we have learned that there are active mechanisms during transcription that promote reassembly of transiently evicted histones or that replace evicted histones with newly synthesized ones. Errors in these chromatin maintenance processes challenge chromatin integrity and lead to transcriptional errors. In addition, histone replacement restores DNA packaging but at the same time leads to loss of epigenetic information and incorporation of specialized histone variants, thereby leading to alterations of the epigenome.

1.3 Chromatin dynamics during gene expression

Many genes in the genome are constitutively expressed. Others are expressed at specific points in the cell cycle or during specific stages of development. Internal and environmental cues from the cell can promote the binding of regulatory proteins to the DNA, at promoter and enhancer regions, to implement dynamic gene regulatory programs (Vihervaara, Duarte, & Lis, 2018). Pioneering factors have the potential to alter the local chromatin environment or recruit polymerases and other proteins of the core transcriptional machinery to initiate gene expression (Zaret & Mango, 2016). Pioneering factors such as Fox A family of transcription factors, can initiate these cooperative interactions by binding directly to specific DNA sequences or interacting with other chromatin components such as specific posttranslational modifications on histone tails (Cramer, 2019b; Iwafuchi-Doi, 2019). Once Pol II has been recruited, gene transcription by RNA Pol II involves three distinct phases: initiation, elongation, and termination. It has been proposed that Pol II after termination can reengage in a subsequent round of mRNA production, completing a transcription cycle (Cramer, 2019a; Lis, 2019).

Initiation

Initiation starts with the formation of the preinitiation complex (PIC) at the promoter, where RNA Pol II assembles with TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, and TFIIH, which are collectively known as the general transcription factors. These assemble into a transcription preinitiation complex (PIC) via a multi-step process that is required to initiate productive RNA synthesis (Hahn & Buratowski, 2016; Z. Zhang & Tjian, 2018). The cooperation with the general transcription allows RNA Pol II to bind to and further open promoter DNA, to initiate messenger RNA (mRNA) synthesis and stimulate the escape of Pol II from the promoter (Cramer, 2019a; S. Sainsbury, C. Bernecky, & P. Cramer, 2015)

Elongation

The entry form of RNA Pol II then associates with a constellation of accessory factors or elongation factors, including of PAF1, Spt4/5 (or DSIF), NELF and FACT that form an elongating Pol II complex (F. X. Chen, Smith, & Shilatifard, 2018; Lavigne, Konstantopoulos, Ntakou-Zamplara, Liakos, & Foustieri, 2017). Transcription elongation proceeds once the elongating Pol II is formed catalyzing substrate selection, phosphodiester bond formation, and enzyme translocation through the gene resulting in repeated cycles of single nucleotide incorporation into a growing mRNA chain. This is a tightly regulated process in most metazoans with environmental cues working via the elongation factors to direct the release of promoter pausing, backtracking and arrest of Pol II (Cramer, 2019a; Fromm, Gilchrist, & Adelman, 2013; Kwak & Lis, 2013). Accompanying the transition from an initiating to productive elongation is the phosphorylation of specific residues on the C-terminal domain (CTD) of the large subunit of Pol II, Rbp1/Rpo21 (Bowman & Kelly, 2014; Cucinotta & Arndt, 2016). A highly conserved feature of the CTD is that it is composed of dozens of repeats of the heptapeptide sequence Y1-S2-P3-T4-S5-P6-S7. All residues within the CTD heptad repeat can be modified but serine 5 and serine 2 phosphorylation seems to be the most indicative of transcription (Komarnitsky, Cho, & Buratowski, 2000). The current model is that Pol II is recruited to genes with a hypophosphorylated CTD; the CTD then becomes phosphorylated on Ser5 during initiation, and subsequently phosphorylated on Ser2 during productive elongation (Egloff & Murphy, 2008; Q. Zhou, Li, & Price, 2012). This differential phosphorylation of Ser2 and Ser5 is thought to recruit transcription associated proteins at the appropriate time of the transcription acting as a “docking site” enabling further regulation of gene expression (Bowman & Kelly, 2014). Structural comparisons reveal that the elongation factors DSIF, NELF, and the PAF1 complex can be accommodated on the Pol II surface while it passages through chromatin (Cramer, 2019b; Farnung, Vos, & Cramer, 2018).

Transcription termination

As RNA Pol II reaches the gene end and transcribes over the polyadenylation site, cleavage and polyadenylation factor (CPF) and cleavage factor (CF) complexes (CPF, CFIA, and CFIB) are recruited and the nascent mRNA is released from the DNA template (Porrua & Libri, 2015; Proudfoot, 2016). Pol II continues to transcribe DNA after the mRNA is released, but this is short-lived, as the exonuclease Xrn2 is recruited and degrades the transcript after which Pol II is released from the DNA template terminating transcription.

mRNA splicing

Typical human genes have introns, and while fewer yeast genes have introns, the intron structure is conserved from yeast to humans (Herzel, Ottoz, Alpert, & Neugebauer, 2017) with a high degree of sequence conservation. Conserved splice sites define the 5' and 3' intron boundaries, while a third conserved sequence upstream of the 3' splice site helps identify the 3' splice site during spliceosome assembly. Yet, despite the sequence conservation, intron ends and alternative splice sites are difficult to define by sequence alone and recent studies suggest that chromatin structure and histone modifications and rates of transcription elongation regulate gene splicing and splice site selection (Dvinge, 2018; Herzel et al., 2017; Luco, Allo, Schor, Kornblihtt, & Misteli, 2011). As the nascent mRNA is generated, the introns are removed by the spliceosome, a megadalton ribonucleoprotein complex. Emerging evidence suggests that the spliceosome travels with Pol II and that spliceosome assembly, splicing catalysis and transcription elongation occur co-transcriptionally, at similar rates and in close proximity (Brody & Shav-Tal, 2011). Indeed, co-transcriptional splicing implies that some introns are removed prior to the completion of mRNA synthesis. Studies have also shown that the Pol II CTD has a role in recruiting splicing factors to the nascent mRNA (Brody & Shav-Tal, 2011; Luco et al., 2011). Yet, while the rate of transcription affects splicing, elongation rates are independent of ongoing splicing and when transcription has completed but splicing has not, the splicing machinery is retained at the site of transcription,

independently of the polymerase (Brody & Shav-Tal, 2011; Herzel et al., 2017). While transcription and spliceosome assembly are interwoven, the question of how many nucleotides downstream of the 5' and 3' splice site need to be transcribed for spliceosome assembly remain unanswered.

Co-transcriptional establishment of histone PTMs

Various of posttranslational modifications are associated with distinct stages of transcription and the establishment of some histone marks are linked to and are the consequence of active transcription (Henikoff & Greally, 2016; Tanny, 2014). While the functional significance of these PTMs is an ongoing topic of investigation, they often provide a memory of recent transcription activity or they signal for additional transcriptional regulation. Active promoters possess high levels of histone H3K4me3 and multiple acetylated H3 and H4 lysine residues, whereas gene bodies of transcribed genes are enriched with monoubiquitinated H2B (H2Bub), H3K36me3, H3K79me2 and H3K79me3 (F. X. Chen et al., 2018). Trimethylation of H3K4 and H3K36 are examples for histone modifications directly resulting from active gene transcription. In yeast, the two histone lysine methyltransferases responsible for methylation of H3K4 and H3K36 are Set1 (as a component of the COMPASS complex) and Set2, respectively. Both Set1 and Set2 are recruited by the Paf1 complex that associates with Pol II during elongation. Set1 binds to the Pol II CTD during the early stages of elongation, when Ser5 is phosphorylated but Ser2 is unphosphorylated (Ng, Robert, Young, & Struhl, 2003). Alternatively, Set2 binds the Pol II CTD during productive elongation when Ser2 is phosphorylated (J. Li, Moazed, & Gygi, 2002). This is reflected in methylation levels, as the level of H3K4me3 peaks at the 5'-ends of actively transcribed genes, while the H3K36me3 level starts low at the 5'-ends of expressed genes and gradually increases toward the 3'-ends. H3K79me3 is another histone mark coupled with active transcription. H3K79me3 is catalyzed by the histone methyltransferase Dot1 and the process is dependent on the Paf1 complex (Paf1C). The Paf1C complex mediates the recruitment of enzymes that monoubiquitinate the C-terminus of histone H2B (H2Bub1) (van Welsem et al., 2018), which in

turn promotes methylation of H3K79 by Dot1 (Anderson et al., 2019; Jang et al., 2019; Valencia-Sanchez et al., 2019; Worden, Hoffmann, Hicks, & Wolberger, 2019; Worden & Wolberger, 2019). Accordingly, H3K79me3 level peaks at the 5'-end and decreases toward the 3'-end. Moreover, H2Bub1 promotes methylation of H3K4 and H3K36 by Set2. While a role for regulators of H2B ubiquitination in regulating transcription at both enhancers and promoters has been identified, no direct role in promoting transcription elongation has been identified (F. X. Chen et al., 2018). In contrast, H3K36me3 in gene bodies has been shown to affect RNA splicing and prevent cryptic transcription (F. X. Chen et al., 2018). The role of the co-transcriptional methylation marks on H3 is still not fully understood (Henikoff & Greally, 2016). The function of Set1/H3K4 methylation in transcription in yeast is still under debate (Woo, Dam Ha, Lee, Buratowski, & Kim, 2017) and positive as well as negative roles have been proposed. H3K36me3 by Set 2 recruits Rpd3 histone deacetylase and works cooperatively with known negative regulators of transcription, Mot1-NC2 and the chromatin remodeler complex Ino80 to maintain a repressive barrier for cryptic transcription (Koster, Yildirim, Weil, Holstege, & Timmers, 2014; Xue et al., 2017). However, there are many histone marks that are not coupled to active transcription (Dong & Weng, 2013).

1.4 Chromatin dynamics during DNA replication

In S phase of the cell cycle the entire genome is copied and the chromatin landscape undergoes dramatic alterations (Alabert et al., 2017). DNA replication initiates from multiple origins of replication to ensure the genome replicates in a timely manner. In *S. cerevisiae*, these origins of replication are defined by a combination of specific sequences and local chromatin (Warner, Azmi, Kang, Zhao, & Bell, 2017). The ORC complex is the first to bind at the origin and serves as a loading platform for the subsequent assembly of the replication machinery. Once ORC binds at the replication origin, it promotes the binding of 2 proteins, CDC6 and CDT1. These proteins facilitate the subsequent loading of the mini-chromosome-maintenance 2–7 (MCM2-7) replicative helicase onto DNA, forming the pre-replicative complex. DNA replication begins once the double

helix is separated into its two component strands, providing DNA polymerase with the single stranded parental template for DNA synthesis. Thus, DNA unwinding is effectively rate limiting for DNA replication initiation and to make sure that each part of the genome is replicated only once, helicase loading and helicase activation is separated into two phases. The first takes place in late mitosis and G1 phase of the cell cycle, where the MCM helicases are loaded onto DNA forming the pre-replicative complex. This is known as origin licensing. The MCM helicases are loaded as a double-hexamers onto DNA ensuring the unwinding of the DNA in a bidirectional manner (Coster & Diffley, 2017; Deegan & Diffley, 2016; Evrin et al., 2014). In S-phase, the second step, helicase activation occurs with the combined activities of cyclin-dependent kinase (CDK), Dbf4-dependent kinase and a host of firing factors that remodel the MCM double hexamer into two active Cdc45–MCM– GINS (CMG) helicases composed of a MCM hexamer, Cdc45 and a GINS heterotetramer comprising Sld5, Psf1, Psf2 and Psf3 (Coster & Diffley, 2017). The high CDK activity in S and G2 phases inhibit the functions of ORC, Cdc6 and Cdt1, and prevents further loading of the MCM helicases, ensuring that individual origins fire just once during each cell cycle (Coster & Diffley, 2017; Y. V. Fu et al., 2011).

The active CMG helicase encircles a single strand of DNA and translocates away from the origin and with a 3' to 5' directionality on the leading strand, allowing for the recruitment of a number of other factors to form the replisome (Deegan & Diffley, 2016; Kose, Larsen, Duxin, & Yardimci, 2019). The unwound DNA becomes a template for primases and polymerases. Primases facilitate the initiation of DNA synthesis by synthesizing small RNA primers that are needed by DNA polymerases to initiate DNA synthesis in a 5' to 3' directionality. Thus, DNA replication on the leading strand differs from the lagging strand and is carried out by polymerase epsilon and delta, respectively. In the lagging strand smaller Okazaki fragments are formed and later processed to complete the synthesis of the lagging strand. The replisome remains stably engaged with DNA at the replication fork throughout elongation until its finally disassembled as two replication forks

converge during termination (Deegan, Baxter, Ortiz Bazan, Yeeles, & Labib, 2019; L. Deng et al., 2019; Mukherjee & Labib, 2019).

Since distinct chromatin states can be inherited through mitotic cell division, chromatin replication cannot be separated from DNA replication. Chromatin replication can be divided into 5 steps. Firstly, disassembly of chromatin is needed ahead of the replisome. Secondly, parental histones need to be recycled onto the two daughter strands. Thirdly, newly synthesized histones need to be supplied to sites of ongoing DNA replication. Nucleosomes are then assembled on both leading and lagging strands from newly synthesized histones and old recycled histones. Finally, the chromatin marks are restored on DNA and histones and the chromatin begins to reassociate with other chromatin components (Alabert et al., 2017). How chromatin is maintained following replication is discussed further in the text.

1.5 Maintenance of the chromatin state during transcription and replication

Since chromatin is instrumental for genome function and proper execution of epigenetically defined developmental programs, efficient maintenance of the chromatin state following the passage of Pol II and following DNA replication is essential for cell survival. Indeed, transcription by Pol II is typically accompanied by nucleosome reassembly and minimal exchange of histones (Kulaeva et al., 2009; Kulaeva & Studitsky, 2010; Petesch & Lis, 2012). During moderate transcription, histones H3/H4 carrying the epigenetic marks are not displaced or exchanged (in contrast to H2A/H2B histones), due to the formation of a novel intermediate: an intranucleosomal DNA loop containing transcribing Pol II, together with the FACT complex (Gurova, Chang, Valieva, Sandlesh, & Studitsky, 2018; F. K. Hsieh et al., 2013), that tightly couples nucleosome disruption ahead of the enzyme with the recovery of DNA-histone interactions in the wake of Pol II. It is hypothesized that the subnucleosome (DNA-bound histone hexamer formed upon release of a H2A/H2B dimer from the octamer) survives Pol II passage and remains at the original position on

DNA. However, in regions of fast and extensive transcription, partial loss and exchange of all core histones at the transcribed regions is reported (de Jong & van Noort, 2019; Dion et al., 2007; Kulaeva et al., 2009; Radman-Livaja et al., 2011; O. J. Rando & Chang, 2009; Teves, Deal, & Henikoff, 2012; Verzijlbergen et al., 2010).

When cells divide and DNA is replicated, the entire genome is separated into single strands as it passes through the replicative helicase, disrupting protein-DNA contacts. Behind the replication fork, proper chromatin organization needs to be re-established on the two daughter strands and maintained for epigenetic information passed on to the next generation. Nucleosome disruption takes place close to the replication fork, as a result of collision with the replicative helicases or due to positive supercoiling ahead of the fork (Alabert et al., 2017; Gasser, Koller, & Sogo, 1996; Groth, 2009). Current evidence supports a model in which the parental (H3–H4)₂ tetramers segregate randomly to the two daughter strands, forming nucleosomes with either new or old H2A–H2B dimers (Ahmad & Henikoff, 2018; Hammond, Stromme, Huang, Patel, & Groth, 2017). How exactly the old histones are transferred to the new DNA is unknown, but the MCM 2-7 helicases and the chromatin chaperone Asf1 and FACT play an important role (Hammond et al., 2017; L. Zhang et al., 2018). Following the passage of the replication fork, nucleosomes need to be assembled, and PCNA is the central component of the replication fork that orchestrates nucleosome assembly by recruiting CAF1 to promote the first step in nucleosome assembly, the deposition of histone H3–H4 dimers onto DNA (Alabert & Groth, 2012; Mattioli et al., 2017; Serra-Cardona & Zhang, 2018). Thereafter, histone H2A–H2B rapidly associates to complete the nucleosome via the actions of NAP1 and FACT (Alabert & Groth, 2012). Interestingly, since DNA replication on the leading strand differs from the Okazaki fragments formed on the lagging strands, DNA replication and nucleosome formation in the two different strands must be adjusted to accommodate the different replicative mechanisms (Alabert et al., 2017). On the leading strand, CAF1 may bind PCNA and act simultaneously with Pol ϵ while on the lagging strand, nucleosomes must be deposited to the

growing Okazaki fragment or immediately after its completion in a process that involves Mcm2-Ctf4-Pol α (Alabert et al., 2017; Gan et al., 2018; Petryk et al., 2018; Yu et al., 2018). Once nucleosome assembly is completed, chromatin maturation takes place, where newly synthesized histones acquire modifications similar to the old ones in the locus in which they are incorporated. Emerging models predict that epigenetic states are not fully restored on newly replicated DNA and suggest that passage through S phase is necessary for chromatin maturation (Alabert & Groth, 2012; Reveron-Gomez et al., 2018). Indeed, S phase may provide an opportunity to reset epigenetic controls while at the same time increasing the risk of unwarranted chromatin changes (Reveron-Gomez et al., 2018).

1.6 Transcription-replication conflicts

As cells grow and divide as part of the cell division cycle, genes are switched on and off on a regular basis (Dolatabadi et al., 2017). At any one time, thousands of different genes act as a template to produce mRNA molecules. For the genes that are transcribed during S phase, transcribing Pol II and the replication machinery use the same DNA template and this can result in unwanted encounters. These encounters can be either co-directional or in head-on orientation and when they occur, the replication fork cannot progress past the elongating Pol II. Transcription-replication conflicts pose a grave threat to genome stability as they can induce replication-fork stalling, DNA recombination events, breaks and mutations (García-Muse & Aguilera, 2016; S. Hamperl, Bocek, Saldivar, Swigut, & Cimprich, 2017). Cells have evolved several strategies to reduce the impact of transcription on replication (García-Muse & Aguilera, 2016; Poli et al., 2016). In eukaryotic cells, highly transcribed regions are either protected by the use of barriers that block replisome progression or by temporarily or spatially separating replication and transcription. In *S. cerevisiae*, replication fork barriers block replisome progression in highly transcribed ribosomal DNA genes in a directional manner, while in mammals, ribosomal DNA is spatially separated in the nucleoli (Canal, Duch, Posas, & de Nadal, 2018; García-Muse & Aguilera, 2016; Ivessa, Zhou,

& Zakian, 2000). Additionally, transcription and replication can be physically and temporarily separated to prevent collisions, for example, genes transcribed during early replication are replicated late in S phase, and vice versa (Meryet-Figuere et al., 2014; Smirnov et al., 2014). When collisions do occur, it is likely that RNA Pol II is released from the DNA to allow passage of the replisome (Million-Weaver, Samadpour, & Merrikh, 2015; Poli et al., 2016). Several lines of evidence suggest that the Pol II, together with Paf1C, is involved in managing transcription-replication conflicts leading to the eviction of Pol II (Felipe-Abrio, Lafuente-Barquero, Garcia-Rubio, & Aguilera, 2015; Poli et al., 2016). Interestingly, there is no clear evidence to suggest that RNA and DNA polymerases ever actually make contact and it is plausible that before the physical connection occurs, the progression of the complexes are attenuated and the complexes fall apart (García-Muse & Aguilera, 2016).

1.7 Decoding the genome: technologies and challenges

Transcription and replication involve the coordination of many proteins and protein complexes. Chromatin proteins have traditionally been identified in genetic screens, where the readout is often indirect. For example, the screen for factors that control telomeric silencing, in which DOT1 was identified, used reporters for silencing and did not probe the chromatin structure directly (Singer et al., 1998). This often leads to indirect effects and many factors may be missed because reporter assays can be biased. In addition, much of our knowledge about chromatin proteins come from biochemical purifications and proteomics approaches. However, such strategies may be biased towards soluble complexes and the result will depend on the exact conditions used. Therefore, translating *in vitro* biochemical interactions and genetic relationships into functions at specific genomic regions is not trivial and important factors might be missed. To understand the composition and regulation of chromatin at a systematic, comprehensive and molecular level it is important to develop assays that directly read out specific chromatin states. Furthermore, as many different types of chromatin occupy the nucleus, there is a need for assays that untangle regulators

and components of single genomic loci. Acquiring this type of direct evidence so far remains challenging due to many technical limitations. Following is a summary of the methods that have expanded our understanding of chromatin and that are relevant for the methods in this thesis.

Chromatin immunoprecipitation (ChIP)

Most of our current knowledge regarding chromatin composition across the genome is based on ChIP (Collas, 2010; Park, 2009; H. S. Rhee & B. F. Pugh, 2012). ChIP involves the selective immunoprecipitation of a protein or modification of interest from a chromatin preparation to determine the associated DNA sequences. Combining of ChIP with high throughput sequencing technologies has been widely used to map the localization of histone modifications, histone variants, transcription factors, and other chromatin proteins in a genome-wide scale. In most cases the first step in ChIP involves *in vivo* crosslinking of proteins that are bound to DNA with formaldehyde. Since DNA-protein interactions are often of dynamic nature, such crosslinking freezes the interaction, allowing for its detection. However, where proteins bind to DNA with higher affinity, as in the case of histones, the crosslinking step can be omitted and native ChIP can be performed. Next, cells are lysed and chromatin is fragmented to a desirable sized product (150–500 bp) and protein–DNA complexes are immunoprecipitated, usually by conjugation of beads with antibodies that recognize specific protein or protein modifications present in the chromatin. Finally, since crosslinking using formaldehyde is reversible, DNA is released from the immunoprecipitated chromatin by reversal of the cross-links by high temperature. Immunoprecipitated DNA sequences are identified by quantitative PCR (ChIP-qPCR) or by high-throughput sequencing (ChIP-Seq) to determine the genomic regions that were originally bound by the protein or protein modification of interest in the living cell. While ChIP has been instrumental in mapping DNA interactions of known chromatin components across an entire genome (McHaourab, Perreault, & Venters, 2018; Vinayachandran et al., 2018), prior knowledge is required for the selection of candidate proteins. Additionally, ChIP experiments are laborious,

which means that scaling the experiments up to include multiple proteins or conditions per experiment is a challenge (Vinayachandran et al., 2018). However, an advantage of ChIP and methods based on ChIP is the PCR amplification step that allows for sufficient detection of binding events even with lower input material, and the ability to add unique molecular identifiers (short DNA barcodes) which allows multiplexing several ChIP samples prior to sequencing and thereby the rapid and simultaneous generation of ChIP-Seq data sets (Chabbert et al., 2015; Lan, Demaree, Ahmed, & Abate, 2017; Rotem et al., 2015).

MS based proteomic approaches

Proteomic approaches from Western blotting to high resolution mass spectrometry (MS) have been powerful tools in the identification of soluble protein complexes in a global and unbiased manner. In addition, affinity purification combined with MS has been invaluable for defining protein interaction networks. Interrogating chromatin-associating or chromatin-bound complexes has been much more challenging, because the complexes bound to chromatin can be difficult to purify owing to the tight integrity of chromatin that needs to be disrupted, without affecting the complex of interest. Further, these proteins may be present at low abundance and the dynamic nature of these protein-DNA interactions only adds to this challenge (Kan, Saksouk, & Dejardin, 2017; Wierer & Mann, 2016). Two significant improvements in MS have allowed for the identification of proteins associated with chromatin. The first mimics DNA-protein interactions *in vitro* using peptide and DNA baits. Examples for such baits include synthesized histone tails containing PTMs, *in vitro* assembled nucleosomes, and DNA baits harboring single nucleotide polymorphisms or DNA modifications (Bartke et al., 2010; Nikolov et al., 2011; Wierer & Mann, 2016). The second resembles classical chromatin immunoprecipitation (ChIP) protocols except that they are followed by MS (Wierer & Mann, 2016). Notable examples include the use of nucleases or mild sonication (Lambert, Mitchell, Rudner, Baetz, & Figeys, 2009) or mild crosslinking with formaldehyde (Kustatscher, Wills, Furlan, & Rappsilber, 2014; Soldi & Bonaldi, 2014; C. I. Wang et al., 2013) to

purify of chromatin-bound proteins and their associated DNA. These methods allow affinity purification of DNA bound protein complexes in quantities sufficient for analysis by MS of chromatin states but do not report on specific genomic loci. Further, improvements in affinity purifications with highly specific antibodies and epitope tags in combination with microfluidics platforms have facilitated proteomic studies with very little starting material (12,000 input cells) (Furlan et al., 2019).

Two purification strategies, isolation of proteins on nascent DNA (iPOND) and nascent chromatin capture (NCC) have been successfully applied to detect proteins associated with replicating DNA (Alabert et al., 2014; Dugrawala & Cortez, 2015). iPOND relies on incorporation of the nucleoside analog 5-ethynyl-2'-deoxyuridine (EdU-labeling) into newly synthesized DNA templates while NCC relies on biotinylating the newly synthesized DNA, by incubating cells in hypotonic conditions to allow the biotin-dUTP to cross the cell membrane. Both techniques allow for purification and examination of proteins associated with nascent DNA and the replisome. Proteins involved in DNA and chromatin replication are then identified by enrichment with nascent DNA compared to bulk chromatin. These technologies have facilitated studies that define the replication fork proteome and identify novel proteins that contribute to DNA and chromatin replication (Alabert et al., 2014; Cortez, 2017; Sirbu et al., 2011; Vasseur et al., 2016). Combining iPOND with different DNA damage conditions, such as exposure to the genotoxic drug hydroxyurea (HU) that leads to stalling of the replication fork and fork collapse with prolonged treatment, has enabled monitoring of the protein recruitment process and modification of proteins at stalled forks (Sirbu et al., 2011). Even though iPOND and NCC are powerful tools to study dynamic chromatin protein interactions, substantial input material is needed to achieve sufficient protein amounts for detection by MS. Furthermore, iPOND and NCC provide information about average binding across multiple replication sites and lacks locus-specific information. Thus, while these studies provide more information about different chromatin states they still lack information about specific genomic loci.

Locus specific proteomes

Identification of proteins that interact and bind at a particular genomic locus would require the isolation of that region and all proteins associated with it (Wierer & Mann, 2016). Methods that combine affinity purification with mass spectrometry have allowed for direct purification of selected genomic loci and their associated proteins. The main challenge is the required sensitivity, as a single-copy binding protein cannot yield more than two molecules per cell for a diploid locus. Thus, with affinity purification techniques, the signal to noise ratio becomes the one important limitation and the signal has to be higher than the non-specific capture of minute amounts of chromatin fragments from the vast excess of the rest of the genome (Kan et al., 2017). This limitation has in large prevented the use of MS based techniques for probing chromatin binders of single-copy loci.

There are positive developments and major achievements in purifying repeated loci and abundant chromatin segments such as telomeric DNA repeat regions and replication forks (Kan et al., 2017). The locus of interest is targeted either by introducing artificial DNA-binding sites (such as LexA-binding sites or the binding site of the tetracycline repressor protein) or by using an endogenous sequencing (Figure 2). The target locus and the associated proteins are purified using (a) specific binding proteins that correspond to introduced DNA binding sites, (b) oligonucleotide probes such as LNA probes (Byrum, Raman, Taverna, & Tackett, 2012; Déjardin & Kingston, 2009) or (c) proteins with sequence specificity such as transcription activator-like (TAL) effector proteins (Byrum, Taverna, & Tackett, 2013) or nuclease deficient Cas9/and specific guide RNAs (Liu et al., 2017; Myers et al., 2018). These methods rely on quantitation of proteins that are significantly enriched over control pull-downs. Efforts to establish locus-specific protein composition have been hampered by the inability to collect material of sufficient amount and purity to allow identification of bound factors (Kan et al., 2017). The main limitation remains the capture of minute amounts of non-relevant chromatin fragments because protein enrichment of a specific target is compared to

any other similarly abundant “negative control”. Proteomics of Isolated Chromatin segments (PiCH) is one such technique that is based on using an affinity handle to capture specific chromatin fragments (Déjardin & Kingston, 2009; Kan et al., 2017). The locus of interest is targeted with desthiobiotinylated oligonucleotide probes containing 50 % of locked nucleic acids (LNA) that have high melting temperatures ensuring strong and stable hybridization to the target sequence. PiCH has been optimized for the purification of proteins associated to abundant sequences such as mammalian telomeres or pericentromeric repeats. The signal to noise ratio is an important limitation to PiCH. Enrichment by PiCH affinity purification is estimated to be ~10,000 fold. A single 1 kb locus of interest represents only ~0.00003 % of the human genome. Thus, to obtain a 50 % pure mixture for downstream mass spectrometric analysis, it would require an enrichment factor of 1,500,000 fold, which is challenging with a single affinity step, limiting the use of PiCH for purification of single copy targets from human cells (Kan et al., 2017).

CRISPR-based methods have recently been used for the purification of specific genomic loci by targeting dCas9-fusion proteins with guide RNAs (gRNAs). These methods have allowed identification of proteins associated nonrepetitive genomic loci, with modest changes to the cellular context and without the need for cross-linking or genomic alterations. Nuclease deficient Cas9 (dCas9) fusion proteins are targeted to specific genomic loci with gRNAs resulting in the introduction of an affinity handle that can be used to purify these regions. Notable examples are (a) dCas9 fused to N-terminal FLAG and biotin-acceptor (FB-dCas9) (Liu et al., 2017), (b) engineered peroxidase APEX2 fused to dCas9 (Caspex) (Myers et al., 2018) and (c) dCas9 fused to the promiscuous biotin ligase BirA (CasID) (Schmidtman, Anton, Rombaut, Herzog, & Leonhardt, 2016)). Once targeted to specific genomic loci with gRNAs, FB-dCas9 and CasID would result in biotinylation of the target chromatin (Liu et al., 2017; Schmidtman et al., 2016), while Caspex labels nearby proteins with biotin derivatives (Myers et al., 2018). The target locus and/or associated proteins are then purified by high affinity streptavidin purification. While these

techniques are promising have identified a range of nuclear and chromatin associated proteins, few specific transcription factors known to recognize these loci were identified. Indeed, further work will be needed to extend the capabilities of these techniques.

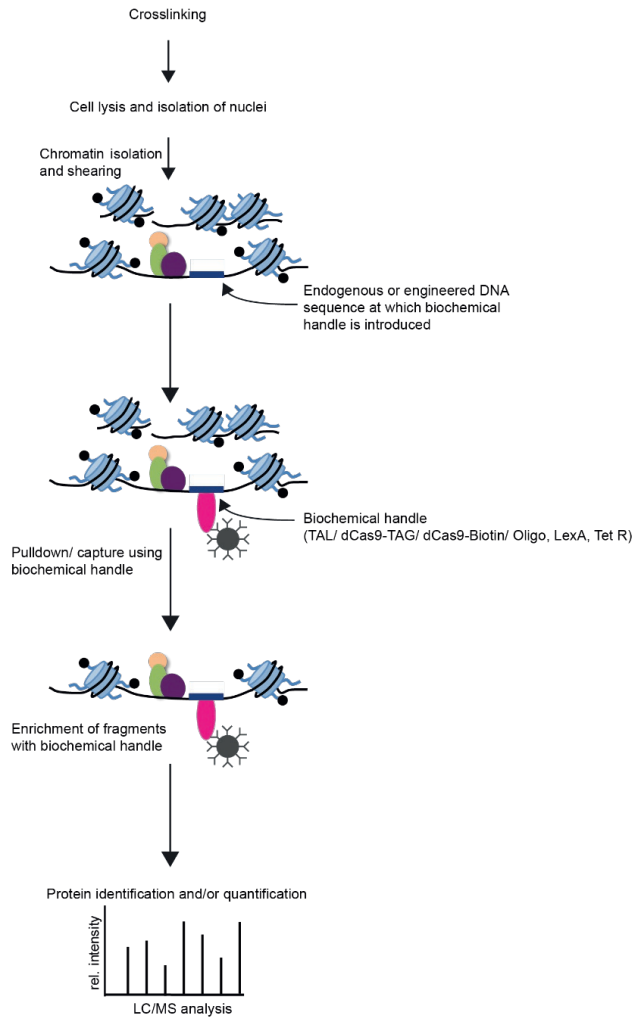


Figure 2. Sequence specific capture of chromatin proteomes by mass spectrometry

Following cell lysis, nuclei are isolated and chromatin is sheared by sonication or enzymatic digestion. To identify chromatin associated protein complexes, individual genomic loci are

enriched using either a complementary DNA probe, an engineered artificial binding site together with a respective binding protein -e.g. LexA, Tet-repressor (Tet-R) or proteins that directly recognize the specific target sequence or recognize and modify the target for affinity purification—e.g. TAL or dCas9-gDNA (dCas9-TAG or dCas9-Biotin, Caspex). The binding proteins or their targets have tags that can be used for affinity purification of the genomic loci or the bound proteins. The proteins are then separated and analyzed by mass spectrometry. Adapted from (Wierer & Mann, 2016).

1.8 Taking advantage of yeast libraries to decode chromatin regulation and proteomes: from yeast deletion collections to Epi-ID and Epi-Decoder

Despite positive strides, locus specific protein binding events are still poorly characterized using mass-spec based methods. For the systematic characterization of locus specific chromatin proteomes, Epi-ID and Epi-Decoder focus on yeast as a model system for alternative approaches. Yeast cells can be maintained in colonies on solid media. When in arrayed libraries, complex bioassays with yeast can be performed in parallel by measuring fitness of each colony in a high-throughput manner (Giaever et al., 2002). The yeast deletion collection, was the first systematic yeast deletion library to be constructed, comprising of around 6000 start-to-stop deletions of non-essential open reading frames of *S. cerevisiae* (Giaever & Nislow, 2014; Pierce, Davis, Nislow, & Giaever, 2007). In addition to the knock-out cassette, two barcodes with unique DNA sequences were introduced next to the knock-out cassettes, resulting in a pair of DNA sequences that serve as identifiers for each knock-out clone. This allows the strains to be pooled in genetic or chemical screens in which cell fitness information is derived from quantitative changes in the abundance of each barcode pair. These collections were crucial in understanding genes that would result in a growth or fitness defect in environmental stress conditions or in drug screens, by comparing barcode abundance before and after exposure. Epi-ID and Epi-Decoder DNA take advantage of the idea that barcodes report on cell identity, and build on the principle that the DNA barcodes are

in a chromatinized context, and therefore can be used to systematically probe for the chromatin state at the barcoded locus.

Epi-ID

Epi-ID is a screening technique for chromatin regulators in yeast that is based on ChIP followed by barcode-sequencing (ChIP-Barcode-Seq). Epi-ID makes use of an arrayed yeast deletion library where each clone contains one specific deletion and at a common location a pair of barcodes flanking a reporter gene. The library is generated by crossing a deletion library (Tong & Boone, 2006) that does not contain barcodes, with a library of Barcoder strains that harbors unique DNA barcodes flanking a selectable marker at a common location, the HO locus (Yan et al., 2008) (Figure 3). Deletion of a protein that regulates or maintains the chromatin environment of the reporter locus will result in changing the chromatin environment, either by decreasing or increasing the abundance of the chromatin feature of interest. With Epi-ID, since each gene deletion corresponds to a specific barcode pair, the effect of the gene deletion on the abundance of the chromatin feature can be assessed by ChIP of the feature of interest (e.g. a histone methylation) followed by high-throughput sequencing and counting of the barcodes. When a gene deletion leads to an increase of the chromatin factor at the barcoded locus, the ChIP efficiency will be greater, and when the gene deletion leads to lower abundance of the chromatin factor, the ChIP efficiency will be lower. This gain or loss of abundance of a chromatin feature is therefore represented in the ChIP efficiency and can be determined by the relative enrichment of each barcode in the immunoprecipitated DNA compared to the input material (ChIP/input) (Figure 3). Thus, ChIP followed by high-throughput sequencing and barcode counting allows for a direct comparison of effects of thousands of gene deletions on a chromatin feature at the barcoded locus. This strategy was first used to screen for regulators of histone H3K79 methylation by Dot1 with ChIP performed against H3K79 monomethylation and H3K79 trimethylation and led to the identification of all known regulators of H3K79 methylation, validating Epi-ID (Vlaming et al., 2016).

A previous study was focused on the identification of genes affecting histone turnover in budding yeast, leading the identification of the HAT complex (Verzijlbergen et al., 2011).

Epi-Decoder

Epi-Decoder builds on the ChIP-Barcode-sequencing technology of Epi-ID. However, instead of screening for regulators of one feature by interrogating gene knock-outs, Epi-Decoder screens for occupancy of all proteins in the cell at the barcoded locus. Thus, Epi-Decoder is a Tag-ChIP-Barcode-Seq technology to systematically measure protein-DNA interactions at a single genomic locus. Epi-Decoder libraries are generated by crossing a library of Barcoder strains with a library of tagged strains where each strain has one protein that is tagged at its endogenous locus with a common epitope tag (Figure 3). With Epi-Decoder, each clone contains one tagged protein and one or a pair of specific barcodes at a common reporter locus. The relative barcode enrichment following ChIP of the common tag (ChIP/Input) can be used to estimate the relative binding of the tagged protein in the proximity of the barcode (Figure 3). With Epi-Decoder, since all proteins are examined using the same tag, under exactly the same conditions, and in the same strain background, the barcode counts and the inferred relative binding efficiencies of all the proteins can be directly compared, in a quantitative manner. The Epi-Decoder library comprises approximately 4000 of the 5600 proteins encoded by the yeast genome, giving a comprehensive view of protein binding at the barcoded locus.

The work in this thesis describes the development and application of Epi-ID and Epi-Decoder technologies with the long-term goal to uncover novel aspects of chromatin biology and understand how chromatin serves as a central platform in the cell to maintain cell identity and to respond to signals to dynamically adjust the epigenome to facilitate chromatin transcription, replication, and maintenance.

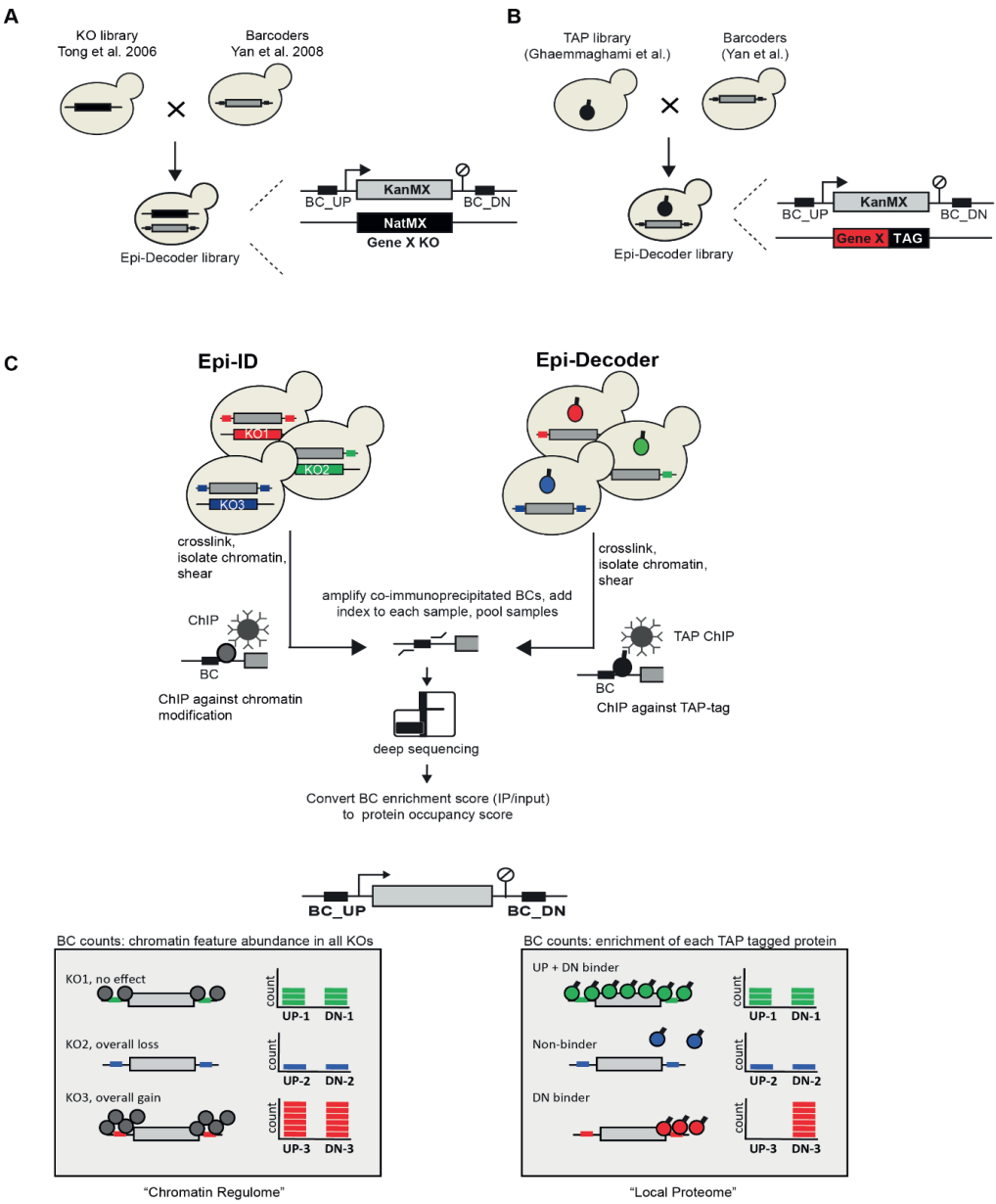


Figure 3. Experimental set up for Epi-ID and Epi-Decoder at the barcoded *HO-KanMX* locus

(A) *Construction of the Epi-ID library: a Deletion collection was crossed to a HO-Barcoder library by using standard SGA procedures. (B) Construction of the Epi-Decoder library: a library of strains encoding TAP-tagged proteins was crossed to a HO-Barcoder library by using standard SGA procedures. (C) Experimental set up for the systematic discovery of epigenetic regulators and chromatin binders with Epi-ID and Epi-Decoder. Colonies of an arrayed library are pooled in a flask, expanded and crosslinked and then used for ChIP with either specific antibodies for Epi-ID or TAG-ChIP for Epi-Decoder. The co-immunoprecipitated barcodes are amplified and counted by massive parallel sequencing. Barcodes of input DNA are counted to control for the presence of each barcode in the pool. Indexed input and ChIP samples of multiple pools can be combined into 1 master pool before sequencing. Protein binding is measured by barcode counting. The HO-Barcoder locus consists of a KanMX resistance marker transcribed by the AgTEF1 promoter flanked by 2 unique barcodes and replacing the HO gene. The BC_UP is promoter-proximal and the BC_DN terminator-proximal and close to (50 bp) an origin of replication (ARS404). BC_DN, downstream barcode; BC_UP, upstream barcode; ChIP, chromatin immunoprecipitation; TAP, Tandem Affinity Purification.*

1.9 Outline of the thesis

Genetic screens have been indispensable for studying chromatin processes. Classical genetic screens are laborious and the readout for chromatin changes is often indirect (Singer et al., 1998).

Chapter 2 describes Epi-ID, a screening strategy in yeast that enables the direct assessment of locus specific chromatin status in thousands of gene mutants in parallel. Epi-ID takes advantage of short DNA sequences called DNA barcodes that are introduced into an array of yeast knock-out mutants, at a common chromosomal location in the genome. Chromatin immunoprecipitation on pools of barcoded mutant strains followed by barcode counting by high throughput sequencing will report on the abundance of the chromatin mark of interest in each mutant strain.

Epi-ID is applicable to a wide range of chromatin proteins and modifications that are present and can be immunoprecipitated at or around a barcoded region. **Chapter 3** describes the application of Epi-ID to identify factors required for maintaining endogenous levels of the histone variant H2A.Z that replaces one or both copies of H2A in nucleosomes downstream of promoters. Epi-ID identified the SWR1, the ATP-dependent chromatin-remodeling complex known for exchanging old nucleosomal H2A for new H2A.Z to be the main regulator of H2A.Z occupancy at promoter nucleosomes. Incidentally, no other histone chaperones or chromatin remodeling complexes were identified as candidate positive regulators. Therefore, we focused on the role of SWR1 in dynamics of H2A.Z in and outside S-phase. Here we combine Recombination-Induced Tag Exchange (RITE) (Verzijlbergen et al., 2010) tagged H2A.Z that allows to discriminate between old and newly synthesized histones and auxin-inducible degron (AID) tag on Swr1 (Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009) in H2A.Z-RITE strains that enables conditional depletion of Swr1. Using RITE and Swr1-AID we show that SWR1 is also responsible for maintaining previously deposited old H2A.Z proteins outside S-phase. Our findings suggest that control of H2A.Z levels in chromatin by SWR1 involves exchanging H2A for H2A.Z when supply of new H2A.Z is high in S-phase and maintaining previously incorporated H2A.Z when supply of new H2A.Z is low outside S-phase.

Transcription, replication, and repair processes all involve interactions of many different proteins and how these interactions are orchestrated at any given location and under changing cellular conditions is largely unknown. Acquiring this type of direct evidence requires systematically measuring protein– DNA interactions at a specific locus. This remains a major challenge due to many technical limitations. To address this problem, **Chapter 4** describes the development of Epi-Decoder, a Tag-ChIP-Barcode-Seq technology in yeast. Epi-Decoder is orthogonal to proteomics approaches and does not rely on MS but instead takes advantage of DNA sequencing.

We apply Epi-decoder to 3 different barcoded genomic loci resulting in comprehensive overviews of local chromatin interactomes. Analysis of the proteome of a transcribed locus proximal to an origin of replication revealed more than 400 interacting proteins. We also show that dynamic chromatin interactions can be identified by changing physiological conditions. Unexpectedly, Epi-Decoder could detect changes in the local chromatin proteome composition when replication stress is introduced prior to local origin firing, affecting replication proteins as well as transcription proteins. Expanding the use of barcode libraries or donor strains for the systematic transfer of barcodes to any yeast library, we demonstrate that barcode libraries of choice can be generated by harnessing clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9)-mediated genome engineering. Thus, we show that Epi-Decoder can be applied to any locus of interest.

In **Chapter 5**, we use improved Epi-Decoder libraries to observe rapid and broad rewiring of local chromatin proteomes at an actively transcribed locus following global inhibition of RNA polymerase. Improved DNA barcoding and construction of a comprehensive chromatin subset enabled efficient monitoring of the temporal dynamics of ~700 chromatin proteins in parallel. We show that treatment with 1,10-phenanthroline, an antifungal agent that inhibits transcription by chelating metals, led to a rapid and broad rewiring of local chromatin proteomes. RNA polymerase II binding was rapidly reduced, which was accompanied by loss of transcription-elongation and -termination factors and gain of chromatin remodelers and metabolic enzymes. Inhibition of transcription elongation by 6-azauracil, which acts by reducing GTP levels, led to changes that were quantitatively and qualitatively distinct from those upon treatment with 1,10-phenanthroline, confirming their different mechanism of action. Finally, we compare inhibition of Pol II by chemical means to inhibition by physiological signals during quiescence. In quiescent cells, eviction of the core transcriptional machinery was accompanied by the appearance of quiescent-cell specific repressors and rewiring of the interactions of protein-folding factors and metabolic enzymes.

Chapter 1

Additionally, we observed that proteins acting in the same complex or annotated to the same process can show dissimilar kinetics, suggesting hierarchies of chromatin binding. Capturing the temporal binding dynamics of chromatin proteins under varying conditions by Epi-Decoder provides a powerful strategy to systematically delineate protein-protein relationships and protein functions.

Overall, the development of Epi-ID and Epi-Decoder have expanded the Epi-discovery tool box, enabling systematic the measurement of chromatin regulomes and local chromatin interactomes.

Chapter 6 further summarizes the findings and focusses on their limitations and horizons.



CHAPTER 2

Epi-ID: systematic and
direct screening for chromatin
regulators in yeast by
Barcode-ChIP-Seq

2

Epi-ID: systematic and direct screening for chromatin regulators in yeast by

Barcode-ChIP-Seq

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Abstract

The assembly and regulation of chromatin requires coordinated activity of multiple mechanisms. Many factors feed into signaling networks that control the epigenome of a cell. It is this complexity that makes understanding the layers of epigenetic regulation a challenge. Genetic screens have been indispensable for studying chromatin processes. However, they can be laborious and the readout for chromatin changes is often indirect. Epi-ID is a screening strategy in yeast that enables the direct assessment of chromatin status in thousands of gene mutants in parallel. Epi-ID takes advantage of DNA sequences called DNA barcodes that are introduced into a library of yeast knock-out mutants at a common chromosomal location in the genome. Chromatin immunoprecipitation on pools of barcoded mutant strains followed by barcode counting by high throughput sequencing will report on the abundance of the chromatin mark of interest in each mutant strain. Epi-ID is applicable to a wide range of chromatin proteins and modifications that are present and can be immunoprecipitated at or around the barcoded region.

2.1 Introduction

All processes taking place on DNA, whether its transcription, replication or repair, involve chromatin. The assembly of DNA into chromatin by histone proteins can be further adjusted by post-translational modifications of histones and interactions with other proteins that bind to DNA and (un)modified histones. Thus, the composition and structure of chromatin is subject to many modifications and they are under the influence of a range of cellular signals and metabolic activities, leading to dynamic chromatin states that vary across the genome, and between cell stages and cell types. Genetic screens have proven to be indispensable for studying chromatin processes and have led to the discovery of many factors involved in the different layers of epigenetic regulation (Bonini & Berger, 2017; Fuchs & Quasem, 2014). Genetic screens can be laborious, however, and often involve functional readouts or reporter assays that can be influenced by indirect effects. To overcome these issues, we developed Epi-ID, a screening method in yeast in which a chromatin state of a reporter locus is directly assessed in a collection of mutants. Genetic screens in budding yeast have been spearheaded by the availability of systematic deletion collections of the ~5000 non-essential genes and the development of Synthetic Genetic Array (SGA) technologies to perform systematic crosses (Boone, Bussey, & Andrews, 2007; Duina, Miller, & Keeney, 2014; Giaever & Nislow, 2014; Tong & Boone, 2006). In addition, barcode technologies have been developed in which short DNA sequences or DNA barcodes serve as unique and quantifiable identifiers, allowing for parallel analysis of fitness in pools of cells (Giaever et al., 2002; Smith et al., 2009; Yan et al., 2008). In Epi-ID these two concepts are combined in a library in which each clone contains a known gene knock-out and a known pair of unique barcodes.

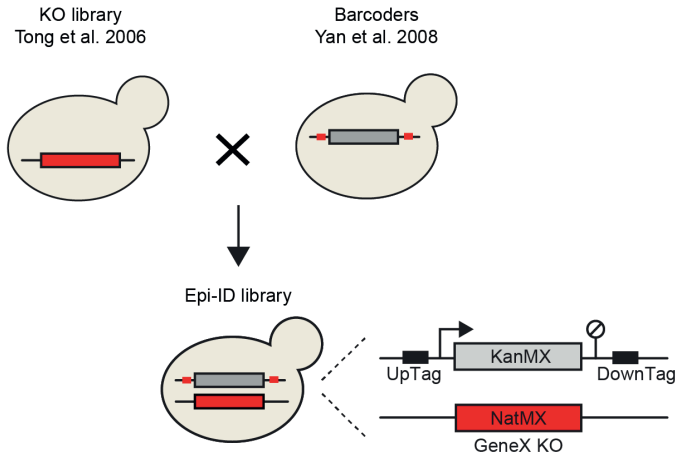


Figure 1

The Epi-ID library was created by crossing a NatMX knock-out library (Tong & Boone, 2006) with a KanMX Barcode collection (Yan et al., 2008) by using SGA methods. The two barcodes are located in distinct genomic contexts: the UpTag is located in close proximity to the promoter region and the DownTag to the terminator region of the KanMX cassette at the *HO* locus.

As depicted in Figure 1, a genome-wide Epi-ID library was created (Vlaming et al., 2016) by crossing a knock-out library (Tong & Boone, 2006) with a Barcode library (~1100 unique pairs of barcodes), which contains in each strain a barcoded KanMX reporter gene containing a G418-resistance marker at a common chromosomal safe-harbor locus, the *HO* gene (Yan et al., 2008). The cross resulted in the generation of approximately 4300 strains divided over 5 plates with unique barcodes (Vlaming et al., 2016). The gene knock-outs contain a nourseothricin (CloNat) resistance marker (NatMX). The KanMX cassette at the *HO* locus is flanked by two unique DNA barcodes: the UpTag and DownTag. The UpTag is located at the promoter region of the *HO*/KanMX gene and the DownTag in the *HO*/KanMX terminator region. Therefore, the two

barcodes are in the same region but in different functional contexts. The Epi-ID library differs from classical yeast knock-out libraries (Giaever et al., 2002) in that the barcodes are placed in a common position in the genome, rather than at the position of the deleted gene. This allows for the assessment of the chromatin feature at a common location in each strain, avoiding possible position effects that are known to influence the chromatin landscape (M. Chen, Licon, Otsuka, Pillus, & Ideker, 2013; X. Chen & Zhang, 2016).

With this Epi-ID library, one can systematically analyze how a knock-out affects the chromatin feature of interest around the DNA barcodes. Briefly, Chromatin Immunoprecipitation (ChIP) is performed on pooled Epi-ID libraries for the chromatin mark of interest, e.g. RNA polymerase or a histone post-translational modification. The DNA barcodes in the input DNA and immunoprecipitated DNA are amplified by a single PCR reaction. During the amplification, the sequence elements necessary for high-throughput sequencing are added and each sample receives a short index sequence to allow multiplexing of samples. Following barcode counting in each sample, barcode enrichment in each ChIP (compared to input) is then used as a measure for the relative abundance of the chromatin mark in each knock-out. For example, a high barcode count of a certain mutant indicates that more of the assessed chromatin feature occurred in that mutant at the barcoded locus (Figure 2).

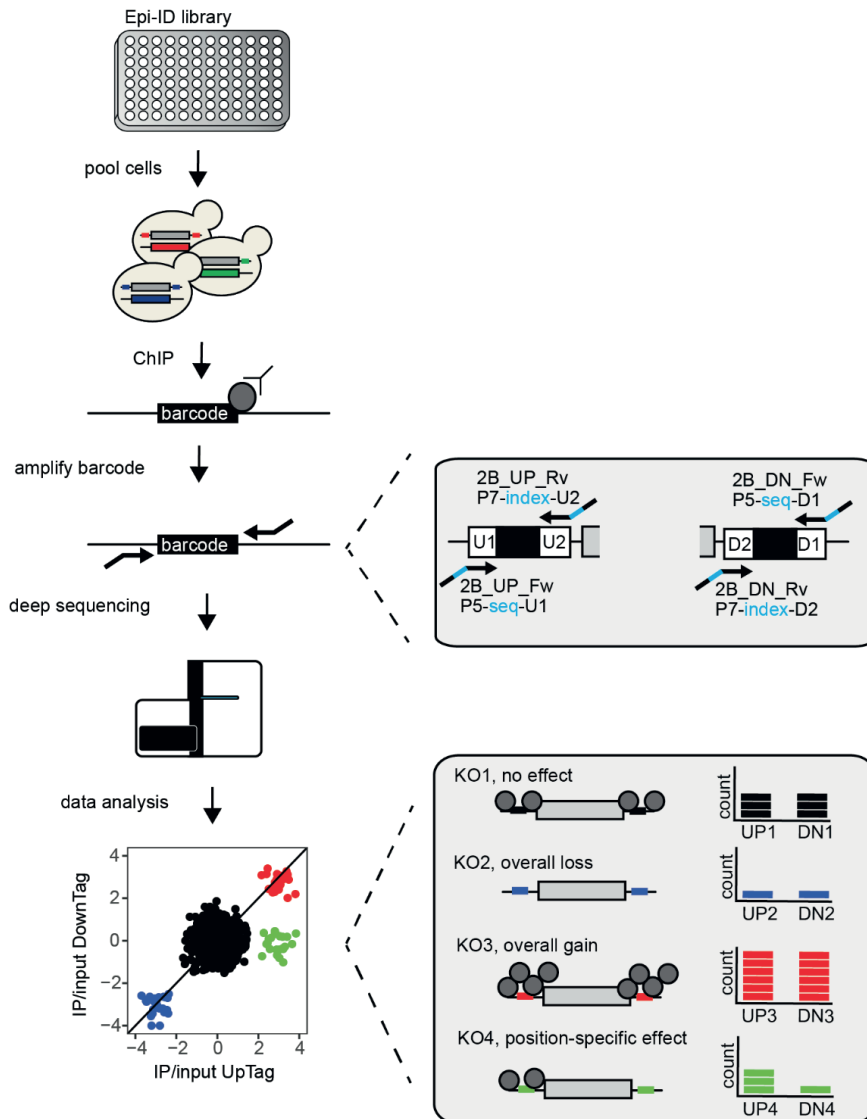


Figure 2

Schematic overview of the Epi-ID screen. The cells from each plate are pooled and a ChIP is performed for the chromatin mark of interest. The UpTag and DownTag are amplified separately with specific primers that also add adaptors for deep sequencing. The reverse primer also

introduces an index to identify each sample and allow for multiplexing. The barcodes are counted by deep sequencing. The majority of the knock-outs do not have an effect on the chromatin mark, so their median-normalized barcode count will be around 0. Increased or decreased counts identify knock-outs with altered chromatin state at the barcode. This can occur at both barcodes or specifically at the UpTag or DownTag.

A key requirement for Epi-ID is that the ChIP against the chromatin feature can successfully immunoprecipitate the barcode DNA. If no antibodies are available for the protein of interest, a gene allele coding for a tagged version of the protein can be introduced in the Epi-ID library using SGA methods. We successfully applied Epi-ID to screen for regulators of H3K79 methylation by Dot1 (Vlaming et al., 2016) and in a small-scale pilot study we combined Epi-ID with the Recombination-Induced Tag Exchange (RITE) assay in a small set of candidate KOs and identified regulators of histone turnover (Verzijlbergen et al., 2011). Finally, the development of advanced genome engineering strategies such as CRISPR-Cas9 will greatly facilitate the development of custom barcoder libraries at chromatin sites other than the HO locus. Such strategies will allow for the determination of the regulatory networks of many other chromatin modifications in the future.

2.2 Materials

Yeast strains and plasmids

The genome-wide Epi-ID library that we recently created consists of five subsets, covering 4921 NatMX gene knock-outs with KanMX barcoders. The strain background is: *MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ leu2Δ ura3Δ0 met15Δ ho::barcoded-KanMX gene::NatMX*. Each library subset is split into 3*384-well plates (with the exception of the 5th subset which is split into 2*384-well plates). The strains are stored and available upon request as 384-well glycerol freezer stock plates (Vlaming et al., 2016). The plates can be handled efficiently by robotics systems such as a RoToR (Singer Instruments). Alternatively, the library subsets can be stored and maintained as liquid pools, which may be preferred when robotics is a limitation in carrying out a screen.

Yeast media and drugs

1. G418 (Geneticin): Dissolve in water at 250 mg/ml, filter-sterilize and store in aliquots at 4°C.
2. CloNat (Nourseothricin): Dissolve in water at 100 mg/ml, filter-sterilize, and store in aliquots at 4°C.
3. YEPD plates: Dissolve 10 g yeast extract, 20 g bacto peptone, 20 g bacto agar in 860 ml water in a 1 l flask. After autoclaving, add 100 ml of 20% glucose solution, 40 ml 1 mg/ml uracil, 10 mg/ml Tryptophan. Mix thoroughly, cool to approximately 65°C, and pour plates.
4. YEPD + G418 + CloNat plates: Cool YEPD medium to approximately 65°C, add 0.8 ml of G418 (final concentration 250 mg/ml), and 1 ml of CloNat (final concentration 100 mg/L) stock solutions, mix thoroughly, and pour plates.
5. YEPD medium: Dissolve 10 g yeast extract, 20 g bacto peptone in a 1 l flask. After autoclaving, add 100 ml of a 20% glucose solution and mix thoroughly.

Yeast plates and accessories

1. Plus Plates for RoToR (PLU-001, Singer Instruments)
2. Pins for RoToR (Singer instruments)
3. Aluminum sealing tape (Nunc, cat. no. 276014) is used for resealing the 384-well plates that contain the frozen stocks of the Epi-ID library.

Buffers

1. TBS: 20 mM Tris-HCL pH 7.9, 150 mM NaCl
2. PBS: 140 mM NaCl, 2.5 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.5
3. BSA: 100 mg/ml in H₂O
4. PBSB: PBS + 5 mg/ml BSA
5. Fix Solution: 50 mM Hepes-KOH, pH 7.5, 100 mM NaCl 5 M, 1 mM EDTA, 11% Formaldehyde
6. Breaking Buffer: 100 mM Tris pH 7.9, 20% glycerol, Protease Inhibitors Cocktail tablets
7. FA buffer: 50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA 1% TritonX-100, 0.1% Na-deoxycholate, H₂O
8. FA-HS: 50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA 1% TritonX-100, 0.1% Na-deoxycholate, H₂O
9. FA+0.8% SDS: similar to FA, for 50 ml, replace 2 ml H₂O with 2 ml 20% SDS
10. FA+0.8% SDS: add 8 ml FA+0.8% SDS to 40 ml FA
11. RIPA buffer: 10 mM Tris pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate 1mM EDTA, H₂O
12. Elution buffer: 50 mM Tris pH 8, 10 mM EDTA, 1% SDS
13. TE: 10 mM Tris pH 8, 1 mM EDTA

Equipment

1. RoToR HDA from Singer Instruments (Watchet, UK)
2. Sorvall fixed angle centrifuge
3. Screw-cap tube for bead beater
4. Bead beater (BioSpec)
5. Silica/zirconia beads (0.5 mm)
6. Bioruptor Pico (Diagenode)
7. 15 ml Bioruptor Tubes and sonication beads Pico (Diagenode)
8. Protein A/G Dynabeads (Thermo Fisher Scientific)
9. Gel imaging system
10. Microcentrifuge.
11. Medium bench swing-out centrifuge.

2.3 Methods

Cell culture and preparation of cell pellets

The number of cells needed to obtain enough barcode copies for preparing a sequencing library depends on the IP efficiency of the antibody. Therefore, the IP efficiency needs to be determined at both the UpTag and DownTag for each new antibody. This can be calculated as percent input by ChIP-qPCR with primers listed in Table 1.

1. Calculate the number of cells needed (see Note 1). Here we assume a culture of 450 ml.
2. Thaw the 384-well library plates and pin directly to 1536 (3*384) YEPD agar plates with the antibiotics (see Note 2).
3. Keep at 30 °C overnight and pin to 1536 plates (YEPD+antibiotics) the following day. Repeat 2 times and prepare for the harvesting of cells on day 3.

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4. On day 3, pipette 10 ml YEPD onto the plate and scrape off the cells with a cell scraper. Transfer the medium with cells into a 50 ml Falcon tube. Repeat 3 times to collect the cells in a total of 30 ml YEPD.
5. Dilute the cell suspension 10 times to measure the OD₆₆₀. Make 450 ml YEPD culture with OD $\pm$ 0.150. Avoid cross contamination between the 5 library subsets.
6. Grow for approximately 5 h until an OD₆₆₀ between 0.500 and 0.700 is reached (an OD₆₆₀ of 0.700 should yield $\sim 1 \times 10^7$ cells).
7. Crosslink the cells by adding 1/10th of the volume fresh Fix solution (45 ml Fix solution for 450 ml culture volume) and slow shaking for 20 min at room temperature. Work in a fume hood, keep the lids closed and use fresh formaldehyde (see Note 3).
8. Add 1/20 volume 2.5 M glycine to get a final concentration of 0.125 M (22.5 ml for a 450 ml culture volume) and shake 5 more minutes at room temperature (see Note 4). Place cells at 4 °C in the subsequent steps.
9. Harvest the cells using GSA buckets in a Sorvall fixed angle centrifuge. Spin at 4000 rpm (2500 x g) for 10 min at 4 °C. If more than one bucket is required for spinning the volume of cells, spin down in multiple steps, each time pouring off supernatant and adding more culture.
10. Resuspend each pellet in 30 ml cold TBS and transfer to 50 ml Falcon tubes. Spin down at 4000 rpm (3300 x g) for 5 min at 4°C.
11. Resuspend in 2 ml TBS + 1:500 PMSF and divide over 4* 2 ml screw-cap tubes for bead beating (see Note 5). Spin down 15 seconds at max speed and remove supernatant.
12. Store cell pellets at -80 °C (at least overnight).

Coupling beads and antibody

Select protein A or G Dynabeads according to the antibody used (see Note 6). The optimal ratio of beads and antibody varies per antibody. If a new antibody is used, it is recommended to titrate the antibody for optimal beads:antibody ratio. Here, we assume a beads:antibody ratio of 20:5.

1. 20 µl of Dynabeads per IP in a 1.5 ml tube (use wide tip or cut the tip of a p200 off at ~2 mm from the tip!).
2. Concentrate the beads on a magnetic particle concentrator (MPC), remove supernatant. Do not leave beads on MPC for more than 2 min.
3. Wash the beads 3 times with 1 ml cold PBS+5 mg/ml BSA (PBSB, should be freshly prepared).
4. Add 100 µl per IP of PBSB, resuspend beads well and add antibody.
5. Rotate 2 hours to overnight at 4°C to bind (see Note 6).
6. Wash the beads 2 times with PBSB, then 1 time with cold FA.
7. Resuspend beads in 100 µl FA per IP.
8. Keep on ice until used.

Chromatin preparation

Chromatin preparation is done in bead beater tubes and 15 ml Bioruptor tubes to prepare for subsequent sonication using the Bioruptor Pico (see Note 7).

Preparing 15 ml Bioruptor tubes for sonication

1. Add 800 mg beads to the tube (this is equivalent to the 3rd line of the 15 ml Bioruptor tube graduation scale).
2. Wash the beads 3 times with PBS (add PBS, quick vortex, 5 min spin at 2000 rpm (835 x g)).
3. Remove all buffer by decanting.

4. Store tubes on ice until used.

Preparing chromatin

All reagents and tubes should be kept at 4°C (on ice). Pre-cool the buffers.

1. Resuspend the pellet in bead beater tube in 200 µl cold breaking buffer (add fresh protease inhibitors to the buffer).
2. Add ~500 µl silica/zirconia beads (0.5 mm).
3. Bead beat in bead beater in a cold block for 4 min. Change the cold block after 2 min (see Note 8).
4. Combine the samples from two bead-beater tubes into one Bioruptor tube already prepared with beads. Add 500 µl of cold FA buffer (+ PIC) to bead beater tubes, invert a couple of times and let beads sink to bottom of the tube and transfer the samples with broken cells to the prepared 15 mL Pico tube with sonication beads (see Subheading 3.3.1). Repeat 2 times with another 500 µl of FA buffer. For smaller culture volumes the reagents can be scaled down (see note 9).
5. Spin 5 min at 4000rpm (3300 x g) at 4°C to pellet chromatin and debris. Discard supernatant.
6. Wash pellet again with 1 ml of cold FA (+PIC), spin 5 min at 4000 rpm (3300 x g) at 4 °C and discard supernatant. The performance of some antibodies can be improved by adding SDS (see Note 10).
7. Add 1 ml of cold FA (+PIC) to the pellet and sonicate using the “Bioruptor Pico” for 10 min at 30 second intervals at high power. Depending on cell counts the DNA should be ~200-2000 bp, but mostly ~600 bp.
8. Add 1 ml cold FA (+PIC) to the suspension to dilute chromatin.
9. Spin 5 min at 4000 rpm (3300 x g) at 4°C to pellet debris and transfer soluble chromatin to a new 2 ml tube.

10. Take an input sample for the chromatin prep (10% of IP volume) and keep at 4 °C.

11. Snap freeze chromatin in liquid nitrogen and store at -80 °C.

Check the chromatin size

The size of the chromatin can be checked by running the purified DNA of the input samples on a gel. It is recommended to confirm that the chromatin was sheared to fragments of ~600 bp before proceeding with the ChIP. Alternatively, the ChIP can be performed immediately and the input sample de-crosslinked simultaneously with the IP samples and analyzed afterwards.

1. Prepare the input sample of the sonicated chromatin. To 20 µl chromatin, add 80 µl elution buffer and 70 µl TE (make volume equal to IP sample). Then add 0.5 µl RNase A (10 mg/ml) and 10 µl ProtK (10 mg/ml).
2. Incubate the samples for 1 h at 50 °C and then overnight (or at least 6 hours) at 65 °C to reverse crosslinks.
3. Clean up DNA using QIAquick PCR Purification Kit (Qiagen). Briefly, to each eluted ChIP sample, add 5 times volume binding buffer and apply ~650 µl to a QIAquick spin column. Spin at 13000 rpm (16,000 x g) for 1 min. Discard the flow through, add the rest of the sample and spin again. Wash with 750 µl wash buffer and spin again to dry. Elute in 30 µl elution buffer at 50 °C.
4. To check input DNA on gel, treat the column purified DNA with RNase A before loading on gel. Add 1 µl of a 1:10 dilution of RNase (10 mg/ml) to 15 µl DNA. Incubate 20 min at room temperature and load fragments on a 1% TAE gel.
5. Depending on the cell counts the DNA should be between 200 and 2000 bp, but mostly ~600 bp (Figure 3).

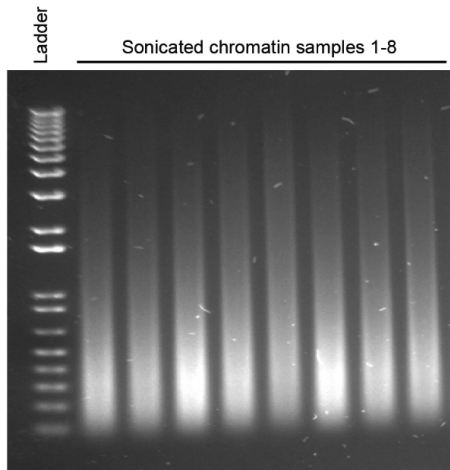


Figure 3

Sonicated chromatin from 1×10^9 cells in 1 ml of cold FA sonicated using the “Bioruptor Pico” (ten minutes with cycles of 30 s intervals on and 30 s off at high power), run on a 1% agarose gel. Depending on cell counts the DNA should be ~200-2000 bp, but mostly ~600 bp.

Chromatin immunoprecipitation

All reagents and tubes should be at 4°C (on ice). Pre-cool the buffers.

1. Use the amount of chromatin calculated previously (see Note 1).
2. Add chromatin to Dynabeads coupled to antibody.
3. Rotate at 4 °C overnight.
4. Concentrate on magnetic particle concentrator (MPC). After 30 seconds invert twice and wait 30 seconds and aspirate off the buffer (e.g. use vacuum pump with clean yellow tip).
5. Wash with 1 ml FA buffer and rotate for 5 min at room temperature (or 4 °C).
6. Repeat wash steps for total of:
 - a. 2x with FA
 - b. 2x with FA-HS

- c. 2x with RIPA
 - d. 1x with TE (when using TE, the beads do not stick well to the wall of the tube on the MPC. Do not aspirate with vacuum pump, but instead use a pipette.)
7. Add 100 µl elution buffer, incubate 10 min at 65°C in a heating block and shake at 650 rpm to prevent the beads from precipitating.
 8. Concentrate on MPC. Take the supernatant (~100 µl), add 70 µl TE, 0.5 µl RNase A (10 mg/ml) and 10 µl ProtK (10 mg/ml).
 9. Incubate the eluted samples for 1 h at 50 °C and then overnight (or at least 6 hours) at 65 °C to reverse crosslinks. Clean up DNA using QIAquick PCR Purification Kit (Qiagen). Briefly, to each eluted ChIP sample, add 5 times volume binding buffer and apply ~650 µl to a QIAquick spin column. Spin at 13000 rpm (16,000 x g) for 1 min. Discard the flow through, add the rest of the sample and spin again. Wash with 750 µl wash buffer and spin again to dry. Elute in 30 µl elution buffer at 50 °C.

Library preparation

The UpTag and DownTag are amplified separately with primers that anneal to a common sequence immediately flanking the barcode (Figure 2). The forward primer introduces the Illumina P5 sequence and extra nucleotides for annealing of the 5' end of custom sequencing primers. The reverse primer introduces the Illumina P7 sequence and a 6-base-pair unique index used as a molecular identifier for each sample to allow for multiplexing. The introduction of this unique index allows for identification of each sample+plate combination. Therefore, the ChIPs and inputs from the 5 subset Epi-ID libraries can be pooled after this point. The unique index allows for extensive pooling of up to at least 150 samples in one Illumina HiSeq lane. As a quality control for the ChIP and the primers, a test PCR is advised before using all IP material for the library preparation.

Test PCR analysis for ChIP efficiency

1. Dilute the ChIP samples for a test PCR to check the approximate efficiency for the ChIP and specificity. Use 2 μ l of the ChIP sample and make a 1:10 dilution.
2. Use 10 μ l of this in a 50 μ l PCR using the 2B_UP_Fw/2B_DN_Fw and one of the unique reverse primers (see Table 1).
3. Use the following cycle program:
 - a. 98°C for 30 sec;
 - b. 10 cycles of 98°C for 15 sec, 55°C for 20 sec and 72°C for 15 sec
 - c. 13 cycles of 98°C for 15 sec, 70°C for 20 sec and 72°C for 20 sec
 - d. final extension of 72°C for 10 min
4. Run 15 μ l on a 1.5% agarose gel to check if the PCR worked. A faint band is a good indication that the ChIP efficiency was sufficient.

Library preparation

1. Repeat the PCR described in the test, but now use as much as possible of the ChIP material (13 μ l of ChIP for UpTag and 13 μ l DownTag). For input, 3 μ l from the undiluted sample is more than sufficient.
2. Run 10 μ l of each reaction on 1.5% agarose gel and use the Gel Doc to generate an image for quantification using Image Lab software. The amplicons from each reaction (UpTag input and UpTag ChIP samples) should be run on one gel for quantification.
3. For quantifying the signal intensity of DNA bands, use the scanner-generated image and analyze using Image Lab software using volume tools. Make sure that the image is not over exposed. Any software that allows for comparing the band intensities can be used for this (see Note 11).
4. Open the image in Image Lab, select volume tools and define the area of interest using a rectangle. Then encircle the rest of the bands using the same rectangle (Figure 4). Click on the local background subtraction method, units=none and regression method=linear.

Click on analysis table to get a list of background adjusted intensity values (Adj.Vol.(int)). This table can be exported into excel and the Adj.Vol.(int) value can then be used to compare the intensities of bands across the gel.

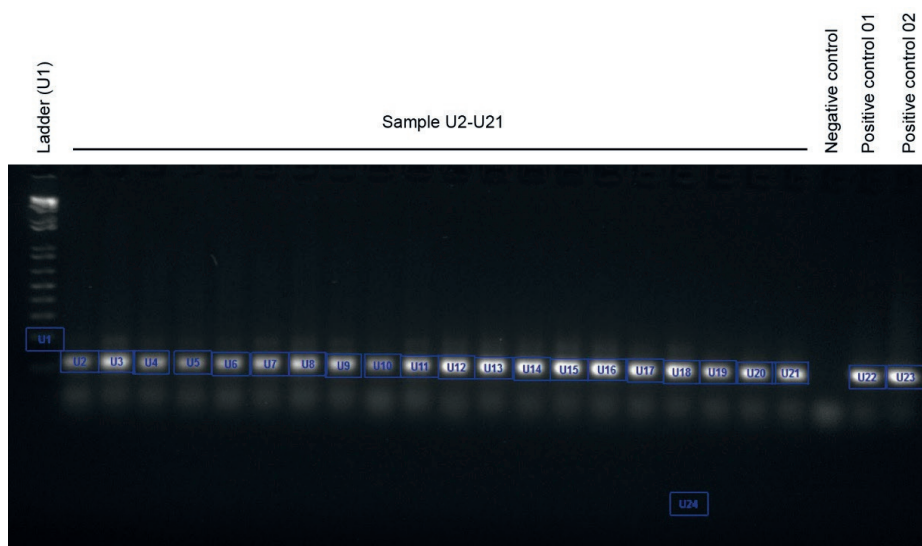


Figure 4

Image Lab quantification of amplified barcodes. 10 µl of barcode PCR reaction is run on a 1.5% agarose gel. Gel doc is used to generate an image and quantification is done with the Image Lab software (volume tools). U2-U21 are UpTag amplified PCR reactions. U1 represents the 200 bp marker from 10 µl of DNA ladder, which can be used to compare the intensity of bands between gels. U22 and U23 are bands from positive control reactions. U24 gives an idea of background intensity.

5. Once the quantification is done, mix the UpTags in equimolar amounts and repeat the steps for the DownTags.
6. Next, run the pooled UpTags and DownTags on one agarose gel. Quantify and mix the UpTags and DownTags in equimolar amounts.

7. Once mixed, run the final pool on a 1.3% agarose gel with large wells. Excise the band (100-150 bp size selection) from the gel and isolate DNA with a QIAquick gel extraction kit (Qiagen): Excise the DNA fragment with a clean scalpel and weigh the gel slice in the tube. Add 3 volumes of binding buffer and incubate in a shaking heat block at 50 °C until the gel has dissolved. Add 1 gel volume of isopropanol to the sample and mix. Place in a QIAquick spin column and centrifuge at 13000 rpm (16,000 x g) to bind DNA. Following the washing steps, elute the DNA with elution buffer to end up with approximately 40 µl purified UpTag and DownTag mix.
8. Run 3 µl on gel to check if there is enough DNA. Even a faint band is sufficient for a good sequencing run.
9. The library is now ready for sequencing with custom UpTag and DownTag sequencing primers (Table 1, 50 µM each in TE).

Sequencing and data analysis

The amplified and pooled barcodes are sequenced using a mix of custom sequencing primers for the UpTag and DownTag. These primers consist of part of the standard Illumina sequencing primers, adjusted to optimize the melting temperature and to include the U1 or D1 sequence present in the UpTag and DownTag amplicon, respectively (Table 1).

Sequencing is done on a HiSeq2500 platform (Illumina, San Diego, CA), single read, >50 bp) with High Output Run Mode. Smaller scale Epi-ID experiments can also be run on a MiSeq (Illumina). The primers described here are compatible with either platform.

To extract the barcodes and indices from each read, we developed the Perl script eXtracting Counting And Linking to Barcode References (xcalibr). This script locates the constant region in each read (U2 or D2, Figure 2) and assigns the flanking sequences to the barcode and index,

respectively. The output of this script is a counts table for each index-barcode combination. The xcalibr source code is available at <https://github.com/NKI-GCF/xcalibr>.

The counts table is further processed by removing counts below ten and dividing by the median per index to normalize for differences in library size of each pooled sample. At this point, several quality control steps could be performed (see Note 12). Finally, barcode-index combinations are converted to ORF names. Further analysis can involve dividing IP values over each other or normalizing for input of reference IPs.

2.4 Notes

1. Pull-down efficiency and calculation of culture quantities

We determined that at least 250 copies per barcode (500 copies for the UpTag and DownTag together) are required to be present in a PCR reaction to minimize jackpot effects. The following formula can be used to calculate the culture quantities required for the respective antibody.

- Required number of copies per barcodes for successful Epi-ID = 250
- Number of barcodes in pool = 1100 (barcode complexity)
- Total number of barcodes in PCR = 250×1100 (based on the two values above)
- ChIP efficiency at UpTag of your antibody = x (depends on the volume of chromatin, antibody and beads used in the ChIP)
- ChIP efficiency at DownTag of your antibody = y (depends on the volume of chromatin, antibody and beads used in the ChIP)
- Input yield (amount of ChIPable DNA that can be extracted from cells) is estimated to be 50%
- Number of cells required for UpTag = $\frac{\text{Total number of barcode loci in PCR}}{(\text{ChIP efficiency UpTag} \times \text{Input yield})}$

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- Number of cells required for DownTag = Total number of barcode loci in PCR/(ChIP efficiency DownTag * Input yield)
 - Number of cells in total ChIP = Number of cells required for UpTag + Number of cells required for DownTag
- At OD660=0.5, the number of cells/ml of culture = $\sim 7 \times 10^6$
- Culture volume required = Number of cells needed in total ChIP/ 7×10^6

2. Cell culture

The Epi-ID library contains a NatMX deletion cassette and the HO locus is replaced by a barcoded KanMX cassette. Thus, the library plates are grown on YEPD plates with CloNat (10 mg/ml) and G418 (250 mg/ml). Once the 384 glycerol plates are thawed, they are immediately arrayed in 1536 format (where 3*384 glycerol plates make one 1536-format plate). When using a library in pelleted cell format, thaw cells in 30 ml rich media and move directly to step 4 in section 3.3.2 Preparing chromatin.

3. Fresh formaldehyde in Fix solution

Fix solution should be prepared freshly with formaldehyde before use as formaldehyde is unstable and will deteriorate with time.

4. 2.5M Glycine

Glycine is added to quench the formaldehyde and is important to prevent the antibody from being crosslinked in the following steps.

5. Cells per bead beater tube

Place a maximum of 1.5×10^7 cells in one bead beater tube and divide pellet equally into an even number of tubes to make it easier for subsequent steps. Adding an excess of cells to a tube will hinder the bead beating process and will result in lower extraction of cell material.

6. Preparing beads and antibody

Coupling of antibody to Dynabeads is done for approximately 2 hours to overnight and should be performed immediately before the IP. For best results, couple beads for ~6 hours and use for IP on the same day. Select protein A or G beads according to the antibody used. For rabbit antibodies use Protein A or Protein G and for mouse and goat antibodies use Protein G beads. Protein G binds better at low pH. Make sure the pH is not higher than pH8 in subsequent steps.

7. Sonication using Bioruptor Pico

Sonication of larger volumes is more efficient when using the 15 ml sonication tubes and beads with the sonicator Pico. Optionally, a Diagenode Bioruptor sonicator can be used, but sonicating time must be adjusted accordingly to ensure that fragments are approximately ~600 bp.

8. Bead beating large cell volumes

Bead beating is done in bead beater tubes using a bead beater. If more cells/tube are used, check under microscope to verify that the cells have lysed. Cells should be almost 100% broken. If not, bead beat a bit longer.

9. Preparing chromatin for smaller culture volumes

For cultures of 150 ml with OD660 of 0.5-0.7, collect pellets into one bead beater tube. Following lysis, continue all steps (step 5 to step 9) with half the volume of reagents.

10. Using SDS in buffers

Some antibodies work best on proteins that are denatured by SDS. For such antibodies, steps 6 to 8 of section 3.3.2 Preparing chromatin should be modified as described below.

6. Wash pellet again with 1 ml of cold FA (+PIC+0.13%SDS), spin 5' 4K at 4°C discard supernatant.

7. Add 1 ml of cold FA (+PIC+0.8%SDS) to pellet and sonicate using the “Bioruptor Pico” 10' at 30 second intervals at high power. Following sonication add 1 ml cold FA (+PIC) to the suspension.
8. Add 1 ml cold FA (+PIC) to the suspension to dilute chromatin. At this point, the SDS concentration is ~0.4%. For ChIP, the SDS concentration needs to be lowered to ~0.12% by adding FA buffer to prevent antibody denaturation.

11. Quantifying DNA with a Bioanalyzer

The protocol described here uses the Gel Doc imaging system to quantify the relative amount of DNA in the ChIP and input library samples. We note here that it is also possible to quantify the DNA samples by using a Bioanalyzer.

12. Quality control by using plate signatures

The five different plates of the genome-wide Epi-ID library use the same barcoder set but each has a specific signature of empty spots corresponding to missing barcode pairs. Visualization of this signature can be done by plotting the values of each column (representing the counts of one indexed sample) of the counts table. This plot allows for an extra check to verify that the correct plate number was used and reveals potential mix-ups. In addition, the barcodes of the empty spots should be completely absent. A low barcode count of empty spots can be an indication of cross-contamination between libraries or samples.

2.5 Acknowledgements

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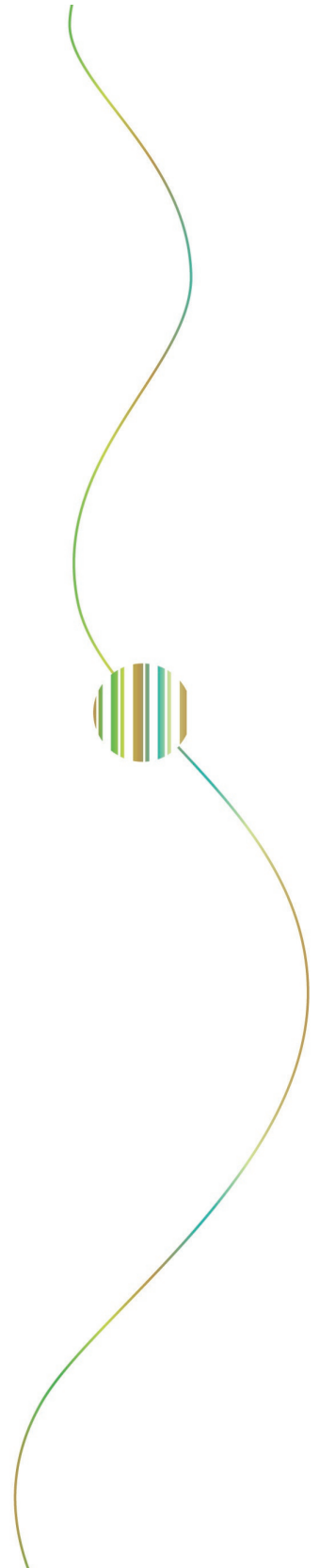
Table 1: List of the primers used to perform an Epi-ID screen.

Name	Sequence
HOpromQfw	GAAGCTTGTTGAAGCATGATGAA
HOpromQrev	TTGCTGCTTATGAGGATATGGATTT
HOtermQfw	GAGTAGAAATACGCCATCTCAAGATACA
HOtermQrev	GGAAAGTTGATCAAGACCCAATAATAA
2B_UP_Fw	AATGATACGGCGACCAACCGAGATCT <u>ACACCTTCCGATCT</u> AGATGTCCACGAGGTCTCT
2B_DN_Fw	AATGATACGGCGACCAACCGAGATCT <u>ACACTCTTCCGATCT</u> ACGGTGTCGGTCTCGTAG
2B_UP_Rv_all	CAAGCAGAAGACGGCATAACGAGATNNNNNNGTCGACCTGCAGCGTACG
2B_DN_Rv_all	CAAGCAGAAGACGGCATAACGAGATNNNNNNAACGAGCTCGAATTCATCGA
2B_UP_Seq	<u>ACACCTTCCGATCT</u> AGATGTCCACGAGGTCTCT
2B_DN_Seq	<u>ACACTCTTCCGATCT</u> ACGGTGTCGGTCTCGTAG

The qPCR primers amplify the HO promoter and terminator. These can be used on the ChIP material to determine the IP efficiency of each antibody. 2B_UP_Fw and 2B_UP_Rv_all amplify the UpTag, and 2B_DN_Fw and 2B_DN_Rv_all the DownTag. 2B_UP_Fw and 2B_DN_Fw introduce the Illumina P5 sequence, a custom sequencing region to anneal sequencing primers (underlined region) and the U1/D1 common region next to the barcodes. 2B_UP_Rv_all and 2B_DN_Rv_all introduce the Illumina P7 sequence, a unique 6-bp index to identify each sample (indicated by N's) and the U2/D2 common region next to the barcodes. A list of the 6-bp indices that we used is available upon request. 2B_UP_Seq and 2B_DN_Seq are custom sequencing primers that consist of the custom sequencing region (underlined region) and the U1/D1 region.

CHAPTER 3

Swr1 controls H2A.Z levels
in *S. cerevisiae* by incorporation
of new and maintenance
of old histones



3

Swr1 controls H2A.Z levels in *S. cerevisiae* by incorporation of new and maintenance of old histones

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Manuscript submitted

Abstract

Histone H2A.Z, the major variant of histone H2A, is a conserved player in gene regulation and is enriched at regulatory regions characterized by dynamic nucleosomes. Together with its effects on nucleosome stability and mechanism of deposition by histone exchange, this suggests that H2A.Z is potentially a highly unstable chromatin feature. Here we investigated the dynamics of H2A.Z in yeast. Although new H2A.Z incorporation can occur independently of replication, in G1-arrested cells little new H2A.Z synthesis was observed compared to cells passaging through S-phase. SWR1, the ATP-dependent chromatin-remodeling complex known for exchanging old nucleosomal H2A for new H2A.Z was found to also be responsible for maintaining previously deposited old H2A.Z proteins outside S-phase. H2A.Z expression, which peaks in S-phase like that of canonical histones, influences the dynamics of H2A.Z because ectopic expression of H2A.Z outside S-phase led to rapid incorporation of new H2A.Z and loss of resident H2A.Z protein. Our findings suggest that control of H2A.Z levels in chromatin by SWR1 involves exchanging H2A for H2A.Z when supply of new H2A.Z is high in S-phase and maintaining previously incorporated H2A.Z when supply of new H2A.Z is low outside S-phase.

3.1 Introduction

The chromatin landscape of the eukaryotic cell is dynamic and influences the gene expression profile in many different ways (Venkatesh & Workman, 2015; K. Zhou, Gaullier, & Luger, 2019). One well known dynamic system is the addition and removal of post-translational modifications on histone proteins, which can alter chromatin structure and influence critical DNA transactions such as DNA repair and gene transcription (Tolsma & Hansen, 2019). An additional dynamic layer of marking chromatin states involves the replacement of canonical histone proteins by histone variants (Avvakumov, Nourani, & Cote, 2011; Filipescu, Szenker, & Almouzni, 2013; Giaimo et al., 2019; Huang & Zhu, 2014; Karolin Luger, Dechassa, & Tremethick, 2012; P. B. Talbert, Meers, & Henikoff, 2019; Weber & Henikoff, 2014; K. Zhou et al., 2019; Zink & Hake, 2016).

While the major wave of incorporation of canonical histones occurs during DNA replication, incorporation of histone variants can occur throughout the cell cycle and is not restricted to S-phase. When a nucleosome is lost outside S-phase, the (H3-H4)₂ tetramer is usually replaced by H4 and the histone variant H3.3. The assembly of H3.3 occurs by a gap-filling mechanism, similar to the replication-coupled deposition of canonical H3 (Filipescu et al., 2013; Weber & Henikoff, 2014). In some organisms, including budding yeast, one H3 type is used for both replication-dependent and –independent H3 assembly (Oliver J. Rando & Winston, 2012). In budding yeast, promoter regions show high turnover of (canonical) histone H3 (Dion et al., 2007; Radman-Livaja et al., 2011). Promoter nucleosomes in yeast and other eukaryotes are also enriched for histone variant H2A.Z, the major variant of canonical histone H2A (Albert et al., 2007; Bagchi, Battenhouse, Park, & Iyer, 2019; Yen, Vinayachandran, & Pugh, 2013). This variant is highly conserved throughout evolution and has a role in a diverse set of cellular processes, such as transcription, splicing, chromosome segregation, heterochromatin silencing, cell cycle progression, and meiosis (Giaimo et al., 2019; González-Arranz et al., 2018; Neves et al., 2017;

Nissen et al., 2017; Skene & Henikoff, 2013; Subramanian, Fields, & Boyer, 2015; Venkatesh & Workman, 2015; Weber & Henikoff, 2014; Zink & Hake, 2016).

The majority of H2A.Z is incorporated by the chromatin-remodeling complex SWR1 in yeast and SCRAP and p400/Tip60 in metazoans (Mattioli, D'Arcy, & Luger, 2015) by an ATP-dependent stepwise exchange mechanism in which H2A-H2B dimers are replaced with H2A.Z-H2B dimers (Clapier, Iwasa, Cairns, & Peterson, 2017; Luk et al., 2010; Mizuguchi et al., 2004; Pradhan et al., 2015; Willhoft et al., 2018). Additionally, the histone chaperones Nap1 and Chz1 are known to interact with SWR1 to deliver H2A.Z for deposition into chromatin (Dronamraju et al., 2017; Y. Wang et al., 2019). On the other hand, two other histone chaperones, FACT and Spt6, prevent accumulation of H2A.Z in gene bodies (Jeronimo, Watanabe, Kaplan, Peterson, & Robert, 2015). The binding of SWR1 to chromatin requires nucleosome-free DNA (Ranjan et al., 2013; Ranjan et al., 2015; Willhoft et al., 2018; Yen et al., 2013), which together with the act of transcription (Bagchi et al., 2019) and the action of FACT and Spt6 provides an explanation for the predominant localization of H2A.Z to the -1 and +1 nucleosomes flanking the nucleosome-depleted region (NDR) around the transcription start site (TSS). There is also evidence for a coordinated deposition of H2A.Z and H3.3 by the p400 ATP-dependent remodeler, functionally connecting two histone variants (Pradhan et al., 2015). During isolation of chromatin from cells, nucleosomes containing both H2A.Z and H3.3 are particularly unstable (Henikoff, 2009; Jin et al., 2009) and H2A.Z has been suggested to favor nucleosome disassembly (Osakabe et al., 2018; Petesch & Lis, 2012; Rudnizky et al., 2016; Weber & Henikoff, 2014; Weber, Ramachandran, & Henikoff, 2014).

The mechanism of its assembly, its presence in dynamic regions, and its role in nucleosome stability together suggest that H2A.Z is potentially a highly dynamic chromatin feature. Although H2A.Z has been associated with dynamic nucleosomes this relationship is not completely understood (Dai, Xu, & Zhou, 2019; de Jong & van Noort, 2019; Giaimo et al., 2019; Paul B. Talbert & Henikoff, 2016; K. Zhou et al., 2019). Here, we took advantage of Recombination-Induced Tag Exchange (RITE) (Verzijlbergen et al., 2010) to discriminate between old and newly synthesized histones and determine the dynamics of H2A.Z under physiological conditions *in vivo* in non-replicating cells.

3.2 Results

Limited replication-independent deposition of H2A.Z in G1- compared to S-phase

To capture the dynamic behavior of H2A.Z, we used RITE to simultaneously monitor old and newly synthesized histones (Verzijlbergen et al., 2010). RITE enables conditional switching of epitope tags by controlling Cre-recombination in a hormone-dependent manner (Fig. 1A) (Radman-Livaja et al., 2011; Terweij et al., 2013; Verzijlbergen et al., 2010). RITE tags were introduced at the C-terminus of either H2A.Z or canonical histone H3 in budding yeast as a control. Upon activation of Cre recombinase, the tags encoded in the genome switch from old V5 (pre-switch) to new HA (post-switch). Strains carrying RITE tags on H2A.Z behaved like wild-type strains when grown in the presence of caffeine or at low temperature (Fig. S1A), confirming that the tags on H2A.Z did not functionally compromise H2A.Z (Morillo-Huesca, Clemente-Ruiz, Andujar, & Prado, 2010). Similarly, RITE tags on the essential H3 protein did not affect the growth of yeast cells under the conditions examined (Fig. S1A). Next, global deposition of H2A.Z was determined using an arrest-release protocol in which replication-independent histone dynamics in G1 could be compared to replication-dependent dynamics of cells passaging through S-phase (Figs. 1B and S1B). Global

levels of old and new histones were examined by immunoblot analysis using an antibody against the LoxP-encoded peptide that simultaneously detects the old and new protein species. As expected, new H3 was readily detectable in replicating cells (Fig. 1C). Also, in non-replicating cells, a substantial amount of new H3 was detected, in agreement with previous studies by us and others (Dion et al., 2007; Jamai, Imoberdorf, & Strubin, 2007; Radman-Livaja et al., 2011; Rufiange, Jacques, Bhat, Robert, & Nourani, 2007; Shukla et al., 2018; Verzijlbergen et al., 2010). In contrast, little new H2A.Z was detected in G1-arrested cells when compared to cell passaging through S-phase while a substantial fraction of old H2A.Z was maintained (Fig. 1C). Immunoblot measures global histone levels in the cell, which may not reflect the chromatin-bound fraction. However, we confirmed that most of the H2A.Z and H3 protein in the cell was associated with chromatin (Figs. S1C-D and see ChIP experiments below), which was expected since histones not incorporated into chromatin are known to be rapidly degraded (Gunjan, Paik, & Verreault, 2006; Singh, Kabbaj, Paik, & Gunjan, 2009). Thus, although we expected H2A.Z to be a highly dynamic chromatin feature with a replication independent assembly mechanism, we observed low levels of new H2A.Z in G1-arrested cells. The majority of new H2A.Z-containing histones were deposited in a replication coupled manner.

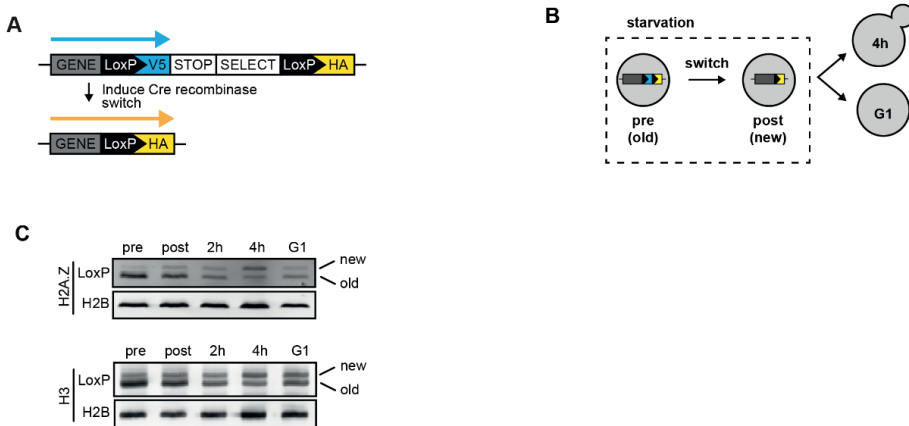


Figure 1. Limited replication-independent deposition of H2A.Z in G1

(A) Outline of Recombination-Induced Tag Exchange: recombination between LoxP sites by hormone-induced Cre recombinase causes a permanent switch from 1xV5 (old tag) to 1xHA-6xHis (HA; new tag) in the coding sequence of the protein of interest. (B) For arrest-release experiments cells were first arrested by nutrient starvation, incubated in the presence of estradiol to activate Cre-recombinase “switch”, and finally released into fresh media with or without alpha factor, which arrests the cells in G1 (see Fig. S1B). (C) Anti-LoxP immunoblot of the old (V5) and new (HA) tag on H2A.Z and H3. The old and new protein isoforms can be distinguished due to their different mobility. An antibody against the spacer-LoxP sequence simultaneously detects old and new RITE-tagged proteins. Histone H2B was used as loading control.

The SWR1 chromatin-remodeling complex is the main regulator of H2A.Z occupancy at promoter nucleosomes

To identify candidate factors and mechanisms responsible for regulating H2A.Z levels in replicating cells, we performed an unbiased genome-wide chromatin-regulator screen. For this purpose we applied Epi-ID (Poramba-Liyanage, Korthout, & Van Leeuwen, 2019; Verzijlbergen et al., 2011; Vlaming et al., 2016) to search for factors required for maintaining endogenous levels of H2A.Z at a barcoded promoter (Figs. 2A). Briefly, in Epi-ID, each knock-out is paired with a unique barcode at a common location. In a pooled ChIP-Seq experiment and following sequencing and counting of DNA barcodes, the relative H2A.Z ChIP efficiency of each barcode (ChIP/input) reports on the H2A.Z abundance around the barcoded locus in each knock-out clone (Figs. 2B and S2A-B). H2A.Z is present at the barcoded promoter, as confirmed by ChIP-qPCR (Fig. S2A) and as shown recently by using a TAP-tagged version of H2A.Z (Korthout et al., 2018). This Epi-ID screen identified SWR1 as the main complex responsible for maintaining H2A.Z levels in this

promoter region (Fig. 2C and S2C) and no other histone chaperones or chromatin remodeling complexes were identified as candidate positive regulators. Therefore, we focused on the role of SWR1 in dynamics of H2A.Z in and outside S-phase.

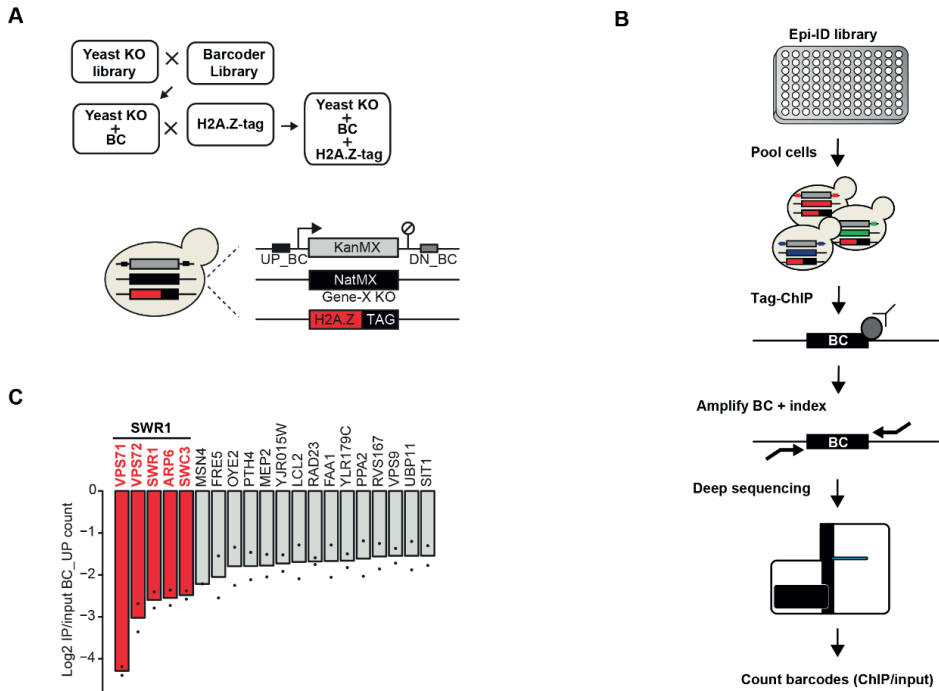


Figure 2. The SWR1 chromatin-remodeling complex is the main regulator of H2A.Z occupancy at promoter nucleosomes.

(A) Generation of the Epi-ID libraries using the SGA technology resulting in a genome-wide library of knock-out mutants, each harboring a unique pair of barcodes and epitope-tagged H2A.Z (eH2A.Z). The Up barcode (dark, BC UP) is located in a promoter containing H2A.Z, the Down barcode (light, BC DN) in a terminator region lacking H2A.Z.

(B) Outline of Epi-ID. In a pool of cells, the ChIP-efficiency, as determined, by the barcode count in high throughput sequencing, is a measure for the amount of H2A.Z at the barcoded reporter locus in each knock-out.

(C) Bar plot of the twenty mutants with the lowest H2A.Z levels at the UP barcode (lowest IP/input barcode counts). The red bars indicate all members of the SWR1 complex that were present in the library. Data are represented as mean $\pm$ range of two biological experiments. See Supplementary Fig. 4B for a scatter plot of the two biological experiments.

H2A.Z deposition in non-replicating cells relies on the enzymatic activity of SWR1

To investigate the role of SWR1 in controlling H2A.Z levels in S-phase and outside S-phase, we introduced an auxin-inducible degron (AID) tag on Swr1 (Nishimura et al., 2009) in H2A.Z-RITE strains. This enabled conditional depletion of Swr1 because the addition of the plant hormone auxin leads to ubiquitination of Swr1-AID by TIR1, which in turn leads to proteasome-mediated degradation (Fig. 3A). With this setting we performed the arrest-switch protocol and added auxin at the time of the H2A.Z tag-switch, resulting in depletion of Swr1 in cells in which Swr1 had deposited old H2A.Z prior to its auxin-induced degradation (Fig. 3B). In the presence of Swr1 (+ Auxin, no TIR1) old H2A.Z was maintained and some new H2A.Z was made in switched cells, as described above. However, upon auxin-induced degradation of Swr1-AID (+ Auxin, + TIR1), no new H2A.Z was incorporated but also previously incorporated old H2A.Z was much reduced in non-replicating cells (Fig. 3C). This indicates that after deposition of H2A.Z an active mechanism involving Swr1 continues to be required for maintenance of old H2A.Z. The Swr1-mediated exchange of H2A-H2B dimers for H2A.Z-H2B dimers is ATP-dependent. Swr1 also interacts with other members of the SWR1 complex and has been shown to possess histone chaperone activity (Hong et al., 2014; V. Q. Nguyen et al., 2013). Therefore, we examined whether old H2A.Z

maintenance by Swr1 is dependent on the ATPase activity or other functional domains of Swr1. For this purpose, an extra copy of wild-type or mutant FLAG-tagged Swr1 (W.-H. Wu et al., 2009) was introduced in a strain harboring a conditional Swr1-AID allele and we asked whether Swr1 could rescue the maintenance of old H2A.Z after auxin-induced depletion of endogenous Swr1-AID (Figs. 3). Wild-type Swr1 and a mutant missing the short N-terminal region that binds the smallest SWR1 subunit Swc7 ($\Delta N1$) showed efficient rescue; expression of these proteins prevented the global loss of old H2A.Z (Fig. 3E). In contrast, a catalytically inactive mutant (Swr1-K727G) or a mutant missing the N-terminal N2 domain that interacts with several SWR1 subunits and H2A.Z-H2B dimers ($\Delta N2$) could not rescue the maintenance of old H2A.Z in the absence of endogenous Swr1 (Fig. 3E), even though the mutant proteins were expressed at equal levels as the wild-type protein (Fig. S3A). Analysis of old H2A.Z by ChIP at different promoters also supported these findings and showed that the rescue by the $\Delta N1$ mutant was less efficient than that of wild-type Swr1 (Fig. 3F) under these conditions. The loss of old H2A.Z upon degradation of Swr1 agrees with the presence of a replication-independent H2A.Z maintenance mechanism that is dependent on the ATPase activity of Swr1 as well as its interactions with other members of the SWR1 complex. Using chromatin-fractionation experiments, we observed that SWR1 activity also affected native chromatin-bound H2A.Z levels more in arrested cells than in replicating cells (Fig. S3B), providing further support for this model in an independent experimental set up.

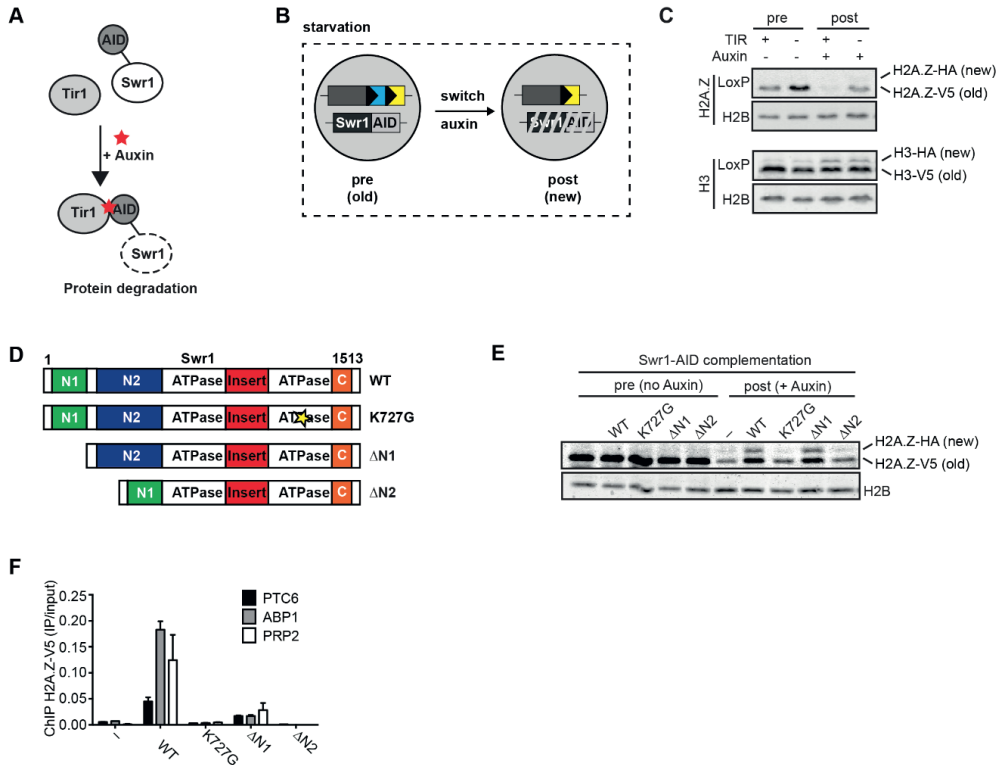


Figure 3. H2A.Z deposition in non-replicating cells relies on the enzymatic activity of SWR1

(A) The Auxin-Inducible Degron System (AID) was applied to Swr1 in strains expressing the Tir1 ubiquitin ligase to target Swr1 for proteasomal degradation upon the addition of auxin. **(B)** For arrest-release experiments cells were first arrested by nutrient starvation and then incubated in the presence of estradiol to activate Cre-recombinase “switch” and auxin for Swr1 degradation. **(C)** Addition of auxin during the H2A.Z-tag-switch of arrested cells led to the loss of old H2A.Z. H3 was not affected by the degradation of Swr1. **(D)** Schematic overview of the Swr1 protein domains, including the locations of the mutations that were present in the plasmids used for the Swr1-AID complementation assay shown in Figure 4E-F. The N1 region of Swr1 is required for the interaction with the Swc7 subunit of the SWR complex. The N2 region interacts with several SWR1

subunits and is important for the integrity of SWR1. The K727G mutation. (E-F) Global levels and chromatin abundance at 3 promoter regions of old H2A.Z in Swr1-AID cells was rescued by plasmids expressing wild-type Swr1 and mutant $\Delta N1$, but not by a catalytically dead mutant (K727G) or the $\Delta N2$ deletion mutant of the N-terminal tail of Swr1 (See Supplementary Fig. 3A for schematic overview of the Swr1 protein domains and Supplementary Table 1 for list of primers).

Maintenance of old H2A.Z and incorporation of new H2A.Z is determined by H2A.Z expression level

The H2A-H2A.Z-exchange reaction by SWR1 can in principle occur throughout the cell cycle. However, our findings suggest that new H2A.Z incorporation is not a constitutive mechanism. Examination of the mRNA levels of H2A.Z showed that they are not constant throughout the cell cycle but peak following S phase, similar to canonical histones (Fig. S4A, and see Cyclebase.org (Santos, Wernersson, & Jensen, 2015) and previous studies (Kudlicki, Rowicka, & Otwinowski, 2007; Pramila, Wu, Miles, Noble, & Breeden, 2006; Spellman et al., 1998; Takahashi et al., 2017)). Since the peak in H2A.Z mRNA expression correlates with the incorporation of new H2A.Z (Fig. 1C), we tested whether supply of H2A.Z can be a determinant of H2A.Z dynamics. To address this question, we modified the RITE assay by adding an extra copy of new H2A.Z under the control of a galactose-inducible *GAL1* promoter (RITE+). When released in galactose-containing media, new histone H2A.Z is synthesized from the endogenous *HTZ1/H2A.Z* locus as well as the constitutively expressed ectopic copy (Fig. 4A). A regular RITE-strain lacking the ectopic copy (RITE) was grown under identical conditions for comparison (Figs. 4A and S4B). Ectopic expression of new H2A.Z led to increased levels of new H2A.Z in the cell. The higher levels of new H2A.Z were accompanied by lower levels of old H2A.Z (Fig. 4B; compare RITE+ with RITE). Analysis of chromatin-bound H2A.Z by ChIP-qPCR confirmed the increase of new and the loss of old H2A.Z upon overexpression of new H2A.Z (Fig. 4C). These results together show that the

expression level of H2A.Z determines the balance between new H2A.Z deposition and old H2A.Z maintenance.

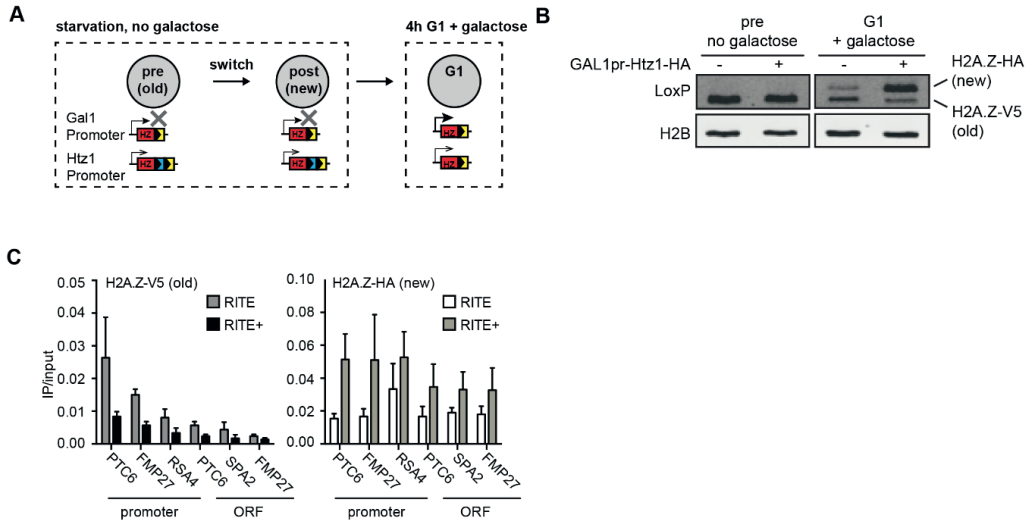


Figure 4. Maintenance of old H2A.Z and incorporation of new H2A.Z is determined by H2A.Z expression level

(A) Histone H2A.Z-RITE tag switch (old V5 to new HA) was combined with a galactose inducible copy of new H2A.Z-HA (GAL1pr-H2A.Z-HA; RITE+) to force expression of H2A.Z in G1. For arrest-release experiments cells were first arrested by nutrient starvation and then incubated in the presence of estradiol to activate Cre-recombinase “switch” and finally released into galactose media with alpha factor, which arrests the cells in G1 and induces the expression of H2A.Z. **(B)** Immunoblot showing H2A.Z before the switch (pre) and the increased levels of new H2A.Z after the switch when cells were arrested in G1 for 4 hours in the presence of galactose (4h G1) compared to a strain without the extra H2A.Z copy (See Fig. S4B). **(C)** ChIP-qPCR for H2A.Z-V5 (old) and new H2A.Z-HA (new) confirmed that ectopic H2A.Z was incorporated in chromatin and

partially replaced old H2A.Z. Data are represented as mean of three biological experiments $\pm$ standard deviation.

3.3 Discussion

Histone dynamics in interphase is a complex but fundamental process involved in controlling chromatin structure and accessibility (Deal & Henikoff, 2010; Deaton et al., 2016; Venkatesh & Workman, 2015). Indeed, histone turnover, the process of evicting and replacing nucleosomal histones by newly synthesized ones, is emerging as an important layer of epigenome control that can influence genome transactions in many different ways. SWR1 has previously been identified as an ATP-dependent complex that incorporates the histone variant H2A.Z in exchange for H2A. Our findings suggest a model in which H2A.Z is subject to two mechanisms of control by SWR1. First, a peak in expression of H2A.Z in S phase allows for the assembly of new H2A.Z by SWR1 in replicating cells. In the absence of SWR1, H2A.Z is incorporated by other mechanisms in cycling cells. Second, the low supply of H2A.Z in non-replicating cells results in the active maintenance of a dynamic pool of old H2A.Z. In the absence of SWR1, a substantial amount of H2A.Z is lost from chromatin outside S phase suggesting that SWR1 is responsible for maintaining H2A.Z in non-replicating cells. Given the critical role of the ATPase activity of SWR1, we expect that both mechanisms involve the H2A-H2A.Z exchange reaction performed by SWR1. An interesting consequence of the maintenance mechanism that we propose here is that information encoded in promoter histones can be retained even when promoter nucleosomes are dynamic and undergo histone disassembly and re-assembly if old histones are maintained at the same nucleosome. Interestingly, changes in H2A.Z incorporation during different cell-cycle phases is also observed in mouse trophoblast cells and pericentric heterochromatin of mouse fibroblasts (Boyarchuk, Filipescu, Vassias, Cantaloube, & Almouzni, 2014; Nekrasov et al., 2012). It will be interesting to

determine whether to what extent these difference are driven by changes in H2A.Z supply and/or H2A.Z maintenance.

3.4 Methods

Yeast strains and growth conditions

Yeast strains were cultured under standard conditions (van Leeuwen, Gafken, & Gottschling, 2002). Strains were grown in YEPD (1% yeast extract, 2% bacto peptone, 2% glucose) in shaking flasks at 30°C. To select for the presence of the RITE cassette, cells were grown in YEPD containing 200 µg/ml Hygromycin. For RITE+ experiments, strains were grown in YEPraf (1% yeast extract, 2% bacto peptone, 3% raffinose) pre- and post-switch, and strains were released in YEPgal (1% yeast extract, 2% bacto peptone, 3% galactose) in shaking flasks at 30°C. Yeast strains used in this study are listed in Supplementary Table S1. UCC7179 was derived from BY4705a (van Leeuwen & Gottschling, 2002) by inserting *ADE2* at TEL-VR using pHR10-6 (van Leeuwen et al., 2002) and replacing *HTZ1* by *URA3* using pRS306 (Brachmann et al., 1998). RITE strain construction was described previously (Terweij et al., 2013). NKI2364 was constructed in the following steps to insert a KanMX-PGAL1-HTZ1-HA-6xHis cassette at the HO safe-harbor locus. First, a piece of the HO promoter was amplified from genomic yeast DNA using primers HOUpFwd1 and HOUpRev1 and cloned into the Sall site of pRS304 (Brachmann et al., 1998) to create pTW090. Second, a KanMX-PGAL1 cassette from pFA6a-KanMX-PGAL1 (Wach, Brachat, Alberti-Segui, Rebischung, & Philippsen, 1997) was inserted in front of the HTZ1-RITE locus of strain NKI8053. Third, following a tag-switch, the KanMX-PGAL1-HTZ1-HA-6xHIS cassette was amplified using primers GZ1 and GZ2 and cloned into the AflII site within the HO promoter fragment of pTW090 to create pTW118. Fourth, a NarI restriction fragment of pTW118 was targeted to the HO promoter to create NKI2364. Strain NKI2346 was generated by inserting a

URA3-P_{ADH1}-OsTIR1-9xMyc cassette at the HO promoter. First, an HO promoter fragment was amplified from pTW090 using primers HD1 and HD2, digested with AatII and cloned into the AatII site of pNHK53 (Nishimura et al., 2009) to generate pTW116. pTW116 was digested with AflII and transformed into strain NKI8053. Strain NKI2389 was generated by inserting an AID-KanMX cassette of pTW115 behind the SWR1 gene in strain NKI2346. pTW115 was made by inserting a 2xT7 tag in the NarI and KpnI sites of pMK43 (Nishimura et al., 2009) using the annealed product of primers NarI 2xT7+G KpnI FW and NarI 2xT7+G KpnI RV. Single copy SWR1 vectors (pRS416-SWR1-2Flag) were introduced into strain NKI2433 and NKI2389 following digestion with StuI and co-transformation with the NatMX cassette amplified from pFvL99 (van Welsem et al., 2008) to replace the *URA3* marker by a NatMX marker. NKI6061 was described previously (Stulemeijer et al., 2015).

Whole-cell extracts in SUMEB

Samples of 2×10^8 cells were harvested and washed with TE (10 mM Tris pH 8, 1 mM EDTA) containing 0.2 mM PMSF. Cell pellets were stored at -80°C until further processing, but at least thirty minutes. Whole-cell extracts were prepared in SUMEB (1% SDS, 8 M Urea, 10 mM MOPS pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) containing protease inhibitors (1 mM PMSF, 1 mM DTT, 5 mM benzamidine, 1 µg/ml pepstatin, 1 µg/ml leupeptin) by glass bead disruption in a mini bead beater (BioSpec). The resulting lysate was incubated for 10 minutes at 65°C and subsequently clarified by centrifuging 5 minutes at 14000 rpm. Samples were stored at -20°C. Prior to gel separation, samples were incubated for 5 min. at 65°C and centrifuged for 1 min. at 14000 rpm at room temperature.

Chromatin Association Assay (CAA)

Samples of 2×10^8 cells were paralyzed with 0.1% final concentration sodium azide. Samples were centrifuged for 2 min at 3000 rpm. Samples were washed with 25 ml H_2O and centrifuged for 2 min at 3000 rpm. Cells were resuspended in 0.5 ml pre-spheroplast buffer (100 mM PIPES KOH pH 9.4, 10 mM DTT) and incubated for 30 min at $30^\circ C$ while shaking. Cells were centrifuged for 2 min at 3000 rpm and incubated in 0.3 ml spheroplast buffer (50 mM KPO_4 , 0.6 M sorbitol, 10 mM DTT, 0.5 mM PMSF) with 27 units of Lyticase for 30 min at $30^\circ C$ while shaking. Formation of spheroplast was determined by microscopy and was at least 95%. Cells were centrifuged 3 min at 3000 rpm at room temperature and resuspended in 150 μl lysis buffer (20 mM PIPES pH 6.8, 150 mM NaCl, 0.4 M sorbitol, 50 mM KOAc, 3 mM $MgAc_2$, 0.5 mM PMSF, Protease Inhibitor Cocktail mini EDTA free (Roche), 1 tablet/5 ml lysis buffer) using a wide-bore tip. Samples were kept on ice from this step onwards. Cells were centrifuged for 2 min at 4000 rpm at $4^\circ C$. Supernatant was discarded and cells were resuspended in 75 μl lysis buffer using a wide-bore tip. To lyse the cells, 0.1% final concentration of Triton-X100 was added. A whole-cell extract sample was taken and samples were centrifuged for 5 min at 14000 rpm at $4^\circ C$. The supernatant (cytoplasm) was transferred to a new 1.5-ml tube. The pellet (nuclei) was washed with 200 μl lysis buffer with 0.1% final concentration Triton-X100 and centrifuged for 5 min at 14000 rpm at $4^\circ C$. Nuclei were resuspended in 75 μl lysis buffer and Triton-X100 was added to 1% final concentration to lyse the nuclei. Samples were incubated for 5 min. on ice. Samples were centrifuged for 15 min. at 12000 rpm at $4^\circ C$. The supernatant (nucleoplasm) was transferred to a new 1.5-ml tube. The pellet (chromatin) was resuspended in 2x Sample Buffer (wide-bore tip) and subsequently sonicated for 3 min in a Bioruptor with 30 sec. on/off cycles at high power. Samples were incubated for 10 min at $95^\circ C$. To whole-cell extract, cytoplasm, and nucleoplasm samples 1x Sample Buffer was added and samples were incubated for 10 min at $95^\circ C$. Samples were stored at $-20^\circ C$. Prior to gel separation, samples were incubated for 5 min at $95^\circ C$.

Reverse transcription

Total yeast mRNA was prepared from 4×10^7 genes using the RNeasy kit (Qiagen) according to the manufacturer's manual using mechanical disruption of the cells. mRNA was treated with DNase and cDNA was made as described previously (Verzijlbergen et al., 2010).

Quantitative PCR

Quantitative real time-PCR (qPCR) was performed with SYBRgreen master mix (Applied Biosystems or Roche) according to the manufacturer's manual. IP and cDNA samples were diluted 10 times, and input samples were diluted 100 times before analyzing by qPCR on a 7500 Fast Real-Time PCR system (Applied Biosystems) or LightCycler 480 II (Roche). qPCR primers are shown in Table 2.

FACS analysis of cell cycle progression

For FACS analysis of DNA content samples of 1×10^7 cells were collected, centrifuged for 10 s at full speed, resuspended in 1 ml 70% ethanol, and stored at -20°C until further processing. The DNA content was measured using SYTOXGreen in flow cytometry using a 530/30 filter on a FACS Calibur (Becton-Dickinson) as described previously (Verzijlbergen et al., 2010). For each measurement 100,000 cells were counted. Analysis was performed using FCS express 2.

Chromatin Immunoprecipitation (ChIP)

For ChIP, cells were grown to mid-log phase in YEPD with 200 µg/ml Hygromycin for pre-switch strains, or YEPD for post-switch strains. Samples of $1-3 \times 10^9$ cells were taken, fixed for 10 minutes (1% formaldehyde, 50 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA) and washed with cold TBS. Pellets were stored in 12 ml flat bottom-tubes or 2 ml screw-cap tubes at -80°C until

further processing. Cells in 12 ml tubes were disrupted in 300 μ l breaking buffer (100 mM Tris pH 7.9, 20% glycerol, protease inhibitor cocktail EDTA-free (Roche) with 400 μ l glass beads in a multivortex for 20 minutes at 4°C. Cells in screw-cap tubes were disrupted in 200 μ l breaking buffer with 500 μ l glass beads in a mini bead beater (BioSpec) in a 4°C cold block for 2 minutes. Lysis was at least 70%, as determined by microscopy. Lysates were transferred to 1.5- or 2-ml tubes and 1 ml FA buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, protease inhibitor cocktail EDTA-free) was added. The mixture was centrifuged for 1 minute at 14000 rpm at 4°C, and the pellet was washed once more with FA buffer. The pellet was resuspended in 450 μ l FA, divided over two 1.5 ml tubes and sonicated for 6-7 minutes in a Bioruptor (Diagenode) with 30-second on-off cycles on high power. Lysates were cleared by centrifugation for 5 minutes at 4°C at 14000 rpm. Supernatant containing chromatin was transferred to 1.5 ml tube and 1 ml FA was added to samples of the 12 ml tubes. Screw-cap tube chromatin samples were treated with Micrococcal Nuclease (MN) to generate mononucleosomes. For these samples, 800 μ l final buffer (15 mM Tris pH 7.4, 50 mM NaCl, 1.5 mM CaCl_2 , 5 mM β -mercaptoethanol, 5 mM MgCl_2) was added. Samples were incubated with 30 units of MN (Fermentas) at 37°C for 20 minutes. The reaction was stopped by adding EGTA and EDTA to a final concentration of 10 mM and placing tubes on ice. The chromatin solution was centrifuged for 15 minutes at 14000 rpm at 4°C; the supernatant was transferred to a new 1.5 ml tube and stored at -20°C. Magnetic Dynabeads coupled with Protein G (Dyna) were incubated in PBS containing 5 mg/ml BSA with antibody for at least four hours at 4°C. 200 (for 12 ml tube samples) or 400 (for screw-cap samples) μ l soluble chromatin was added to prepared Dynabeads and incubated rotating overnight at 4°C. Then 1 ml of FA buffer was added and samples were incubated rotating for 5 minutes at RT. The samples were washed twice with each of the buffers FA, FA-HS (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate), RIPA (10 mM Tris pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA). Finally, the samples were washed once with TE (10 mM Tris pH8, 1 mM EDTA). 100 μ l

elution buffer (50 mM Tris pH 8, 10 mM EDTA, 1% SDS) was added to the samples and incubated for 10 minutes at 65°C. Subsequently the samples were centrifuged 1 minute at 14000 rpm and 80 µl supernatant was collected. 70 µl TE was added to samples and cross links were reversed in 0.625 mg/ml Proteinase K and 3 µg/ml RNaseA incubated for 1 hour at 50°C and subsequently overnight at 65°C. For input samples, 20 (for 12 ml tube samples) or 40 (for screw-cap samples) µl chromatin solution was combined with 60 µl elution buffer and 70 µl TE, and treated in the same manner as IP samples to reverse cross links. DNA was purified by using the High Pure PCR Product Purification Kit (Roche).

Protein detection by immunoblot and antibodies

Prior to immunoblotting, 3-8 µl of lysate was separated on a 16% polyacrylamide gel. Separated proteins were transferred to a 0.45 µm nitrocellulose membrane for one (H3 and H2B) or 1.5 (when Pgf1 was detected on the same membrane) hours at 0.1 A. Membranes were blocked with PBS containing 2% or 5% Nutrilon (Nutricia) for one hour, first antibody incubations were done either for two hours at room temperature or overnight at 4°C in TBS containing 0.05% Tween-20 (TBST) with 2% Nutrilon. After washing three times in TBST, secondary antibody incubation was performed in TBST with 2% Nutrilon and LI-COR Odyssey IRDye 800CW or IRDye 700CW antibody at 1:10,000 for 45 minutes at room temperature in the dark followed by two times 10-minute wash in TBST and one time 10-minute wash in PBS. Membranes were scanned using a LI-COR Odyssey IR Imager (Biosciences) and analyzed using the Odyssey LI-COR software package version 3.0 or the image studio software package. Antibodies used in this study for blots and ChIP are Pgf1 (A-6457, Invitrogen), histone H2B (39238, Active Motif), histone H2A.Z (39647/39648 Active motif), histone H3, HA (12CA5), V5 (R960-25, Invitrogen), Flag and LoxP (Verzijlbergen et al., 2010).

Epi-ID

Experiments were performed essentially as described previously (Poramba-Liyanage et al., 2019; Vlaming et al., 2016). For the generation of the H2A.Z-tag Epi-ID libraries, we combined a genome-wide yeast knock-out collection (Tong & Boone, 2006) with a Barcode library (Yan et al., 2008), essentially as described previously (Vlaming et al., 2016) and subsequently crossed in a tagged version of H2A.Z (V5 (NKI8519) or Flag (NKI8520)). This resulted in the H2A.Z-V5-Epi-ID (NKI8532) and H2A.Z-Flag-Epi-ID (NKI8533) libraries. For Epi-ID, cells were pooled in five Barcode subsets and ChIP was performed as described above with antibodies against either V5 or Flag. The barcodes (BC_UP and BC_DN) in the input and IP samples were amplified using primers containing index sequences to allow for multiplexing. Primers are shown in Table 2. Barcode counting was performed by deep sequencing using the HiSeq2000 platform (Illumina).

3.5 Acknowledgements

We thank the members of our laboratory for critical reading of the manuscript, Carl Wu for providing the Swr1 plasmids, Charles Boone, Michael Costanzo, Brenda Andrews and Corey Nislow for providing the NatMX yeast knock-out and KanMX yeast Barcode collections, Hiroyuki Arai for help with analyses of H2A.Z levels in mutant strains, the Japanese National BioResource Project for distributing AID plasmids.

3.6 FUNDING

This work was supported by the Netherlands Organisation for Scientific Research (NWO-VICI-016.130.627 to FvL).

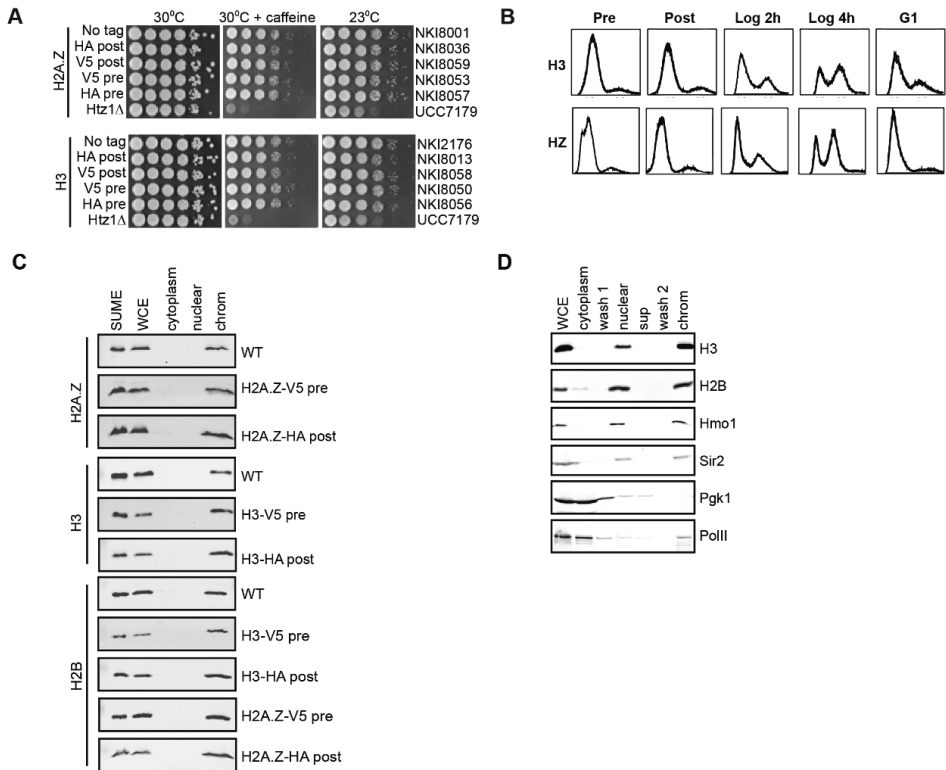
3.7 Author contributions

DPL, TK, MT, and FvL designed the studies and analyzed the data. DPL, TK, MT and FvL wrote the paper. MT, DPL, KFV and TvW performed strain constructions, turnover assays, ChIP and blot analyses. DPL and IJES performed chromatin-association assays. DPL performed the Epi-ID screen. TK performed bioinformatics analyses. All authors reviewed the results and approved the final version of the manuscript.

3.8 Conflict of interest

The authors declare that they have no conflict of interest.

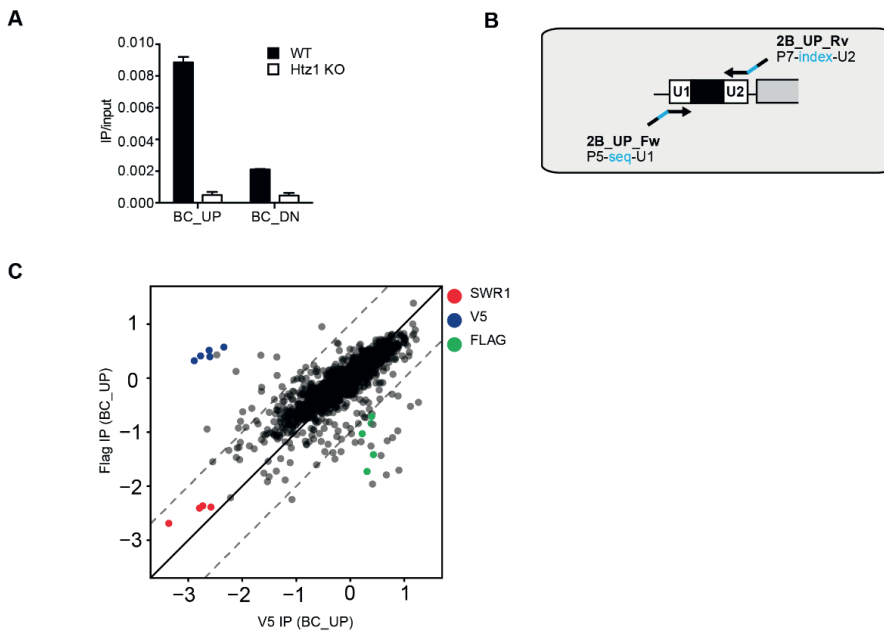
3.9 Supplementary Data



Supplemental Figure 1

(A) Spot test to examine fitness of strains containing the RITE tags. Strains were spotted in a 10-fold dilutions series on YEPD or YEPD containing 10 mM caffeine. Plates were grown at 30°C or 23°C for three days. An *htz1Δ* strain was used as control. **(B)** Analysis of DNA content by flow cytometry of H3-RITE and H2A.Z-RITE during an arrest-switch-release experiment. **(C)** Immunoblot comparison of H3 and H2A.Z fractionation in RITE strains. Fractionation of each histone was determined in a WT strain and V5 (old) and HA (new) strains for both H3 and H2A.Z.

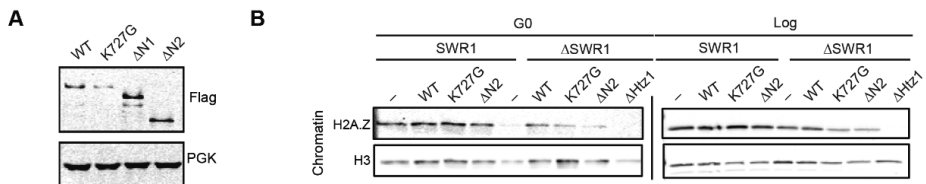
For all samples tested, the histones H3, H2A.Z and H2B were only present in the whole cell extracts and chromatin fractions. **(D)** Immunoblots of a Chromatin Association Assay using a WT strain. H3, H2B, Hmo1, and Sir2 were used as controls for chromatin bound proteins; P_{gk1} is a cytoplasmic protein; RNA polymerase II (PolII) has been reported to be present both in the cytoplasm and bound to DNA.



Supplemental Figure 2

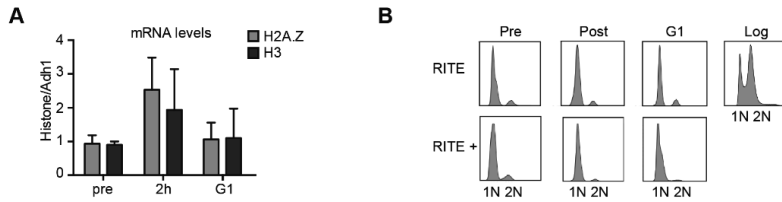
(A) H2A.Z occupancy at the barcoded HO locus that was used in the Epi-ID screen, measured by ChIP-qPCR. An *htz1Δ* strain (H2A.Z KO) was used as a negative control. Data are represented as mean $\pm$ standard deviation of three biological experiments. **(B)** Schematic overview of the primers used to amplify BC_UP at the promoter, which is flanked by the constant regions U1 and U2. The Illumina P5 sequence and extra nucleotides for annealing of the 5' end of the custom sequencing primers is introduced by the forward primer. The Illumina P7 sequence and a 6 bp

index is introduced by the reverse primers. **(C)** Scatterplot of the two biological Epi-ID screen replicates, in which two different tags were used (FLAG and V5). Each dot represents a knock-out strain. Factors with variation of more than 1 log2 fold change were removed (dotted line, removed 51 genes). Wild-type strains with either FLAG or V5 tags were used as positive and negative controls (green and blue dots). The members of the SWR1 complex present in the library were the strongest outliers (red dots).



Supplemental Figure 3

(A) Immunoblot of the plasmids expressing wild-type and mutant versions of the Flag-tagged Swr1 protein from experiments of Fig 3E-F. P_{gk1} was used as a loading control. **(B)** Immunoblots of a Chromatin Association Assay using a WT strain and SWR1 KO strains in replicating cells and in cells arrested in G1. The decrease in global levels of H2A.Z seen in the chromatin fraction was rescued by plasmids expressing wild-type Swr1, but not by a catalytically dead mutant (K727G) or the $\Delta N2$ deletion mutant of the N-terminal tail of Swr1. H3 is used as loading control.



Supplemental Figure 4

(A) mRNA levels of H3 and H2A.Z measured by RT-qPCR and normalized to ADH1, which is constitutively expressed, showing that H2A.Z levels peak in S phase coinciding with the replication coupled deposition of H2A.Z. Data are represented as mean $\pm$ standard deviation of three biological experiments. **(B)** Analysis of DNA content by flow cytometry of H2A.Z-RITE and H2A.Z-RITE+ during an arrest-switch-release experiment as described previously. A sample of replicating cells was added as a control. Data shown are one out of three biological experiments.

Table S1. List of yeast strains

Name	Relevant genotype	Ref*	Notes	Fig
BY4733	MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0	CB		1S, 2S
NKI8001	MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 bar1Δ::HISG his3Δ200::HIS3-P _{TDH3} -CRE-EBD78			1S
NKI2176	NKI8001; hht1-hhf1::MET15	MT		1S
NKI8050	NKI2176; hht2::HHT2-LoxP-V5-T _{ADH1} -HphMX-LoxP-HA-6xHis	MT		1, 1S, 4S
NKI8056	NKI2176; hht2::HHT2-LoxP-HA-6xHis- T _{ADH1} -HphMX-LoxP-V5	MT		1S
NKI8013	NKI8050; hht2::HHT2-LoxP-HA-6xHis			1S
NKI8058	NKI8056; hht2::HHT2-LoxP-V5			1S
NKI8053	NKI8001; htz1::HTZ1-LoxP-V5-T _{ADH1} -HphMX-LoxP-HA-6xHIS	MT		1, 1S, 4S
NKI8057	NKI8001; htz1::HTZ1-LoxP-HA-6xHis-T _{ADH1} -HphMX-LoxP-V5	MT		1S
NKI8036	NKI8053; htz1::HTZ1-LoxP-HA-6xHis			1S
NKI8059	NKI8057; htz1::HTZ1-LoxP-V5			1S
NKI2364	NKI8053; P _{HO} ::P _{GAL1} - HTZ1-HA-6xHis			4, 4S
UCC7179	MATa his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 ade2Δ::hisG ADE2-TEL-VR htz1Δ::URA3			1S, 2S
NKI2389	MATa his3Δ200 leu2d0 met15d0 trp1d63 ura3d0 bar1::HISG is3::HIS3-Pgpd-CRE-EBD78 htz1::HTZ1-LoxP-V5-HPH-LoxP- HA-6xhis swr1::SWR1_2xt7_Auxin_Induced_Degron_KanMX HO::URA3_pADH1-OsTIR1			3, 3S
NKI2397	MATa his3d200 leu2d0 met15d0 trp1d63 ura3d0 bar1::HISG is3::HIS3-Pgpd-CRE-EBD78 htz1::HTZ1-LoxP-V5-HPH-LoxP- HA-6xhis Swr1::SWR1_2xt7_Auxin_Induced_Degron_KanMX			3, 3S
NKI2391	MATa his3d200 leu2d0 trp1d63 ura3d0 met15d0 hht1-hhf1::MET15 bar1::HisG his3::HIS3-Pgpd-CRE-EBD78 hht2::HHT2-LoxP-V5-ADHterm-HPH-LoxP- HA+6xHis SWR1_2xt7_Auxin_Induced_Degron_KanMX pHO::URA3_pADH1-OsTIR1			3, 3S
NKI2399	MATa his3d200 leu2d0 trp1d63 ura3d0 met15d0 hht1-hhf1::MET15 bar1::HisG his3::HIS3-Pgpd-CRE-EBD78 hht2::HHT2-LoxP-V5-ADHterm-HPH-LoxP- HA+6xHis SWR1_2xt7_Auxin_Induced_Degron_KanMX			3, 3S
NKI2433	MATa his3d200 leu2d0 met15d0 trp1d63 ura3d0 bar1::HISG his3::HIS3-Pgpd-CRE-EBD78 htz1::HTZ1-LoxP-V5-HPH-LoxP- HA-6xhis pHO::URA3_pADH1-OsTIR1 SWR1_2xt7_Auxin_Induced_Degron_KanMX pRS_NatMX_SWR1_2xFlag			3S
Barcode library	MATa his3d1 leu2d0 met15d0 ura3d0 HO::barcodedKanMX	YN		2
NatMX KO library	MAT@ can1d::STE2pr-Sp_his5 lyp1d his3d1 leu2d0 ura3d0 met15d0 GOI::NatMX	TN		2
NatMX KO x Barcoders collection	MATa can1d::STE2pr-Sp_his5 lyp1d his3d1 leu2d0 ura3d0 met15d0 HO::barcodedKanMX GOI::NatMX	HV		2
NKI8519	MAT@ his3d200 leu2d0 met15d0 trp1d63 hht1-hhf1::URA3_Nar1_CRE-EBD78 htz1::HTZ1-LoxP-V5-HPH-LoxP- HA-6xhis			2
NKI8520	MAT@ his3d200 leu2d0 met15d0 trp1d63 hht1-hhf1::URA3_Nar1_CRE-EBD78 htz1::HTZ1-LoxP-2xFlag-HPH-LoxP-V5			2
NKI8532	MATa can1d::STE2pr-Sp_his5 lyp1d his3d1 leu2d0 ura3d0 met15d0 LYS2+ TRP+ HO-BC-KanMx-BC Ygf::NatMx Hht1-hhf1::CRE(URA3-CRE) htz1::HTZ1-LoxP-V5-HPH-LoxP- HA-6xhis		H2A.Z-V5 Epi-ID library	2, 2S
NKI8533	MATa can1d::STE2pr-Sp_his5 lyp1d his3d1 leu2d0 ura3d0 met15d0 LYS2+ TRP+ HO-BC-KanMx-BC Ygf::NatMx Hht1-hhf1::CRE(URA3-CRE) htz1::HTZ1-LoxP-2xFlag-HPH-LoxP-V5		H2A.Z-Flag Epi-ID library	2, 2S

* (CB=Brachmann et al., 1998; MT=Terweij et al., 2013, TN= Tong et al., 2006, YN=Yan et al., 2008, HV=Vlaming et al., 2016)

Table S2. List of primer sequences

Primer	Sequence
PTC6_Qfor	ATCGGGGCAATTAAGCATC
PTC6_Qrev	CCCGTAACAAGTCCAGCTTC
RSA4_Qfor	TCTCTGGGAAGTTGAGCCTCTT
RSA4_Qrev	CACAATATACAGATGTCCACCCTGAT
ABP1_Qfor	CCTATCAATCACAATGGCTTTGG
ABP1_Qrev	TCGGAGCCTCTGACAATCTTC
PTC6orf_Qfor	TGCAACTCGAAACAAGAAGT
PCT6orf_Qrev	TAATATCCCCATTTTATCAAAAATCA
SPA2orf_Qfor	ATCAAGAGAAGAGGGTTCGACAAG
SPA2orf_Qrev	CATCGCTGCGGTAATGG
SpLoxP_Qfor	GGTGGATCTGGTGGATCTATAA
V5_Qrev	AGACCGAGGAGAGGGTTAGG
HAHIS_Qrev	TGCGCATAGTCAGGAACATC
HTZ1orf_Qfor	GGATCCAAAGCTGCCATTTA
HTZ1orf_Qrev	ATCCTTGGCTGCATTACCAG
HHT2_Qfor2	AAGCCCCAAGAAAACAATTAGC
HHT2_Qrev2	TGGCTTATATCTGTGAGGCTTCTAA
ADH1 ORF II fwd	TAGGTTCTTTGGCTGTTCAATACG
ADH1 ORF II rev	CGGAAACGGAACGTTGATGACACCG
HOUpFwd1	GATCGTCGACGACGTCGGCGCCTCGAGCATGCATATACGTACCAGGTTACAG
HOUpRev1	GATCGTCGACGACGTCGGCGCCTCGAGCATGCATGGATTTAGAGCTAATAGTTG
GZ1	AGTCATGATCCTTAAGCGAGCTCGTTAAACTGGATG
GZ2	AGCTTAAGTCTCATGATACGTATGGAAATTTATCGTC
HD1	CATGGTACCGACGTCATATACGTACCAGGTTACG)
HD2	GTAGGTACCGACGTCGATGAATAGTGC GTTTAGAAC
Narl 2xT7+G KpnI FW	CGCCATGGCTTCTATGACAGGAGGTCAACAGATGGGAAT- GGCAAGCATGACTGGTGGACAGCAAATGGGTGGTAC
Narl 2xT7+G KpnI RV	CACCCATTTGCTGTCCACCAGTCATGCTTGCCATTCCCATCTGTTGACCTCCTGTCATAG- AAGCCATGG
2B_UP_Fw	AATGATACGGCGACCACCGAGATCTACACCTTCCGATCTAGATGTCCACGAGGTCTCT
2B_UP_Rv_all	CAAGCAGAAGACGGCATACGAGATNNNNNNNGTCGACCTGCAGCGTACG
2B_UP_Seq	ACACCTTCCGATCTAGATGTCCACGAGGTCTCT
2B_DN_Fw	AATGATACGGCGACCACCGAGATCTACACTCTTCCGATCTACGGTGTGGTCTCGTAG
2B_DN_Rv_all	CAAGCAGAAGACGGCATACGAGATNNNNNNAACGAGCTCGAATTCATCGA
2B_DN_Seq	ACACTCTTCCGATCTACGGTGTGGTCTCGTAG
HOpromQfw	GAAGCTTGTTGAAGCATGATGAA
HOpromQrev	TTGCTGCTTATGAGGATATGGATTT
HOtermQfw	GAGTAGAAATACGCCATCTCAAGATACA
HOtermQrev	GGAAAGTTGATCAAGACCCAATAATAA

CHAPTER 4

Decoding the chromatin
proteome of a single
genomic locus by
DNA Sequencing



4

Decoding the chromatin proteome of a single genomic locus by DNA sequencing

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Abstract

Transcription, replication, and repair involve interactions of specific genomic loci with many different proteins. How these interactions are orchestrated at any given location and under changing cellular conditions is largely unknown because systematically measuring protein-DNA interactions at a specific locus in the genome is challenging. To address this problem, we developed Epi-Decoder, a Tag-ChIP-Barcode-Seq technology in budding yeast. Epi-Decoder is orthogonal to proteomics approaches because it does not rely on mass spectrometry but instead takes advantage of DNA sequencing. Analysis of the proteome of a transcribed locus proximal to an origin of replication revealed more than 400 interacting proteins. Moreover, replication stress induced changes in local chromatin-proteome composition prior to local origin firing, affecting replication proteins as well as transcription proteins. Finally, we show that native genomic loci can be decoded by efficient CRISPR/Cas9-assisted construction of barcode libraries. Thus, Epi-Decoder is an effective strategy to identify and quantify in an unbiased and systematic manner the proteome of an individual genomic locus by DNA sequencing.

4.1 Introduction

The chromatin at any given location in the genome is a dynamic entity that likely involves the interaction of many different proteins and non-protein factors. The essential and complex processes of transcription, replication and repair require major chromatin rearrangements to access and use the genome (Lai & Pugh, 2017; Venkatesh & Workman, 2015). Furthermore, the genome's chromatin is under the influence of signals that can relay information of cellular events or states to the genome and vice versa (Badeaux & Shi, 2013; Dutta, Abmayr, & Workman, 2016). Fully understanding the chromatin-regulatory mechanisms in the cell will require comprehensive knowledge of the full set of proteins that bind at individual genomic loci. Although many chromatin factors have already been identified by genetics and protein-protein interaction studies, direct, unbiased, and comprehensive analyses of chromatin interactions at specific genomic loci has remained a major challenge (Kan et al., 2017; Wierer & Mann, 2016). A commonly considered strategy towards solving this problem is introducing an affinity handle at a locus of interest, purifying the locus (capture) and analyzing the co-purified proteins by mass spectrometry (MS) (Déjardin & Kingston, 2009; Stephan Hamperl et al., 2014). However, a major challenge with capture-MS is the need for very high levels of enrichment of the locus of interest versus the rest of the large genome while at the same time obtaining sufficient amounts of material for comprehensive and quantitative MS analysis (Kan et al., 2017; Wierer & Mann, 2016). For example, in a model organism with a small genome such as yeast, purification of a 1 kb locus with its associated proteins from an entire genome of ~30 Mb requires a 30,000-fold purification.

Here, we present an independent approach, Epi-Decoder, with which the interactome of a single-copy locus is determined by DNA sequencing instead of mass spectrometry. In this approach developed in yeast, a library of clones is generated in which each clone harbors at least one DNA barcode at a fixed locus and one protein tagged at its endogenous locus with a common epitope tag. Following chromatin immunoprecipitation (ChIP) for the common tag on a pool of cells, the

barcodes of the co-immunoprecipitated DNA are counted by high-throughput sequencing and the amount of barcode recovered serves as a read-out for the amount of the corresponding specific protein binding at the barcoded locus. Importantly, in contrast to proteins, DNA barcodes can be amplified prior to counting, boosting the sensitivity of detection.

Epi-Decoder of a single transcribed locus in yeast identified more than 400 chromatin-interacting proteins, enabled the identification of differences in protein binding between the 5'- and 3'-end of a gene, and demonstrated a chromatin rewiring in response to physiological changes. We also demonstrate that Epi-decoder can be applied to a locus of interest by harnessing CRISPR/Cas9-mediated genome engineering. Thus, Epi-Decoder provides an efficient method to provide a comprehensive map of the dynamic proteome of a single-copy locus. Moreover, it is an orthogonal approach to capture-MS because acquisition of quantitative and qualitative information on protein binding does not involve mass spectrometry but is obtained by DNA sequencing.

4.2 Results

Epi-Decoder: Measuring protein binding by DNA barcode counting

To develop a strategy for systematic and comprehensive decoding of the interactome of a single genomic locus, we asked whether DNA barcode technology can be harnessed to solve this challenging proteomics problem. We and others previously showed that short DNA barcodes (≤ 20 bp) integrated in the genome and embedded in chromatin can serve as molecular identifiers of the chromatin state they are in (Akhtar et al., 2013; U. T. Nguyen et al., 2014; Vlaming et al., 2016). Building on that notion, we used Synthetic Genetic Array (SGA) technologies (Giaever & Nislow, 2014; Tong & Boone, 2006) to create arrayed yeast libraries that allow for high-throughput and direct assessment of chromatin states of many cell clones in parallel. In these libraries, each clone contains a pair of known unique barcodes flanking a constitutively transcribed kanamycin resistance gene (*KanMX*) under control of the *Ashbya gossypii* *AgTEF1*-promoter and -terminator

at the *HO* locus (Yan et al., 2008) as well as a known Tandem Affinity Purification (TAP)-tagged protein expressed from its endogenous locus (Ghaemmaghami et al., 2003) (Fig 1A). The Epi-Decoder libraries used here cover ~4000 TAP-tagged proteins (S1 Table) of the ~5600 proteins encoded in the yeast genome (Byrne & Wolfe, 2005). Following pooling of the barcoded TAP-tag strains, cells are incubated with formaldehyde to crosslink proteins to DNA and subjected to ChIP (Fig 1B). From the co-immunoprecipitated DNA and the input samples, the barcoded regions are amplified (S1 Fig), and the barcodes identified and quantified by parallel sequencing (Fig 1B). In this set-up, which we call Epi-Decoder, the abundance of a barcode (ChIP/input) reports on the cross-linking of the corresponding TAP-tagged protein to its own barcoded region for every TAP-tagged clone in the pool (Fig 1C). The reporter gene at the *HO* locus is flanked by two barcodes that can be analyzed in parallel but that have different chromatin contexts: the upstream barcode (BC_UP) is located in the promoter region whereas the downstream barcode (BC_DN) lies in the terminator region and in close proximity to an origin of replication (Fig 1C). Thus, Epi-Decoder analysis of the barcoded *KanMX* gene at the *HO* locus results in an inferred binding score for the vast majority of the proteins present in the yeast genome at two proximal but distinct genomic loci.

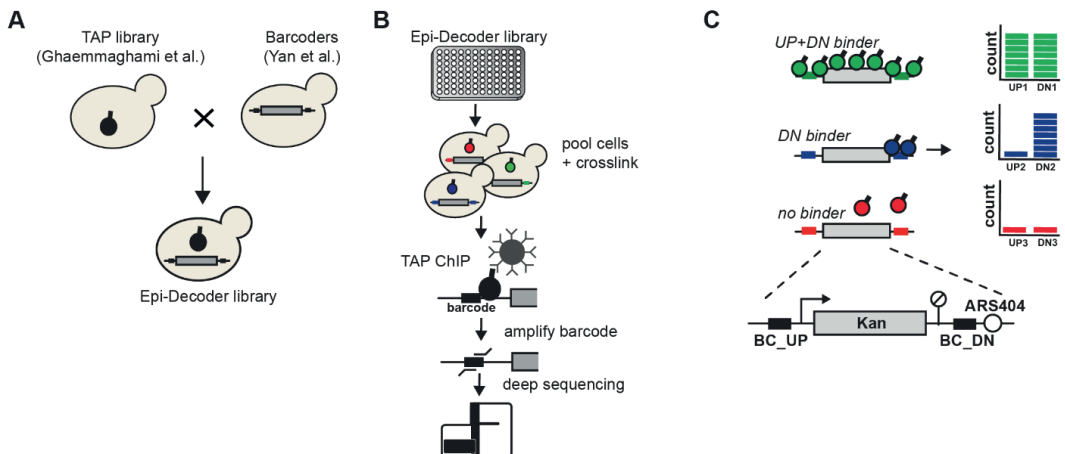


Figure 1. Outline of Epi-Decoder

(A) Construction of the Epi-Decoder library: a library of strains encoding TAP-tagged proteins was crossed to a Barcoder library by using standard SGA procedures. (B) Experimental set-up of Epi-Decoder: colonies of an arrayed library are pooled in a flask, expanded, subjected to cross-linking, and used for a TAP ChIP. The co-immunoprecipitated barcodes are amplified and counted by massive parallel sequencing. Barcodes of input DNA are counted to control for the presence of each barcode in the pool. Indexed input and ChIP samples of multiple pools can be combined into one master pool before sequencing. (C) Protein binding is measured by barcode counting. The Barcoder locus consists of a KanMX resistance marker transcribed by the AgTEF1 promoter flanked by two unique barcodes and replacing the HO gene. The BC_UP is promoter-proximal, the BC_DN terminator-proximal and close to (50 bp) an origin of replication (ARS404).

Epi-Decoder identifies many chromatin-associated factors

For BC_UP and BC_DN we identified the immunoprecipitated barcodes with significantly different counts compared to background (red dots in Fig 2A), based on six biological replicates (S2A Fig). The vast majority of significantly different barcodes showed a positive binding score (Fig 2A, red dots, IP/Input > 0). Here we refer to the factors associated with those barcodes as binders. Together, we identified 469 binders of which 18 were specific for BC_UP and 273 for BC_DN (Fig 2B, S1 Table). Significantly depleted factors (Fig 2A, red dots IP/input<0) were not expected because proteins cannot bind less to their barcodes than negative controls such as non-expressed or non-tagged proteins. The low number of significantly depleted factors indicates a low rate of false discovery, in agreement with our stringent cut-off (FDR<0.01). Among the binders, the four canonical histone proteins (represented by seven histone genes in the library) were among the most-enriched proteins at both barcodes. Furthermore, 267 out of the 469 binders had GO terms related to DNA binding (Fig 2C, S2B Fig), confirming that Epi-Decoder provides an effective

approach for identifying chromatin-interacting factors. In addition to known DNA-interacting proteins, we found a substantial number of factors involved in RNA processing and cellular metabolism. RNA processing events such as capping, splicing, cleavage, and polyadenylation are all processes known to occur in close conjunction with transcription and hence in proximity to DNA (Bentley, 2014), providing an explanation for the recovery of barcodes associated with RNA processing proteins. The presence of factors involved in cellular metabolism cannot be explained by general associations with transcription, although recent studies have suggested various roles of metabolic enzymes in the nucleus (Dutta et al., 2016; Reid, Dai, & Locasale, 2017; Su & Pillus, 2016) (and see discussion). Given the fact that some of these factors are known to be highly expressed, we investigated the possibility that their chromatin interactions were determined by protein abundance. Overall, chromatin binders were generally more highly expressed than non-binders (Fig 2D). However, high protein expression level alone was not sufficient for binding: many chromatin binders were not highly abundant and many highly abundant proteins were non-binders. For example, ribosomal proteins are highly abundant but most of them were not detected as chromatin interactors even though ribosomal proteins traffic through the nucleus to form ribonucleoprotein complexes for ribosome assembly.

Furthermore, for a selected panel of factors reflecting different classes of binders and different expression levels, we could quantitatively confirm the positive and negative Epi-Decoder results by ChIP-qPCR and immunoblot analysis of individual clones (Fig 2E, S2C-D Fig). Finally, we excluded the possibility that leaking of cytosolic factors into the nucleus due to the presence of methanol in formaldehyde solutions was a cause of the crosslinking of metabolic enzymes to chromatin. Using formaldehyde with or without methanol as a stabilizer resulted in nearly identical binding profiles (S2E Fig). Whether or not the binding of metabolic enzymes to chromatin is biologically significant will require further in-depth studies and functional experiments. Together, our results suggest that the barcode counts obtained by Epi-Decoder accurately and quantitatively

report on the efficiency of protein crosslinking at the barcoded loci and captures histones, other core chromatin proteins, as well as additional factors. We note that in the Epi-Decoder set-up, proximity to DNA, genomic distance from the barcode, protein abundance, and cell-to-cell variation are among the factors that can influence the measured quantitative binding scores.

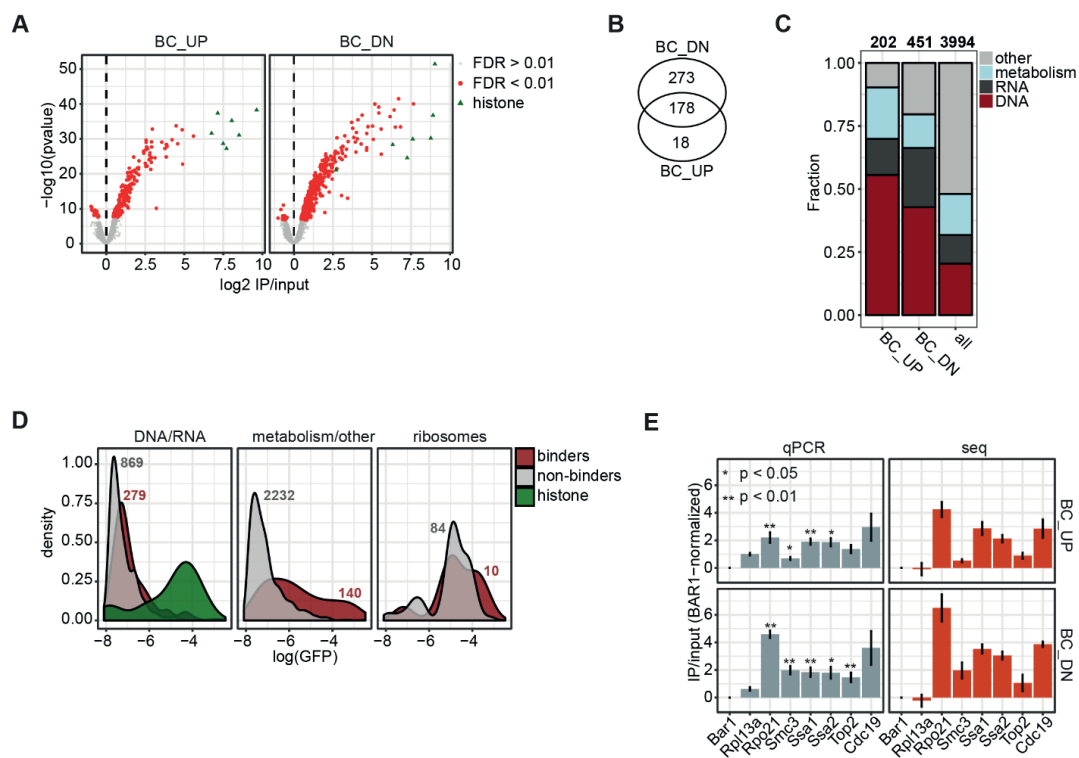


Figure 2. Identifying chromatin binders with DNA barcode counting

(A) Volcano plot showing the average $\log_2$ ChIP/input versus significance (p -value) calculated for 3,994 factors for BC_UP and 3,995 factors for BC_DN across 6 independent biological experiments. The red color indicates factors with a Benjamini-Hochberg adjusted p -value < 0.01 . Green triangles represent the barcodes associated with the TAP-tagged histone proteins in the library. (B) Venn diagram indicating the number of factors that are significant ($FC > 0$; $FDR < 0.01$)

at BC_UP, BC_DN, or both. (C) Bar plots showing the fraction of factors in four different categories that were determined by using GO annotations (see Methods). The first two bars show the fractions in the group of binders (at either BC_UP or BC_DN). The right bar shows the fraction in the background set (the entire library). (D) Density plot of GFP abundance using data from the Cyclops database (<http://cyclops.cabr.utoronto.ca/>). The binders were defined as before ($FC > 0; FDR < 0.01$), and the categories are similar as in C. The ribosome category was defined based on the CYC2008 database (<http://wodaklab.org/cyc2008/>). Histones are shown separately; they bind at the DNA and are highly abundant. The numbers indicate the size of each group. (E) ChIP-qPCR of selected TAP-tag strains from the barcoded KanMX Epi-Decoder library, with specific primers in proximity to BC_UP and BC_DN. To compare the barcode counts with ChIP-qPCR signal, the samples were normalized to the Bar1-TAP signal (Bar1 is not expressed in these cells) before calculating ChIP/input. The average of three biological replicates is shown; the error bars indicate the standard deviation. Rpl31A - a highly expressed ribosomal subunit frequently used in anchor away studies to deplete proteins from the nucleus - was used as a negative control and as a reference to determine binding specificity in the qPCR analysis. Rpo21 is the largest subunit of RNA polymerase II, Smc3 is a cohesin subunit, Ssa1 and Ssa2 are HSP70 chaperones, Top2 is topoisomerase 2, and Cdc19/Pyk1 is pyruvate kinase. The p-values from a two-sided t-test compared to Rpl31A are indicated by asterisks.

Different interactomes of the promoter and terminator regions

To determine whether Epi-Decoder can be used to identify locus-specific proteins, we next compared the significantly enriched factors for BC_UP to those of BC_DN. For this purpose, we plotted the binding scores for BC_UP versus BC_DN and color coded enriched factors according to the main chromatin functions or complexes expected at this locus (Fig 3A). Strikingly, we observed that proteins within the same complex or process tend to cluster together. This strongly

suggests that Epi-Decoder does not just detect binding events but also provides quantitative information about protein occupancy.

Several factors and complexes were shared between the two locations. Besides histones, strongly-enriched factors at both barcodes include subunits of RNA polymerase II and several other factors and complexes with a well-known role in transcription elongation such as DSIF, Elf1, FACT, Spt6, and the PAF-C complex (Fig 3A) (Oliver J. Rando & Winston, 2012; Sarah Sainsbury, Carrie Bernecky, & Patrick Cramer, 2015). However, BC_UP and BC_DN also showed substantial quantitative interactome differences reflecting their different functional states and suggesting that many proteins show locus-specific binding behavior (Fig 3B). At BC_UP at the 5'-end of the gene, histone variant H2A.Z (Htz1) was strongly enriched, which is in agreement with the enrichment of H2A.Z in promoter regions (Albert et al., 2007), which we confirmed for this locus using an antibody that recognizes the endogenous H2A.Z protein (S3A Fig). In addition, BC_UP showed enrichment for general regulatory factors Reb1 and Rap1. This is in agreement with the known Rap1 binding site in the *TEF1* promoter of the *KanMX* gene (M. Chen et al., 2013) and the role of Reb1 as a general chromatin organizer of regulatory regions (Oliver J. Rando & Winston, 2012). BC_UP also showed enrichment for the basal transcription factors TFIID, -E, -F and -H, which form the pre-initiation complex together with RNA polymerase II (Ho Sung Rhee & B. Franklin Pugh, 2012; Sarah Sainsbury et al., 2015).

At the 3'-end, BC_DN showed enrichment for factors involved in transcription termination and mRNA cleavage and polyadenylation, an important step in mRNA 3'-end formation (Tous et al., 2011). The Cohesin complex was also enriched at BC_DN. Finally, we observed strong binding of the evolutionary conserved ORC and MCM complexes at BC_DN, which is in agreement with the proximal origin of replication sequence at which ORC and then MCM are loaded to assemble the pre-replication complex (S. P. Bell, Kobayashi, & Stillman, 1993; Yuan et al., 2017) BC_DN-specific binding of Orc1 was confirmed by ChIP-qPCR (S2C Fig). Thus, in addition to common

binders, Epi-Decoder revealed locus-specific enrichment of factors (Fig 3C). This confirms that while the barcodes are in the same genomic region, they are in distinct functional contexts that can be separated by this approach even though they are only 1.5 kb apart. This level of resolution was robust since the BC_UP- and BC_DN-specific binding pattern was maintained when the average chromatin-fragment size was increased or decreased by applying shorter and longer shearing time, respectively (S3B-C Fig).

Interestingly, not all factors followed the expected distribution (Fig 3A). Tfa2, the small subunit of the heterodimeric basal transcription factor TFIIIE, and Ssl2, the dsDNA translocase subunit of the basal transcription factor TFIIH, were found at BC_UP but were also strongly enriched at BC_DN. 3'-binding of Tfa2 and Ssl2 has been observed at other genes as well (Ho Sung Rhee & B. Franklin Pugh, 2012; Soares & Buratowski, 2012) but the significance remained uncertain. Since in Epi-Decoder all proteins have the same tag and are assessed simultaneously in a pool in a quantitative manner, the deviant binding pattern observed here cannot be explained by antibody issues or experimental and strain differences and therefore strongly suggests that Tfa2 and Ssl2 have special roles or positions within their complex or have non-canonical functions outside their complex.

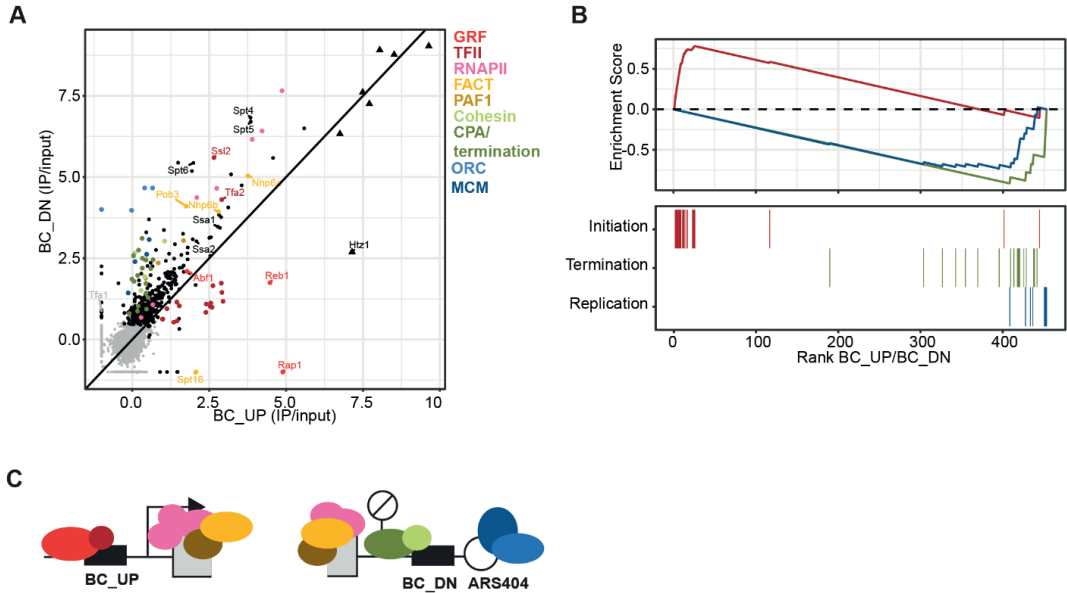


Figure 3. Capturing the chromatin interactome with Epi-Decoder

(A) Scatter plot of ChIP/input values, which can be interpreted as binding scores, of BC_UP versus BC_DN. For some factors, only the BC_UP or BC_DN value was available. In these cases, the missing value was set to -1 in order to still visualize the remaining value. Factors are colored based on known functions or complexes. Triangles represent histone proteins. The average values of six replicates are shown. (B) Enrichment plot for three categories: initiation (TFIID-E and general regulatory factors (GRFs)), termination (Cleavage and Polyadenylation and THO complex) and replication (ORC and MCM) factors. Binders were ranked based on the BC_UP/BC_DN ratio. The top part shows the running sum enrichment for each category. Initiation factors were significantly enriched at BC_UP ($p=3.18^{-3}$), and termination and replication at BC_DN ($p=2.03^{-7}$ and $p=1.06^{-5}$). The bottom lines indicate the factors represented in the categories. (C) Illustration of the different protein complexes that Epi-Decoder identified at the barcoded KanMX gene at the HO locus.

Conditional local proteomes: Chromatin rewiring in hydroxyurea

The chromatin interactome defined by Epi-Decoder confirmed that the barcodes flank an actively transcribed gene in close proximity to and transcribing towards an origin of replication that is licensed with the pre-replicative complex. This conformation is of special interest because upon firing of the origin, replication fork progression will require negotiation with the transcription machinery, potentially leading to collisions (W. Feng, Di Rienzi, Raghuraman, & Brewer, 2011; García-Muse & Aguilera, 2016; Stephan Hamperl & Cimprich, 2016; Hobson, Wei, Steinmetz, & Svejstrup, 2012; E. A. Hoffman, McCulley, Haarer, Arnak, & Feng, 2015). It has been proposed that in order to avoid collisions, RNA polymerase II is degraded at a subset of genes that is about to be replicated (Poli et al., 2016). Since this recently described process of chromatin adaptation is still poorly understood, we used Epi-Decoder to determine in a comprehensive and unbiased manner how the barcoded HO locus interactome changes when cells are arrested in early S-phase in hydroxyurea (HU; S4A Fig), the condition in which RNA polymerase II degradation has been observed (Poli et al., 2016). HU affects replication-fork progression by reducing the supply of dNTPs and by the generation of reactive oxygen species (Somyajit et al., 2017).

We first confirmed previous observations (Alvino et al., 2007) that in HU early origins have fired but that the middle to late barcoded HO origin (ARS404) has not fired yet (Fig 4A). Despite the absence of initiation of replication, Epi-Decoder revealed multiple changes in the chromatin proteome; 40 proteins showed a significantly different binding score in HU at BC_UP and 79 were significantly different at BC_DN and their altered abundance could not be explained by changes in protein levels (S4B Fig). Firstly, we observed lower mRNA levels of the barcoded *KanMX* gene compared to the *HphMX* gene, which expresses a different mRNA (Hph instead of Kan) from the same *AgTEF1* promoter but at a different genomic location (*HphMX* replaces the *CAN1* gene at chromosome V) and not in proximity to an origin of replication (Fig 4B). This was accompanied by a reduction in occupancy of multiple RNA polymerase II subunits at the barcoded gene, extending

the previous findings that RNA polymerase II is degraded at a subset of genes that has been or is about to be replicated (Fig 4C). Secondly, the chromatin response was not restricted to RNA polymerase II because other general transcription proteins involved in capping, initiation, elongation and termination were also reduced, showing that the reduction of transcription involves a broad range of changes in the (co)transcriptional machinery (Fig 4C). Thirdly, we observed additional changes that were not directly related to transcription but indicate topological alterations (Fig 4C and S4C Fig). Topoisomerase II (Top2) binding was strongly increased at both ends of the *KanMX* gene in HU. Top2 is the main enzyme releasing topological stress during S phase (Baxter & Diffley, 2008) and targeted to nucleosome free DNA during replication stress (Sperling, Jeong, Kitada, & Grunstein, 2011). Pds5, the cohesin maintenance factor was also increased at BC_DN, and a closer inspection of the cohesin complex indicates that three of the four cohesin subunits in the library showed moderately increased occupancy as well, suggesting stabilization of cohesin binding in this region (S2 Table). Finally, all the subunits of the ORC complex in the library showed decreased binding at BC_DN, while occupancy of the MCM complex was unaltered. ORC proteins have been suggested to remain bound to origins throughout the yeast cell cycle (Stephen P. Bell & Labib, 2016; DePamphilis, 2005), possibly being negatively influenced by MCM proteins in G1 (Aparicio, Weinstein, & Bell, 1997). The direct and quantitative comparison of all origin-proximal factors by Epi-Decoder suggest that the interaction of ORC proteins with the origin is compromised in S-phase prior to firing but that this cannot be explained by increased MCM protein occupancy. Therefore, our results show that ORC subunits interact more dynamically with chromatin throughout the yeast cell-cycle than expected but are in agreement with the behavior of ORC in other organisms (Bleichert, Botchan, & Berger, 2017; DePamphilis, 2005). How lower ORC binding influences origin firing and subsequent fork progression of this locus remains unknown. Interestingly, recent *in vitro* reconstitution experiments demonstrated that the MCM complex is stably bound to DNA once assembled and also competent

for replication, even after removal of ORC proteins, suggesting that origin firing per se may not be affected (Yeeles, Deegan, Janska, Early, & Diffley, 2015).

In summary, Epi-Decoder uncovered condition-dependent composition of a local chromatin-proteome. At a transcribed gene next to a licensed origin, arrest in HU led to tuning down of transcription by a general reduction of transcription protein binding, which is accompanied by increased occupancy of topology factors, and altered stoichiometry of replication proteins.

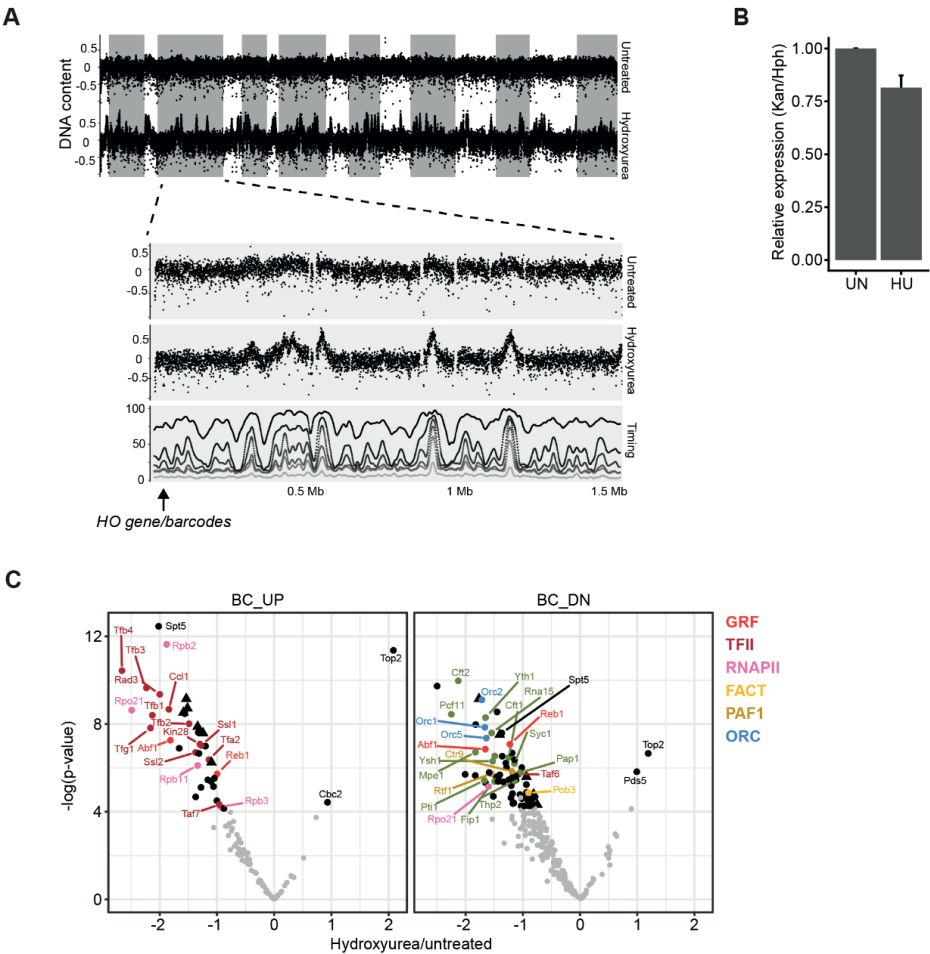


Figure 4. Chromatin rewiring upon hydroxyurea treatment

(A) DNA regions replicated in the HU arrest were determined by DNA copy number assessment. The DNA coverage in bins of 200 bp is plotted across the genome for untreated and hydroxyurea treated samples. The lower panel shows a zoom-in of chromosome IV, which contains the barcoded locus on the left arm (indicated by the arrow). Replication timing in the absence of HU was obtained from Alvino et al (Alvino et al., 2007). The percentage of replicated DNA is plotted for each bin at 10, 12.5, 15, 17.5, 25 and 40 minutes after release from a G1 arrest, as indicated by the shades of grey. (B) RT-qPCR shows relative mRNA expression of KanMX/HphMX in untreated and hydroxyurea-treated samples. The untreated samples were set to 1. The average of three biological replicates is shown; error bars indicate standard deviation. (C) Volcano plot showing the ratio of binding in hydroxyurea/untreated for factors that were significantly enriched in either one or both of the conditions. The colored dots are factors with significantly different binding scores ($FDR < 0.05$).

Application of Epi-Decoder to a native locus

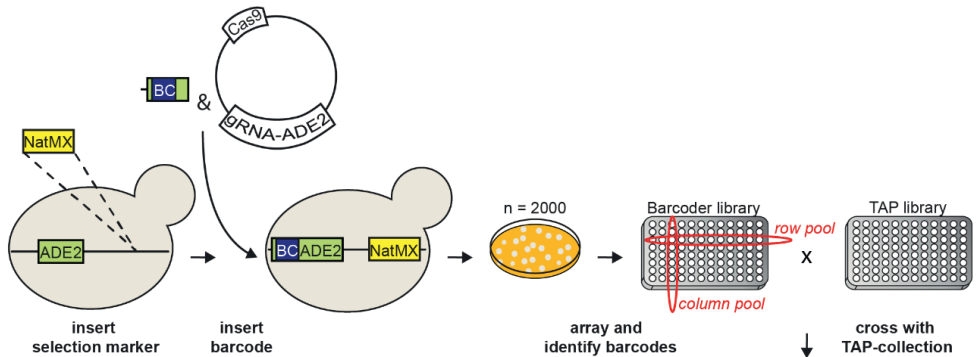
We showed that Epi-Decoder effectively measures DNA-protein interactions at a barcoded reporter gene. Next, we aimed to establish versatility, i.e. the ability to apply Epi-Decoder to any native genomic locus of interest. To this end we set up a protocol that facilitates the generation of new barcoded libraries by implementation of CRISPR/Cas9 (Fig 5A). In *S. cerevisiae*, a double-strand break induced by targeted Cas9 is generally lethal unless it can be repaired by homologous recombination (DiCarlo et al., 2013; B. X. Fu, St Onge, Fire, & Smith, 2016; Mans et al., 2015; Ryan et al., 2014). Therefore, we expected that transforming a strain with Cas9, gRNA and a BC-containing repair template would result in efficient barcode incorporation (Fig 5A). For a proof-of-concept we chose to insert a 15-bp barcode in the transcribed region of the *ADE2* gene, 57 bp downstream of the start of the *ADE2* coding sequence. Since disruption of *ADE2* causes the

accumulation of a red pigment in the cell, it provided a direct visual read-out to confirm the efficiency of the BC insertion (DiCarlo et al., 2013; Roy et al., 2018). Random colonies were selected and transferred to an arrayed format to enable crossing to the TAP-tag library and, in parallel, to identify the integrated barcode at each location. The vast majority of the colonies turned red and contained a barcode insertion, confirming that CRISPR/Cas9 can provide a highly efficient barcoding strategy. The new BC_5'-ADE2 collection contained 1974 strains with a unique barcode at the 5'-end of *ADE2*. It was crossed with the TAP-tagged protein collection, which resulted in 3604 barcode-TAP-tagged protein combinations. A NatMX cassette integrated downstream of the *ADE2* gene was used to select for the barcoded locus during the genetic crosses without interfering with the chromatin context of the barcode.

Here, the Epi-Decoder screen for BC_5'-ADE2 was performed on diploid cells, in which the TAP-tag allele and the barcoded allele are both present in a heterozygous fashion. As expected, histones and other general chromatin factors were detected (Fig 5B and S5 Table). Furthermore, we detected at BC_5'-ADE2 proteins associated with active transcription such as RNAPII subunits, transcription initiation factors, and transcription elongation factors, consistent with the promoter-proximal location of the barcode. Indeed, the binding pattern of BC_5'-ADE2 was very similar to that of BC_UP, which is also in a promoter-proximal location (Fig 5B). Together, this confirmed the successful application of Epi-Decoder to a new, native barcoded locus. Interestingly, BC_UP and BC_5'-ADE2 also showed some differences. Rap1, which is a strong binder at the Rap1-controlled *AgTEF1* promoter (M. Chen et al., 2013) was not detected at the 5'-end of the *ADE2* gene, which is in agreement with what is known about Rap1 binding across the genome (Gutin et al., 2018). Furthermore, transcription-initiation and -elongation proteins were generally recovered with lower scores at BC_5'-ADE2 than at BC_UP, which is in agreement with the lower transcript levels found for *ADE2* versus *TEF1* and *AgTEF1* promoter-controlled transcripts (S5B Fig and (Holmes et al., 2015)). Thus, by taking advantage of efficient

CRISPR/Cas9-mediated barcode library construction, Epi-Decoder can uncover the local chromatin-proteomes of native loci of interest.

A



B

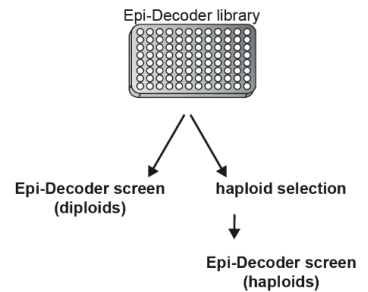
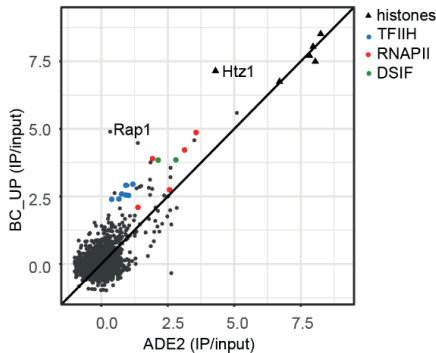


Figure 5. Application of Epi-Decoder to a native genomic locus

(A) Procedure to generate a custom Epi-Decoder collection. A selection marker (*NatMX*) was inserted in proximity to the locus of interest, downstream of the to-be-barcode *ADE2* gene. Subsequently, a Cas9 and gRNA expressing vector was introduced in this strain together with a repair template that is composed of a 15 bp random sequence (BC) flanked by 40 bp arms with homology to the gRNA target site, here at the 5'-end of the *ADE2* coding sequence (details see S5A Fig). Transformants, the vast majority of which represent barcoded clones, were transferred

to an arrayed format. Rows and columns were pooled for identifying barcodes and their location on the array by high throughput sequencing. The barcoder library was mated and crossed with the TAP-tagged protein library to generate heterozygous diploids and then haploids (see Fig 1), after which Epi-Decoder screens could be performed. For diploid screens, the insertion of a selectable marker genetically linked to the barcode can be omitted. (B) Epi-Decoder results of BC_5'-ADE2 (n=2, average) were compared to BC_UP (Fig 2). Binding scores were visualized in a scatterplot with histones shown as triangles and classes of proteins coloured: TFIIH, RNAPII, DSIF.

4.3 Discussion

Strategies aimed at identifying proteins bound at specific genomic loci frequently involve affinity-capture combined with mass spectrometry (Kan et al., 2017; Wierer & Mann, 2016). Affinity handles generally involve engineering the locus by introducing binding sites for a tagged protein, capturing a native locus by using oligo-capture, or targeting inactive tagged Cas9 proteins. Important progress has been made for multi-copy DNA loci such as repetitive DNA elements (Déjardin & Kingston, 2009; Stephan Hamperl et al., 2014). Recently, oligo-capture (Guillen-Ahlers et al., 2016) and CRISPR/Cas9 technology (Liu et al., 2017; Tsui et al., 2018) have been applied to identify proteins at single copy genomic loci. However, the problem of assessing a unique locus in a comprehensive and quantitative manner by mass spectrometry has not been solved and many challenges remain, a major one being the high degree of purification of the locus of interest while obtaining sufficient material for good coverage in MS analysis (Wierer & Mann, 2016). Epi-Decoder provides a powerful and orthogonal strategy in which the very challenging proteomics problem of decoding the chromatin proteome of a single genomic locus is addressed by DNA sequencing. We demonstrate that Epi-Decoder enables an unbiased, comprehensive and quantitative analysis of protein occupancy of a single locus in budding yeast.

In addition to known and expected core chromatin proteins, we observed significant binding scores for many proteins that do not have canonical DNA-related functions. Among the unexpected factors are several metabolic enzymes, oxidative-stress response factors, the yeast ubiquitin activating enzyme, chaperones, proteasome subunits, and RNA-processing factors (S1 Table). Some of these unexpected factors are highly expressed; their binding score may reflect non-specific binding. On the other hand, not all unexpected interactors are highly expressed or show equal binding scores. In addition, for a substantial number of the identified metabolic enzymes (e.g. glyceraldehyde 3-phosphate dehydrogenase/Tdh3, pyruvate kinase/Cdc19, homocitrate synthase/Lys20,Lys21) and protein chaperones (e.g. the HSP70 chaperones Ssa1 and Ssa2), there is evidence that they have active roles in transcription and DNA repair (Floer, Bryant, & Ptashne, 2008; Gancedo, Flores, & Gancedo, 2016; S. Li et al., 2015; Ringel et al., 2013; Torres-Machorro, Aris, & Pillus, 2015). Furthermore, the binding scores of some metabolic enzymes changed upon treatment with HU (S2 Table), indicating that their interaction with chromatin is not constitutive. Indeed, there is a growing body of evidence that enzymes in the cytoplasm might have moonlighting functions in the nucleus, for example by local supply of co-factors of histone modifying enzymes (Boukouris, Zervopoulos, & Michelakis, 2016; S. Li et al., 2015; Linder & Mostoslavsky, 2017; Monaghan & Whitmarsh, 2015; Sivanand et al., 2017; Su & Pillus, 2016). Alternatively, the interaction with chromatin could somehow influence the activity of metabolic enzymes. The protein interactions that we describe here for three barcoded locations provide a rich resource for further unraveling the biological meaning of non-canonical chromatin interactions.

Epi-Decoder is more than a discovery tool. It offers the possibility to compare two loci or to analyze one locus under different physiological or genetic conditions in a systematic and quantitative manner. Since all proteins are examined in a pooled fashion and using the same affinity handle, differences in purification and cell backgrounds can be excluded and binding scores can be directly compared. This is exemplified by the basal transcription factors Tfa2 and Ssl2, which are

quantitative outliers compared to their complex members (TFIIE and TFIIH, respectively), suggesting non-canonical functions. Another example is the chromatin rewiring we observed upon treatment with HU, which uncovers large scale quantitative changes in interactions of transcription and replication proteins, as well as other factors.

We note that Epi-Decoder generates independent binding scores for proteins with (nearly) identical protein sequences but encoded by different genes, such as the histone proteins or other protein paralogs such as Ssa1 and Ssa2, which are 98% identical. This can provide extra information about the differential use of related proteins that cannot easily or not be distinguished by mass spectrometry methods. Protein isoforms that arise through post-translational modification cannot be distinguished by Epi-Decoder.

The Epi-Decoder strategy will be applicable to a broad range of fundamental epigenetic questions. A few requirements need to be met for successful application. First, Epi-Decoder requires a library of tagged proteins. For budding yeast, several tagged protein libraries are already available in addition to the carboxy-terminal TAP-tag library used here (Douglas et al., 2012; Huh et al., 2003; Suter et al., 2013). This will allow for extending the current Epi-Decoder analyses to a nearly complete coverage of the proteome as well as to alternative (amino-terminal) tags, together enabling an unprecedented deep analysis of chromatin proteomes. Importantly, tagged protein libraries are also becoming available for many other organisms (Dewari et al., 2018; Hein et al., 2015; Huttlin et al., 2017; Luck, Sheynkman, Zhang, & Vidal, 2017; Yang et al., 2011; Yazaki et al., 2016). A second requirement for Epi-Decoder applications is the integration of DNA barcodes proximal to the locus of interest. Given their small size, DNA barcodes generally only minimally disrupt a locus but this needs to be verified for the locus of interest, as is the case for strategies involving targeting affinity handles by Cas9 or other approaches. Fortunately, genomic barcoding has come within reach for many research questions due to the availability emerging genome engineering strategies, in yeast and other organisms (B. X. Fu et al., 2016; Mitsunobu, Teramoto,

Nishida, & Kondo, 2017; Ryan et al., 2014; Wright et al., 2016). Indeed, we demonstrate that CRIPSR/Cas9 can be used to generate markerless barcoded DNA libraries at high efficiency for a new locus of interest (Ryan et al., 2014). Furthermore, our findings with the barcoded native *ADE2* locus demonstrate that Epi-Decoder is not restricted to haploid cells but can also be used in diploid cells in which the tagged allele of a protein and the barcoded allele are present in a heterozygous state. This greatly facilitates creating Epi-Decoder libraries in yeast as well as other cell systems.

In summary, Epi-Decoder is powerful and versatile strategy for decoding the protein interactome of a single genomic locus. We expect that the Epi-Decoder strategy and derivatives thereof will enable the decoding of dynamic proteomes of different loci in different organisms and will be of high value for addressing a broad range of important chromatin-biology questions in future applications.

4.4 Methods

Media and growth conditions

Yeast media was prepared as described previously (Tong & Boone, 2006; van Leeuwen & Gottschling, 2002). For screening in untreated conditions, yeast strains were grown in YEPD (1% yeast extract, 2% bacto peptone, and 2% glucose) in log phase. S phase arrest was achieved by adding to log phase cells (OD₆₆₀ of 0.4) in YEPD one volume of YEPD + 360 mM HU to achieve 180 mM HU final concentration and cells were harvested after 2 hours. The arrest was verified by flow cytometry.

Yeast strains and library manipulations

Yeast strains used in this study are listed in S6 Table. Library manipulations on solid media were performed using synthetic genetic array (SGA) technology (Tong & Boone, 2006) combined with

robotics using a RoToR machine (Singer Instruments, Watchet, UK). Library manipulations in liquid media to generate pools for barcode identification were performed using a Hamilton Microlab Star (Hamilton, Germany). A collection of barcoded Tandem Affinity Purification (TAP)-tagged strains (library NKI4217) was generated as follows. In order to cross the TAP-tag and Barcoder libraries, the TAP-tag collection (Ghaemmaghami et al., 2003) was made compatible for SGA and converted to mating type α . This was done by crossing the TAP library with NKI4212, which was derived from strain Y8205 by replacing the STE2pr-Sp_his5 cassette at the *CAN1* locus by *HphMX* to enable selection during SGA for the TAP-His3MX6 alleles by using histidine prototrophy. This resulted in the *MAT α* TAP collection (NKI4214) divided over four different 1536-plates. Each of the TAP-tag plates was mated with the set of 1140 barcoder *MATa* strains containing a pair of known unique barcodes flanking a constitutively transcribed kanR marker gene (*KanMX*) under control of the *Ashbya gossypii* *TEF1* promoter and terminator at the *HO* locus (Yan et al., 2008). Diploids were obtained by G418 and Hygromycin double selection on rich media and transferred to sporulation media. After sporulation, the combination library was obtained by selecting twice for *MAT α* haploids and then twice for *MAT α* haploids containing a TAP-tag, the barcoded *KanMX* cassette and the *HphMX* marker. The barcodes and TAP tags were verified for a few selected strains. The effects of sonication time and the presence of methanol in formaldehyde solutions were assessed by performing three biological replicate analyses of the pool of plate 2 of the *KanMX* Epi-decoder library. Five extra barcoded control strains (TAP-tagged versions of Hht2, Htb2, Rpl13a, Ste2 and Bar1) were generated by using the respective clones from the NKI4214 library and introducing unused barcodes from *MATa* haploid gene knockout library (Open Biosystems, Huntsville, AL) at the *HO* locus of these strains.

Generation of the BC_5'-ADE2 barcoded collection**Preparation of the Cas9/gRNA vector**

The Cas9 and gRNA expression vector pML104 (Addgene plasmid #67638 (Laughery et al., 2015)) was used to introduce a double strand break at the *ADE2* locus, 57 bp into the coding sequence. pML104 was isolated from dam- INV110 *E. coli* (ThermoFisher #C717103) and digested with *Swa*I (NEB #R0604) and *Bcl*I (NEB #R0160). DNA oligonucleotides gRNA_Ade2_39a and gRNA_Ade2_39b (designed using <http://wyrickbioinfo2.smb.wsu.edu/crispr.html>) were hybridized in T4 ligation buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH 7.5) cycling through: 95°C for 6 minutes; decrease 70x 1°C/1 minute (final 25°C). The hybridized oligonucleotides were directly ligated into the digested pML104 vector (molar ratio: 100 hybridized nucleotide insert / 1 cut vector) by T4 ligase (NEB #M0202S) for 20 hours at 16 °C. Alpha-Select Silver Competent Cells (Bioline #BIO-85026) were transformed by heat shock with the ligation product for amplification of the ADE2-gRNA containing pML104 vector.

Barcode containing repair template

The barcode containing repair template was generated from a long DNA oligonucleotide (Repair_Guide_39; final concentration 0.001 µM) using MyTaq Red Mix (Bioline #BIO-25043) and two primers (Ade2_BC_39_fw and Ade2_BC_39_rv; final concentration 0.5 µM) in a PCR reaction: 96°C 1 minute, 14 x (96°C 20 seconds, 50°C 20 seconds, 72°C 20 seconds), 72°C 1 minute. The PCR product was purified using CleanPCR beads (CleanNA #CPCR-0050).

Preparation of *S. cerevisiae* with a selection marker

Before introducing a barcode, the selection marker NatMX was introduced 140 bp downstream of the *ADE2* ORF to enable selection for the barcoded locus during genetic crosses with the TAP-tagged protein collection. This step can be omitted for screens with diploid cells. NatMX was amplified from pFvL99 (Verzijlbergen et al., 2010) (primers: pRS_to_ADE2_1 and pRS_to_ADE2_2) and integrated in BY4741 (Brachmann et al., 1998) by homologous recombination followed by CloNat (100 µg/mL) resistance selection, resulting in strain NKI2558.

Construction of the BC_5'-ADE2 collection

NKI2558 (4×10^6 cells) was transformed with the ADE2-gRNA containing pML104 vector (50-250 ng) and repair template (12.5-125 pM) via the LiAc/SS carrier DNA/PEG method (Gietz & Schiestl, 2007). Transformed cells were plated on synthetic complete uracil drop-out plates and colonies were grown for four days at 30°C. Single colonies were moved to liquid YEPD + CloNat in 384-plates using toothpicks. These arrayed strains were plated using the RoToR from Singer Instruments (Watchet, UK) on solid medium containing 5-fluoroorotic acid to select for the loss of the plasmid. The new BC_5'-ADE2 collection (NKI2559) was crossed with the TAP-tag collection as described in method section 'Yeast strains and library manipulations, with the exception that CloNat was used instead of G418 to select for the barcoded allele in this library. The diploid library (NKI2560) was used for Epi-Decoder analysis.

Decoding the barcode sequences and locations of the BC_5'-ADE2 collection

The BC_5'-ADE2 collection was grown in 384-plates in liquid YEPD + CloNat and a Hamilton robotics liquid handling workstation (Hamilton Microlab Star, Hamilton, Germany) was used to make pools of strains that were located in the same row or in the same column, so that yeast from

each position on the plate was present in one row-pool and one column-pool (Zuzarte et al., 2014). DNA was extracted from each pool using phenol-chloroform-isoamyl alcohol (method section: 'Genomic DNA isolation and sequencing'). Barcodes from all the pools were amplified by PCR to prepare sequencing libraries for the MiSeq platform; similar as described in method section 'Epi-Decoder (TAG-ChIP-Barcode-Seq)'. During this barcode amplification a pool-specific unique 6 or 12 bp index was incorporated in the PCR products (S7 Table). High throughput sequencing was performed using a custom sequence primer following the guidelines from <http://nextgen.mgh.harvard.edu/CustomPrimer.html>. After high throughput sequencing, reads were sorted by the pool-specific index. The first 15 bp sequence-read that occurred at least 50 or 100 times with the same index were appointed to be the barcodes. Barcodes identified in a single row-pool were searched for in all single column-pools; a match between row and column reflects a coordinate, a position on the original 384-plate. Barcodes for which more than one matching row-column combination was detected were rejected. Locations to which more than one barcode was assigned were also rejected.

Epi-Decoder (TAG-ChIP-Barcode-Seq)

Epi-decoder libraries were grown on YEPD plates overnight and the colonies of each plate were pooled together in liquid culture. The cultures were grown until log phase (OD₆₆₀ of ~0.4) and cross-linked for 20 minutes with 1/10th of the volume of freshly prepared Fix Solution (1% formaldehyde (Sigma-Aldrich 252549-500ML), 50 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA) and subsequently quenched for 5 minutes with Glycine (125 mM final concentration). Where indicated, methanol-free formaldehyde (Thermo Scientific; 28908) was used for cross-linking. Cells were washed once in cold TBS with 0.2 mM PMSF and the pellet was frozen at -80°C. Cells from frozen pellets of $\sim 1.5 \times 10^9$ cells were lysed by bead beating in 200 μ L breaking buffer (100 mM Tris pH 7.9, 20% glycerol, protease inhibitor cocktail EDTA-free) with

Zirconia/silica beads. The lysate was washed twice in 1 ml FA buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, protease inhibitor cocktail EDTA-free) and sonicated using the Bioruptor PICO (Diagenode) for 10 minutes at 30 s intervals. Where indicated, chromatin was sonicated for 5 or 20 minutes. Chromatin was cleared by centrifugation for 5 minutes at 4 °C at 4000 rpm. One hundred microliter chromatin was used as input material. For ChIP of the Barcoded KanMX gene, IgG Sepharose 6 Fast Flow beads (GE healthcare) were washed 3 times with PBSB (PBS containing 5 mg/mL BSA), and incubated with 1 ml chromatin for 6 hours on a turning wheel at 4 °C. For ChIP of the barcoded *ADE2* locus, Dynabeads M-270 Epoxy beads (ThermoFisher #14301) were used. Rabbit Immunoglobulin G (IgG, 0.2 mg) from serum (Sigma, I5006-100MG) was coupled to 10 mg epoxy-activated Dynabeads in phosphate buffer with ammonium sulphate according to the manufacturers protocol. After a PBS wash, IgG-couples beads were resuspended in 1.2 ml PBS containing 0.02% sodium azide. Of a prepared-beads solution 80 µL was used for 800 µL chromatin. Samples were washed twice in FA buffer, twice in high salt FA buffer (500 mM NaCl), twice in RIPA buffer (10 mM Tris pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA) and once with TE buffer (10 mM Tris pH8, 1 mM EDTA). With each wash step, the Sepharose beads were spun for 2 minutes at 3000 rpm at 4 °C. IP samples were eluted for 10 minutes at 65°C in 100 µL elution buffer (50 mM Tris pH 8, 10 mM EDTA, 1% SDS). IP and input samples were digested with 0.5 µL RNase A (10 mg/mL) and 10 µL ProtK (10 mg/mL) in 70 µL TE for 1 hour at 50 °C and subsequently kept overnight at 65°C to reverse crosslinks. DNA was purified using the QIAquick PCR purification kit (Qiagen). BC_UP, BC_DN and BC_5'_ADE2 were amplified separately with specific primers (S7 Table: 2B_UP_Fw + 2B_UP_Rv_all; 2B_DN_Fw +2B_DN_Rv_all; P5-XL-ADE2_g39_REV + P7L-IDn-ADE2_g39_REV). PCR products were mixed in an equimolar fashion and purified from an agarose gel with a size selection of 100-150 bp. The purified DNA was sequenced (single read, >50 bp) on a HiSeq2500/MiSeq platform (Illumina, San Diego, CA), using

one or a mix of custom sequencing primers (S7 Table: 2B_UP_Seq, 2B_DN_Seq or Seq-XL-ADE2_g39_REV).

Barcode counting

Barcodes were extracted from the sequencing reads by using the Perl script eXtracting Counting and Linking to Barcode References (XCALIBR). The code and detailed descriptions of the functions are available at <https://github.com/NKI-GCF/xcalibr>. Briefly, XCALIBR locates the constant regions (U2 and D2 of the barcoded *KanMX* gene or the constant region next to the barcode at *ADE2*) in each amplicon and reports the 6 bp upstream of the index, and 20 bp downstream of the *KanMX* barcode or 15 bp downstream of the *ADE2* barcode. The resulting table contains counts for each barcode-index combination. Counts below 10 were removed from this table before further pre-processing and filtering steps.

Barcode data pre-processing and filtering

Input (IN) and immunoprecipitated DNA (IP) of each plate was amplified separately with a unique index. Even though we aimed to mix each sample equimolarly, plate-specific differences in counts could still occur. This was corrected by normalizing each plate by its median. The counts table was log2 transformed and barcode-index combinations were matched with the ORF names. Factors with low input counts were removed since these were likely to be missing from the library or the barcode failed to amplify due to technical reasons. We manually validated several factors for the presence of a TAP tag by PCR with primers in the specific ORF and the TAP tag. This revealed that ORC4, MCM3, MCM7 and RAD6 were not properly tagged; we therefore removed these strains for further analysis. The final set of the barcoded *KanMX* library contained information on 3,994 BC_UP and 3,955 BC_DN barcode clones. We noticed that the dynamic range was slightly different between biological replicates. To overcome this problem, we

performed quantile-normalization for the replicates of BC_UP and BC_DN separately. This method is used to generate similar distributions by first ranking each replicate based on the counts and then replacing the counts of each rank by the mean count of that rank.

Barcode statistical analysis

Factors with IP counts that were significantly enriched over input were identified by using the Limma R/Bioconductor software package (Ritchie et al., 2015). P-values were adjusted for multiple testing by converting them to false discovery rates using the Benjamini-Hochberg procedure. Factors with a positive fold-change and an $FDR < 0.01$ were selected as significantly enriched. For untreated conditions, six independent biological samples were used. The running sum scores for the enrichment plot (Fig 3B) were calculated with the `gseaScores` function from the HTSanalyzeR package. For identifying differential binders upon HU treatment, three biological samples were used for treatment and no treatment. Here, we only considered factors that were significant binders in at least one of the conditions ($FC > 0$ and $FDR < 0.05$). Limma was used to select factors with significantly different IP counts ($FDR < 0.05$). Barcode counts were compared with protein abundance data measured by GFP intensity. This data was obtained from the CYCLOPs database (Koh et al., 2015).

GO slim process enrichment

GO slim process terms are condensed versions of the full GO ontology (Ashburner et al., 2000; Gene Ontology, 2015). GO slim process terms were downloaded from the *Saccharomyces* Genome Database (Cherry et al., 2012) and enrichment analysis was performed by using the `fisher.test` function in R with option `alternative="greater"`. We manually assigned the following categories based on GO slim terms listed here. DNA binding: DNA-templated transcription,

initiation, DNA-templated transcription, elongation, DNA-templated transcription, termination, transcription from RNA polymerase I promoter, transcription from RNA, polymerase II promoter, chromatin organization, histone modification, DNA replication, DNA recombination, DNA repair, cellular response to DNA damage stimulus, regulation of DNA metabolic process, nuclear transport and chromosome segregation. RNA binding or processing: mRNA processing, rRNA processing, tRNA processing, RNA modification, RNA splicing and RNA catabolic process. Metabolism: carbohydrate metabolic process, cofactor metabolic process, nucleobase-containing small molecule metabolic process, monocarboxylic acid metabolic process, cellular amino acid metabolic process, generation of precursor metabolites and energy, oligosaccharide metabolic process and lipid metabolic process.

ChIP-qPCR

ChIP experiments were performed similar to Tag-ChIP-Barcode-Seq, but with 20 µl bed volume of IgG Sepharose 6 Fast Flow beads and 200 µl chromatin. For untagged-H2A.Z ChIP, the Htz1-specific antibody (Active Motif, 39647) was coupled to Protein G Dynabeads (Thermo Fisher Scientific). ChIP was performed with 20 µl Dynabeads and 200 µl chromatin. Factors were selected such that they reflect different classes of binders and different expression levels, in addition to negative controls Bar1 (not expressed in these cells) and Rpl13A (a ribosomal subunit). Quantitative PCR was performed on the purified DNA with SYBR green master mix (Applied Biosystems or Roche) or SensiFAST SYBR master mix (Bioline) according to the manufacturer's protocol and analyzed on LightCycler 480 II (Roche). The binding was analyzed with specific primers in close proximity to the BC_UP and BC_DN (S7 Table). Each sample was measured in two technical duplicates in the qPCR and the average value of these two was taken as one value when combining biological replicates. For comparison of the ChIP-qPCR and BC-seq results in a quantitative manner, the negative control (Bar1) was used to normalize the raw values.

Flow Cytometry

Flow cytometry samples were prepared to monitor cell cycle progression and verify S phase arrest by HU. 1×10^7 Cells were collected and fixed with 70% ethanol and stored at -20°C . Flow cytometry was performed as previously described (Verzijlbergen et al., 2010), after staining DNA with Sytox green (Molecular Probes). Flow cytometry measurements were taken on a FACSCalibur with CellQuest software (Becton Dickinson) and further analyzed with FlowJo software (Treestar).

Genomic DNA isolation and copy-number determination by sequencing

Two independent strains (NKI8529 and NKI8587) were selected for genomic DNA isolation to determine the replication status of genomic loci. Genomic DNA was isolated as described previously (C. S. Hoffman & Winston, 1987). Briefly, 2×10^7 cells were spun down and resuspended in 200 μl of 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA. Then, 200 μl phenol-chloroform-isoamyl alcohol (25:24:1) was added and cells were lysed by bead beating with 300 μl Zirconia/silica beads. 200 μl TE was added, the cells were spun for 5 minutes at max speed and the aqueous layer was transferred to a new tube. The pellet was washed in 1 ml 100% ethanol and re-suspended in 400 μl TE. To precipitate the DNA 10 ~14 M ammonium acetate and 1 ml 100% ethanol was added and the tube was inverted to mix contents and spun 2 minutes at 13000 rpm. The pellet was air dried and suspend in 50 μl TE. The gDNA was then purified by sending through an Isolate II genomic DNA kit (Bioline). 20 μl of ethanol precipitated gDNA was resuspended in 180 μl lysis buffer GL and 1 μl RNaseA (10 mg/ml) and kept at room temperature for 20 minutes before proceeding with step 3 of the manufacturers protocol. DNA was eluted in 50 μl elution buffer G. The gDNA was sheared with a Covaris E220 ultrasonicator (Covaris) to obtain fragments of 150-200bp and sequenced on the HiSeq2000 platform (Illumina, San Diego, CA).

Genomic DNA isolation and sequencing data analysis

Single-end reads of 65 bp were aligned to the *S. cerevisiae* reference genome version R64 (UCSC SacCer3) by using BWA (H. Li & Durbin, 2009). The BAM files with aligned reads were filtered for mapq 37 and converted to bedgraph with bins of 250 bp by using deepTools (Ramirez et al., 2016). By using custom R scripts, the region of unstable transcript XUT_12F-188 was removed because it contains repetitive sequences. The average coverage in each 250 bp bin was plotted across the entire genome by using custom R scripts. The replication timing in the absence of HU was obtained from a density transfer experiment (Alvino et al., 2007), and downloaded from Table S1 in that study. This table contains the percentage of the genome that had become hybrid in density (%HL DNA) in cells collected at different time points (Alvino et al., 2007). The plots were generated for both samples separately and showed similar patterns.

Reverse Transcription

RNA was isolated using the RNeasy Mini Kit (QIAGEN) using the protocol for Yeast cells, with a few modifications. Briefly, 2×10^7 cells were spun down (5 minutes at 3000 rpm) and pellets were dissolved in 600 μ L cold RLT buffer. Cells were broken by bead beating with 400 μ L Zirconia/silica beads and debris was separated by centrifuging 2 minutes at 13000 rpm. The supernatant (~350 μ L) was collected and mixed with one volume (~350 μ L) 70% EtOH and transported to RNeasy columns. Following the buffer RW1 and buffer RPE wash steps; RNA was eluted in 50 μ L elution buffer. Eluted RNA was treated with DNase I (QIAGEN) to remove genomic DNA. Next, cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen). RT-PCR was performed with the primers in S7 Table. Each sample was measured in two technical duplicates and the average value of these two was taken as one value when combining biological replicates.

Quantitative data used for graphs in figures

Quantitative observations that underlie the data summarized in the graphs are shown in S1 Data.

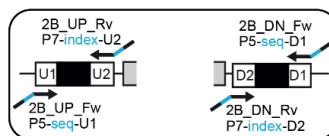
Data Availability and accession numbers

Tables S1-S5, S1 Data and data accession numbers can be accessed on the journal website, <https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.2005542>

4.5 Acknowledgements

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4.6 Supporting Data

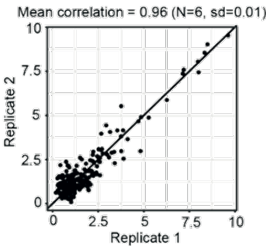


S1 Fig. Relates to Fig 1.

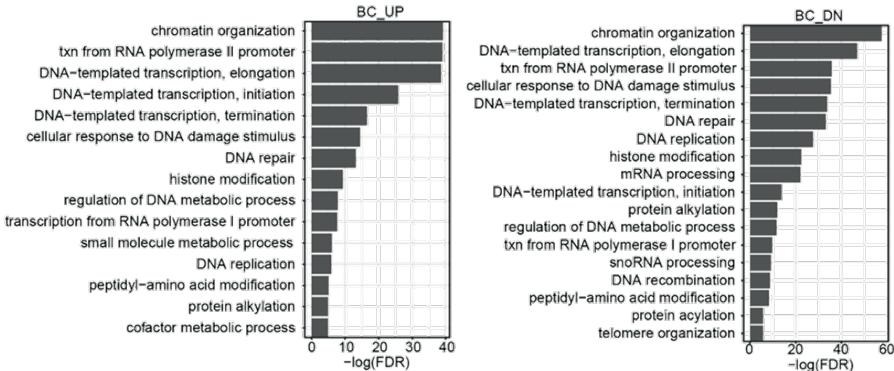
Schematic overview of the primers used to amplify the HO barcodes, which are flanked by constant regions U1, U2, D1 and D2. The forward primers introduce the Illumina P5 sequence and

extra nucleotides for annealing of the 5'-end of the custom sequencing primers. The reverse primers introduce the Illumina P7 sequence as well as a 6 bp index.

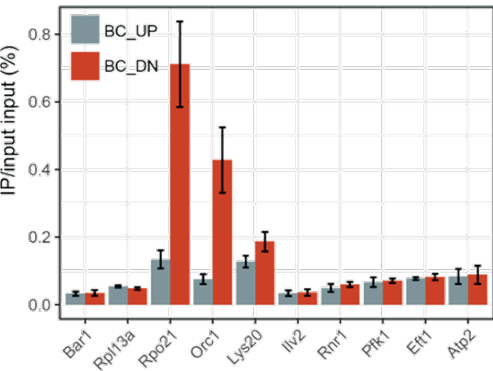
A



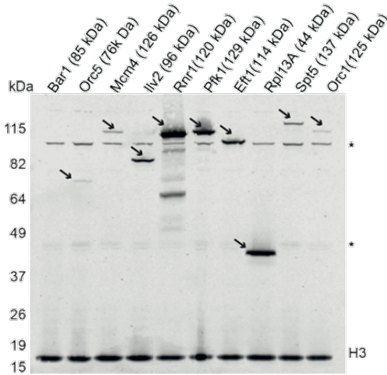
B



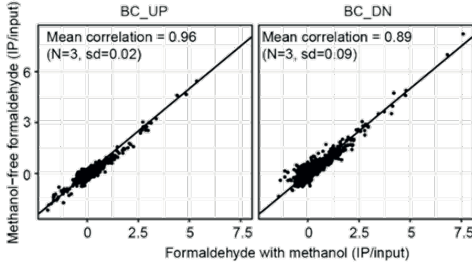
C



D

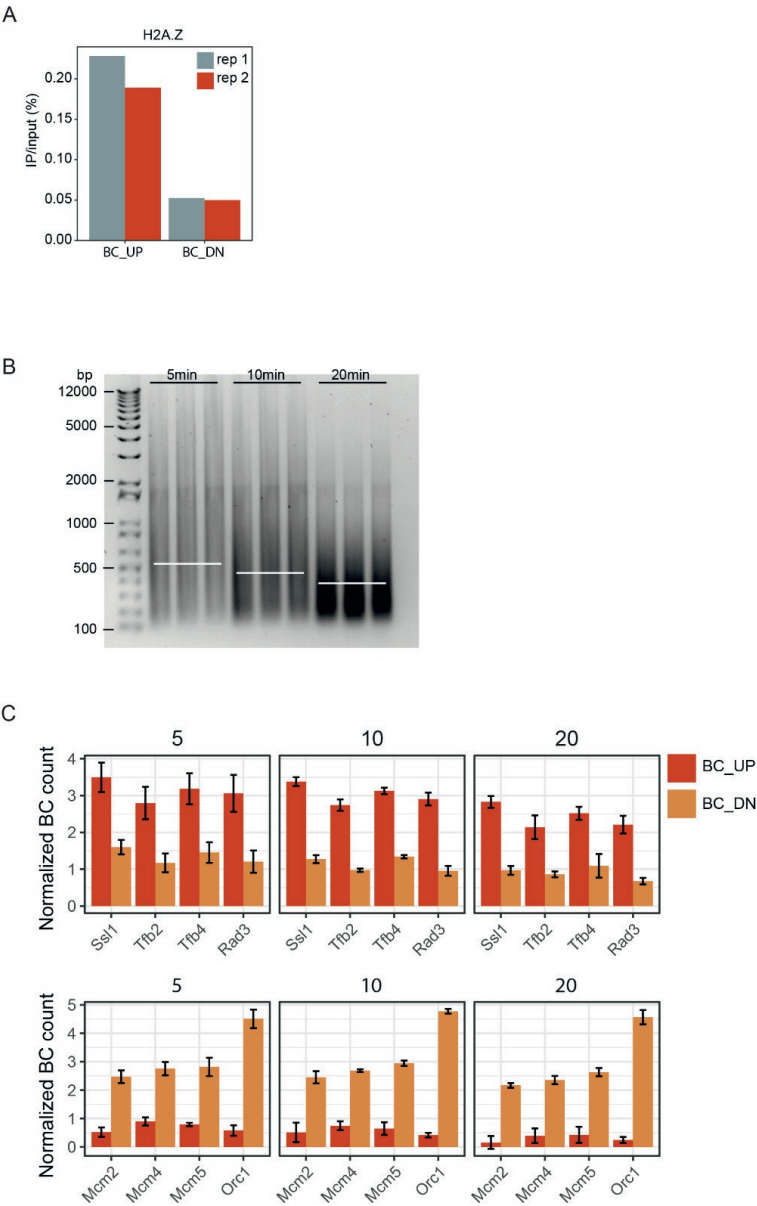


E



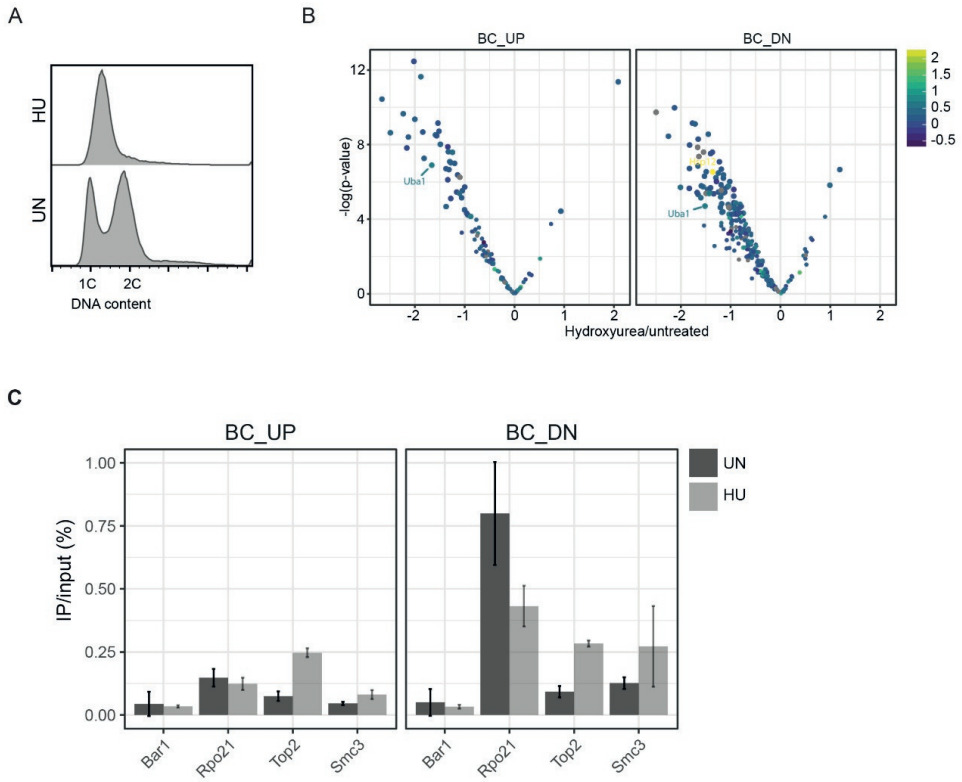
S2 Fig. Relates to Fig 2.

(A) Scatter plot showing the normalized BC-UP and BC-DN barcode counts for the binders of biological replicates. Two samples were randomly chosen for a representative figure. The mean correlation and standard deviation was calculated for all six replicates. (B) Bar plot showing the GO slim terms (process) enriched at BC_UP and BC_DN. Terms with a Fisher Exact p -value < 0.01 were considered to be enriched. The x-axis shows the number of factors associated with each term, and the non-transparent color indicates the proportion of binders. (C) ChIP-qPCR analysis of selected TAP-tag strains with specific primers in proximity to BC_UP and BC_DN (S7 Table). The average of three biological replicates is shown; the error bars indicate the standard deviation. Bar1 (not expressed) and Rpl13a (a highly-expressed ribosomal subunit) were included as negative controls. Rpo21 (the largest subunit of RNA polymerase II), Orc1 (the largest subunit of the ORC complex) and Lys20 (homocitrate synthase isozyme) were identified as binders by Epi-Decoder. Ilv2 (acetolactate synthase), Rnr1 (a subunit of ribonucleotide reductase), Pfk1 (phosphofructokinase), and Eft1 (a translation elongation factor) are highly-expressed proteins (see panel D) that were not identified as binders by Epi-Decoder. (D) Expression levels of proteins shown in panel C were verified by immunoblot analysis. The indicated protein sizes (kDa) include the TAP tag. Untagged histone H3 was used as a loading control; the asterisks indicate non-specific bands that are also detected in the negative Bar1 control. (E) Scatter plot showing the normalized barcode counts for one subset of the HO Barcoder library (see Methods and S3 Table), comparing regular methanol-containing with methanol-free formaldehyde. Values shown are average across three replicates for both BC_UP and BC_DN.



S3 Fig. Relates to Fig 3.

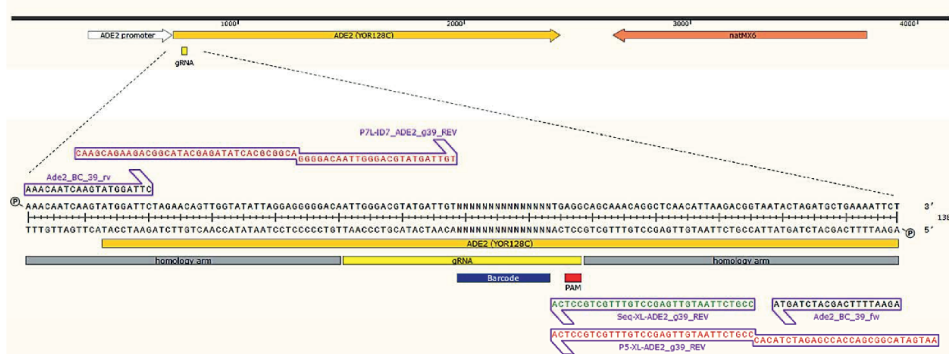
(A) ChIP-qPCR analysis of endogenous (untagged) H2A.Z with specific primers in proximity to BC_UP and BC_DN (S7 Table). Two independent biological replicates are shown of strain NKI3504. (B) DNA gel showing the distribution of the DNA fragment sizes after 5, 10, and 20 minute sonication, with average values of 520, 470, and 390 bp. (C) Bar plot showing normalized Epi-Decoder barcode counts in the 5, 10, and 20 minute sonication samples. Shown are factors of the TFIIH complex that were specifically enriched at BC_UP (promoter), and subunits of the ORC and MCM that were specifically enriched at BC_DN (proximal to the origin of replication). Bars represent average across three biological replicates and error bars represent standard deviation.



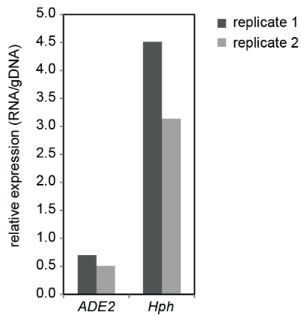
S4 Fig. Relates to Fig 4.

(A) Flow cytometry analysis showing the DNA content of cells with (HU) and without hydroxyurea (UN) treatment (180 mM for 2 hours). (B) Volcano plot similar to Figure 4b, but here the dots are colored based on their protein abundance changes in HU based on data from (Tkach et al., 2012). (C) ChIP-qPCR for selected strains, with specific primers in close proximity to BC_UP and BC_DN. Bar1-TAP was used as a negative control because it is not expressed in these cells. To compare the barcode counts with ChIP-qPCR signal, the samples were normalized by the Bar1-TAP signal before calculating ChIP/input. The average of three biological replicates is shown; the error bars indicate the standard deviation.

A



B



S5 Fig. Relates to Fig 5.

Design and expression of the barcoded ADE2 locus. (A) NatMX6 (pink) was inserted downstream of the ADE2 (YOR128C) coding sequence (orange) after which 15 bp barcodes were inserted 57 bp downstream of the start codon of ADE2 by template-directed repair of a CRISPR/Cas9-induced break. The zoom-in visualizes the repair template that was transformed in to *S. cerevisiae* along with the Cas9+gRNA expression vector via which the barcode was inserted: the orange bar is the 5'-end of ADE2; 15x 'N' is the barcode; the yellow bar (minus the 15x 'N') is complementary to gRNA + the PAM sequence (red bar); the grey bars are the homology arms. The purple delineated arrows are primers used for: amplifying the repair template (black text, Ade2_BC_39_fw and Ade2_BC_39_rv); amplifying an Epi-Decoder_5'_ADE2 library (red text, P5-XL-ADE2_g39_REV

and P7L-ID7_ADE2_g39_REV); sequencing an Epi-Decoder BC_5'-ADE2 library (green text, Seq-XL-ADE2_g39_REV). (B) The relative activity of the ADE2 and AgTEF1 promoter was derived from transcript levels of the mRNAs expressed from the two promoters (ADE2 and Hph) as determined by RT-qPCR. Quantitative PCR was also performed for gDNA samples (n=9) to correct for differences in primer efficiency. The bars represent two biological replicates (strains NKI8587 and NKI8588; arbitrary values). ADE2 mRNA (expressed by the ADE2 promoter) was lower than Hph mRNA (expressed by the AgTEF1 promoter of the HphMX cassette). The lower expression of ADE2 is in agreement with RNA-Seq measurements of relative ADE2 and ScTEF1 expression levels (Holmes et al., 2015).

S6 Table. List of yeast strains used in this study.

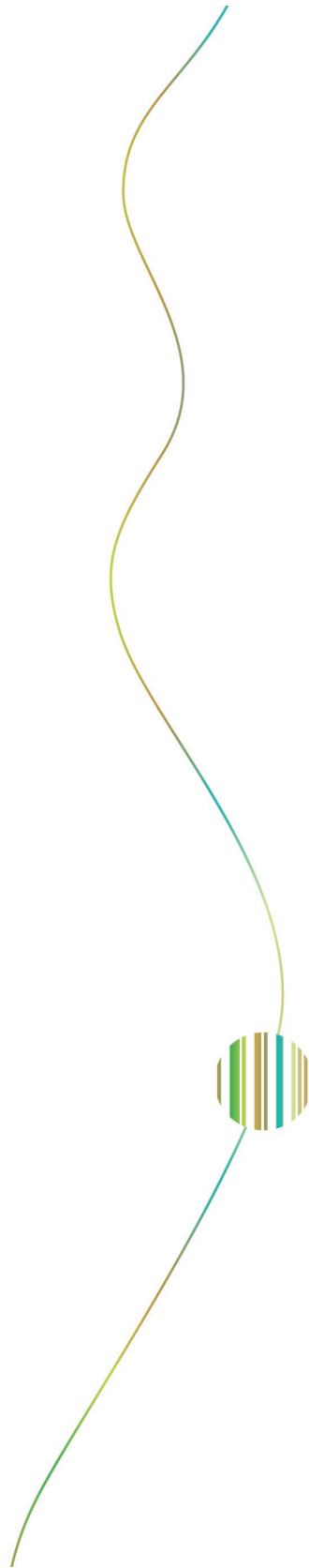
Strain	Ref	Genotype
TAP-tag library	1	MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 gene-TAP-His3MX6
Y8205	2	MATα can1Δ::STE2pr-Sp_his5 lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0
Barcode library	3	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ho::BC-KanMX-BC
NKI4212	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0
NKI4214 library	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 gene-TAP-His3MX6
NKI4217 library	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC gene-TAP-His3MX6
NKI8525	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC(sir2) HHT2-TAP-His3MX6
NKI8526	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC(sir3) RPL13A-TAP-His3MX6
NKI8527	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC(sir4) HTB2-TAP-His3MX6
NKI8528	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC(caf40) STE2-TAP-His3MX6
NKI8529	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC(mot2) BAR1-TAP-His3MX6
NKI8589	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC SSA1-TAP-His3MX6
NKI8582	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC SSA2-TAP-His3MX6
NKI8587	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC RPO21-TAP-His3MX6
NKI8588	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC TOP2-TAP-His3MX6
NKI8590	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC SMC3-TAP-His3MX6
NKI3515	This study	MATα can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC CDC19-TAP-His3MX6
NKI2558	This study	MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ADE2-NatMX
NKI2559 library	This study	MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 BC-ADE2-NatMX
NKI2560 library	This study	MATa/α his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 CAN1/can1Δ::STE2pr-Sp_his5 LYP1/lyp1Δ::STE3pr-LEU2 ADE2/BC-ADE2-NatMX GENE/gene-TAP-His3MX6
NKI3504	This study	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ho::BC-KanMX-BC

S7 Table. List of the DNA oligonucleotides used in this study

Name	Sequence
HOpromQfw	GAAGCTTGTTGAAGCATGATGAA
HOpromQrev	TTGCTGCTTATGAGGATATGGATTT
HOtermQfw	GAGTAGAAATACGCCATCTCAAGATACA
HOtermQrev	GGAAAGTTGATCAAGACCCAATAATAA
2B_UP_Fw	AATGATACGGCGACCACCGAGATCTACACCTTCCGATCTAGATGTCCACGAGGTCTCT
2B_DN_Fw	AATGATACGGCGACCACCGAGATCTACACTCTTCCGATCTACGGTGTCCGGTCTCGTAG
2B_UP_Rv_all	CAAGCAGAAGACGGCATACGAGATNNNNNNNGTCGACCTGCAGCGTACG
2B_DN_Rv_all	CAAGCAGAAGACGGCATACGAGATNNNNNNAACGAGCTCGAATTCATCGA
2B_UP_Seq	ACACCTTCCGATCTAGATGTCCACGAGGTCTCT
2B_DN_Seq	ACACTCTTCCGATCTACGGTGTCCGGTCTCGTAG
KanMX_Qfw	TGATGCGCTGGCAGTGTT
KanMX_Qrev	CGCGATCGCTGTTAAAAGGA
HygroFwd	TCGGTCAATACACTACATGG
hphmidR	TTTGCCAGTGATACACATGG
gRNA_Ade2_39a	GATCATTGGGACGTATGATTGTTGGTTTTAGAGCTAG
gRNA_Ade2_39b	CTAGCTCTAAAACCAACAATCATACGTCCCAAT
Repair_Guide_39	AGAATTTTCAGCATCTAGTATTACCGTCTTAATGTTGAGCCTGTTTGCTGCCTCANNNNNNN NNNNNNNNNACAATCATACGTCCCAATTGTCCCCCTCCTAATATACCAACTGTTCTAGAATC CATACTTGATTGTTT
Ade2_BC_39_fw	AGAATTTTCAGCATCTAGTA
Ade2_BC_39_rv	AAACAATCAAGTATGGATTG
pRS_to_ADE2_1	ATAGAAATCAACGCTTACAGGTGTCCTTTTTTAAGAATTTAGATTGTAAGTGCAC
pRS_to_ADE2_2	CTGGATATGTATGTATGTATAATAAGTGATCTTATGTATGCTGTGCGGTATTTACACCG
P7L-IDn-	CAAGCAGAAGACGGCATACGAGATNNNNNNNNNNNGGACAATTGGGACGTATGATTGT
ADE2_g39_REV	
P5-XL-	AATGATACGGCGACCACCGAGATCTACACCCGTCTTAATGTTGAGCCTGTTTGCTGCCTCA
ADE2_g39_REV	
Seq-XL-	CCGTCTTAATGTTGAGCCTGTTTGCTGCCTCA
ADE2_g39_REV	
ADE2_ORF_Qfor	TTGGGTTTTCCATTCGTCTTG
ADE2_ORF_Qrev	CAACGAAGTTACCTCTTCCATCGT

CHAPTER 5

Inhibition of transcription leads
to rewiring of locus-specific
chromatin proteomes



5

Inhibition of transcription leads to rewiring of locus-specific chromatin proteomes

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Abstract

Transcription of a chromatin template involves the concerted interaction of many different proteins and protein complexes. Analyses of specific factors showed that these interactions change dramatically during stress and upon developmental switches. However, how the binding of multiple factors at any given locus is coordinated has been technically challenging to investigate. Here we employed Epi-Decoder in yeast to systematically decode, at one transcribed locus, the chromatin binding changes of hundreds of proteins in parallel upon perturbation of transcription. Taking advantage of improved Epi-Decoder libraries, we observed broad rewiring of local chromatin proteomes following chemical inhibition of RNA polymerase. Rapid reduction of RNA polymerase II binding was accompanied by reduced binding of many other core transcription proteins and gain of chromatin remodelers. In quiescent cells, where strong transcriptional repression is induced by physiological signals, eviction of the core transcriptional machinery was accompanied by the appearance of quiescent-cell specific repressors and rewiring of the interactions of protein-folding factors and metabolic enzymes. These results show that Epi-Decoder provides a powerful strategy for capturing the temporal binding dynamics of multiple chromatin proteins under varying conditions and cell states. The systematic and comprehensive delineation of dynamic local chromatin proteomes will greatly aid in uncovering protein-protein relationships and protein functions at the chromatin template.

5.1 Introduction

Many proteins assemble on to DNA to implement gene regulatory programs and ensure the expression of a subset of genes in agreement with the state and environmental cues of the cell. RNA polymerase II (Pol II) is an integral part of this assembly as it catalyzes the DNA dependent synthesis of messenger RNA (mRNA). Regulation of Pol II occurs at different stages of transcription and involves concerted actions of many proteins and protein complexes and dynamic post-translational modifications of histones and the carboxy-terminal domain (CTD) of Pol II (F. X. Chen et al., 2018; X. Li, Egervari, Wang, Berger, & Lu, 2018; S. Sainsbury et al., 2015).

The process of transcription involves distinct stages: initiation, elongation and termination (F. X. Chen et al., 2018; X. Li et al., 2018; S. Sainsbury et al., 2015). The wrapping of DNA by histones into nucleosomes presents a barrier for each of these steps. Chromatin remodelers recruited to promoters can displace nucleosomes to open up crucial recognition elements for transcription factor (TF) binding (Chereji & Clark, 2018; Kujirai et al., 2018; R. Prasad, D'Arcy, Hada, Luger, & Bartholomew, 2016; Struhl & Segal, 2013; Tramantano et al., 2016). For initiation of transcription, Pol II assembles with basal TFs (TFIIB-H) at the promoter to form the pre-initiation complex, which opens up the DNA, initiates RNA synthesis, and stimulates the escape of Pol II from the promoter (Y. Feng, Zhang, & Ebright, 2016; Hantsche & Cramer, 2017; S. Sainsbury et al., 2015). Productive elongation involves recruitment of Paf1C (Polymerase II Associated Factor complex), DSIF (DRB Sensitivity Inducing Factor; Spt4/5), Spt6 and FACT (Facilitator of Chromatin Transcription) (Ehara & Sekine, 2018; Vos et al., 2018; Xu et al., 2017). Towards the end of genes transcription is terminated and the RNA is processed by the recruitment of cleavage and polyadenylation factor (CPF) and cleavage factor (CF) complexes (CPF, CFIA and CFIB), which results in the release of Pol II and the nascent mRNA from the DNA template (Porrua & Libri, 2015). Biochemical and genetic studies have provided a rich catalog of factors involved in the

different stages of transcription. However, how the many different interactions are coordinated at any given chromatin locus in time and under changing conditions is still poorly understood (Qiu & Kaplan, 2019).

Unraveling the relationships between proteins and the hierarchies among them at the chromatin template will require measuring the chromatin interactome and monitoring the changes upon perturbation. A common and convenient strategy to perturb transcription is the use of inhibitors of Pol II (Bensaude, 2011). Some of the commonly used inhibitors of transcription initiation or elongation are not active in live yeast cells because of uptake deficiency or because the target protein or target site on the protein is not conserved (Bensaude, 2011). However, the addition of the metal chelators thiolotin and 1,10-phenanthroline (PH) leads to rapid loss of transcription in yeast. This has, for example, enabled the determination of mRNA half-lives and establishing the role of Pol II activity in nucleosome positioning (Adams & Gross, 1991; Grigull, Mnaimneh, Pootoolal, Robinson, & Hughes, 2004; T. H. Hsieh et al., 2015; Lauinger et al., 2017; M. Sun et al., 2013; Vasseur et al., 2016). In addition, 6-Azauracil (AU), which perturbs the supply of ribonucleotides and thereby indirectly affects RNA polymerases, is frequently used to assess transcription-elongation phenotypes in yeast (Exinger & Lacroute, 1992; Handschumacher & Welch, 1956; Mason & Struhl, 2005; Shaw & Reines, 2000; H. Zhou, Liu, Shi, Yu, & Lu, 2015). Although these chemicals and related compounds have been used extensively and are convenient and powerful tools to block transcription, little is known about how the direct and indirect inhibition of transcription affects the interactions of Pol II and its protein partners with the chromatin template.

Transcription is also regulated under physiological conditions, such as during development and differentiation. In budding yeast, a dramatic global change in transcription is observed when cells enter quiescence, a survival mode that involves a G₁-like cell-cycle arrest, with increased

resistance to stress, metabolic rewiring, and large-scale reorganization of the genome and cellular machineries (Aragon et al., 2008; Broach, 2012; Guidi et al., 2015; Kuang et al., 2014; Kuang, Pinglay, Ji, & Boeke, 2017; McKnight, Boerma, Breeden, & Tsukiyama, 2015; Mews et al., 2014; Miles, Li, Davison, & Breeden, 2013; Radonjic et al., 2005; Rutledge, Russo, Belton, Dekker, & Broach, 2015; Sagot & Laporte, 2019a, 2019b; S. G. Swygert & Tsukiyama, 2019; Young et al., 2017). Quiescence is central to many important biological processes and is conserved from unicellular eukaryotes to multicellular organisms (Cheung & Rando, 2013; Sagot & Laporte, 2019a). We recently demonstrated that quiescent cells show a 30-fold drop in mRNA levels and that this massive transcriptional shutoff is dependent on the conserved histone deacetylase (HDAC) Rpd3 (McKnight et al., 2015). Rpd3 is recruited to the majority of gene promoters in quiescent cells, leading to global hypoacetylation of chromatin and gene repression (McKnight et al., 2015). Transcriptional shutdown correlates with loss of Rpb3, one of the subunits of Pol II (McKnight et al., 2015; Young et al., 2017). Another highly conserved chromatin binding factor, condensin is also involved in quiescence-specific chromatin domain structure and transcriptional repression (Sarah G. Swygert et al., 2018; S. G. Swygert & Tsukiyama, 2019).

To systematically interrogate the chromatin changes at a transcriptionally active locus upon inhibition of transcription, we employed Epi-Decoder, a Tag-chromatin immunoprecipitation-Barcode-Sequencing (TAG-ChIP-Barcode-Seq) technology in budding yeast (Korthout et al., 2018). Epi-Decoder enables the decoding of the proteome of a single barcoded genomic locus by DNA sequencing and barcode counting. It takes advantage of the power of cellular DNA barcoding (Chabbert, Adjalley, Steinmetz, & Pelechano, 2018; Fowler & Fields, 2014; Keschull & Zador, 2018; Matreyek, Stephany, & Fowler, 2017; Roy et al., 2018; Vlaming et al., 2016; Weile et al., 2017; Yan et al., 2008) and yeast genetics (Bonini & Berger, 2017; Costanzo et al., 2019; Duina et al., 2014) and provides a quantitative approach orthogonal to capture-mass-spectrometry efforts (Kennedy-Darling et al., 2014; Kustatscher et al., 2014; Myers et al., 2018; Raschle et al.,

2015; Schmidtman et al., 2016; Sirbu et al., 2011; Waldrup et al., 2014). Here we describe an expanded library of yeast strains carrying a double-barcoded transcribed reporter gene integrated at the HO locus (Epi-Decoder-HO). We first used this improved library to verify that the chromatin interactions measured by barcode counting are not biased by barcode sequences. Using the expanded library, we then constructed a dedicated library of ~ 700 (putative) chromatin proteins (for more details see Methods) of which the binding at the reporter locus can be assessed in parallel and in triplicate with three independent barcodes in a single sample. Furthermore, binding in the promoter region and terminator region of the locus can be assessed in parallel since the reporter gene contains a barcode at the 5' (BC_UP) as well as the 3' end (BC_DN), providing two independent and complementary proteomics measurements. This library, which facilitates comparing experimental conditions, was used to simultaneously measure the binding kinetics of ~700 proteins at the promoter and terminator region of the transcribed locus in response to transcription inhibition by chemical perturbation and during quiescence.

5.2 Results

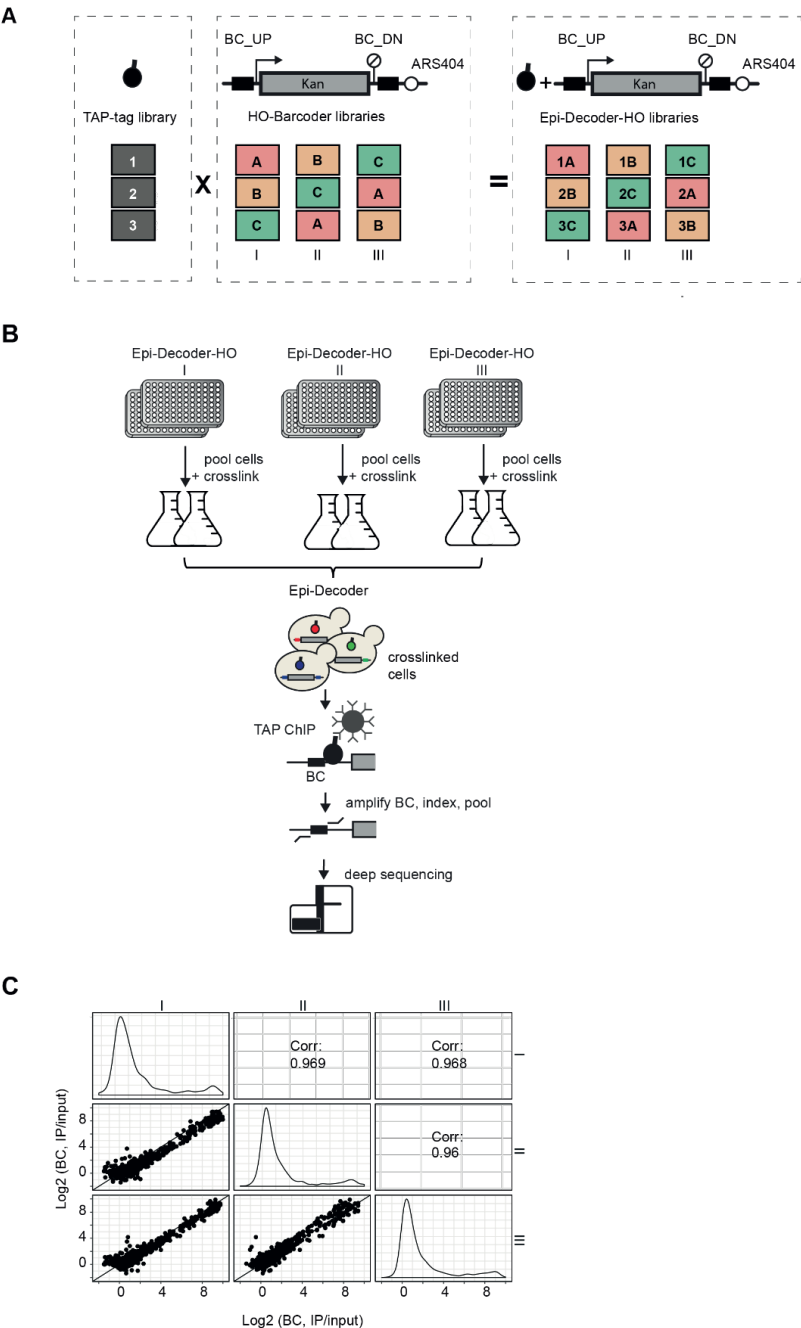
Generation of improved Epi-Decoder-HO libraries

To measure local proteome dynamics at a promoter and terminator region upon transcriptional inhibition, we first improved the previously reported Epi-Decoder-HO library to optimize the comparison of multiple time points and conditions. Epi-Decoder is a strategy for decoding the local proteome of a single genomic locus (Korthout et al., 2018). It relies on short (~16-20bp) DNA barcodes (BCs) integrated at a common locus in the genome, here a constitutively expressed KanMX marker gene integrated at the HO locus and flanked by two barcodes BC_UP and BC_DN that are 1.5 kb apart (HO-Barcoders). The barcoded KanMX cassette is a Kanamycin gene controlled by the heterologous *AgTEF1* promoter and terminator from *Ashbya gossypii*, a yeast related to *S. cerevisiae*. This 1.5 kb reporter-gene cassette replaces the coding sequence of the *HO* gene and is therefore flanked by the endogenous *HO* promoter and terminator sequences as well as an origin of replication (ARS404) 53 bp downstream of BC_DN. This reporter locus has previously been used in screens for various chromatin- and transcription-related features (M. Chen et al., 2013; Korthout et al., 2018; Verzijlbergen et al., 2011; Vlaming et al., 2019; Vlaming et al., 2016). The library of HO-Barcoders was combined with a genome-wide library of proteins tagged with a common epitope tag, a Tandem Affinity Purification (TAP) tag (Figs. 1A, and S1A). Upon pooling, cross-linking, chromatin immunoprecipitation (ChIP), amplification of the barcoded regions, and counting the barcodes by massive parallel sequencing (Fig 1B), the abundance of each BC (ChIP/input) reports on the occupancy of each tagged protein at its barcoded locus (Korthout et al., 2018; Verzijlbergen et al., 2011; Vlaming et al., 2016). Integral for high-throughput assessments like this is the ability to multiplex many individual TAP-tag clones (~4800 in total) in the ChIP assays using unique BCs and indexing strategies. Here we expanded the previous set of HO-Barcoders from ~1100 to ~2500 (Douglas et al., 2012), enabling coverage of the full Epi-Decoder library with only two separate subsets (Fig 1B and Supplemental Fig S1A). The expanded

Barcoder-HO library was combined with the TAP-tag library in three different ways (see Methods) such that every TAP-tag is linked to three independent HO-Barcoders (I, II and III) and creating a triplicate 700 Chromatin TAP-tag subset (Chrom-3xBC) that can be processed as one pool due to non-overlapping barcode pairs in the replicates.

Figure 1. Outline of expanded and optimized Epi-Decoder analysis

(A) Three Epi-Decoder-HO libraries were generated by crossing the TAP-tag library to an expanded HO-BC library in three different ways to combine each TAP-tag protein with three independent barcodes (see also Supplemental Fig S1a). The HO-BC locus consists of a constitutively expressed 1.5 kb KanMX resistance gene integrated at the HO locus, controlled by the heterologous AgTEF1 promoter and terminator from *Ashbya gossypii*, and flanked by a promoter-proximal BC_UP and a terminator-proximal BC_DN. Downstream of BC_DN lies an origin of replication (ARS404). **(B)** Clones of each Epi-Decoder-HO library are combined and processed in two separate pools and used for ChIP of TAP-tagged proteins (spheres with black handle). The BCs (colored lines), which flank the KanMX reporter gene (grey box) at the HO locus are amplified from ChIP and Input and indexed, allowing for the pools to be combined and counted by massive parallel sequencing. The relative barcode count (IP/input) reports on protein abundance of each TAP-tagged protein (~4250) at the barcoded locus. **(C)** Comparison of the binding scores (IP/input) of both BC_UP and BC_DN of chromatin binders (as determined previously in (Korthout et al., 2018)) in the three Epi-Decoder-HO libraries. Indicated are the Spearman correlation coefficients and the diagonal line represent $x=y$. Density plots show the distribution of the barcode counts in each of the three replicates. See Supplemental Table S1 for counts of all proteins examined. The results for BC_UP and BC_DN separately are shown in Supplemental Fig S1B-C.



Interrogation of protein binding with multiple DNA barcodes

Epi-Decoder uses DNA BC counting as a reporter for protein binding (Korthout et al., 2018). With the three independent Epi-Decoder-HO libraries (Fig 1B), we first assessed to what extent variation is caused by the barcode sequences. The protein-binding patterns in each of the three Epi-Decoder-HO libraries strongly correlated between the replicates (Fig 1C). This was true for both BC_UP and BC_DN (Supplemental Fig S1B, S1C and Supplemental Table S1) and was further confirmed by the analysis of individual clones (see below). Although it is possible that specific sequences could cause a bias for certain proteins, in general our results show that the inferred protein binding scores are largely independent of the short barcode sequences. This is in agreement with our previous observation that proteins belonging to the same complex (e.g. ORC, MCM, DSIF, Pol II) tend to show similar binding scores while binding of each subunit is detected with a different barcode sequence (Korthout et al., 2018). The use of multiple Tag-Barcode combinations further increases the confidence of single-barcode measurements. For example, we have previously shown that Ssl2 and Tfa2, two factors known for their roles in transcription initiation, unexpectedly show very high binding at the HO terminator region (Korthout et al., 2018). By replicating this finding with three independent barcodes, we can now negate the possibility that BC effects dictated the unexpected binding pattern of Ssl2 and Tfa2 (Supplemental Table S1). The consistent and robust binding of Ssl2 and Tfa2 at the terminator region in the absence of other basal transcription factors suggests a non-canonical function of these factors.

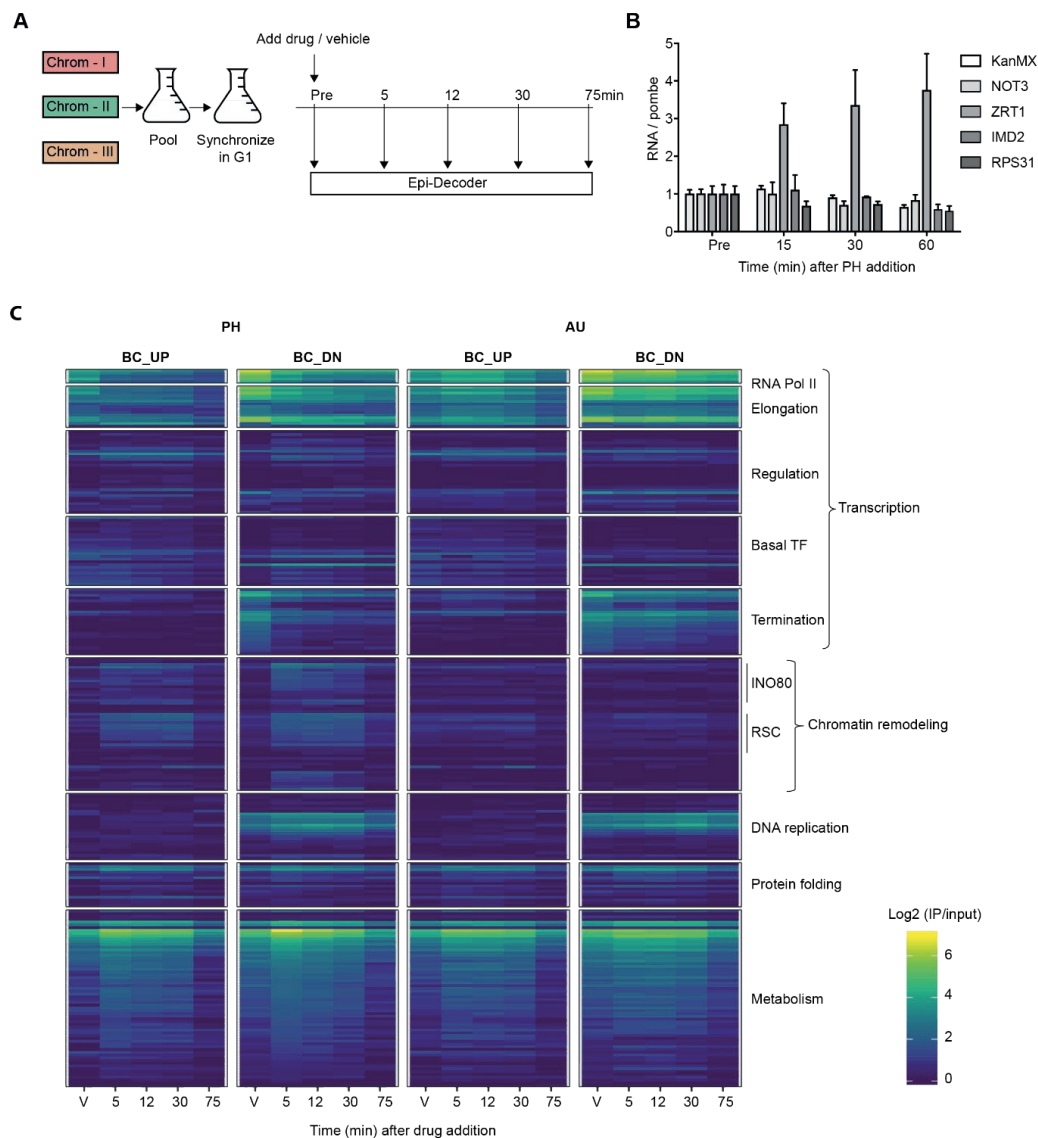
A Chromatin-TAP-tag subset library for capturing dynamic local chromatin proteomes

Having confirmed that Epi-Decoder provides a robust assay to quantitatively measure protein-binding at a single locus of thousands of proteins in parallel, we focused on the Chromatin TAP-tag subset (Chrom-3xBC; Fig 2A and see Methods) to determine the dynamics of the local chromatin proteome following chemical inhibition of Pol II. The antifungal agent PH is a metal ion chelator that sequesters Zn^{2+} ions, which are essential for the activity of RNA polymerases

(Cramer et al., 2000; Markov, Naryshkina, Mustaev, & Severinov, 1999; McCann, Kellett, Kavanagh, Devereux, & Santos, 2012; Scrutton, Wu, & Goldthwait, 1971). Addition of PH to cells rapidly affects mRNA levels (Grigull et al., 2004; Miller et al., 2011; Wada & Becskei, 2017). AU is an inhibitor of IMP dehydrogenase (IMPDH), the rate-limiting enzyme in *de novo* GTP synthesis (Grigull et al., 2004; Kaplan, Jin, Zhang, & Belyanin, 2012; Ljungdahl & Daignan-Fornier, 2012). Treatment of cells with AU, results in depletion of intracellular nucleotide pools, thereby affecting transcription elongation (Exinger & Lacroute, 1992; Handschumacher & Welch, 1956; Mason & Struhl, 2005; Shaw & Reines, 2000; Shaw, Wilson, Smith, & Reines, 2001). To investigate the consequences of inhibition of Pol II at the chromatin template, the Chrom-3xBC Epi-Decoder library was incubated at with PH and AU and the local proteomes were determined at several subsequent time points (Fig 2A). All experiments were carried out at low temperature (16°C) to facilitate the capturing of dynamic binding events. In addition, cells were arrested G₁ to avoid cell-cycle dependent effects (Supplemental Fig S2A) (O'Duibhir et al., 2014). Independent triplicate measurements in the Chrom-3xBC pool and sample multiplexing enabled efficient analysis of the dynamics of known chromatin proteins across multiple conditions and time points.

We confirmed that PH and AU are active under these conditions: both compounds inhibited cell growth (Supplemental Fig S2B) and addition of PH led to induction of *ZRT1* mRNA while addition of AU led to induction of *IMD2* mRNA (Fig 2B and Supplemental Fig S2C), which is in agreement with previous observations (Grigull et al., 2004; Shaw & Reines, 2000) and which shows that inhibition of transcription by these drugs is not complete. Under these experimental conditions and time points, we observed little decay of mRNA levels of the barcoded KanMX gene or other genes examined (Fig 2B and Supplemental Fig S2C), which is in agreement with the observed temperature effects on RNA decay kinetics (Lotan et al., 2005). In contrast, global inspection of binders at the barcoded KanMX gene showed that the addition of PH or AU led to many rapid and pronounced changes at the chromatin level at BC_UP and BC_DN (Fig 2C). Generally, changes

in protein binding were stronger in PH than in AU, confirming that the observed effects are specific for the treatment.



(A) Three versions of the Chromatin-TAP-tag subset, each containing a unique set of barcode pairs, were combined in one pool (Chrom-3xBC). The culture was incubated with *a-factor* to synchronize the cells in G₁, after which PH or AU was added and samples were collected at the time points indicated for Epi-Decoder analysis, RNA analysis, and flow cytometry. A sample treated with vehicle was collected at the last time point and a control sample was taken of a G₁-arrested culture without treatment. All experiments were carried out at 16°C to facilitate capturing dynamic binding events. **(B)** Analysis of mRNA changes over time by RT-qPCR confirmed the decay of most transcripts and an increase in the PH-responsive ZRT1 gene under conditions shown in panel A. RNA levels are relative to untreated (Pre) and normalized to a transcript from a spike-in of untreated *S. pombe* cells (see Materials and Methods) to correct for global changes (mean of three biological replicates $\pm$ SD). **(C)** Heatmap of the binding scores (mean IP/input of three biological replicates) of selected proteins with a binding score >0.5 at any of the four local proteome time series indicated. See Supplemental Table S2 for mean binding scores of all proteins examined in the Chrom-3xBC library. Proteins were manually clustered and ranked in functional subcategories.

Rewiring of the core transcription machinery at chromatin upon inhibition of Pol II

The global overview of the dynamic chromatin proteomes (average of three replicates, Fig 2C) revealed broad rearrangements, especially of protein complexes related to transcription. Other proteins were unaffected, such as the replication proteins specifically associated with BC_DN proximal to the origin of replication ARS404. This was further illustrated by the analysis of individual replicate measurements of proteins representing different aspects of chromatin biology (Fig 3A). Importantly, the three replicates, based on different barcode pairs, showed very similar results, further indicating that barcode sequences do not generally affect the protein-binding measurements in Epi-Decoder. As observed previously (Korthout et al., 2018), Pol II and transcription elongation factors were found at BC_UP and were more abundant at BC_DN,

initiation factors and basal transcription factors (TFs) were more enriched at BC_UP, while transcription termination factors and replication proteins were specifically bound to BC_DN (Figs 2C, 3A, and Supplemental Table S2). To determine the consequences of transcription inhibition for the transcription machinery in more detail, we focused on Pol II and transcription elongation factors. All the Pol II subunits present in our Epi-Decoder library showed a pronounced and progressive loss of binding upon addition of PH at BC_UP and BC-DN. In AU, Pol II binding was also reduced but to a lesser extent (Fig 3B). Therefore, we here focused on the changes following treatment with PH. Loss of binding can be caused by lower levels of the protein due to protein degradation or by redistribution of the protein. To distinguish between these two possibilities, we determined the protein level of Rpo21, the largest subunit of Pol II, by immunoblot analysis and found that treatment with PH did not lead to reduced Rpo21 protein levels (Fig 3C and Supplemental Fig S3A) while treatment with AU showed a modest decrease (Fig 3D and Supplemental Fig S3B). Therefore, the reduced binding of Pol II subunits in PH was not accompanied by increased Pol II protein degradation.

Finally, the strong reduction in Pol II binding observed in Epi-Decoder in PH at the barcoded KanMX gene under the control of the *AgTEF1* promoter and terminator could be validated by ChIP-qPCR at the 5' and 3' end of the endogenous *TEF1* gene (Figs 3E and Supplemental Fig S3C). Thus, in addition to the current knowledge that Pol II activity is inhibited directly by PH and indirectly by AU (Cramer et al., 2000; Markov et al., 1999; McCann et al., 2012; Scrutton et al., 1971), our results show that chemical inhibition of Pol II also leads to partial eviction of the core Pol II machinery from the chromatin template.

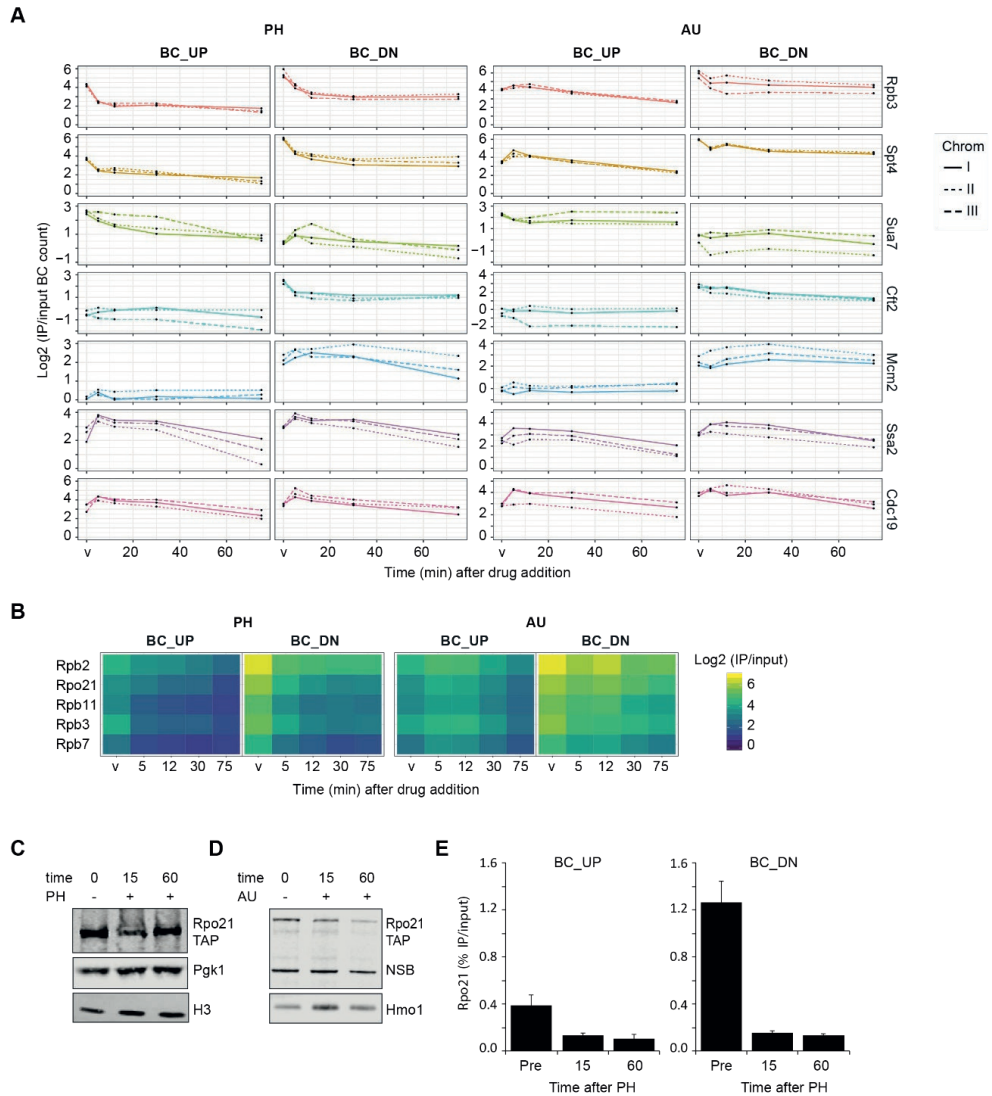


Figure 3. Treatment with phenanthroline leads to rapid loss of Pol II and transcription-associated proteins

(A) Differential and dynamic binding behavior of proteins representing different chromatin processes upon treatment with PH and AU. The lines indicate the three different barcode pairs of the indicated TAP-tagged proteins in the Chrom-3xBC library (Log2 IP/ input at time points

indicated in Fig 2). **(B)** Zoom in of heat map of Fig 2 showing the Pol II subunits present in the library. **(C-D)** Immunoblot analysis of the largest subunit of Pol II (Rpo21-TAP) with and without PH and AU treatment in G_1 -arrested cells at 16°C. Pgk1, H3, Hmo1, and a non-specific band (NSB) were used as loading controls. **(E)** ChIP-qPCR analysis of Rpo21 binding at the BC_UP and BC_DN regions in G_1 -arrested cells treated with (15 and 60 min) and without (Pre) PH at 16°C (average of three biological replicates $\pm$ SD).

Epi-Decoder uncovers protein-protein relationships

Several proteins and protein complexes have been biochemically or genetically linked to the process of transcription elongation (F. X. Chen et al., 2018; Cucinotta & Arndt, 2016; Koerber, Rhee, Jiang, & Pugh, 2009; Kwak & Lis, 2013; Pufall & Kaplan, 2013; Van Oss, Cucinotta, & Arndt, 2017; Venters & Pugh, 2009; Vinayachandran et al., 2018). However, how the interactions between the core transcriptional machinery and elongation factors are dynamically orchestrated on the chromatin template is still incompletely understood (Martin, Chruscicki, & Howe, 2018; Mayer et al., 2010; Vinayachandran et al., 2018). Inspection of the dynamic proteomes at the HO locus showed that elongation factors did not all respond equally to inhibition of Pol II by PH (Fig 4A) and AU (Supplemental Fig S4A). The conserved elongation factors Elf1, Spn1, Spt6, and DSIF (Spt4/5) were rapidly and progressively evicted from chromatin, closely resembling the dynamics of Pol II upon PH treatment (Figs 4A and 3B). This indicates that most elongation factors depend on Pol II for recruitment and maintenance. The treatment with AU, performed and processed in parallel, showed more modest effects on binding of Pol II and the elongation factors (Supplemental Fig S4A-B). The FACT complex, composed of Spt16 and Pob3, is known to be recruited to chromatin by the act of transcription and its associating factors Nhp6a and Nhp6a, two High Mobility Group (HMG) proteins (Carvalho et al., 2013; Kwak & Lis, 2013; Martin et al., 2018; McCullough, Connell, Petersen, & Formosa, 2015; Pathak et al., 2018; Ruone, Rhoades, & Formosa, 2003).

At BC_UP, FACT eviction was delayed compared to eviction of Pol II upon PH treatment (Figs 4A-B), suggesting that FACT might depend on more stable factors at transcribed genes such as histones or histone modifications (Martin et al., 2018; Vinayachandran et al., 2018). At BC_DN, the two core FACT subunits Spt16 and Pob3 were rapidly lost upon addition of PH, closely resembling the behavior of Pol II and other elongation factors. In contrast, the HMG proteins Nhp6a and Nhp6b did not show reduced binding after Pol II inhibition (Figs 4A-B). This difference may reflect the ability of HMG proteins to bind DNA and nucleosomes directly, independently of other chromatin proteins. However, the persistent binding of Nhp6a/b was not sufficient to maintain Spt16/Pob3 at transcribed chromatin when Pol II was evicted.

Recent structural studies on Paf1C and reconstituted macromolecular transcription assemblies show that DSIF (Spt4/5), Paf1C, and Spt6 can form an intricate protein network around elongation-permissive Pol II (Vos et al., 2018; Xie et al., 2018). Indeed, like DSIF and Spt6, most Paf1C subunits showed reduced binding to chromatin when Pol II was inhibited and evicted by PH (Fig 4A-B) and a similar, albeit more modest response was observed in AU (Supplemental Fig S4A-B). However, binding of Ctr9, the largest subunit of Paf1C, was largely unaffected in PH, with a small decrease only observed at later time points (Figs 4A-B). This was unexpected because Ctr9 forms a central part of Paf1C: it acts as a key scaffold protein during Paf1C assembly and makes many contacts with Pol II and the other subunits (P. Deng et al., 2018; Vos et al., 2018; Xie et al., 2018). The distinct kinetics of Ctr9 compared to Pol II and the other Paf1C subunits was not caused by barcode effects because it was observed with three independent HO-barcode pairs (Fig 4B). We confirmed the delayed loss of Ctr9 by ChIP-qPCR analysis of the barcoded promoter and terminator regions (Fig 4C) as well as the endogenous *TEF1* locus (Supplemental Fig S4C). The differences in kinetics were not caused by differences in protein abundance, because Ctr9 showed very similar global expression levels as Paf1 and Pol II subunits (Supplemental Fig S4D). We also tested whether the delayed loss of Ctr9 was caused by a difference in crosslinking

sensitivity (De Jonge, Brok, Kemmeren, & Holstege, 2019; Zaidi, Hoffman, Shetty, Bekiranov, & Auble, 2017). However, Paf1 and Rpo21 were not more sensitive to shorter cross-linking conditions than Ctr9 (Supplemental Fig S4E). Finally, addition of PH did not lead to major changes in the expression level of Rpo21 and Paf1, compared to Ctr9, the latter remaining bound when Rpo21 and Paf1 had already been evicted upon PH treatment (Fig 4D). Together, our results show that maintenance of Ctr9 at transcribed chromatin is independent of Pol II and Paf1C subunits, suggesting that Ctr9 has a transcription elongation-independent mechanism to bind to DNA. A recent study showed that Ctr9 can bind to DNA independent of its association to Paf1C by its ability to bind single-strand-containing DNAs (P. Deng et al., 2018) and Ctr9 has been shown to bind to triple-helical DNA (Musso, Bianchi-Scarra, & Van Dyke, 2000). Ctr9 has also been suggested to bind to nucleosomes (Vos et al., 2018). These binding activities may be responsible for anchoring Ctr9 to PH-perturbed chromatin in the absence of its complex partners and Pol II. Of note, under conditions of transcriptional repression in Q cells, Ctr9 followed the pattern of the other Paf1C members (see below), showing that its dynamics are context dependent.

In addition to uncovering unexpected protein-binding dynamics within well-known protein complexes, the parallel analysis of hundreds of proteins also offers the possibility to obtain more insight into proteins of which the chromatin functions have been less well annotated. Here we more closely examined Srm1 (also known as Prp20), a homologue of human Regulator of Chromosome Condensation 1 (RCC1). Srm1/RCC1 is a guanine nucleotide exchange factor that localizes to the nucleus, binds nucleosomes in a dynamic way, and occupies a large part of the yeast genome (Bierbaum & Bastiaens, 2013; Koerber et al., 2009; Makde, England, Yennawar, & Tan, 2010; McGinty & Tan, 2016; Nemergut, Mizzen, Stukenberg, Allis, & Macara, 2001; S. Tan, 2012; F. Wu et al., 2011). In the Epi-Decoder analysis, all proteins are examined in one pool and with the same tag, thereby delivering qualitative as well as relative quantitative information. The high barcode counts observed for Srm1 show that it binds very efficiently to chromatin, in the same

range as the abundant transcription elongation factors mentioned above (Fig 4A and Supplemental Fig S4A). Indeed, analysis of Srm1 by ChIP-qPCR confirmed the efficient binding of this protein and an increase after PH treatment at the reporter locus as well as endogenous loci, including a non-transcribed region (Fig 4E). This, together with the genome-wide binding pattern (Koerber et al., 2009), indicates that Srm1 should be considered as a common component of yeast chromosomes. However, the distribution but also the kinetic behavior of Srm1 does not mirror that of the transcription elongation factors: upon treatment with PH, Srm1 binding increased (Fig 4AE) while binding of Pol II and most elongation proteins decreased. Although not quantitatively the same, the behavior of Srm1 is more similar to that of the chromatin remodelers INO80 and RSC, several metabolic enzymes (Fig 2C), the heat shock protein Ssa2 (Fig 3A and Supplemental Fig S4F) and high-mobility-group protein Hmo1 (Supplemental Fig S4F). In contrast, the high-mobility group-like protein Spt2, a chaperone involved in histone recycling over transcribed regions of active genes (S. Chen et al., 2015; Nourani, Robert, & Winston, 2006), was decreased in active regions (Supplemental Fig S4F). The dynamic behavior of Srm1, Ssa2, Hmo1, and Spt2 after pH treatment was not caused by changes in global protein levels (Supplemental Fig S4G). Together, these results suggest that Srm1 is an abundant chromatin protein with the potential to affect chromatin structure and function but it that it might not act as a canonical transcription elongation factor.

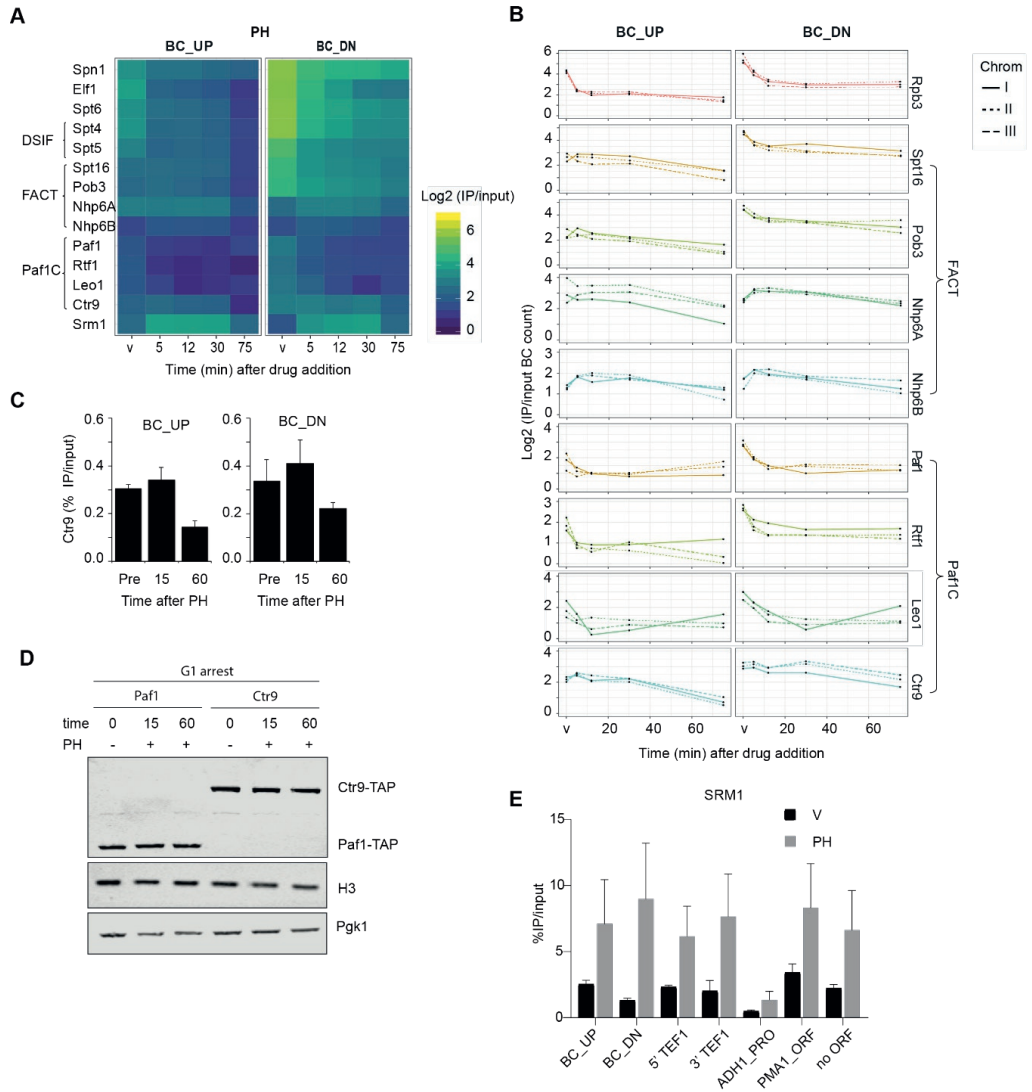


Figure 4. Differential response of transcription elongation factors to chemical inhibition of *Pol II*

(A) Zoom in on the heat map of Fig 2 (PH treatment), showing proteins annotated to transcription elongation. (B) Independent replicates of proteins related to FACT and Paf1C (Log2 IP/input at time points as in Fig 2). The lines show the three different barcode pairs of the indicated TAP-

tagged proteins in the Chrom-3xBC library. (C) ChIP-qPCR analysis of Ctr9 binding at the BC_UP and BC_DN regions in G₁-arrested cells treated with (15 and 60 min) and without (Pre) PH at 16°C (average of three biological replicates +/- SD). (D) Immunoblot analysis of Ctr9-TAP and Paf1-TAP with and without PH treatment in G₁-arrested cells at 16°C. Pgk1 and H3 were used as loading controls. (E) ChIP-qPCR analysis of Srm1 binding in G₁-arrested cells at 16°C, treated for 15 min with vehicle (V) or phenanthroline (PH) (average of three biological replicates +/- SD). Analyzed loci are the BC_UP and BC_DN regions, the 5' and 3' end of the endogenous TEF1 gene, the ADH1 promoter, the PMA1 open reading frame, and a non-transcribed locus (S. Chen et al., 2015; van Welsem et al., 2018) (see Supplemental Table S5 for more details).

Rearrangement of the chromatin proteome of the barcoded HO locus upon entry in quiescence

Finally, we investigated how the chromatin proteome of the barcoded HO locus was altered by the strong physiological transcriptional shut down during quiescence. Chrom-3xBC Epi-Decoder library pools were grown in YEPD at 30°C for seven days, which causes cells to arrest in saturation with G₁ DNA content. Stationary phase cultures consist of two populations: quiescent (Q) cells that are more uniform, long-lived, and highly stress-resistant, and a nonquiescent (NQ) cells that are more heterogeneous, short-lived, and stress-sensitive (Allen et al., 2006; Aragon et al., 2008; L. Li et al., 2013; Sagot & Laporte, 2019a, 2019b; Young et al., 2017). NQ cells were also isolated and processed as a reference.

Analysis of the RNA Pol II machinery, transcription elongation factors, basal transcription factors, and transcription termination and RNA processing factors showed a pronounced loss of the core transcription machinery in Q and NQ cells (Fig 5A-B, Supplemental Fig S5A-E; Supplemental Table S2). Overall, the magnitude of the changes was higher than that observed in the G₁ cells treated with PH with several exceptions. The FACT-associating factors Nhp6a and Nhp6a showed

increased chromatin binding in Q cells (Figs 5B and Supplemental Fig S5A), in contrast to PH treated cells (Figs 4 and Supplemental Fig S4). Tho1, a protein associated with transcribed chromatin, showed increased abundance in Q and NQ cells but not in PH (Figs 5B and Supplemental Fig S5A), suggesting that Tho1 might have Q/NQ-cell specific functions and that its recruitment is independent of ongoing transcription, in contrast to canonical transcription elongation proteins.

To gain more insight into the possible origin of the transcriptional repression of the barcoded HO locus, we inspected transcriptional regulators at the BC_UP promoter region (Figs 5C and Supplemental Fig S5B). Q cells showed binding of the quiescence-specific transcription repressor Xbp1, which was absent from mid-log cells and during PH treatment. This protein is known to recruit the histone deacetylase Rpd3, mediating the global shutdown of gene expression in Q cells (McKnight et al., 2015). Indeed, Rpd3 and members of the Rpd3L complex were mostly undetectable in mid-log but readily detected in Q cells. Members unique for the Rpd3S complex were not detected (Supplemental Table S3). This well-established repression pathway was accompanied by several additional changes in transcription regulators. Q cells showed increased abundance of other TFs like Mcm1, Mbf1, Tbf1, Abf1, and Swi6 (Fig 5C and Supplemental Fig S5B). Many of these factors were also altered in NQ cells but generally to a lesser extent than in Q cells.

Besides the core transcriptional machinery, TFs, and Rpd3L, we observed several other striking changes in the chromatin proteomes (Figs 5C-E and Supplemental Fig S5B-G). Yeast Histone H1 (Hho1) showed strong increased abundance in Q and NQ cells (Figs 5C and Supplemental Fig S5B). High-mobility group protein 1 (Hmo1), which has been shown to function as a linker histone and promote chromatin compaction (Panday & Grove, 2016) showed the same trend (Figs 5C and Supplemental Fig S5B). Paradoxically, the negative regulator of transcription Spt2 left the

chromatin in Q and NQ cells, as it did in PH treated cells. These changes were paralleled by the recruitment of the ATP-dependent chromatin remodeling complexes INO80 and RSC, as well as several heat shock proteins and other factors involved in protein folding (Figs 5E and Supplemental Fig S5C and S5F). The recruitment of INO80 is especially relevant in the light of a recent study showing that INO80 acts in the MINC complex to negatively regulate transcription across the yeast genome (Xue et al., 2017). In addition, striking changes were seen in the binding of metabolic enzymes (Supplemental Fig S5G).

Finally, quiescence also affected the organization of replication proteins at the origin of replication (ARS404) proximal to BC_DN (Fig 5D). While perturbation of transcription by PH did not lead to major changes, cells in quiescence showed a complete loss of the minichromosome maintenance complex (MCM), the replicative helicase. The loss of MCM was accompanied by increased abundance of the members of the origin-recognition complex (ORC) (Fig 5D). This altered balance is consistent with a competition model that has been proposed for MCM and ORC (Aparicio et al., 1997). Replication origin licensing, which involves the recruitment of ORC and subsequently the assembly of an inactive form of the replicative helicase MCM, occurs during late mitosis and G₁ phase of the cell cycle. After formation of a pre-replication complex, during S-phase the helicase is activated and the replication initiates (Stephen P. Bell & Labib, 2016; Bellush & Whitehouse, 2017; Siddiqui, On, & Diffley, 2013). While cells arrested in G₁ by mating pheromone maintain MCM (Liang & Stillman, 1997) (Fig 5D), MCM was lost in Q cells, which are arrested in a G₁-like state (Fig 5D). It is possible that clearing MCM from origins in Q cells is important for eliminating any inappropriate activation of replication origins during the prolonged state of arrest. The mechanism causing the absence of MCM and the role thereof in Q cell biology will require further study but it should be noted that similar dynamics have been observed in mouse and *S. pombe* (Madine et al., 2000; Namdar & Kearsey, 2006; W. Sun et al., 2000). Clearly, our findings do show that replication origin licensing must be re-established when cells re-enter the cell cycle.

Together, these results show that upon loss of transcription during quiescence, the chromatin proteome of the barcoded HO locus undergoes a major rewiring that affects transcriptional proteins and replication complexes and also involves the recruitment of structural proteins, chromatin remodelers, and protein-folding machineries, as well as changes in metabolic enzymes.

5.3 Discussion

Determining the changes to chromatin in response to cell signaling or stress can reveal insights into common mechanisms of gene regulation. Obtaining a comprehensive understanding of chromatin changes, although highly informative (Kim et al., 2010; Vinayachandran et al., 2018; Weiner et al., 2012), is often laborious and therefore not applicable to multiple conditions. Epi-Decoder provides a strategy for identifying and quantifying in an unbiased and systematic manner the proteome of an individual genomic locus by DNA sequencing. Here we used a dedicated comprehensive chromatin library (Chrom-3xBC) to determine the changes in local chromatin proteomes upon inhibition of Pol II by chemical means or by physiological signals during quiescence.

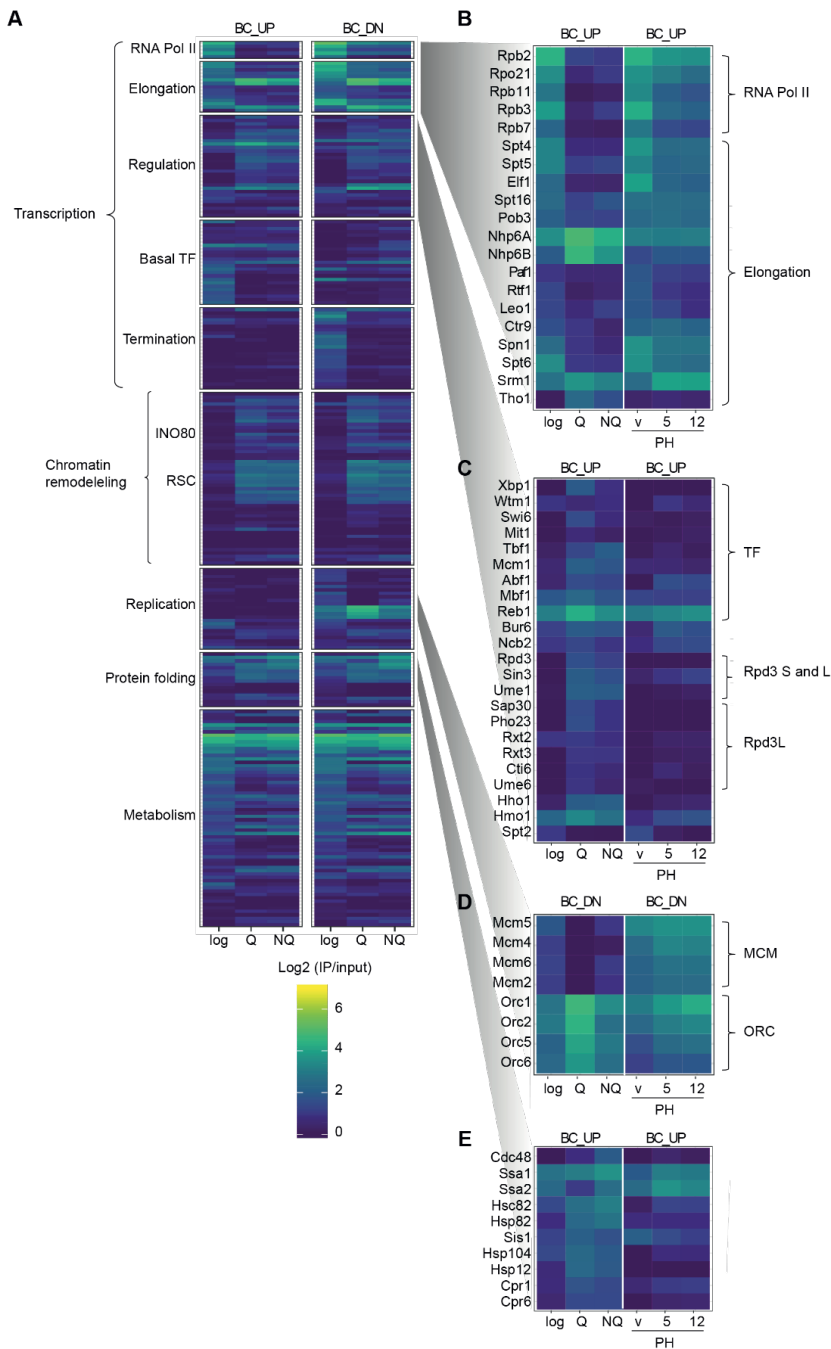


Figure 5. Chromatin proteome rewiring upon transcriptional repression in quiescence

(A) Heatmap of the HO Epi-Decoder binding scores in mid-log, Q, and NQ cells (mean IP/input of three biological replicates). See Supplemental Table S3 for mean binding scores of all proteins examined in the Chrom-3xBC library. Proteins were manually clustered and ranked in functional subcategories as in Fig 2C. **(B-E)** Zoom in of heat map of panel A (promoter region BC_UP, except replication for which terminator BC_DN next to the origin of replication was used) showing proteins in the indicated annotated clusters. PH treatment during G1 arrest (vehicle, 5 and 12 minutes) is shown for comparison.

PH is a potent inhibitor of Pol II and has fungistatic activity against a broad range of pathogenic fungi and bacteria (McCann et al., 2012). PH and thiolutin, which acts in a similar way, are often used to determine consequences of perturbation of transcription in yeast. However, the consequences at the chromatin level have not been well characterized. This is surprising since RNA processing, RNA export, and other downstream processes are linked to the act of transcription and influenced by chromatin modifying factors. Inhibition of Pol II with PH resulted in rapid remodeling of the local chromatin proteomes at the HO locus (Fig 2c). Pol II was evicted, probably reflecting a structural rearrangement caused by PH. This idea is in agreement with observations that mutants of Pol II with altered catalytic activity show reduced Pol II occupancy at genes (Malik, Qiu, Snively, & Kaplan, 2017). Interestingly, we did not observe changes in overall abundance of the evicted proteins Rpo21 and Paf1 and several other proteins, indicating that the factors evicted in PH were not subject to degradation. Besides inhibiting Pol II, PH - a metal chelator - is known to also affect the activity of metalloproteases, including a proteasome-associated deubiquitinating enzyme (McCann et al., 2012; Verma et al., 2002). Therefore, the stability of the proteins evicted by PH may at least in part be explained by inhibition of proteasome-mediated degradation. The nature and scale of the changes observed after PH treatment, particularly the loss of the core transcription machinery, suggests that inhibition of Pol II can have

widespread effects on many nuclear processes in the cell. This should be considered when interpreting downstream effects of chemical inhibitors of transcription, such as mRNA processing and stability.

During quiescence, global changes in histone modifications have been observed (Mews et al., 2014; Young et al., 2017) as well as global repression by Rpd3 (McKnight et al., 2015), chromatin compaction by condensin (Sarah G. Swygert et al., 2018), and reduced Pol II binding (Young et al., 2017). However, the extent to which the epigenome of quiescent cells is remodeled to support this important developmental state and to allow re-entry into vegetative growth remains poorly understood (S. G. Swygert & Tsukiyama, 2019). Application of Epi-Decoder to the barcoded HO locus revealed several insights. We confirmed the recruitment of the quiescence-specific transcription repressor Xbp1 and the Rpd3L complex and found that this coincides with the loss of the core transcription machinery, spanning all the phases of transcription from initiation, to elongation, termination and RNA processing. This broad loss of core transcription proteins was accompanied by increased abundance of several structural proteins, chromatin remodelers, heat shock proteins, and changes in metabolic enzymes. Together, these observations show that Q cells undergo a major rewiring at the level of chromatin. Some of the changes in Q cells were also observed in PH. Even though the changes were sometimes quantitatively different, these changes most likely reflect secondary effects of transcription repression in Q cells. Factors in this category include Srm1, Hmo1, INO80, and RSC, which all increased in abundance. In Q cells we also observed increased occupancy of other transcription-related proteins (Fig 5C), yeast histone H1 (Hho1), specific metabolic enzymes (e.g. Tdh1, Arg1; Supplemental Fig S5E), and several heat shock proteins (Fig 5E). While Ssa1, an HSP70 family ATP-binding protein, was maintained in Q cells compared to mid-log and NQ cells, binding of its paralog Ssa2, which is 98% identical to Ssa1, was reduced (Supplemental Fig 5E). Ssa1 and Ssa2 have recently been shown to have distinct roles in protein quality control in the yeast nucleus (Ramon D Jones et al., 2019; Rupali

Prasad, Xu, & Ng, 2018). Determining whether Q-cell specific changes involve factors recruited to or evicted from chromatin in response to transcription loss in Q cells or whether they have an active role in transcriptional repression will require further studies. We note that some of the observed dynamics in Q and NQ cells might, at least in part, be driven by altered expression of the proteins in the different conditions. For example, budding yeast expresses two acetyl-coenzyme A synthetases that are present in mitochondria, the cytosol and the nucleus (Falcón, Chen, Wood, Aris, & Biochemistry, 2009; S. Li et al., 2015). Acs1, which is subject to glucose repression and expressed aerobic conditions, is bound in Q cells, while Acs2, which is expressed in glucose-containing media, is bound in mid-log cells (Supplemental Fig S5E).

While Q cells and NQ cells showed overlap in the changes compared to mid-log cells, we also observed important differences. This was not unexpected given the different fate of the long-lived Q and short-lived NQ cells (L. Li et al., 2013) and the differences in global histone modifications (Young et al., 2017). NQ cells lacked efficient recruitment of Xbp1, which correlated with lower levels of Rpd3L and INO80. In addition, the loss of the MCM complex was incomplete in NQ cells. Together these changes may contribute to the poor fitness of these cells. Furthermore, the binding of Cdc48 at the BC_UP promoter region and BC_DN terminator region in NQ cells is a possible indicator of the general short-lived nature of NQ cells (Fig 5E). Cdc48, known as VCP or p97 in humans, is an essential and highly conserved AAA+ ATPase. It functions as an unfoldase or segregase facilitating the extraction of proteins from macromolecular complexes, including chromatin, to enable subsequent degradation by the proteasome (Dantuma, Acs, & Luijsterburg, 2014; Franz, Ackermann, & Hoppe, 2016; Lafon et al., 2015). The presence of Cdc48 in chromatin at the barcoded HO locus together with the presence of several other factors involved in protein folding (Fig 5E) suggests that the chromatin in NQ cells elicits stress signals that lead to the recruitment of folding and unfolding activities in an effort to maintain chromatin protein homeostasis. The recruitment of protein quality-control factors to the chromatin is in agreement

with recent observations that effective and diverse protein quality control mechanisms are active in the nucleus (Frottin et al., 2019; Ramon D Jones et al., 2019; Ramon D. Jones & Gardner, 2016; Rupali Prasad et al., 2018). Interestingly, our results suggest that inhibition of transcription with PH or the major rearrangements in Q cells do not elicit these signals.

In summary, our results show that Epi-Decoder provides a powerful strategy for capturing the temporal binding dynamics of chromatin proteins under varying conditions. Using Epi-Decoder we obtained information on what proteins bind to an active gene locus and how the binding of each protein is affected by perturbation of transcription. Our data provide a new view on how quiescence leads to major chromatin rearrangements affecting the chromatin proteome at different levels. We expect that future studies on local chromatin proteome maps under different conditions and at other genomic loci will offer powerful resources for detailed molecular and functional annotation of chromatin proteins and their interactions and relationships at the genome.

5.4 Methods

Yeast strains and library manipulations

Yeast strains used in this study are listed in Supplemental Table S4. Library manipulations on solid media were performed using synthetic genetic array (SGA) technology (Tong & Boone, 2006) and a RoToR instrument (Singer Instruments, Watchet, UK). Rearranging of yeast libraries and generation of pools for barcode verification and identification were performed in liquid media using a Hamilton Microlab Star (Hamilton, Germany). The HO locus barcoder library (Korthout et al., 2018) was expanded from ~1100 strains to ~2500 strains using additional barcoder strains kindly provided by the Andrews lab (Douglas et al., 2012). The strains that could be validated were included for Epi-Decoder analysis. The barcoder strains were arrayed over 6 x 384 well plates (Supplemental Fig S1A). The previous and newly acquired barcoder strains were validated by sequencing pools of rows and columns (Korthout et al., 2018) to ensure that the plate location

matched with the expected BC_UP and BC_DN sequences. Strains with incorrect or uncertain annotations were removed from further analysis. The updated barcoder library (NKI8591) was combined with a re-arrayed version of the MAT α TAP-tag collection (NKI8592) with four key design principles. First, a subset of ~700 chromatin associated factors was allocated to 2 dedicated plates of the 12 x 384 plates (Supplemental Fig S1A, asterisk). The Chromatin-TAP-tag subset contains factors that were either (a) considered a binder at BC_UP or BC_DN, in untreated or HU-treated cells in previous Epi-Decoder experiments at the HO locus (Korthout et al., 2018), or (b) had a GO or GO Slim term related to chromatin, chromatin organization, DNA replication, DNA repair, or transcription (in publicly available datasets from yefasco (<http://yefasco.cabr.utoronto.ca>), yeasttract (<http://www.yeasttract.com>), or in published literature). Factors specifically involved in RNA PolI or RNA PolIII transcription were omitted. The highly-abundant histones were also omitted to increase the sequencing depth of the barcodes associated with other chromatin proteins. Finally, factors that appeared to be incorrectly tagged or barcoded were removed from the analysis. Second, the barcoder library can be re-shuffled to obtain three libraries in which each TAP-tagged protein is coupled with three different barcode pairs, I (NKI8598), II (NKI8599) and III (NKI8600). Third, each full library of ~4000 strains can be processed in two subset pools (Fig 1A, Supplemental Fig S1). Fourth, the three barcode versions of the 700 Chromatin TAP-tag subset (Chrom-3xBC; I (1A and 10D), II (1C and 10F) and III (1B and 10E)) can be processed together in one pool due to non-overlapping barcode pairs. The barcodes and TAP tag were verified for a few selected strains. Strains with incorrect or uncertain barcode or tag status were removed from further analysis.

Culture media, growth conditions and inhibitors

Yeast media were prepared as previously described (Korthout et al., 2018; Tong & Boone, 2006; van Leeuwen & Gottschling, 2002). For Epi-Decoder analysis of the full libraries, strains were grown in YEPD (1% yeast extract, 2% bacto peptone, and 2% glucose) to log phase at 30°C and harvested. For screening with transcription inhibitors, the Chrom-3xBC library was grown on YEPD plates overnight and the colonies were pooled in liquid culture. The cultures were grown until log phase (OD₆₆₀ of ~0.4) at 16° C and then a-factor (final concentration of 0.02 µg/ml from a stock solution of 0.2 mg/ml in methanol (O'Reilly, Charbin, Lopez-Serra, & Uhlmann, 2012) was added for 3 hours to arrest the cells in G₁. The arrest was verified by flow cytometry as described below. Synthetic a-factor was synthesized as described (O'Reilly et al., 2012). Samples were collected for Epi-Decoder (150 mL) and RNA isolation (10 mL). 1,10-Phenanthroline (PH; 100 µg/mL final concentration from a 100 mg/mL stock solution in ethanol, Aldrich, Cat No. 131377-5G), 6-azauracil (AU) (2 mg/mL final concentration from a 100 mg/mL stock solution in DMSO) or vehicle (ethanol 0.1% or DMSO 2%) was added to G₁ arrested cells and samples for Epi-Decoder (150 mL), flow cytometry (1 mL) and RNA extraction (10 mL) were collected at 5 min, 12 min, 30 min and 75 min (for drugs) and 75 min (for vehicle control). All cultures were maintained at 16°C. Drug concentrations were chosen based on previous studies (Grigull et al., 2004). For validation experiments, RNA extraction and protein extraction, individual strains from fresh plates were grown until log phase (OD₆₆₀ of ~0.4) at 16°C prior to arresting in G₁ and exposure to drug treatment. For growth curves, PH and AU were added to asynchronous cultures of three independent strains (NKI8528, NKI8529, NKI8613) grown in YEPD at 16°C, and samples for OD measurement at 660 nm were collected every 30 min. For isolation of quiescent cells, frozen aliquots of the pooled Chrom-3xBC library were thawed, expanded in YEPD and further processed as described previously (Spain, Swygert, & Tsukiyama, 2018).

RNA isolation and reverse transcription

RNA was isolated using the RNeasy Mini Kit (QIAGEN) using the protocol for Yeast cells, with a few modifications, essentially as in (Korthout et al., 2018) and with a spike-in reference. Samples of 10 mL were collected at each time point for RNA extraction. A reference *S. pombe* strain (A8545; a gift from R. Allshire) was grown to OD₆₆₀ = 0.5 and pellets of aliquots were frozen. The equivalent of 5 mL reference culture was added to each 10 mL sample prior to cell lysis. The combined cells were spun down and pellets were resuspended in 600 µL cold RLT buffer. Cells were broken by bead beating with Zirconia/silica beads and debris was removed by centrifugation. The supernatant was collected and mixed with one volume 70% EtOH and transferred to RNeasy columns. Following the buffer RW1 and buffer RPE wash steps RNA was eluted in 50 µL elution buffer. Eluted RNA was treated with DNase I (QIAGEN) to remove genomic DNA. Next, cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen). RT-PCR was performed with the primers described in (Supplemental Table S5). Each sample was measured in two technical duplicates and the average value was taken when combining biological replicates. The *S. pombe* gene *ACT1* was used for normalization. To confirm effects of drug treatment, RNA was isolated from Chrom I, Chrom II and Chrom III libraries and the clonal line NK18619.

Epi-Decoder (TAG-ChIP-Barcode-Seq)

Epi-Decoder was done essentially as described previously (Korthout et al., 2018). Following arrest and treatment with drugs, samples (150 mL) were immediately crosslinked with 1/10th of the volume of freshly prepared Fix Solution (1% methanol free formaldehyde (Thermo Scientific; 28908), 50 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 1 mM EDTA) for 20 minutes (unless specified otherwise) and subsequently quenched for 5 minutes with Glycine (125 mM final concentration). Cells were washed once in cold TBS with 0.2 mM PMSF and the pellet was frozen at -80°C. Cells from frozen pellets were lysed by bead beating in 200 µL breaking buffer (100 mM Tris pH 7.9, 20% glycerol, protease inhibitor cocktail EDTA-free) with Zirconia/silica beads and lysates were

collected after washing twice in 1 ml FA buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, protease inhibitor cocktail EDTA-free). Lysates were sonicated using the Bioruptor PICO (Diagenode) for 10 minutes at 30 second intervals and soluble chromatin was separated by centrifugation. For ChIP, 50 μ L chromatin was used as input material and 1 mL was used for IP using IgG Sepharose 6 Fast Flow beads (GE healthcare). Beads were washed 3 times with PBSB (PBS containing 5 mg/mL BSA), and incubated with chromatin overnight on a turning wheel at 4°C. Samples were washed twice in FA buffer, twice in high salt FA buffer (500 mM NaCl), twice in RIPA buffer (10 mM Tris pH 8, 250 mM LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA) and once with TE buffer (10 mM Tris pH8, 1 mM EDTA). For washing, the Sepharose beads were spun for 2 minutes at 3000 rpm at 4°C. DNA was eluted from IP samples using 100 μ L elution buffer (50 mM Tris pH 8, 10 mM EDTA, 1% SDS), at 65°C, for 10 minutes. IP and input samples were treated with 0.5 μ L RNase A (10 mg/mL) and 10 μ L ProtK (10 mg/mL) in 70 μ L TE for 1 hour at 50°C and subsequently kept overnight at 65°C to reverse crosslinks. DNA was purified using the QIAquick PCR purification kit (Qiagen). BC_UP, BC_DN were amplified separately with specific primers (Supplemental Table S5). PCR products were mixed in an equimolar fashion and purified from an agarose gel with a size selection of 100-150 bp. The purified DNA was sequenced (single read, >50 bp) on a HiSeq2500/MiSeq platform (Illumina, San Diego, CA), using one or a mix of custom sequencing primers (Supplemental Table S5).

Barcode counting

The barcode counts were obtained as in (Korthout et al., 2018). Briefly, the Perl script eXtracting Counting and LInking to Barcode References (XCALIBR, <https://github.com/NKI-GCF/xcalibr>) was used to locate the barcodes in the reads and generate a count table. The median of each sequence library (every tag and time-point combination is a separate library) was used to normalize the raw barcode counts. Log2 IP/input values were used for heatmaps and line plots.

ChIP-qPCR

ChIP experiments on individual clones (NKI8587, NKI8619, NKI8625, NKI2608, NKI2609, NKI2611, NKI2612) were performed similar to Tag-ChIP-Barcode-Seq, but with 20 μ L bed volume of IgG Sepharose 6 Fast Flow beads or 40 μ L epoxy-activated Dynabeads coupled with rabbit IgG and 200 or 400 μ L chromatin (as described previously (Korthout et al., 2018)). Each ChIP was done in triplicate. Quantitative PCR was performed on the purified DNA with SensiFAST SYBR master mix (Bioline) according to the manufacturer's protocol and analyzed on LightCycler 480 II (Roche). The binding was analyzed with primers specific for regions in proximity to the BC_UP and BC_DN or for endogenous regions (Supplemental Table S5). Each sample was measured in two technical duplicates and the average value of these two was taken as one value when combining biological replicates.

Flow Cytometry

Flow cytometry samples were prepared to verify G₁ arrest by a-factor and to monitor cell cycle progression in the presence of drugs. Approximately 1×10^7 cells were collected and fixed with 70% ethanol and stored at -20 °C. Flow cytometry was performed as previously described (Haase, 2004), after staining DNA with Sytox green (Molecular Probes). Flow cytometry measurements were taken on a FACSCalibur with CellQuest software (Becton Dickinson) and further analyzed with FlowJo software (Treestar).

Protein detection by immunoblot and antibodies

For immunoblotting, strains (NKI8587, NKI8619, NKI8625, NKI8628) were grown to mid-log phase (OD₆₆₀ 0.6–0.9). Samples of 2×10^8 cells were harvested and washed with Tris-EDTA (TE; 10 mM Tris pH 8, 1 mM ethylenediaminetetraacetic acid (EDTA)) containing 0.2 mM phenylmethane sulfonyl fluoride (PMSF). Cell pellets were stored at -80°C until further processing, but at least 30 min. Whole-cell extracts were prepared in SUMEB (1% sodium dodecyl sulfate (SDS); 8 M urea;

10 mM 3-(N-morpholino)propanesulfonic acid, pH 6.8; 10 mM EDTA; 0.01% bromophenol blue) containing protease inhibitors (1 mM PMSF, 1 mM dithiothreitol, 5 mM benzamidine, 1 µg/ml pepstatin, 1 µg/ml leupeptin) by bead beating. The resulting lysate was incubated for 10 min at 65°C and subsequently clarified by centrifuging 5 min at 21 × g. Prior to immunoblotting, 4–10 µl of lysate (~2 × 10⁶ cells) was separated on a polyacrylamide gel (16% for histones, 8-10% for other proteins, or gradient gels). Separated proteins were transferred to a 0.45-µm nitrocellulose membrane for 1 h (histones) or 2 h (other proteins) at 1 A. Membranes were blocked with phosphate-buffered saline (PBS) containing 2% or 5% Nutrilon (Nutricia) for 1 h, and first antibody incubations (dilutions see below) were performed overnight at 4°C in 4 ml Tris-buffered saline containing 0.05% Tween-20 (TBST) with 2% Nutrilon. After washing three times in TBST, secondary antibody incubation was performed in TBST with 2% Nutrilon and LI-COR Odyssey IRDye 800CW antibody at 1:10 000 for 45 min at room temperature in the dark followed by 10 min washes twice in TBST and once in PBS. Membranes were scanned using an LI-COR Odyssey IR Imager (Biosciences) and analyzed using Image Studio 2.0 (LI-COR). For density scans, the signal was the sum of the individual pixel intensity values for a shape minus the product of the median intensity values of the pixels in the background (with a border width top/bottom of 3) and the total number of pixels enclosed by the shape (Area): Signal = Sum – (Background × Area). Primary antibodies and their dilutions used in this study are Pgk1 (459250, Invitrogen, RRID:AB_221541; 1:4000), Histone H2B (39238, Active Motif, RRID:AB_2631110; 1:2000), Histone H3 (RRID:AB_2631108 (43); 1:2000), TAP (CAB1001, ThermoFisher Scientific, RRID:AB_10709700; 1:1000), Hmo1 (Abcam; ab71834, 1:1000). Secondary antibodies used are IRDye 800CW goat anti-Mouse IgG (0.5 mg) 926-32210 Li-COR (RRID:AB_621842) and IRDye 800CW goat anti-Rabbit igg (0.5 mg) 926-32211 Li-COR (RRID:AB_621843).

Data Availability

All relevant data are within the paper and its Supporting information files.

5.5 Acknowledgements

We thank Brenda J Andrews and Charles Boone for kindly providing the expanded yeast Barcode Collection. The authors thank the RHPC facility of the Netherlands Cancer Institute for providing computational resources, the NKI Genome Core Facility for barcode-sequencing, Ben Morris of the NKI high-throughput center for help with rearraying yeast libraries by robotics. We thank Craig D. Kaplan, Thom Molenaar, Eliza Mari Kwesi-Maliepaard, Bas van Steensel, Tineke Lenstra, Marlize van Breugel and Anne Kappert for insightful discussions and valuable suggestions.

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5.7 Author contributions

DWP-L, TK, FvL: Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting and revising the article; CEC and TT: Q cell library preparation; DWP-L: Methodology and Validation; TvW: Acquisition, analysis and interpretation of data; IvK: data analysis; DEA, HO: Generation of reagents.

5.8 Supplemental Tables

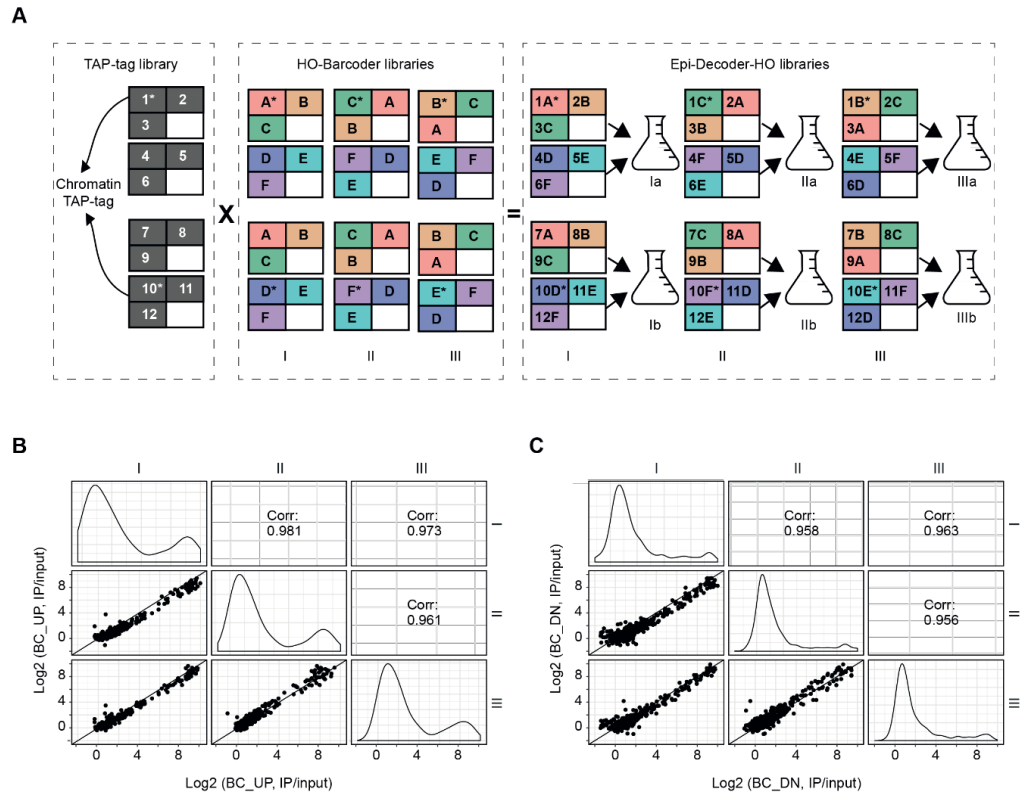
Supplemental tables S1-S3 can be found on the online version of the publication.

Supplemental Table S1: Epi-Decoder-HO full library barcode counts

Supplemental Table S2: Epi-Decoder-HO Chrom-3xBC inhibitor barcode counts

Supplemental Table S3: Epi-Decoder-HO Chrom-3xBC Q and NQ barcode counts

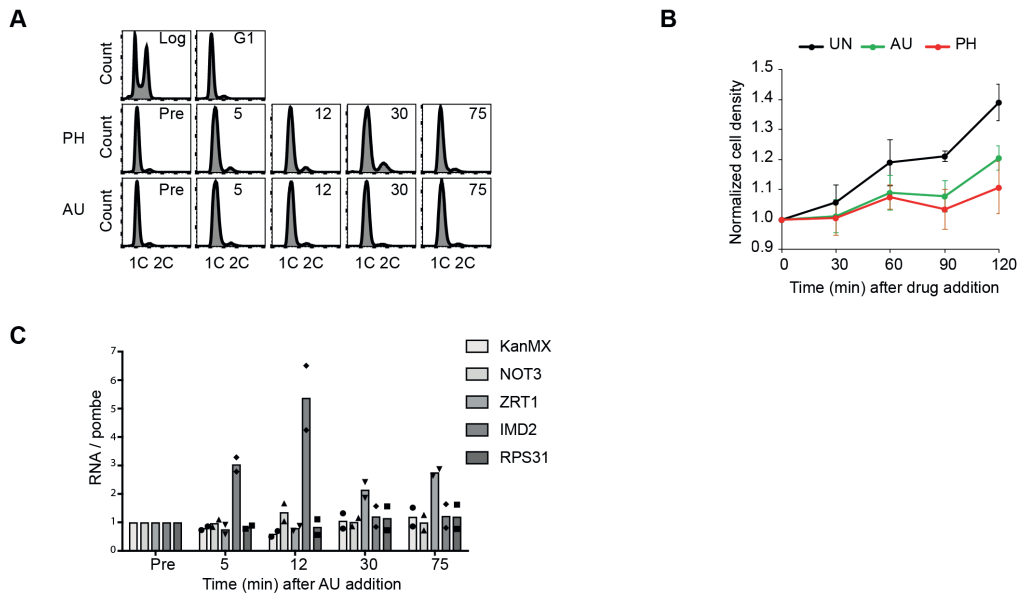
5.9 Supplemental Data



Supplemental Figure S1

(A) Details of the construction of triplicate Epi-Decoder-HO libraries. A re-arrayed MAT α TAP-tag library and an expanded MAT α BC library were crossed in three different combinations. In the re-arrayed TAP-tag library, a subset of chromatin proteins was allocated to two 384-well plates (*). The HO-BC library consists of ~2250 BCs. For each cross, the HO-BC library was re-shuffled to

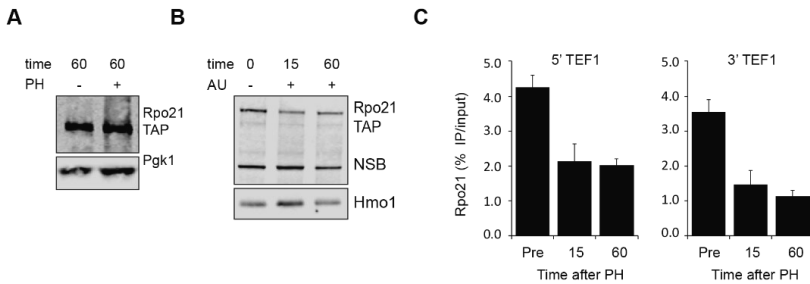
obtain three Epi-Decoder-HO libraries, I, II and III with different TAP-tag-BC combinations. Each library was processed in two pools, 'a' and 'b'. The three versions of the Chromatin-TAP-tag subset (*; Chrom-3xBC) have non-overlapping barcodes and were processed in one pool. **(B-C)** Comparison of the binding scores (IP/input) of BC_UP and BC_DN separately for chromatin binders (as determined previously in (Korthout et al., 2018)) in the three full Epi-Decoder-HO libraries. Indicated are the Spearman correlation coefficients and the diagonal line represent $x=y$. Density plots show the distribution of the barcode counts in each of the three replicates. See Table S1 for binding scores of all proteins examined.



Supplemental Figure S2

(A) Flow cytometry analysis of DNA content to verify the G_1 arrest by a- factor during the course of the experiment show in Fig. 2A. Samples were collected before treatment (Pre) and at the post-treatment time points indicated. **(B)** Growth curve of cells treated with PH and AU at 16°C in the

absence of *a-factor* (average of three independent strains $\pm$ SD). **(C)** Analysis of mRNA expression changes over time by RT-qPCR upon addition of AU. The gene *IMD2* is known to respond to AU and taken along as an indicator of the experimental condition. RNA levels in two *Chrom-3xBC* libraries are shown relative to an untreated library (Pre) and normalized to a transcript from a spike-in of untreated *S. pombe* cells (see Materials and Methods).



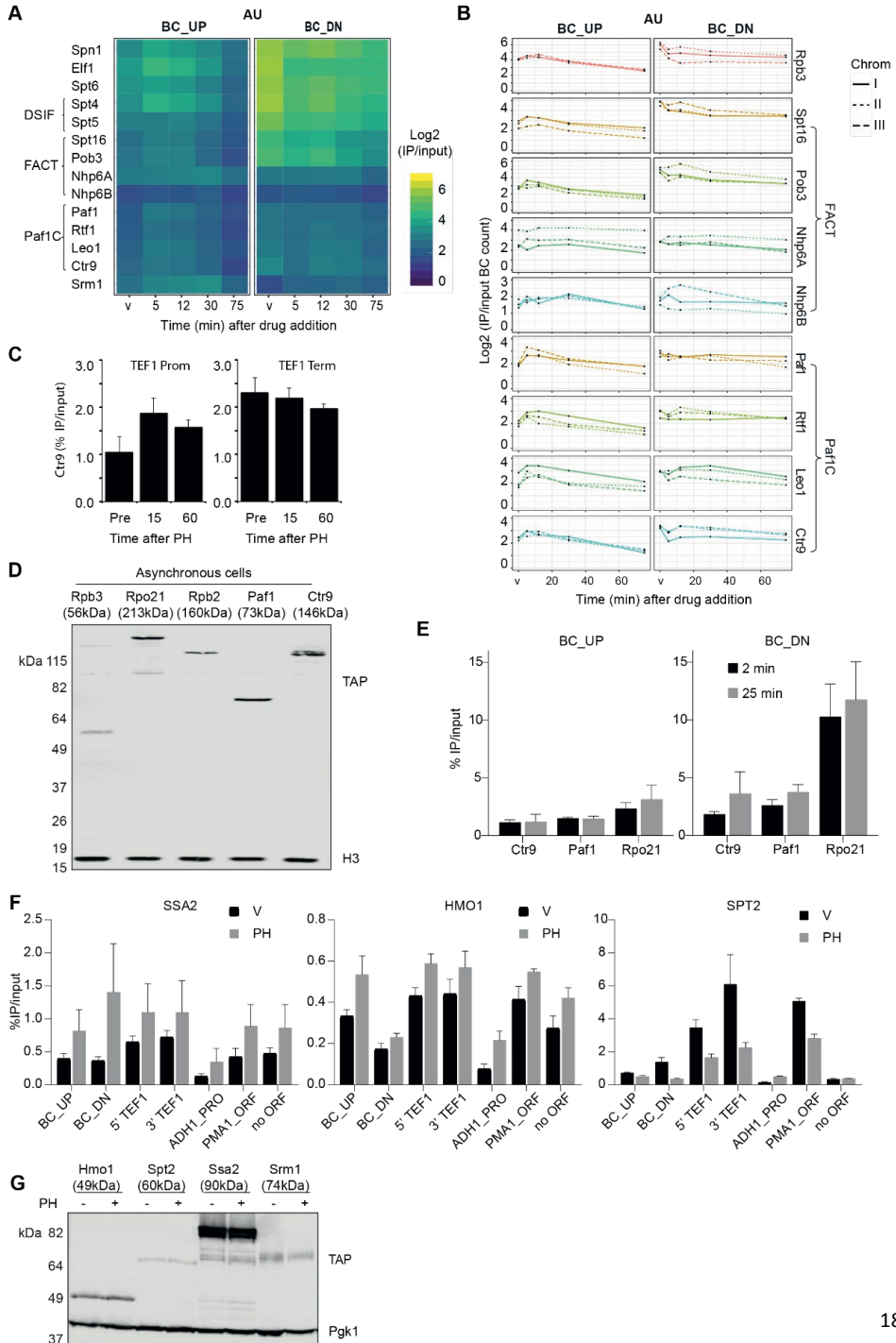
Supplemental Figure S3

(A-B) Replicates of samples shown in Fig. 3C-D. Immunoblot analysis of Rpo21-TAP with and without PH and AU treatment in *G*₁-arrested cells at 16°C. Pgk1, H3, Hmo1, and a non-specific band (NSB) were used as loading controls. **(C)** ChIP-qPCR analysis of Rpo21 binding at the 5' and 3' end of the endogenous *TEF1* gene in *G*₁-arrested cells treated with (15 and 60 min) and without (Pre) PH at 16°C (average of three biological replicates $\pm$ SD).

Supplemental Figure S4

(A) Zoom in on heat map of Fig 2 (AU treatment), showing proteins annotated to transcription elongation. **(B)** Independent replicates of proteins related to FACT and Paf1C (Log₂ IP/input at time points as in Fig. 2; the lines show the three different barcode pairs of the indicated TAP-tagged proteins in the *Chrom-3xBC* library). **(C)** ChIP-qPCR analysis of Ctr9-TAP binding at the 5' and 3' end of the endogenous *TEF1* gene in *G*₁-arrested cells treated with (15 and 60 min) and

Inhibition of transcription leads to rewiring of locus-specific chromatin proteomes

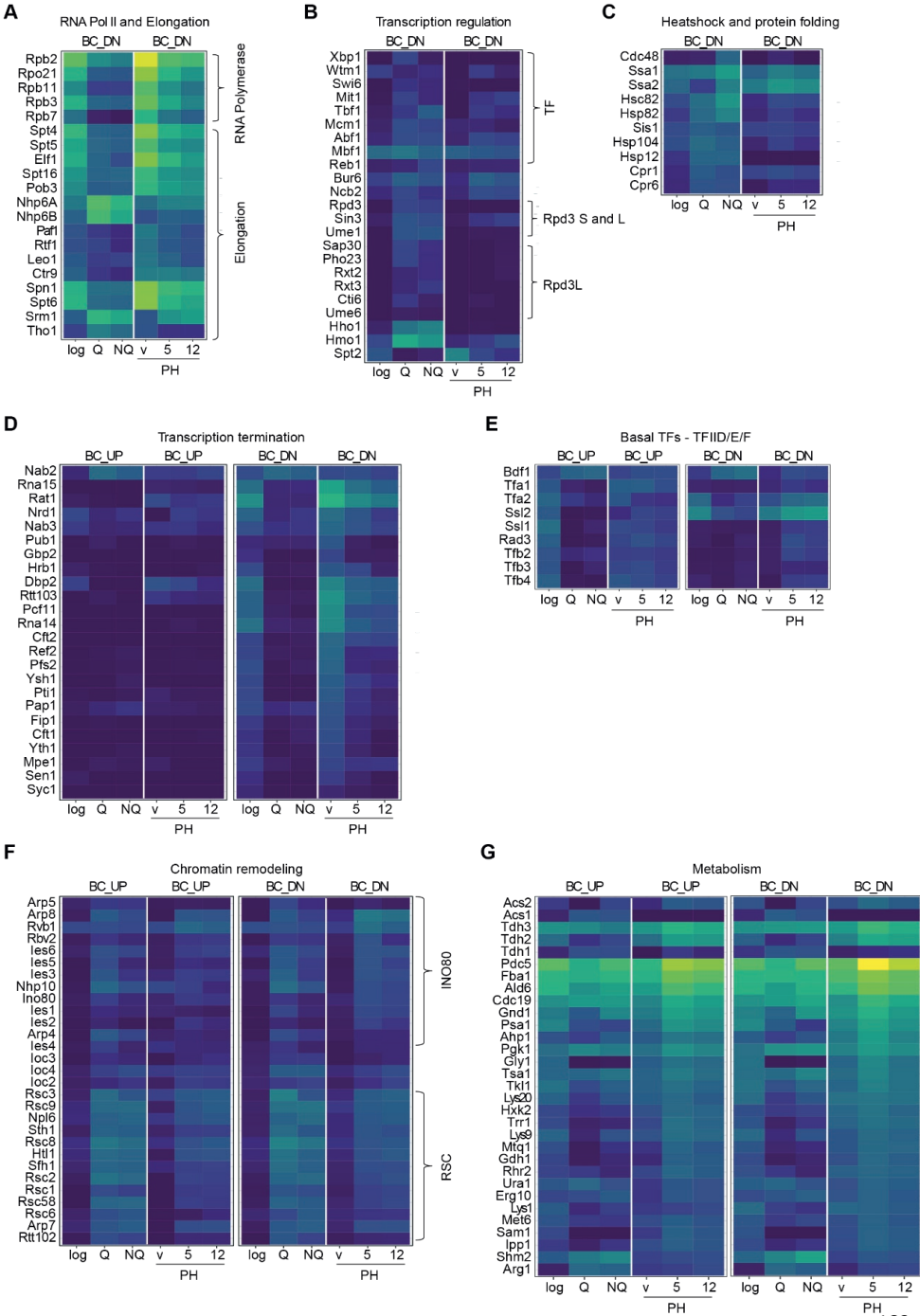


without (Pre) PH at 16°C (average of three biological replicates +/- SD). (D) Immunoblot analysis of TAP-tagged Rpb3, Rpo21, Rpb2, Paf1 and Ctr9 in asynchronously growing cells. H3 was used as loading control. The indicated protein sizes (kDa) include the TAP tag (21kDa). (E) ChIP-qPCR analysis of cross-linking efficiency of Ctr9, Paf1, and Rpo21 at the BC_UP and BC_DN regions after 2 and 25 minutes of crosslinking in asynchronous cells grown at 30°C. (F) ChIP-qPCR analysis of binding of Ssa2, Hmo1 and Spt2 in G₁-arrested cells at 16°C, treated for 15 min with vehicle (V) or phenanthroline (PH) (average of three biological replicates +/- SD). Analyzed loci are as in Fig. 4E. (G) Immunoblot analysis of TAP-tagged Hmo1, Spt2, Ssa2, and Srm1 in G₁-arrested cells treated for 15 minutes with vehicle or PH at 16°C. Pgk1 was used as loading control. The indicated protein sizes (kDa) include the TAP tag (21kDa).

Supplemental Figure S5

(A-E) Zoom in on heat maps of Fig 5 showing proteins in the indicated annotated clusters. PH treatment during G₁ arrest (vehicle, 5 and 12 minutes) is shown for comparison.

Inhibition of transcription leads to rewiring of locus-specific chromatin proteomes



Supplemental Table S4: Yeast strains

Strain/library	Genotype	Ref
NKI8591	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ho::BC-KanMX-BC	This study
NKI8592	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 gene-TAP-His3MX6	This study
NKI8598	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC gene-TAP-His3MX6	This study
NKI8599	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC gene-TAP-His3MX6	This study
NKI8600	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC gene-TAP-His3MX6	This study
NKI8528	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC STE2-TAP-His3MX6	1
NKI8529	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC BAR1-TAP-His3MX6	1
NKI8613	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC POL2-TAP-His3MX6	This study
NKI8587	MATa can1Δ::HphMX lyp1Δ::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC RPO21-TAP-His3MX6	1
NKI8619	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC CTR9-TAP-His3MX6	This study
NKI8625	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC PAF1-TAP-His3MX6	This study
NKI8628	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC RPB3-TAP-His3MX6	This study
NKI8647	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC RPB2-TAP-His3MX6	This study
NKI2609	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC SRM1-TAP-His3MX6	This study
NKI2608	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC SSA2-TAP-His3MX6	This study
NKI2611	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC HMO1-TAP-His3MX6	This study
NKI2612	MATa can1Δ::HphMX lyp1d::STE3pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ho::BC-KanMX-BC SPT2-TAP-His3MX6	This study
A8545	h+ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18 cnp1:cnp1-loxP-HA-HYG-loxP-T7 (S. Pombe)	Robin Allshire Lab

References 1: Korthout, T., et al. (2018). PLoS Biol. 16, e2005542.

Supplemental Table S5: Primers

Primer Name	Sequence
HOpromQfw	GAAGCTTGTTGAAGCATGATGAA
HOpromQrev	TTGCTGCTTATGAGGATATGGATTT
HOtermQfw	GAGTAGAAATACGCCATCTCAAGATACA
HOtermQrev	GGAAAGTTGATCAAGACCCAATAATAA
YeastTef1_qfw1	CGGTCATGTCGATTCTGGTA
YeastTef1_qRev1	ACCTAATTCAGCGGCTTCCT
3'YeastTEF1_qFw1	GCTTTGGTCAAGTTCGTTCC
3'YeastTEF1_qRev1	TAGCGGCCTTTTCAGTCTTG
PMA1_ORF_Qfor1	AAATCTTGGGTGTTATGCCATGT
PMA1_ORF_Qrev1	CCAAGTGTCTAGCTTCGCTAACAG
ADH1 PRO ii fwd	CCGTTGTTGTCTCACCATATCC
ADH1 PRO ii rev	GTTTCGTGTGCTTCGAGATACC
NoORF_Qfor	GGCTGTCTAGAATATGGGGCCGTAGTA
NoORF_Qrev	CACCCCGAAGCTGCTTTCACAATAC
2B_UP_Fw	AATGATACGGCGACCACCGAGATCTACACCTTCCGATCTAGATGTCCACGAGGTCTCT
2B_DN_Fw	AATGATACGGCGACCACCGAGATCTACACTCTTCCGATCTACGGTGTCCGCTCTCGTAG
2B_UP_Rv_all	CAAGCAGAAGACGGCATAACGAGATNNNNNNNGTCGACCTGCAGCGTACG
2B_DN_Rv_all	CAAGCAGAAGACGGCATAACGAGATNNNNNNNAACGAGCTCGAATTCATCGA
2B_UP_Seq	ACACCTTCCGATCTAGATGTCCACGAGGTCTCT
2B_DN_Seq	ACACTCTTCCGATCTACGGTGTCCGCTCTCGTAG
KanMX_Qfw	TGATGCGCTGGCAGTGTT
KanMX_Qrev	CGCGATCGCTGTTAAAAGGA
ZRT1_qFW	GCCATCGGTTTGGGTGTTT
ZRT1_qRev1	CCAGAGATAACAAGCGCAGTGT
not3_qFwd	AAAGGTCTCGTGATATGTGTGAAA
not3_qRev	CCAAGGGAGATGGTGGATTA
IMD2_2f	TTGGATTCATCCCAAGGAAA
IMD2_2r	AGCAATCAAATTGGCAGCTT
RPS31-1	GATTGATCTTTGCTGGTAAGC
RPS31-rev	CCAAGGTAGAGTAGATTCC
Pom_qact1 fw	CCCAAATCCAACCGTGAGAAGATG
Pom_qact1 rev	CCAGAGTCCAAGACGATACCAAGTG

CHAPTER 6

Summary and
general discussion



6

Summary and general discussion

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6.1 Summary of the thesis

The basic packaging of DNA into nucleosomes is more or less the same across the genome. However, the specific distribution of nucleosomes, their post-translational modifications, and the proteins that interact with specific chromatin structures and DNA sequences vary, leading to different types of chromatin across the genome. Each of these specific chromatin structures may have its own properties and functions. Therefore, this complexity gives rise to challenges in understanding general principles of chromatin such as the maintenance of chromatin states during transcription and DNA repair and how chromatin maintenance is coordinated spatially and temporally with DNA replication.

Chapter 1 describes how the packaging of DNA into chromatin intimately associates with the regulation of the gene expression. The packaging of chromatin into nucleosomes and the positioning of nucleosomes can help organize a gene unit such that transcription starts at the intended transcription start site. The wrapping of DNA into nucleosomes can prevent transcription initiation if the histones occupy binding sites for transcription factors or access to DNA by the basal transcription machinery. This can be modulated by post-translational modifications that can loosen histone-DNA contacts or promote histone re-assembly in the wake of RNA and DNA polymerase. During transcription and replication, histones are transiently evicted from the DNA and need to be reassembled once the process is finished. Transcription typically is associated with histone acetylation and dedicated machineries are in place to remove the acetyl groups and reset the chromatin state to prevent ectopic transcription from hyperacetylated sites.

In the presence of external signals or during cell cycle progression or development, specific gene regulatory programs need to be implemented. Such programs are initiated by the binding of regulatory proteins to specific elements at regulatory regions such enhancers and promoters and their binding is further aided by the inherently dynamic nature of chromatin which provides an

opportunity for the binding of pioneering transcription factors. A cascade of binding events is then initiated, with the recruitment of chromatin remodelers that can dispel the nucleosomes and open up the chromatin. This permits the synergistic binding of general transcription factors that initiate transcription allowing the cell to implement its gene regulatory programs. While chromatin modifications regulate gene transcription, the process of transcription also changes the chromatin. Various enzymes are recruited to transcribing Pol II, for example histone acetyltransferases, ubiquitinating and de-ubiquitinating enzymes, and histone methyltransferases, which together lead to a broad spectrum of transcription-coupled histone post transcriptional modifications (PTMs). Thus, whether such modifications are a cause or consequence of transcription is an ongoing topic of interest (Henikoff & Greally, 2016; P. B. Talbert et al., 2019). Similarly, there is much interest in uncovering the underlying mechanisms that allow for the replication fork to move through chromatin and leave properly packaged daughter strands behind, without disturbing the epigenetic state of the cell. It is to be expected that chromatin modification and restoration processes are in part loci specific.

To understand the dynamics of chromatin at a detailed level, it is important to know the different players at the different loci across the genome and how chromatin states are regulated. To move towards this higher-level understanding, this thesis has focused on dissecting locus-specific chromatin regulatory pathways and protein binding events. **Chapter 2** and **Chapter 3**, describe the application of Epi-ID to determine regulatory mechanisms, while **Chapter 4** and **Chapter 5** describe Epi-Decoder to decode local chromatin proteomes. These Epi-discovery techniques use chromatin immunoprecipitation followed by barcode sequencing (ChIP-Barcode-Seq) in budding yeast to probe for locus-specific chromatin regulomes and chromatin interactomes.

6.2 Epi-ID: Interrogating locus-specific chromatin regulomes

Yeast libraries in arrayed format have been indispensable for performing complex bioassays in high throughput (Giaever et al., 2002). Yeast deletion collections with DNA barcodes that are introduced in each genome replacing the gene that is deleted, have been pivotal in high throughput screening. The digital counting of DNA barcodes as a proxy for the relative abundance of each deletion strain has allowed for the yeast gene deletion strains to be pooled in genetic or chemical screens (Giaever & Nislow, 2014; Pierce et al., 2007). As a result, complex screens can be conducted in parallel and in high throughput. In **Chapter 2** we describe a detailed protocol for Epi-ID, which builds on the principle that DNA barcodes are part of the genome, in a chromatinized context and therefore can be used for the systematic and direct interrogation of the chromatin state in the vicinity of the DNA barcodes, essentially providing a locus-specific readout of chromatin states in thousands of mutants in parallel.

In Epi-ID, the chromatin status is interrogated in pools of mutants by ChIP followed by barcode sequencing and allows for direct comparison of effects of the deletion mutants on the chromatin status. This concept of Epi-ID was first tested in a small-scale screen to determine the molecular mechanisms of histone turnover in the 'classical' barcoded deletion collection (Verzijlbergen et al., 2011). A potential caveat of using the classical deletion collection (Giaever & Nislow, 2014) for interrogating chromatin states was that the barcodes were integrated at the sites of deleted genes, and therefore in a different genomic location in each deletion mutant. Therefore, the readout could suffer from position effects. The issue of position effects was solved by generating a novel library where non-barcoded deletion strains were crossed with the yeast HO-Barcoder library in which each strain is wild type but carries a unique pair of barcodes flanking a selectable marker at a common location, the *HO* locus (Figure 1). Barcodes are introduced at a safe harbor locus of *S. cerevisiae* strain BY4741, replacing the *HO* open reading frame with two unique bar codes flanking the *KanMX4* cassette (Yan et al., 2008). The HO gene encodes for a site-specific endonuclease

that is required for gene conversion or mating-type switching at the MAT locus. *HO* expression is highly regulated and is confined to haploid cells at late G1 phase of the cell cycle. In laboratory yeast strains, the *HO* allele is often mutated to prevent mating type switching and *HO* deletion is generally accepted to be phenotypically neutral and is considered not to cause a fitness effect (Baganz, Hayes, Marren, Gardner, & Oliver, 1997; Pierce et al., 2007; Yan et al., 2008). HO-Barcoders retain the flanking sequences of *HO*, and thus retain the chromatin context of the *HO* locus, including the cis-regulatory regions that are upstream of the *HO* promoter. Thus, the chromatin context at the barcodes are influenced both by the *HO* locus regulation and the continuously expressed *AgTEF1* promoter of the KanMX cassette. At the 3' end, the barcodes are influenced by the *HO* terminator as well as the *AgTEF1* terminator. Further, the terminator proximal barcode is in close proximity (50 bp) to the *HO* origin of replication (ARS404) (Korthout et al., 2018). The barcoding strategy used has an added advantage, that it actually harbors two barcodes that are in two different chromatin contexts, the *HO* promoter, and *HO* terminator, enabling the interrogation of chromatin at two very different genomic contexts in parallel. The first use of this library was to screen for regulators of histone H3K79 methylation by Dot1 (Vlaming et al., 2016). Antibodies against H3K79 monomethylation and H3K79 trimethylation were used for the parallel analysis of these methylation states, and this led to the identification of all known regulators of H3K79 methylation, and several novel regulatory mechanisms, validating Epi-ID and demonstrating the power of it.

Epi-ID has been used to study histone turnover and H3K79 methylation and can be used for many other chromatin features. Key requirements for Epi-ID are that the feature of interest is present at the barcoded locus and that a ChIP against the chromatin feature of interest can pull down the barcoded regions. Therefore, Epi-ID relies on the availability of efficient ChIP antibodies for the chromatin feature studied or for a barcoder library around the feature of interest. In **Chapter 3** we explored the use of Epi-ID to probe for candidate factors and mechanisms responsible for

regulating promoter levels of the histone variant H2A.Z. For this screen, we generated a library in which tagged versions of H2A.Z were introduced to the Epi-ID-HO library. The tags on H2A.Z resulted in efficient pull-down of H2A.Z and the screen successfully identified SWR1C, the ATP-dependent chromatin-remodeling complex known for exchanging nucleosomal H2A for H2A.Z, as the main regulator of H2A.Z levels at promoter nucleosomes. These results show that tagged versions of chromatin factors can be efficiently crossed into Epi-ID libraries, bypassing the need for efficient antibodies against endogenous proteins for chromatin regulome screens.

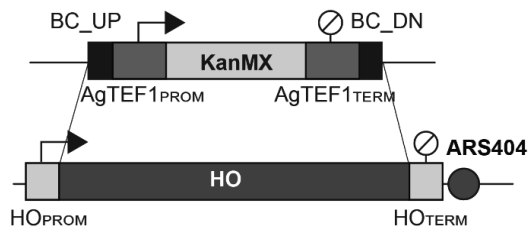


Figure 1. The barcoded *HO-KanMX* locus

Schematic diagram of the barcoded *HO-KanMX* locus where barcodes are introduced at a safe-harbor locus of *S. cerevisiae* strain BY4741 by replacing the *HO* open reading frame with two unique bar codes flanking the *KanMX4* cassette. The *BC_UP* is promoter-proximal and the *BC_DN* terminator-proximal and close to (50 bp) an origin of replication (*ARS404*).

Opportunities and challenges with yeast libraries

In **Chapter 3**, we successfully cross in H2A.Z-tag alleles into the Epi-ID-HO library. Tagged versions of H2A.Z increased the ChIP efficiency compared to using an antibody against H2A.Z/Htz1. A C terminal or N-terminal tag can affect the function of the protein of interest. However, with H2A.Z we could confirm that the tagging did not affect some of the important functions of H2A.Z in dealing with stress. While tags can be used to bypass the need for efficient antibodies, they cannot be used for studies of post-translational modifications. However, the repertoire of validated antibodies and engineered reader proteins recognizing differentially modified states of histones is expanding rapidly (Albanese et al., 2020).

Another potential limitation of crossing in tagged alleles is that each genetic cross can lead to introduction of errors in the libraries. This issue lies within the construction of complex libraries using the Synthetic Genetic Array (SGA) procedure of creating complex barcoded *S. cerevisiae* collections. SGA is a method for systematically constructing double mutants or transferring DNA barcodes from donor strains into an existing *S. cerevisiae* collection (Tong et al., 2001; Yan et al., 2008). Using SGA, the Epi-ID-HO library was generated by crossing the NatMX knock-out library (Baryshnikova et al., 2010; Tong & Boone, 2006) with the Barcoder collection (Yan et al., 2008). This cross generated haploid strains that combine the genetic characteristics of both parent strains. SGA works by eliminating “unwanted genotypes” at consecutive rounds of selection. This is done by linking each allele of interest to a selectable marker such as the dominant nourseothricin-resistance marker, NatMX or the G418-resistance marker KanMX. In addition, selection for MATa haploids is performed by using the SGA MATa haploid marker, *can1d::STE2pr-Sp_his5* (*STE2pr-Sp_his5* is integrated into the genome deleting the open reading frame (ORF) of the *CAN1* gene, which confers sensitivity to canavanine) and by selection for haploids using deletion of the *LYP1* gene, which confers resistance to thialysine (Baryshnikova et al., 2010).

Yeast mating occurs between MATa and MAT α cells, and results in a diploid zygote, which is an a/ α cell, capable of producing spores. Upon induction of sporulation, yeast spores are formed, and they can develop into haploid yeast cells. Each cross will generate four spores of which two are MATa and two are MAT α . The chance of having the mating type of choice, MATa is 1/2. In parallel, selection occurs for the other markers: Barcode locus (KanMX), tagged-protein locus (HIS3), can1d::STE2pr- Sp_his5 (selection for MATa haploids), and lyp1d (selection against diploids). Together, only 1/64 spores will have the desired combination of alleles. Since sporulation on the standard lab yeast strains is not very efficient, this requires optimal sporulation conditions and efficient colony replication. While this is a significant challenge, in **Chapter 3** and further in **Chapters 4 and 5** (see below) we demonstrate that with careful selection and constant checks for strains that could escape, it is indeed possible to generate complex yeast libraries using SGA. However, it should be kept in mind that each genetic cross can introduce errors such as cross-contamination, or escaper colonies in which all or a subset of the cells lacks one of the desired alleles.

Combining the Epi-ID (Barcode + KO) library with H2A.Z-Tag

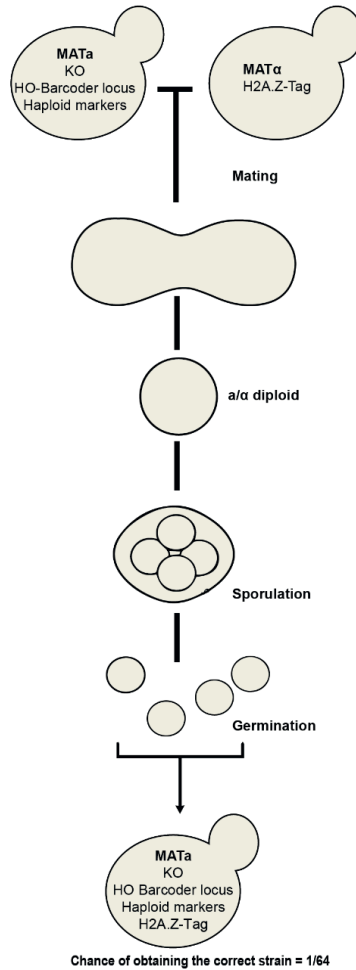


Figure 2. Combining the Epi-ID (Barcode + KO) library with H2A.Z-Tag using synthetic genetic array

SGA is a method for systematically combining alleles in yeast by eliminating “non-ideal strains” and selecting for the desired genotypes by consecutive rounds of selection (see text). In the above example, a MAT α strain carrying a tagged version of H2A.Z linked to a selectable marker (NatMX)

is crossed to an ordered array of MATa Barcoders each containing a specific gene deletion and SGA selection markers. Adapted from (Baryshnikova et al., 2010). Using a combination of subsequent selection schemes results in a new library in which the alleles are combined.

Potential applications of Epi-ID:

Regulome screens using Epi-ID have so far depended on KO collections. These inherently have the problem of excluding gene KOs that affect strain viability. One way of avoiding this is to generate Epi-ID libraries with other types of mutant alleles besides knock-outs. For example, Yen et al described the combination of barcoders with “decreased abundance by mRNA perturbation” (DAmP) alleles (Yan et al., 2008). If systematic libraries of degron alleles (e.g. Auxin-Induced Degradation; AID) (Nishimura et al., 2009) or anchor away alleles (AA) (Haruki, Nishikawa, & Laemmli, 2008) would be available, this would further expand the possibilities of probing gene functions of essential genes and of measuring short-term responses. However, in their current form, these methods require multiple genetic elements, making them less suitable for systematic genetic crosses in SGA. Epi-ID screens as outlined in **Chapter 2** and **Chapter 3** have focused on the effect of knock-outs on the barcoded HO-KanMX locus. For probing the regulome of chromatin features that are not present at the HO-KanMX locus (e.g. heterochromatin, rDNA, tRNA, transposons, subtelomeric chromatin, etc), or for probing a different chromatin locus altogether, the yeast KO collection could potentially be crossed with different or multiple Barcode libraries. Advances in this area and other potential applications such as humanizing Epi-ID are discussed further in the text.

6.3 Epi-Decoder: Decoding locus-specific chromatin binding events

Epi-ID identifies regulators of chromatin features. What Epi-ID does not uncover is whether the regulators act directly or indirectly at the locus. For example, adenosine kinase promotes H3K79 methylation by co-factor metabolism and was identified by Epi-ID (Vlaming et al., 2016). However, it is not bound at the barcoded locus. In contrast, Rtf1, a binder at the barcoded locus (Korthout et al., 2018), was identified as a positive regulator of H3K79 methylation (Vlaming et al., 2016). Rtf1 recruits Bre1/ Rad6 to chromatin of transcribed regions and thereby promotes Dot1 activity (Vlaming et al., 2016). This missing information in Epi-ID led us to ask whether the DNA barcoding technology could be harnessed to determine which proteins occupy the barcoded locus.

In **Chapter 4** we describe the development of Epi-Decoder, a TAG-ChIP-barcode sequencing technology to systematically measure protein-DNA interactions proximal to the barcoded loci, essentially enabling us to take a snapshot of chromatin-associated factors in a locus-specific manner. For the pilot screen described in **Chapter 4**, we generated a library of strains where each clone harbors a pair of DNA barcodes at the HO-KanMX locus, and one protein tagged at its endogenous locus with a common epitope tag, the TAP tag. Similar to Epi-ID, ChIP is performed for the common tag in pooled cells and barcodes are counted in the immunoprecipitated DNA by high-throughput sequencing. The barcode counts are then used as a readout for the binding of the specific protein to the barcoded locus. Since Epi-Decoder examines all proteins using the same affinity handle under exactly the same conditions in the same strain background in parallel, binding scores can be directly compared in a quantitative manner. Of the approximately 5600 proteins encoded by the yeast genome, approximately 4200 TAP-tagged proteins were included in the Epi-Decoder analysis of the barcoded KanMX gene at the HO locus (Epi-Decoder-HO). In total, more than 400 proteins were identified to bind at the barcoded HO locus. While many proteins were binding to both the promoter and terminator proximal barcodes, ~20 were specific for the promoter, while ~250 were specific for the terminator. The four canonical histone proteins

were the most enriched of the shared binders at the two loci. The vast majority of binders had gene ontology (GO) terms related DNA binding or were involved in the downstream RNA processing, such as capping, splicing, cleavage and polyadenylation, all of which occur together with transcription and is in close proximity to DNA.

Metabolic enzymes cross-link to chromatin

In addition to well-known proteins involved in chromatin, replication, transcription, and RNA processing, we identified a substantial number of factors involved in cellular metabolism (e.g., glyceraldehyde 3-phosphate dehydrogenase/Tdh3, pyruvate kinase/ Cdc19, homocitrate synthase/Lys20) that bind at the barcoded HO locus. The binding of these metabolic enzymes is dynamic, changing upon HU treatment and after exposure to transcription inhibitors, indicating that the binding of these factors is not constitutive. It is possible that binding of these metabolic factors may depend on their high expression level. Nevertheless, in **Chapter 4** we could show that not all highly expressed metabolic factors bind at the locus. Using methanol-free formaldehyde did not alter the binding of these metabolic factors, suggesting that leaking of cytoplasmic contents to the nucleus during fixation extraction does not explain these findings. It will be important in future experiments to determine whether cross-linking is efficiently blocked before the cells and nuclei are lysed and chromatin is prepared.

In **Chapter 4**, we barcoded a native genomic locus, *ADE2*, by generating a new barcoded library using CRISPR/Cas9 genome editing (Figure 3). The barcoded *ADE2* locus was generated by inserting a 15-bp barcode in the transcribed region of the *ADE2* gene (BC_5'-*ADE2*), 57 bp downstream *ADE2* transcription start site. *ADE2* is involved in the *de novo* purine nucleotide biosynthetic pathway. Disruption of *ADE2* causes the accumulation of a red pigment in cells, and provided a direct visual read-out to confirm the efficiency of the barcode insertion. Barcoded donor

strains were generated using CRISPR-Cas9 genome editing. Random colonies were selected and transferred to an arrayed format to identify the integrated barcode in each clone and to enable crossing to the TAP-tagged library using SGA, generating the BC_5'-ADE2 Epi-Decoder library. The binding pattern of this promoter-proximal native locus (BC_5'-ADE2) was very similar to that of barcoded *HO*-KanMX promoter locus (BC_UP), confirming its promoter-proximal location and validating the pilot study at the *HO* locus. Further, expected differences between BC_UP and BC_5'-ADE2 were detected by Epi-Decoder with Rap1, a strong binder at the Rap1 controlled AgTEF1 promoter at BC_UP (M. Chen et al., 2013), undetected at the 5' end of the ADE2 gene. Additionally, the lower binding scores for transcription-initiation and -elongation proteins seen at BC_5'-ADE2 was consistent with the lower transcript levels found at the *ADE2* versus *TEF1* promoter-controlled transcripts. As for the binders associated with cellular metabolism, we could now compare binding of cellular metabolites at the three different loci, the barcoded promoter and terminator of the *HO*-KanMX gene, and the 5'-*ADE2* locus. Interestingly, the metabolic binders did not show preferential binding to either of the loci, suggesting that they interact in a non-specific manner. Therefore, it will be important to examine other genomic loci, such as loci without ongoing transcription, regions of heterochromatin, or regions transcribed by Pol I and Pol III. While there is evidence that some cytoplasmic enzymes might have moonlighting functions in the nucleus (Boukouris et al., 2016; S. Li et al., 2015; Linder & Mostoslavsky, 2017; Monaghan & Whitmarsh, 2015; Sivanand et al., 2017; Su & Pillus, 2016), an interesting study would be to compare the chromatin contexts in different metabolic states, and Epi-Decoder would be an ideal platform to this end. In future studies it will be important to determine what the physiological relevance of the interaction of metabolic enzymes with the chromatin is. Their binding to chromatin may affect the local chromosome environment (e.g. by the local production of co-factors or by acting as a scaffold) or the activity of metabolic enzymes may be influenced by the interactions in the nucleus. Testing these models will require more insights into the modes of interaction and the design of separation-of-function alleles to separate the metabolic function from the 'moonlighting' function

in the nucleus. One possible strategy is to add an nuclear export signal to a metabolic enzyme to exclude it from the nucleus, while maintaining its normal metabolic function.



Figure 3. The barcoded 5'-ADE2 locus (5'-ADE2 Barcoders)

A schematic diagram of the barcoded 5'-ADE2 locus where a 15-bp barcode is inserted in the transcribed region of the ADE2 gene (BC_5'-ADE2), 57 bp downstream of the start of the ADE2 coding sequence.

Dynamic local proteomes

When combined with altering conditions or cell cycle progression, Epi-Decoder can capture temporal binding dynamics of chromatin proteins. In **Chapters 4 and 5** we show that Epi-Decoder can successfully report on the broad chromatin rewiring that follows replication stress, transcription stress, or entry into quiescence. In **Chapter 4**, we focus on the barcoded HO replication origin (ARS404) which is in close proximity to the actively transcribing KanMX gene in the Epi-Decoder-HO library. The KanMX gene transcribes towards the HO replication origin that is licensed with the pre-replicative complex (Figure 1). Upon firing of the origin, the replication fork will have to negotiate with the transcription machinery, to avoid collisions. When exposed to replication stress induced by HU, multiple changes in the chromatin proteome were revealed by Epi-Decoder, with 40 proteins at the promoter and 79 proteins at the terminator showing significantly different binding

that could not be explained by altered expression level alone. Many of these binding changes relate to proteins involved in transcription. The reduction of Pol II subunits extends previous findings of displacement or degradation of RNAPII during HU-induced replication stress (Poli et al., 2016). The accompanied reduction of mRNA levels at the barcoded KanMX gene upon HU-induced stress further supports an extensive chromatin rewiring geared at suppressing transcription. Chromatin rearrangements following replication stress extend beyond their effects on transcription. Topoisomerase II binding was strongly increased at both ends, while all the subunits of ORC in the library showed decreased binding at the terminator while occupancy of MCM was unaltered. While these changes may indicate extensive reorganization of the locus in preparation for transcription-replication conflicts, interestingly, changes at the locus occur even before the firing of the adjacent HO origin (ARS 404). Early replication origins have typically fired before replication is stalled by the presence of HU, while middle to late origins, including the barcoded HO origin have not yet fired in HU (Yabuki, Terashima, & Kitada, 2002). Thus, the chromatin rearrangements observed occur prior to any possibility of conflict. We hypothesize that these events prepare the locus for potential replication-transcription conflicts and current consensus favors a model where Pol II is released to enable completion of DNA replication (García-Muse & Aguilera, 2016). Yet, how does this work? What mechanisms could sense and relay this information leading to the rewiring of the locus? Interestingly, while there is a loss of binding at the locus, there is very little gain in binding, with the binding of Topoisomerase II being the prominent exception. Topoisomerase II is the main enzyme releasing topological stress during S phase and in replication stress. One explanation is that these changes merely reflect a response to the cell being in S phase. However, when we compared two genes that have the same AgTEF1 promoter, we observed higher mRNA levels in HU for the gene that is not in proximity to an origin of replication. Thus, the reduction in transcription cannot be a simple reflection of the cell being in S-phase, but is clearly linked to the proximity to the origin of replication.

At the heart of epigenetics is the inheritance of chromatin states and Epi-Decoder can be a valuable asset to gain insights into how licensed origins are maintained, how the replisome is assembled over time, and how chromatin is reassembled in the wake of replication. Such a study would provide clues about reassembly factors that might assist inheritance of histones or other chromatin proteins. Further, insights gained will help delineate the binding events associated with replication and those that are specific for S phase. To this end, the Epi-Decoder libraries can be arrested in G1 and then released into fresh media, and chromatin rewiring can then be assessed at regular intervals. However, the Epi-Decoder-HO library is MAT α , which means that a G1 arrest requires the use of mating pheromone A-factor (O'Reilly et al., 2012). SGA markers *can1d::STE2pr-Sp_his5* and *lyp1 Δ ::STE3pr-LEU2* in principle allow for selection of MATa and MAT α cells, respectively. However, the TAP-tag is linked to a HIS3 marker, which precludes the use of the MATa selection marker. Therefore, TAP-tag libraries and Epi-Decoder libraries are all of the MAT α mating type. The use of the mating pheromone α -factor for arresting MATa cells is well documented. However, the effect of A-factor on MAT α cells is less well documented. Similarly, there is less experience with arrest-release protocols using A-factor. During the work described in this thesis we performed pilot studies to determine the optimal conditions for A-factor arrest and release. We collected cells over time following release from A-factor arrest and determined DNA copy number at the replication origins by qPCR. Interestingly, unlike with α -factor, we found that the release from A-factor was slow and the firing of the replication origin ARS404 was asynchronous (data not shown). We next considered the use of HU, which can also be used to arrest replication forks or slow them down to allow detection. However, only early origins fire in HU. The firing of late origins, such as ARS404, is suppressed in HU due to reduced nucleotides that activate the S-phase checkpoint, blocking the firing of the middle to late origins (Yekezare, Gomez-Gonzalez, & Diffley, 2013; Zegerman & Diffley, 2010). Dbf4 and Sld3 are directly inhibited by activation of the S-phase check point. This S-phase checkpoint can be bypassed by overexpressing mutant versions of *DBF4* (*dbf4-4A*) and *SLD3* (*sld3-A*) (Zegerman & Diffley,

2010), which allows for late origins to fire in the presence of HU. Therefore, we incorporated the dbf4-4A and sld3-A mutants (integrated into a single locus) into the Epi-Decoder-HO library. This involved an additional crossing step to incorporate the mutants into the library (unpublished). However, while the HO replication origin did fire in the presence of HU, the passage of the replication fork was very slow, taking over four hours for fork passage over the barcode following addition of HU. This delay also leads to asynchrony in origin firing. For Epi-Decoder to be successful, the barcoded origin needs to fire synchronously in the population. To this end, a solution would be to barcode an early and more faithfully replicating origin, such as ARS607, a commonly studied, early-firing replication origin (Yabuki et al., 2002) and follow the passage of the replication origin over time. Indeed, barcoded donor strains with barcodes at ARS607 can be efficiently generated using CRISPR-Cas9 genome editing following the procedure described in **Chapter 4**.

6.4 Expanding the possibilities of Epi-ID and Epi-Decoder

Single pool ChIP-barcode sequencing

A cornerstone of ChIP-barcode-sequencing techniques such as Epi-Decoder, that sets them apart from conventional ChIP methods, is the ability to interrogate all proteins directly in one sample. In addition, the focus on a single locus reduces the need for high sequencing depth, allowing for efficient and extensive multiplexing of samples in each sequencing run. This makes ChIP-barcode-sequencing methods cost-effective, and allows for high-throughput chromatin profiling. The availability of the libraries in arrays allows the construction of different formats of the same library or the construction of smart sub-libraries. We demonstrate this in Chapter 5, where we use the Epi-Decoder-HO library to follow changes in the local chromatin proteome as Pol II is inhibited. Our goal was to create one library pool to interrogate binding of the full yeast proteome. In Chapter 4, the original Epi-Decoder-HO library was split over four sub libraries which were processed

separately. This was needed because of the availability of only ~ 1100 unique Barcoders. Even after expanding the HO-Barcoders from ~1100 strains to ~2500 strains using additional Barcode strains provided by the Andrews lab, we were still short of ~1700 Barcoders as the TAP-tagged library covers approximately 4200 proteins of the 5600 proteins encoded by the yeast genome (Xie et al., 2018). However, it did allow us to split the Epi-Decoder-HO library over two sub libraries. In addition, we decided to construct a smaller TAP-tag library of ~700 (putative) chromatin proteins (Chrom-Tap) by re-arraying the full Tap-tagged library, such that the Chrom-Tap subset can be efficiently and easily separated from the full library. We next crossed the Chrom-TAP subset library with the ~2500 HO-Barcoders generating the Chrom-3xBC library where binding at the *HO* locus can be assessed in triplicate, i.e. binding of each TAP-tag protein is interrogated with three independent barcodes. This enables efficient data collection of multiple time points or multiple conditions.

An advantage of the single-pool Epi-Decoder approach is that protein binding can be directly compared in a quantitative manner across all replicates since all proteins and their replicates are examined in exactly the same condition, using the same affinity handle, in the same strain background. We demonstrate this in **Chapter 5**, where we use the Chrom-3xBC library to determine the changes in local chromatin proteomes upon inhibition of Pol II by chemical means or by physiological signals during quiescence. Inhibiting transcription either by the antifungal agent 1,10-phenanthroline (PH), a small molecule inhibitor Pol II or by 6-azauracil, which acts by reducing GTP levels, led to rapid and broad rewiring of the local chromatin proteome. Changes seen were not limited to transcription elongation and termination factors that are known to depend on Pol II, but also included gain of chromatin remodelers and metabolic enzymes. In quiescent cells, Pol II is repressed by strong physiological signals and we could confirm the recruitment of the quiescence-specific transcription repressor Xbp1 and the Rpd3L complex that accompanied the loss of the core transcription machinery. The loss of core transcription proteins was

accompanied by increased abundance of several structural proteins, chromatin remodelers, heat shock proteins, and changes in metabolic enzymes showing that Q cells undergo a major rewiring at the level of chromatin. Indeed, some of the changes in Q cells were also observed in PH and most likely reflect secondary effects of transcription repression in Q cells. We also observe proteins acting in the same complex or annotated to the same process that show dissimilar kinetics, suggesting hierarchies of chromatin binding. Examples are the general transcription factors Ssl2 and Tfa2, which bind strongly at the promoter as well as the terminator region, showing that their behavior is different to that of the other general transcription factors, which bind predominantly at the promoter region only. The Paf1C transcription elongation complex is another example that shows dissimilar kinetics. Where most Paf1C subunits depend on Pol II for binding, levels of the largest subunit Ctr9 deviated from this. This is of particular interest due to the known role of the Pol II CTD in recruiting Paf1C and structural studies showing the central role of Ctr9 in the assembly of Paf1C ((P. Deng et al., 2018; Vos et al., 2018; Xie et al., 2018). How does Ctr9 escape eviction from chromatin in the presence of Pol II eviction? Indeed, recent studies have shown that Ctr9 can bind to DNA independent of its association to Paf1C by its ability to bind single-strand-containing DNAs, triple-helical DNA and nucleosomes (P. Deng et al., 2018; Musso et al., 2000; Vos et al., 2018). Alternatively, it is possible that once Ctr9 is recruited to the chromatin, another protein is involved in stabilizing its binding. Yet, with the Chrom-3xBC library, we only screen with 700 putative chromatin binders and it is possible that we fail to identify new or unexpected proteins, whose binding might be context dependent.

Generating new barcoded libraries using CRISPR/Cas9 technology

While screening with the Chrom-3xBC library has its advantages (e.g. screening for two barcodes in parallel for 700 factors), its main disadvantage is that we only screen with 700 chromatin binders and at a non-native locus. Indeed, one way to bypass this is to increase the Barcode library size. However, to cover every TAP-tag protein we require 4200 Barcoders, or 12,600 if we want to cover every protein in triplicate with independent barcodes. In **Chapter 4**, we set up a 2-step protocol to generate new and larger Barcode libraries using CRISPR/Cas9 technology, making it possible to generate comprehensive proteome maps of many genomic loci (Figure 4). We demonstrate this by introducing barcodes at the *ADE2* locus (Figure 3). The first step is to insert a selection marker (NatMX) in proximity to the locus of interest, downstream of the to-be-barcoded *ADE2* gene. This is to ensure that the newly formed 5'-*ADE2*-Barcoders could be crossed into the TAP-tagged libraries and the Epi-Decoder libraries created could be efficiently selected using SGA procedure. In *S. cerevisiae*, introducing a double-strand break by targeted Cas9 is generally lethal unless it can be repaired by homologous recombination (DiCarlo et al., 2013; B. X. Fu et al., 2016; Mans et al., 2015; Ryan et al., 2014). Therefore, we next introduce a Cas9- and guide RNA (gRNA)-expressing vector into this strain, together with a repair template library composed of a random barcode sequence flanked by 40 bp arms with homology to the gRNA target site, at the 5' end of the *ADE2* coding sequence. Since the vast majority of the transformants represent repaired and barcoded clones, random colonies can be picked and transferred to an arrayed format. After generating smart pools, the integrated barcode in each clone can be identified by high-throughput sequencing. Thus, the 2-step protocol now allows for barcoding of any native genomic locus of interest, allowing for Epi-ID and Epi-Decoder to be applied at sites other than barcoded reporter genes.

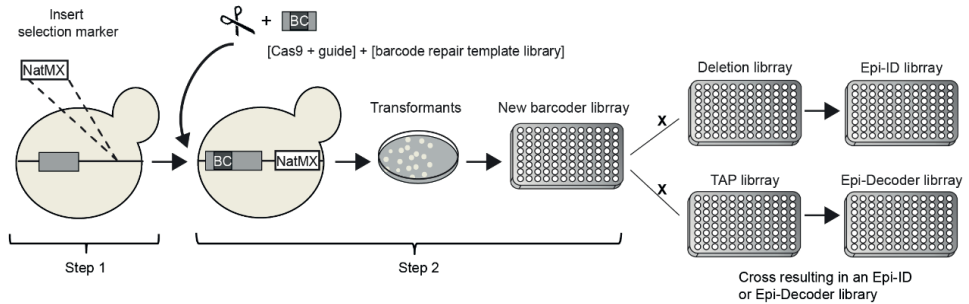


Figure 4. Schematic diagram of the 2-step protocol for barcoding of a native genomic locus of interest with CRISPR-Cas9

In step 1, a selection marker (*KanMX*) is introduced in the proximity of the locus of interest to enable crossing the barcoded allele into the TAP library using SGA procedure. In step 2, barcodes (BC) are introduced by creating a double strand break at the locus of interest by transforming strains with a plasmid encoding Cas9 and a guide RNA. This break is then repaired by a BC-containing repair template library that has homology to the sites flanking the double-strand break and that is co-transformed with the Cas9-guide plasmid. The majority of transformants represent barcoded strains and they can be picked and placed in arrayed format. This new arrayed library can then be sequenced to identify the barcodes at each clone location. The barcoder library can then be crossed with either a TAP-tagged ORF library or a collection of deletion strains creating Epi-Decoder or Epi-ID libraries.

Effects of barcode sequences on Epi-Decoder binding scores

Smart barcoding strategies and CRISPR-Cas9 mediated protocols for generating barcoder libraries increase the versatility of Epi-Decoder libraries. One aspect we wanted to investigate further is the extent to which these short, 20bp sequences that are inserted affect protein binding. With single pool Epi-Decoder, protein binding can be directly compared across the three replicate barcodes and in **Chapter 5** we show that a high correlation exists between the binders of the

triplicate libraries. This indicates that the insertion of the 20 bp barcodes generally does not affect protein binding scores. Yet, for a few factors, one of the triplicate barcodes would show binding scores that deviated in an unexplainable manner from the other two. It is possible that certain barcode sequences, while limited to 20bp, could prevent or promote binding, either by removing a favorable binding sequence or by enhancing the binding of different proteins. Such outliers can be identified and removed from further analysis of preferred.

Expanding Epi-Discovery tools

Epi-ID and Epi-Decoder are powerful tools for describing regulomes and static and dynamic local proteomes. In addition, they can be used as discovery tools. The availability of multiple barcoder libraries will be of great benefit for future studies and the SGA methods allow for further engineering of the libraries for follow-up studies. Firstly, for Epi-Decoder, the comparison of multiple loci under multiple conditions can provide a powerful resource to identify correlations between proteins or protein-hierarchies in binding to chromatin. As illustrated in Figure 5A, and demonstrated in **Chapter 4** with the use of the HO-barcoders and the BC_5'-ADE2 barcoders, the ability to generate new Barcoder libraries and the relative ease of crossing them to tagged-protein libraries allows the interrogation of multiple loci in parallel. In Figure 5A, protein binding to the arbitrary locus 1 and 2, can be analyzed under multiple physiological or genetic conditions. Here, the binding of the arbitrary factors A and B at the locus 1 is condition dependent while binding of factor B is retained at locus 2 in both conditions. Additionally, existing Epi-Decoder libraries can be used to further insert additional barcodes by crossing them to new Barcoder libraries with careful consideration for mating type and using different selection markers that would allow for efficient SGA (Figure 5B). This would allow for the interrogation of additional loci in a single pool, increasing the efficiency of sample collection and processing. Screening for chromatin regulators with Epi-ID will also benefit by generating libraries with multiple tagged loci. For example, the Epi-ID screen for regulators of histone H3K79 methylation used barcodes at the HO-KanMX gene

promoter and terminator for its readout, while H3K79 trimethylation is enriched within transcribed gene regions (A. T. Nguyen & Zhang, 2011; Vlaming et al., 2019). These screens can now be repeated at loci where the barcode is placed within coding regions, such as the 5'-ADE2 locus. Finally, Epi-ID and Epi-Decoder can be combined to untangle chromatin networks as illustrated in Figure 5C. For example, in **Chapter 4** we identify several chromatin proteins with increased abundance at the HO locus upon HU treatment. One example is Wtm1, a known negative regulator of ribonucleotide reductase (RNR), the protein that is inhibited by HU (represented by unknown Factor A in Figure 5C). Wtm1 binds to RNR and retains it within the nucleus, thereby preventing its sequestering in the cytoplasm, thus regulating its function. To understand how Wtm1 is recruited, we could TAP-tag Wtm1 and introduce this allele in an Epi-ID library. This would point to proteins that are required to target Wtm1 to the locus. To determine the role of Wtm1 binding at *HO* locus, the Epi-Decoder analysis could be repeated in a library in which Wtm1 is absent or over expressed. Alternatively, CRISPR mediated gene targeting of Wtm1 at ectopic loci targeting can be used to further identify its role in the chromatin proteome. These are just a few examples of the many possibilities.

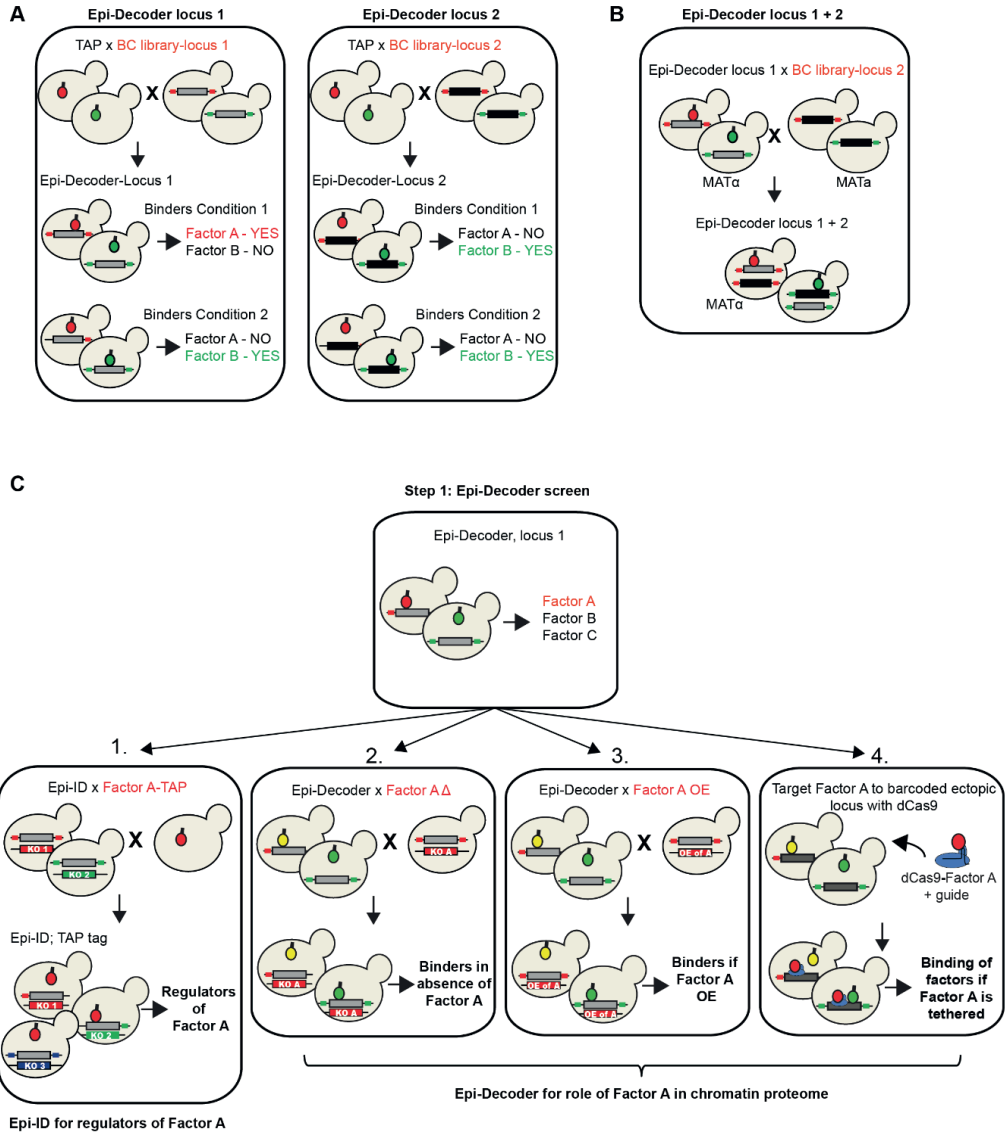


Figure 5. Epi-Discovery combinations

(A) In this thesis we describe the comparison of two barcoded loci (*HO-KanMX* and *ADE2*) under multiple conditions with Epi-Decoder. Protein binding can vary between loci and conditions. (B) If linked to unique markers, two barcoded loci can be combined in one Epi-Decoder library. These

loci can be analyzed in the parallel in the same pools. (C) A chromatin binder identified by Epi-Decoder (Factor A) can be further studied by subsequent Epi-discovery screens to untangle chromatin networks. 1) To understand how binding of Factor A is recruited and regulated, the TAP-tagged allele (Factor A – TAP) can be introduced in an Epi-ID library. Epi-ID for Factor A-TAP would point to proteins that regulate Factor A occupancy at the locus. 2) The Epi-Decoder analysis can be repeated after crossing in a deletion of Factor A (Factor A Δ) to determine how the absence of Factor A affects the local proteome 3) Similarly, Epi-Decoder analysis can be repeated in a background where Factor A is overexpressed (Factor A OE) or 4) where Factor A is targeted to an ectopic location via CRISPR targeting.

6.5 Epi-ID and Epi-Decoder beyond yeast?

The budding yeast, *S. cerevisiae*, is a powerful model system for defining and discovering fundamental mechanisms of many cellular processes and the genetic tools enable novel screening methods such as Epi-ID and Epi-Decoder. Apart from the ease of performing genetic screens and arrayed yeast libraries that further facilitate this, it has a short generation time, and a well-characterized genome. Further, they are the ideal model system for gene editing with CRISPR-Cas9 because yeast have a preference for homologous recombination over non-homologous end joining for the repair of double strand breaks and cells in which breaks are not repaired by homologous recombination will die ((DiCarlo et al., 2013; Mans et al., 2015). Therefore, integration of DNA into the yeast genome is highly efficient using CRISPR-Cas9, and no selectable markers are needed. Yet, adopting Epi-ID and Epi-Decoder to mammalian systems would enable the discovery of additional cellular processes that are unique to human cells.

Realizing human Epi-Decoder will be technically challenging. The basic elements needed are a tagged ORF library and a barcoded locus library and they will have to be combined. Firstly, for human Epi-Decoder, a collection of tagged human ORFs is needed. But, unlike in the yeast TAP-

tagged ORF library, where each ORF is tagged and the protein is expressed from its natural chromosomal location, such human ORF collections are not readily available. Mammalian ORF collections are often cDNA collections that can be cloned into expression vectors (Yang et al., 2011) and introduced in the cell of interest. Secondly, a library of barcodes is needed at a common location. With the rapidly improving efficiency of genome engineering, making such a library may be possible in the future. However, a major hurdle will be to combine the barcodes with tagged ORFs in such a way that the complexity remains limited and the unique barcodes can be assigned to unique tagged ORFs. In yeast this is greatly facilitated by using arrays on solid media, in which each clone, i.e. each tagged ORF and each barcoder has a known address. The advance of single cell sequencing may offer possibilities in the future to link tagged ORF and barcode in individual cells in a pooled library. Although it is preferable to have barcodes in a native locus, the second-best option would be to place the tagged ORF and barcoded reporter libraries at model locus that can be engineered for efficient targeting of ORF-barcode libraries. In this way, the ORF and barcode are in the same locus, which will facilitate linking them to each other. Such strategies would help in realizing human Epi-Decoder but it will require multiple challenging steps of engineering and sequencing. Therefore, it is expected that budding yeast will in the future still be highly valuable for defining the fundamental principles of chromatin regulation.

ADDENDUM



Addendum

Bibliography

Nederlandse samenvatting

PhD Portfolio

Acknowledgements

Curriculum vitae

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NEDERLANDSE SAMENVATTING

Uitbreiding van de 'Epi-Discovery' toolbox:

Het systematisch identificeren van chromatine-regulatoren en chromatine-binders met behulp van barcodes.

Het eukaryotische genoom zit verpakt in chromatine: een structuur bestaande uit DNA, histonen en bijbehorende eiwitten. Er bestaan 4 kernhistonen, H2A, H2B, H3 en H4, en een linkerhiston, H1. De vier kernhistonen vormen samen een octamere histonkern, waar het DNA omheen gewikkeld zit. Samen vormen het DNA en de histonkern een nucleosoom; de structurele basiseenheid van chromatine. Histon H1 bindt vervolgens aan zogeheten 'linkergebieden' tussen de nucleosomen. Door deze binding wordt elk nucleosoom aan zijn burens verbonden waardoor een geordende fiber ontstaat: de chromatinefiber. De basisstructuur van de chromatinefiber is min of meer gelijk in het hele genoom. Wel kunnen de dichtheid van nucleosomen, hun post-translationele modificaties, en de eiwitten die aan chromatine gebonden zijn variëren, waardoor verschillende chromatinetoestanden kunnen ontstaan. **Hoofdstuk 1** beschrijft deze variatie en de daarmee gepaard gaande uitdagingen bij het begrijpen van algemene principes rondom chromatine, zoals de rol van chromatine en genexpressie, het behoud van de structuur van chromatine tijdens DNA transcriptie en DNA reparatie, en de ruimtelijke en temporale coordinatie van chromatinestructuur en DNA replicatie.

Voor een goed begrip van chromatine is het essentieel om te weten hoe de chromatinetoestanden op verschillende plekken in het genoom worden gereguleerd en welke factoren daarbij een rol spelen. Daarom richt dit proefschrift zich op het ontrafelen van locus-specifieke chromatineregulatie en chromatine-eiwitdynamiek.

In **hoofdstuk 2** wordt Epi-ID beschreven. Met deze techniek, ontwikkeld voor gebruik in gist, kan in parallel voor duizenden genetische mutanten worden bepaald wat de chromatinetoestand is. Epi-ID maakt gebruik van korte DNA sequenties – DNA barcodes. In een collectie giststammen, met elk een specifieke mutatie, wordt in elke stam op dezelfde genomische locatie een unieke barcode geïntroduceerd. Vervolgens wordt op de hele collectie chromatine-immunoprecipitatie uitgevoerd. Door in het geprecipiteerde materiaal met behulp van 'high-throughput sequencing' te tellen hoe vaak elke barcode voorkomt, wordt bepaald hoe vaak een bepaalde chromatinekenmerk in elk van de mutanten voorkomt. Epi-ID kan voor een breed scala aan chromatinemodificaties en chromatineeiwitten worden toegepast. **Hoofdstuk 3** beschrijft hoe Epi-ID gebruikt kan worden om factoren te identificeren die verantwoordelijk zijn voor het reguleren van de hoeveelheid H2A.Z. Dit is een histonvariant die een of beide kopien van het H2A kernhiston vervangt in nucleosomen die zich naast promotors van genen bevinden. Met Epi-ID werd bepaald dat SWR1 de belangrijkste regulator is van de verhouding van H2A versus H2A.Z. SWR1 is een ATP-afhankelijk chromatineremodelleringscomplex waarvan bekend is dat het H2A kan vervangen door H2A.Z. Interessant genoeg werden geen andere histon-chaperones of chromatineremodelleringscomplexen gevonden. Nader onderzoek naar de rol van SWR1 in H2A.Z-regulatie wees uit dat SWR1 ervoor zorgt dat, tijdens S-fase (als er relatief veel nieuwe H2A.Z wordt aangemaakt) H2A wordt vervangen door H2A.Z, terwijl SWR1 er buiten S-fase (als er weinig H2A.Z wordt aangemaakt) voor zorgt dat eerder aanwezige H2A.Z behouden blijft.

Processen als DNA transcriptie, replicatie en reparatie komen allen tot stand uit een veelvoud van interacties tussen een groot aantal eiwitten. Wat deze processen reguleert op verschillende plekken in het genoom is grotendeels onbekend omdat tot nu toe de technieken ontbraken om dat op systematische wijze te bestuderen. **Hoofdstuk 4** beschrijft Epi-Decoder, een op ChIP en barcoding gebaseerde sequencing technologie waarmee dergelijke vragen in gist beantwoord kunnen worden. In plaats van gebaseerd te zijn op massa-spectrometrie, zoals eerdere methodes,

wordt gebruik gemaakt van DNA-sequencing. Vervolgens wordt Epi-Decoder toegepast op 3 verschillende loci in het genoom, waarmee een uitgebreid overzicht gegenereerd wordt van welke eiwit-interacties er op deze specifieke plekken plaatsvinden. Door gebruik te maken van veranderende fysiologische omstandigheden laten we zien dat we in staat zijn om dynamische chromatine-interacties te identificeren. Daarnaast hebben we laten zien dat met Epi-Decoder elk gewenste locus onderzocht kan worden. We laten daarvoor zien dat de benodigd barcode-libraries kunnen worden gegenereerd door gebruik te maken van de CRISPR/Cas9-technologie. In **hoofdstuk 5** gebruiken we verbeterde Epi-Decoder-libraries om te laten zien hoe chromatine-eiwitinteracties op een actief getranscribeerd locus snel kunnen veranderen als RNA-polymerase II (PolII) geremd wordt door chemische of fysiologische signalen. We laten zien dat chemische remming van PolII leidt tot een snelle verandering in de lokale chromatine-eiwit-interacties. De binding van RNA-polymerase II nam snel af, waardoor factoren die een rol spelen bij transcriptie-elongatie en transcriptie-terminatie afnamen, terwijl metabole eiwitten en chromatineremodelleringscomplexen juist toenamen. Als transcriptie geremd wordt met fysiologische signalen, gaat dit gepaard met cel-specifieke repressie en verandering van metabole eiwitten en factoren die een rol spelen bij eiwitvouwing. Daarbij hebben we laten zien dat verschillende eiwitten uit hetzelfde complex of waarvan bekend is dat ze in hetzelfde proces een rol spelen, soms verschillende kinetiek vertonen. Dit duidt op een hiërarchie tussen chromatinebindingsprocessen. Het blootleggen van dergelijke bindingsdynamiek van chromatine-eiwitten onder veranderende omstandigheden laat zien dat Epi-Decoder een krachtige strategie is om eiwit-eiwitinteracties en eiwitfuncties systematisch in kaart te brengen.

De hier ontwikkelde methodes Epi-ID en Epi-decoder zijn een aanvulling op het 'Epi-discovery' gereedschap, waarmee op een systematische manier chromatine-interacties in kaart gebracht kunnen worden.

PORTFOLIO

Name PhD student: Deepani W. Poramba-Liyanage

PhD period: September 2013 – September 2018

Name PhD supervisor: Prof. Dr. Fred van Leeuwen

Prof. Dr. Maarten van Lohuizen

1. PhD training

	Year	Workload (ECTS)
General courses		
- Scientific Writing in English for Publication, OOA	2017	1.5
- Postgraduate course advanced immunology, VUmc.	2017	2.9
Specific courses		
- Image J/Fiji image processing and analysis	2018	0.6
- 10 th course on Epigenetics, Institute Curie	2014	2
Seminars, workshops and master classes		
- Weekly international seminars at the NKI	2013-2018	4
- NKI meet the speaker lunches	2013-2018	4
- Annual OOA PhD Retreat	2013-2017	4

Presentations

- EpiGeneSys 4 th annual meeting, Barcelona (Oral)	2014	0.5
- Dutch Chromatin meeting (Poster)	2013-2016	2.0
- CHAINS (Oral)	2018	0.5
- Dutch Chromatin meeting (Poster)	2018	0.5
- EpiGene2Sys meeting, Munich, Germany (Poster)	2018	0.5
- Dutch replication meeting (Poster)	2018	0.5

(Inter)national conferences

- Epigenomics as a discovery tool (EpiGeneSys), Mainz, Germany	2014	0.5
- EpiGeneSys 4 th annual meeting, Barcelona, Spain	2014	0.5
- CHAINS	2018	0.5
- Dutch chromatin meeting	2013-2016	1
- Dutch replication meeting, Delft	2018	0.25
- EpiGene2Sys meeting, Munich, Germany	2018	0.5

2. Teaching

	Year	Workload (ECTS)
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Supervising

- Dominique Morais – Thesis project	2017	2
- Anouk Ide – Thesis Project	2018	1.5

3. Parameters of Esteem

	Year
Abstracts selected for talk	
- Epigenesys 4 th annual meeting, Barcelona	2014
- CHAINS	2018

4. Publications

	Year
Peer reviewed	
- Poramba-Liyanage, D. W. , Korthout, T., Cucinotta, C. E., van Kruijsbergen, I., van Welsem, T., El Atmioui, D., . . . van Leeuwen, F. (2020). Inhibition of transcription leads to rewiring of locus-specific chromatin proteomes. <i>Genome Res.</i> 10.1101/gr.256255.119	2020
- Korthout, T., Poramba-Liyanage, D. W. , van Kruijsbergen, I., Verzijlbergen, K. F., van Gemert, F. P. A., van Welsem, T., & van Leeuwen, F. (2018). Decoding the chromatin proteome of a single genomic locus by DNA sequencing. <i>PLoS Biol</i> , 16(7), e2005542.	2018
- Vlaming, H., Molenaar, T. M., van Welsem, T., Poramba-Liyanage, D. W. , Smith, D. E., Velds, A., . . . van Leeuwen, F. Direct screening for chromatin status on DNA barcodes in yeast delineates the regulome of H3K79 methylation by Dot1. (2016). <i>eLife</i> , 5, 10.7554/eLife.18919.	2016
Other	
- Poramba-Liyanage, D. W. , Korthout, T., & Van Leeuwen, F. (2019). Epi-ID: systematic and direct screening for chromatin regulators in yeast by Barcode-ChIP-Seq. <i>Methods in Molecular Biology</i> , 2049, 87-103.	2019

Other

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CURRICULUM VITAE

Deepani W. Poramba-Liyanage was born on the 15th of May, 1984 in Colombo, Sri Lanka. She completed her A levels in 2003 from Ladies' College, Colombo. She then started her degree in Human Biology from the Faculty of Medicine, University of Sri Jayewardenepura, Sri Lanka.



After graduating with a Class and distinctions in Biochemistry, Physiology, Pharmacology and Pathology in 2011, she was awarded the Utrecht Excellence Scholarship to continue her master's education at Utrecht University, Utrecht, the Netherlands. The program included research projects at the Utrecht Medical Center and the Netherlands Cancer Institute, Amsterdam. After graduating from her master's degree in 2013, she joined the lab of Fred van Leeuwen where she conducted research on the development of DNA barcode screens for epigenetic analysis. She will pursue her career as a Postdoc in the lab of Marianna Tryfonidou at the Regenerative Medicine Center Utrecht. Here she will continue to study epigenetics in a Horizon2020 funded project that works on generating an advanced therapeutic product based on induced pluripotent stem cells for the treatment of lower back ache.

