Identifying and characterizing regulators of histone acylation and replication stress

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Genetic screening in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae as a model organism

The unicellular eukaryote budding yeast or *Saccharomyces cerevisiae* has been widely deployed as a model organism to answer biological questions using classical genetic approaches. The growth of yeast in both diploid and haploid forms and its easy manipulation are considerable advantages of yeast as a model organism. The high level of homologous recombination provides efficient means to introduce specific mutations or disrupt genes in the genome. At the dawn of the sequencing age, an international effort was devoted to sequence the full genome of *S. cerevisiae*. As a result, yeast was the first eukaryote to have its complete genome sequenced (Goffeau et al., 1996). Subsequent analysis revealed the yeast genome is very compact and a protein-coding gene can be found every 2 kb with only 5% of genes containing introns. In contrast, in the lower gene-dense human genome, a protein-coding gene is found in only every 30 kb of sequence (Dujon, 1996). In total, yeast has around 6000 genes of which 1100 (20%) are essential for growth (Winzeler et al., 1999). A comparison between the genomes of budding yeast and humans reveals that even though their gene numbers differ radically (~6000 versus ~20,000 genes), a considerable amount of yeast genes have a human orthologue (Kachroo et al., 2015). This conservation is also evident by the ability of ~1000 human disease genes to functionally complement their budding yeast orthologs (Heinicke et al., 2007). In this thesis, we will further illustrate how yeast can serve as a powerful experimental model for chromatin and DNA replication studies.

Creating the yeast deletion collection

The full genome-sequencing of budding yeast inspired the creation of the yeast deletion project, one of the earliest genome consortia. The goal of this project was to create a collection of strains in which all non-essential open reading frames were systematically knocked out and replaced by a marker gene. This haploid collection was constructed by the yeast community and would serve as a resource for functional genomics (Giaever et al., 2002). To create a single deletion, the gene of interest in a haploid strain was replaced with the KanMX selection marker gene by integrating a PCR-generated cassette through homologous recombination. Two molecular identifiers, consisting of 20 base pair molecular barcodes unique to each deletion, flank the KanMX marker. This enables the identification of a strain by sequencing and has proven highly beneficial in high-throughput genomic experiments. The final haploid yeast knockout collection (YKO) consists of deletions for nearly all (96%) of the non-essential ORFs, thus, comprising around 5000 deletions. From the entire YKO, 80% of the mutants display fitness defects under

basal growth conditions (Giaever et al., 2002). In 2006, the YKO was updated to its current 2.0 version and has not been upgraded since. To study essential genes, additional libraries have been constructed such as a diploid heterozygous deletion library and the *d*ecreased *a*bundance by *m*essenger RNA *p*erturbation (DAmP) library, which disrupts the 3' untranslated region (UTR) of a gene by insertion of an antibiotic resistance cassette (Schuldiner et al., 2005).

Measuring the yeast deletion collection by colony growth or barcode sequencing

The arrayed growth of colonies from YKO strains on agar plates is a simple and highly applied practice in yeast library analysis. The colonies can be combined from 96 up to 6144 strains growing on a single plate (Bean et al., 2014). Since the location of each deletion strain on the plate is known, the growth of a colony can be determined by taking high-resolution images of the plates. By overlaying a colony grid over the plate image, individual colony size can be measured. In the subsequent analysis steps, these colony sizes are normalized and spatially corrected to adjust for growth effects that are caused by nutrient availability or plate location. The growth of a colony serves as a proxy for the relative fitness of each strain. This is measured over time or between experimental conditions. By quantitively measuring colony growth, this method also maps the fitness of single or double mutants in high-resolution across various environmental stressors (Baryshnikova, 2018; Costanzo et al., 2016; Guénolé et al., 2013; Srivas et al., 2013).

Apart from measuring the growth of individual colonies, the YKO collection of strains can grow competitively in a pooled fashion and single strain fitness can be measured by barcode identification (Hillenmeyer et al., 2008; Lee et al., 2005; Ooi et al., 2001; Pierce et al., 2007; Winzeler et al., 1999). The presence of the molecular identifiers enables the identification of a strain. Therefore, the abundance of a barcode is used as a read-out for the growth of the corresponding deletion strain in various conditions. Barcodes can be identified by tag hybridization on microarrays or by next-generation sequencing.

Gene-environment interactions

As mentioned previously, under basal growth conditions, 20% of protein-coding yeast genes are essential for growth. However, after a change in growth conditions, such as a switch in carbon source, a particular set of genes that was dispensable will become essential for growth (Giaever et al., 2002; Weinhandl et al., 2014). This is an example of gene-environment interactions, many of which have been profiled with the help of the yeast deletion collection (Figure 1A). By exposing yeast mutants to over 400 chemicals or experimental growth conditions, it became clear that ~97% of the non-essential yeast genes can affect fitness when deleted (Hillenmeyer et al., 2008). This means that, in certain conditions, the redundancy provided between all non-essential genes can be limiting. Therefore, environmental changes revealed many chemical-genetic interactions in yeast.

High-throughput genetic interaction experiments

Early genetic experiments revealed that most genes do not function as sole regulators in the cell, but rather operate together in complex networks. One of the most powerful applications of the yeast deletion library was to investigate and unearth these genetic interaction networks. Genetic interactions can manifest in several ways. For instance, a negative genetic interaction occurs when the combined loss of two genes has a stronger additive effect than was expected from their individual fitness effects. This occurs when the two tested genes form a protein complex or operate in redundant pathways. When the combined loss of two genes is inviable, this is referred to as 'synthetic lethality'. Conversely, a positive genetic interaction occurs when the combined loss of two genes is phenotypically stronger than is to be expected. For instance, when a second mutation fully alleviates the effect of a first mutation, this is called a suppressor mutation. A positive genetic interaction can also take place when a mutant masks the effect of the first mutation, a phenomenon called epistasis (Guarente, 1993; Hartman et al., 2001).

Many high-throughput genetic interaction studies are powered by synthetic genetic array (SGA) technology. SGA technology facilitates the systematic crossing of a single gene deletion, a query, with a yeast library to generate double mutants on a large scale (Tong et al., 2004; Tong, 2001). This technology was initially used to determine synthetic lethal interactions measured by loss of colony growth after haploid double-mutant selection. However, subsequent screening methods became highly quantitative by measuring colony growth of all double mutants. This enabled the systematic identification of negative and positive genetic interactions between gene-pairs (Baryshnikova, 2018; Collins et al., 2007; Parsons et al., 2004; Tong, 2001). This application was further developed into a method called epistatic mini-array profiling (E-MAP) (Figure 1B). With E-MAP, the statistical power of the interaction screen is increased by selecting multiple queries that are all connected to one or more biological processes (Collins et al., 2007; Schuldiner et al., 2005). Because these studies were limited by their selection in query genes, many E-MAPs were centered on a single process, for instance the early secretory pathway (ESP), small nucleolar RNAs (snoRNA) and chromosome biology (Collins et al., 2007; Puchta et al., 2016; Schuldiner et al., 2005; Tong et al., 2004). The study of genetic interactions in yeast reached its apotheosis with the establishment of all possible double deletion mutant combinations, resulting in 23 million mutant pairs (Costanzo et al., 2016). Of all the non-essential yeast gene combinations, almost 1 million genes genetically interact with another gene, showing ~550,000 negative and ~350,000 positive interactions of which 10,000 interactions were synthetic lethal. A limitation of these genetic interaction studies is their culturing under regular laboratory conditions. Many genes function in response to various environmental stimuli and, therefore, a number of genetic interactions are context-dependent (St Onge et al., 2007). To identify these dynamic interaction networks, double mutants can be grown under different

conditions (Figure 1C). This differential epistasis mapping (dE-MAP) has mostly been performed in the context of DNA damage (Bandyopadhyay et al., 2010; Guénolé et al., 2013; Srivas et al., 2013). dE-MAPs have both confirmed previous interactions and revealed new genes in the DNA damage response, for example the involvement of the RSC complex in nucleotide excision repair (NER) or the involvement of histone acetyltransferase Rtt109 in the mutagenic bypass of DNA lesions (Guénolé et al., 2013; Srivas et al., 2013). Taken together, these high-throughput genetic interaction studies suggest a high number of (differential) genetic interactions exist and identifying them uncovers complex genetic networks and new insights into biological processes.

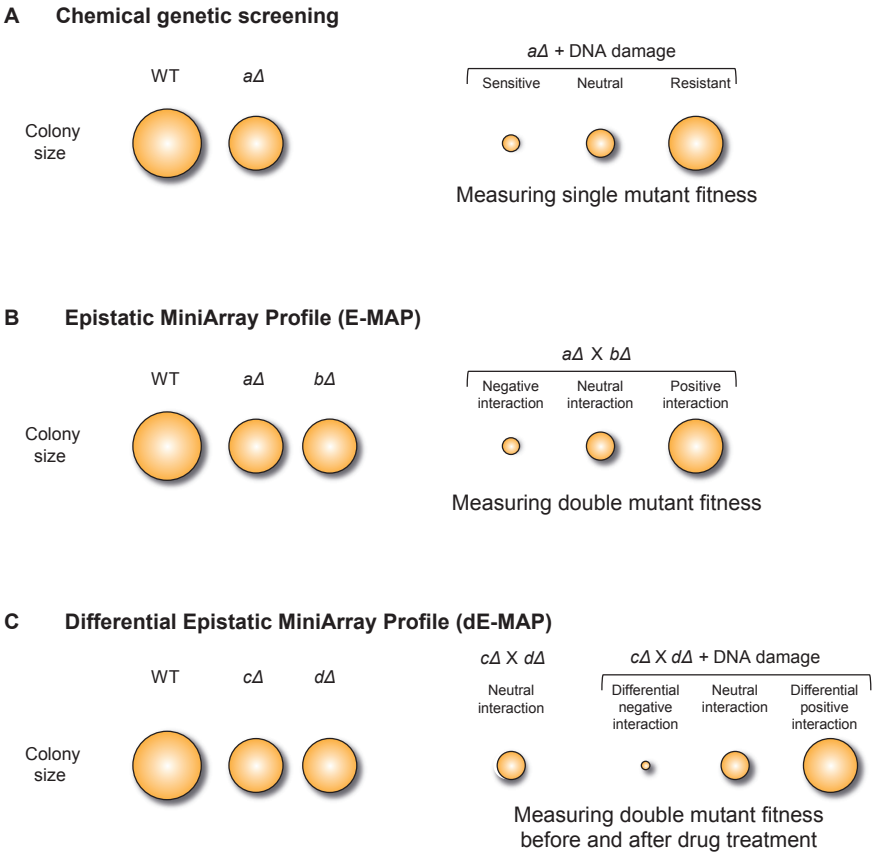

Figure 1. Interpretation of yeast colony growth during high-throughput screening.
(A) In chemical genetic screening, yeast colony growth is compared between the average colony size across the library, the neutral colony size. A colony smaller than neutral is deemed sensitive to DNA damage while a colony larger than neutral is deemed resistant. (B) During E-MAP analysis, the colony size of a double mutant is compared to expected effect of the two separate single mutants. A larger or smaller colony than expected can be interpreted as a genetic interaction between two mutants. (C) During dE-MAP analysis, the approaches of (A) and (B) are combined to determine whether double mutants have a genetic interaction that is only observed by treating them with a chemical.

Screening for chromatin regulators

Chromatin and posttranslational modifications

Eukaryotic cells contain genetic information in the form of DNA, which is stored in the nucleus and mitochondria. To compact the DNA in the nucleus, a 147 base pair stretch of DNA is wrapped around octamers, made up of two copies of each of the four core histone proteins H2A, H2B, H3 and H4. These histone octamers are tightly packaged together with DNA into nucleosomes, thereby forming a higher-order structure called chromatin. Through this packaging, chromatin not only contributes to organizing DNA in the nucleus but also to the regulation of transcription, DNA repair and recombination. There are several processes known to regulate chromatin structure. For instance, ATP-dependent chromatin remodeling complexes, like the INO80 complex, promote the exchange of the H2A-H2B histone dimer or the SWI/SNF family which can slide or evict nucleosomes from chromatin (Clapier et al., 2017). In addition, both canonical and variant histones are deposited on chromatin by various histone chaperones during replication as well as outside of S-phase (Hammond et al., 2017). Histone proteins can also undergo posttranslational modifications (PTMs) that alter the charge of the histone protein and change the nucleosome-DNA interaction. These histone modifications are recognized by specialized reader proteins, which, in turn, recruit protein complexes such as chromatin-modifying enzymes or ATP-dependent chromatin remodelers. The most abundant modification is histone acetylation, one of the first histone modifications to be identified (Allfrey et al., 1964; Simithy et al., 2017). To establish acetylation, an acetyl group from an acetyl-CoA donor is covalently linked to the positively charged ε-amino group of a lysine. In an unmodified form, the positively charged lysine is drawn to negatively charged DNA. This affinity is weakened when the lysine charge is neutralized through acetylation. Consequently, the nucleosome becomes destabilized, chromatin opens up and histone exchange is facilitated. Therefore, acetylation promotes DNA accessibility, and this facilitates processes like transcription (Hebbes et al., 1988; Tse et al., 1998). Other well-studied modifications are acetylation, methylation, phosphorylation, ubiquitylation, and ADP-ribosylation, which are involved in various processes like transcription, DNA repair and DNA replication (Zhang et al., 2015).

Acetylation

The regulation of acetylation was elucidated by the identification of histone acetylation writer proteins, the first of which was Gcn5 in yeast (Brownell and Allis, 1995; Brownell et al., 1996; Kleff et al., 1995). Gcn5 is a histone acetyltransferase (HAT), an enzyme that catalyzes the acetylation reaction. HATs are divided into three families based on their sequence and structural properties. These are the MYST (Moz, Ybf2, Sas2 and Tip60), p300/CREP-binding protein (p300/CBP) and GNAT (Gcn5-related N-acetyltransferase) families (Marmorstein, 2001). In yeast, many HATs

are part of larger complexes that play a role in transcription. For instance, Gcn5 is present in three complexes, SAGA, ADA and SLIK, and acetylates specific lysine residues on the N-terminal tails of H3 and H2B. The H4 writer protein Esa1 is part of the NuA4 complex while Sas3 is part of the NuA3 complex and acetylates H3. Conversely, histone deacetylases (HDACs) are eraser proteins, capable of removing acetylation marks from chromatin (Taunton et al., 1996; Vidal and Gaber, 1991). As opposed to HATs, HDACs have an inhibitory effect on transcription (Braunstein 1993). HDACs are divided into four families based on their homology in yeast. Class I (HDAC1, 2, 3 and 8) is related to yeast Rpd3, class II (HDAC4, 5, 6, 7, 9, and 10) is related to yeast Hda1, while class III contains seven NAD^+-dependent sirtuins and class IV only contains HDAC11 with no yeast counterpart (Kurdistani and Grunstein, 2003). These HDACs also often function in protein complexes. In contrast to HATs, HDACs do not have a specific affinity for a particular acetyllysine residue, while they can have some preference for a particular histone.

Acylations

During the last decade, the repertoire of acetylation-like modifications, referred to as acylations, has been expanded by their identification in various mass spectrometry studies (Figure 2) (Huang et al., 2018b; Tan et al., 2011; Tan et al., 2014; Xie et al., 2012; Xie et al., 2016; Zhang et al., 2019; Zhang et al., 2009). While these acylations are structurally similar to acetylation, various acylations such as propionylation, butyrylation and crotonylation contain longer carbon chains than acetylation. In addition, succinylation contains a carboxylgroup, an acidic group that chances the lysine charge from positive to negative. Other large additions to lysines are benzoylation and lactylation, which contain an aromatic acyl group and a lactyl group, respectively. Their corresponding short-chain fatty acid acyl-CoAs (e.g. propionyl-CoA, butyryl-CoA, crotonyl-CoA and succinyl-CoA) function as the co-factor for these modifications. Because the identification of the first HAT was vital to understand the role of histone acetylation, numerous studies have focused on the identification of writers for histone acylation. This revealed that several canonical HATs possess broad acylation activities. For instance, histone crotonylation and/or butyrylation activity was detected for the HATs p300, Gcn5 and Esa1/MOF (Chen et al., 2007; Kollenstart et al., 2019; Liu et al., 2017b; Sabari et al., 2015) and this thesis). Succinyltransferase activity was, so far, only found in KAT2A (hGCN5) (Wang et al., 2017). Currently, p300 is the suggested transferase for lactylation, while no HAT has been described to possess histone benzoylation activity (Huang et al., 2018b; Zhang et al., 2019). However, the enzymatic transferase activities for these last two modifications are, in all probability, inefficient since the affinity of various HATs for acyl-CoAs decreases concomitant with the increasing length of the acyl group (Kaczmarska et al., 2016; Montgomery et al., 2015; Ringel and Wolberger, 2016; Simithy et al., 2017). In case of crotonylation, the preference of

acetyl-CoA over crotonyl-CoA by various HATs in combination with the low abundance of crotonyl-CoA, likely results in a low stoichiometry of crotonylation (Simithy et al., 2017). So far, no other enzymes than HATs have been shown to possess broad histone acylation activities.

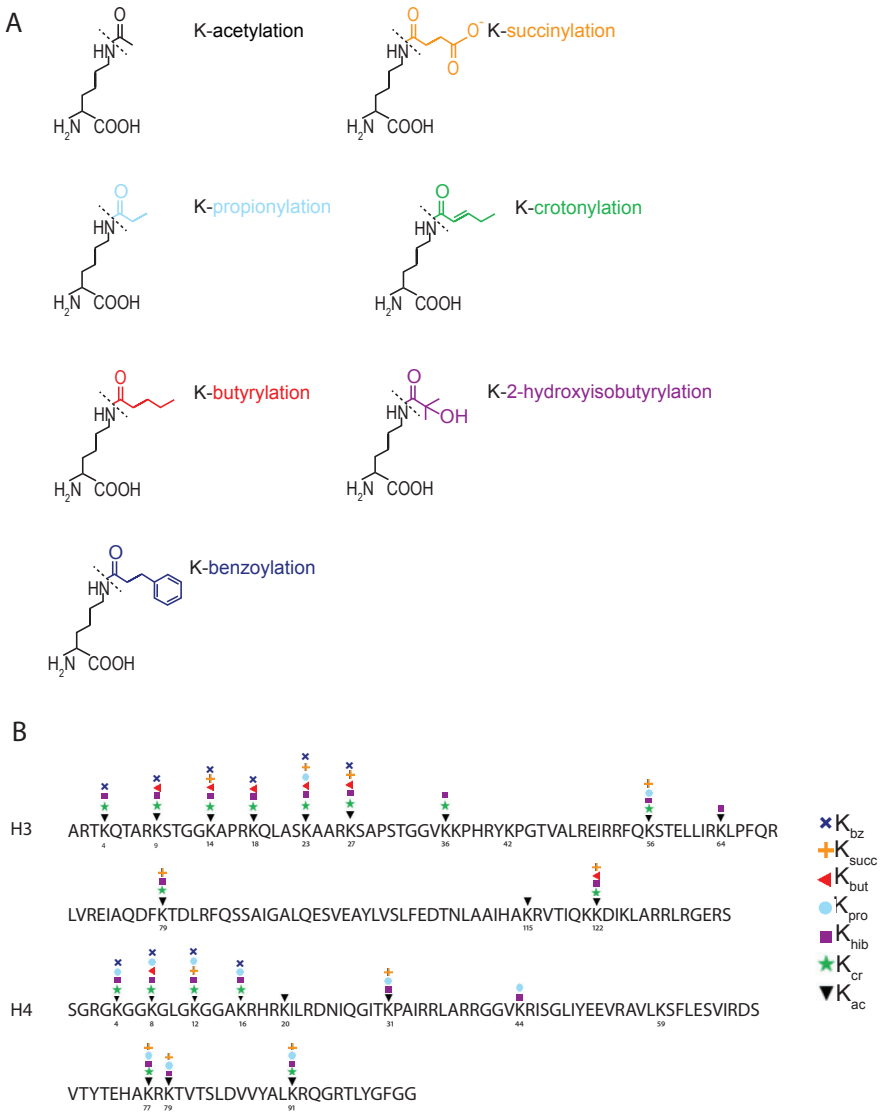

Figure 2. Histone acylation structures and acylated lysines on H3 and H4.
(A) The chemical structures for various acylations. (B) Overview of acyllysine modifications on H3 and H4.

Acylation and transcription

Transcriptionally active chromatin is typically characterized by a high degree of acetylation on the tails of histone H3 and H4 (Kurdistani et al., 2004; Pokholok et al., 2005). High levels of acetylation increase chromatin accessibility, which subsequently leads to the binding of the transcription machinery through its associated reader proteins. Consequently, the acetylation of histones by HATs is a prerequisite for gene activation and transcription (Kuo et al., 1998; Reid et al., 2000). To initiate transcription, transcriptional activators recruit HAT-containing complexes like SAGA or NuA4 to upstream activating sequence (UAS) elements (Kuo et al., 2000; Reid et al., 2000). HATs like Gcn5 then acetylate specific lysine residues on H3 and H4 located at promoters and regions near the transcription start site (TSS). Gene activation can also be promoted through H3K56 acetylation by the HAT Rtt109. This precedes the activation of the *PHO5* gene and subsequently favors chromatin disassembly and transcription activation (Williams et al., 2008). Apart from gene regulation, histone acetylation is present throughout the genome and both Gcn5 and Esa1 function in maintaining global levels of acetylation (Vogelauer et al., 2000). While this global regulation is important for the basal state of histone acetylation, it does not affect the entire transcriptome as only a subset of genes depends on SAGA or NuA4 for their expression (Holstege et al., 1998; Lee et al., 2000; Reid et al., 2000). Thus, HATs mostly act in a global manner by maintaining a basal level of histone acetylation. However, they can also act in a targeted manner by adopting locus-specific co-activator functions to create docking sites for the binding of transcription-promoting proteins. These proteins bind acetylated chromatin through their specialized reader domains such as bromodomains, double PHD finger (DPF), BET or YEATS domains (Gong et al., 2016). These domains are also found in HAT proteins. For instance, Gcn5 contains a bromodomain that is responsible for promoting cooperative acetylation whereby Gcn5-containing complexes are recruited to chromatin (Li and Shogren-Knaak, 2009). Protein complexes like chromatin remodelers SWI/SNF and RSC also bind to acetylated histones through their bromodomain-containing subunits (Hassan et al., 2002; Kasten et al., 2004).

Similar to acetylation, histone crotonylation, butyrylation and propionylation are all associated with active chromatin in both yeast and human cells (Fellows et al., 2018; Goudarzi et al., 2016; Gowans et al., 2019; Kebede et al., 2017; Liu et al., 2017b; Sabari et al., 2015; Tan et al., 2011). In cell-free systems, p300 stimulates transcription through histone butyrylation propionylation, as well as by 2-hydroxyisobutyrylation and lactylation (Goudarzi et al., 2016; Huang et al., 2018a; Kebede et al., 2017; Zhang et al., 2019). Moreover, in this system, histone crotonylation by p300 stimulates transcription to a greater extent than histone acetylation (Sabari et al., 2015). This effect is recapitulated *in vivo*, where an acetylation-dead but crotonyltransferase-proficient p300 mutant is capable of stimulating transcription (Liu et al., 2017b). Apart from transcription, histone crotonylation has been shown to play a role in several processes, including histone replacement

during spermatogenesis, acute kidney injury and pluripotency maintenance in mouse embryonic stem cells (Liu et al., 2017a; Ruiz-Andres et al., 2016; Wei et al., 2017). Histone succinylation has also been linked to increased gene transcription through its writer KAT2A (Wang et al., 2017).

How acylations affect transcription can partially be explained by how reader proteins recognize and bind to these modifications. Histone crotonylation is recognized by a subset of acetylation readers. Two domains, double PHD finger (DPF) and YEATS domain proteins, even preferentially bind to histone crotonylation when compared to other acylations (Andrews et al., 2016; Flynn et al., 2015; Li et al., 2016; Shanle et al., 2015; Xiong et al., 2016). In addition, YEATS domain protein GAS41 can bind histone succinylation modifications in a pH-dependent manner (Wang et al., 2018). Most other acylation reader proteins, however, preferentially bind acetylation. Due to steric hindrance, most bromodomain proteins bind histone crotonylation marks with a lower affinity than acetylation marks (Xiong et al., 2016). Of these bromodomain proteins, CECR2 and BRD9 can recognize acetylation and butyrylation with similar affinities *in vitro*, while being repelled by crotonylation (Flynn et al., 2015). Reader protein BRDT is another example of this phenomenon as the bromodomain of BRDT binds to acetylated, but not butyrylated H4K5 and H4K8 marks. Alternating levels of histone acetylation and butyrylation during sperm cell differentiation induce the dynamic binding of BRDT to gene promoters. Even though butyrylation drives transcription *in vitro*, here, it prevents the BRDT-driven gene expression (Goudarzi et al., 2016). In conclusion, acylation reader proteins generally function in transcriptional regulation and translate dynamic acylation levels into various transcriptional responses.

The link between acylation and metabolism

Chromatin modifications are highly affected by changes in metabolism because metabolic pathways supply the precursors for histone acylations (Choudhary et al., 2014; Fellows and Varga-Weisz, 2019; Gut and Verdin, 2013; Trefely et al., 2020). In case of acetylation, the precursor, acetyl-CoA, is generated through various compartmentalized metabolic pathways, including the fatty acid β-oxidation in peroxisomes and the tricarboxylic acid (TCA) cycle in mitochondria (Flikweert et al., 1996; Kunau et al., 1995). Besides these pathways, nuclear and cytosolic acetyl-CoA is directly generated from acetate by specialized acetyl-CoA synthases (ACS) (Takahashi et al., 2006). Shuttling of acetyl-CoA between cellular compartments requires specialized transport pathways since many organelles are impenetrable for acetyl-CoA (van Roermund et al., 1995). Because acetyl-CoA generation heavily relies on glucose availability, the intracellular levels of acetyl-CoA fluctuate depending on the nutrient supply of a cell. Consequently, high glucose levels increase acetyl-CoA production and result in high levels of histone acetylation (Cai et al., 2011; Takahashi et al., 2006; Wellen et al., 2009). Generally, growth conditions in yeast laboratories are nutrient-rich, which results in high levels of acetyl-CoA

and histone acetylation. When yeast cells run out of glucose, they switch from glycolysis to respiration whereupon acetyl-CoA and global histone acetylation levels decline. However, during continuous, glucose-limiting growth, the levels of acetyl-CoA are oscillating between three distinct metabolic phases, namely OX (oxidative, respiratory), RB (reductive, building), and RC (reductive, charging), collectively called yeast metabolic cycles (YMC). When yeast enters the YMC and begins the growth phase, also called OX phase, acetyl-CoA levels increase, which results in acetylation of histone H3 by Gcn5 and expression of pro-growth genes. A premature entry into growth phase can be triggered by supplementing the medium with additional acetate, which is subsequently converted into acetyl-CoA (Cai et al., 2011).

In contrast to acetyl-CoA, the metabolic regulation of other acylation precursor CoA's, such as crotonyl-CoA and butyryl-CoA, remains poorly understood, although they also originate from metabolic pathways. For instance, the conversion of α-ketoglutarate to succinyl-CoA, which is an intermediate of the TCA cycle, by a nuclear localized α-ketoglutarate dehydrogenase (α-KGDH) complex stimulates histone succinylation levels locally (Wang et al., 2017). Although elevated succinate levels lead to increased histone succinylation, an acyl-CoA synthase that converts succinate to succinyl-CoA has yet to be identified (Smestad et al., 2018). Under physiological pH, the negatively charged succinyl-CoA will likely also cause non-enzymatic succinylation of (histone) lysines ((Wagner et al., 2017; Wagner and Payne, 2013) and this thesis).

Slightly more is known about the production of crotonyl-CoA through the fatty acid β-oxidation pathway or the catabolism of amino acids. Crotonyl-CoA makes up less than 5% of the total short-chain acyl-CoA pool in various human cell types and is 1,000 times less abundant than acetyl-CoA (Sabari et al., 2015; Simithy et al., 2017). Like acetate, extracellular crotonate can be converted into crotonyl-CoA, which, subsequently, increases histone crotonylation levels (Kollenstart et al., 2019; Sabari et al., 2015). Conversely, the presence of chromodomain Y-like transcription corepressor (CDYL) on chromatin can locally lower crotonyl-CoA levels by converting crotonyl-CoA to β-hydroxybutyryl-CoA (Liu et al., 2017a). While it remains unclear how crotonate is endogenously produced, increased peroxisomal fatty acid β-oxidation during the YMC is associated with increased histone crotonylation. During these limited-glucose conditions, histone acetylation levels are low while histone crotonylation levels peak. In contrast to previous observations (Kollenstart et al., 2019; Sabari et al., 2015), these high crotonylation levels were associated with a reduction in the expression of pro-growth genes. Their expression was even further reduced after the addition of external crotonate (Gowans et al., 2019). By contrast, other reports state that external crotonate rather stimulates gene expression (Kollenstart et al., 2019; Li et al., 2016; Sabari et al., 2015).

Like crotonyl-CoA, butyryl-CoA is also a product of the fatty acid β-oxidation pathway. However, high butyrate levels do not solely convey into high histone butyrylation levels. This is because butyryl-CoA can also be metabolized into acetyl-CoA, thereby contributing to histone acetylation (Donohoe et al., 2012). Besides this, butyrate also functions as a class I HDAC inhibitor, thereby indirectly stimulating histone acetylation levels (Candido et al., 1978; Davie, 2003). So far, no mechanisms are known to regulate butyryl-CoA levels in the nucleus.

In addition to the endogenous acyl-CoA production, many acylation precursors are produced by exogenous sources. These precursors are often a class of bacterial metabolites. For instance, gut bacteria generate abundant levels of short-chain fatty acids, such as acetate, propionate and butyrate (den Besten et al., 2013). These, in turn, are taken up by various tissues and metabolized into acyl-CoAs. Because of the link between gut bacteria and acyl-CoAs, dietary changes or antibiotic treatment also affect short-chain fatty acids and, subsequently, histone acylation (Carrer et al., 2017; Donohoe et al., 2012; Fellows et al., 2018). In conclusion, metabolic pathways play an important part in regulating chromatin changes through the production of key precursor metabolites required for different histone acylations.

Screening for chromatin regulators

To increase our understanding of histone acylation, various screening methods have been developed to identify acylation regulators. For instance, by using SGA technology, genetic interactions around histone acetylation regulators were identified en masse (Lin et al., 2008). In this study, 38 acetylation-related query genes were selected to generate genome-wide genetic interaction profiles. This revealed that the SAGA and NuA4 complexes, together with the HDA and RPD3 complexes, are key regulators of the dynamic balance between histone acetylation and deacetylation that is pivotal for cell survival. In addition, insight into the functional modules of the NuA4 complex highlighted the complex' function in regulating histone acetylation at DNA double-stranded breaks (DSBs) (Lin et al., 2008).

One of the elegant methods to identify chromatin regulators is based on high-throughput chromatin immunoprecipitation (ChIP) experiments. To enable such a high-throughput approach, the yeast deletion collection is combined with additional barcodes at a common genomic locus. In this ChIP-based method, termed Epi-ID, the chromatin status of a single genomic locus is interrogated by screening thousands of yeast mutants simultaneously (Poramba-Liyanage et al., 2019). This has been successfully used to identify regulators of histone turnover, H3K79 methylation and histone acetylation ((Verzijlbergen et al., 2011; Vlaming et al., 2016) and this thesis). A similar approach has been used in Epi-Decoder to study the chromatin-associated proteome. Here, DNA barcode integration in the TAP-tagged yeast library allows

the identification of TAP-tagged proteins that bind the barcoded chromatin region (Korthout et al., 2018).

In human cells, genome-wide screening for regulators of PTMs is not as straightforward as in yeast. For instance, the Epi-ID method is challenging to implement in mammalian systems due to difficulties with integrating molecular identifiers, e.g. guide RNAs from CRISPR libraries, in a locus of choice. A promising tool is the application of cell sorting-based technologies combined with barcode or mutant identification. For instance, fluorescence-activated cell sorting (FACS) can separate intracellular phenotypes in pools of barcoded cells in which mutations can be identified using high-throughput sequencing. Using such a concept, several genome-wide screens have been performed in a haploid human cell line to identify regulators of PTMs. After sorting cells using an antibody against the PTM of choice, sequencing the gene trapping-induced mutations identified regulators of methylation (H3K27) and crotonylation (H2AK119) (Brockmann et al., 2017). While CRISPR screens can also be combined with FACS-based sorting, this has thus far not been applied to interrogate chromatin status.

Screening for regulators of DNA replication

DNA replication and DNA replication stress

During cell division, specialized and dedicated protein complexes coordinate and execute DNA replication in an efficient and mostly error-free manner. This faithful DNA replication is important to maintain genome stability and ensure cell survival. However, replication forks can encounter several obstacles that impede replication fork progression and cause replication stress (Zeman and Cimprich, 2014). For instance, obstacles such as interstrand crosslinks (ICLs), DNA-RNA hybrids or DNA adducts block progression of the replication machinery. Also, drugs like hydroxyurea (HU) can induce replication stress by depletion of dNTPs, which severely slows down fork progression. Stalled replication forks require specialized proteins that stabilize and protect the DNA from degradation, induce cell cycle arrest and promote replication fork restart (Branzei and Foiani, 2009; Pardo et al., 2017; Zeman and Cimprich, 2014). Collectively, this response is called the replication stress response. When the restart of replication forks is unsuccessful, forks can collapse. This can lead to DSB formation, genome instability and may promote the onset of cancer.

There are different types of damage that activate the replication stress response. For example, depletion of the dNTP pool inhibits replication fork progression and leads to the formation of stretches of single stranded DNA (ssDNA) close to the replisome. Unlike HU, MMS-induced lesions can be bypassed with the help of DNA damage tolerance (DDT) mechanisms, where specialized DNA Translesion Synthesis (TLS) polymerases are recruited that can bypass the

lesion (Marians, 2018). Bypassing DNA damage comes at the cost of replication accuracy and can lead to the formation of ssDNA gaps behind the fork. These replication stress responses are mostly conserved from yeast to human.

In yeast, but also in higher eukaryotes, the RPA-coated ssDNA accumulating near replication forks results in the recruitment of checkpoint kinase Mec1 (hATR) and its co-factor Ddc2 (hATRIP) to trigger a replication stress response (Rouse and Jackson, 2002; Zou and Elledge, 2003). After Mec1 recruitment, the 9-1-1 (Ddc1-Rad17-Mec3) complex activates Mec1 aided by DNA helicase/nuclease Dna2 and scaffold protein Dbp11 (hTopBP1) (Figure 3A) (Kumar and Burgers, 2013; Majka et al., 2006; Mordes et al., 2008). Next, activated Mec1 phosphorylates multiple replisome targets to prevent fork collapse and amplifies the stress signal by activating the Rad53 effector kinase (Figure 3B) (Furuya et al., 2004; Paciotti et al., 1998). How Rad53 activity is further stimulated depends on the type of lesion. During replication fork stalling, the main mediator of Rad53 activity is Mrc1 (hClaspin), which fully activates Rad53 together with replisome components Sgs1 (homologue of WRN helicase) and RFC (hCtf18) (Alcasabas et al., 2001; Bjergbaek et al., 2005; Cejka et al., 2010; Cobb et al., 2003; Hegnauer et al., 2012; Katou et al., 2003; Osborn and Elledge, 2003; Tanaka and Russell, 2001; Tercero et al., 2003). Contrary to stalled forks, damaged temples with ssDNA gaps behind the fork, such as those induced by MMS, activate Rad53 through Rad9 (Bacal et al., 2018; García-Rodríguez et al., 2018). Once Rad53 is fully activated, it elicits a myriad of responses (Figure 3B). To slow down the S-phase progression, late origin firing is repressed by Rad53-mediated phosphorylation and inactivation of origin initiation factors Sld3 and Dbf4 (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). Inhibition of late origin firing prevents the replication of damaged templates and allows initiating DNA replication at later timepoints. Rad53 also evokes a wide transcriptional response that promotes expression various DNA damage-inducible genes, such as RNR gene transcription, thereby upregulating the dNTP pool (Branzei and Foiani, 2009; Pardo et al., 2017). Lastly, Mec1 and Rad53 phosphorylate replisome components among which are various DNA polymerases, Mrc1, Tof1 and the replicative CMG (Cdc45/Mcm2-7/GINS) helicase complex to stabilize stalled replication forks (Can et al., 2019; De Piccoli et al., 2012; Smolka et al., 2007; Zhou et al., 2016). This stabilization prevents the uncoupling of the CMG helicase and DNA polymerases and enables DNA synthesis to continue after replications stress is relieved. In the absence of functional Rad53, uncoupling of DNA synthesis and helicase activities leads to excessive DNA unwinding by the CMG helicase (Cobb et al., 2003; Gan et al., 2017; Katou et al., 2003; Sogo et al., 2002; Tercero and Diffley, 2001). However, how exactly this targeting and stabilization of replication forks is orchestrated remains unclear. Taken together, preventing and resolving replication stress requires a highly complex system of which the many levels of regulation are not yet fully understood.

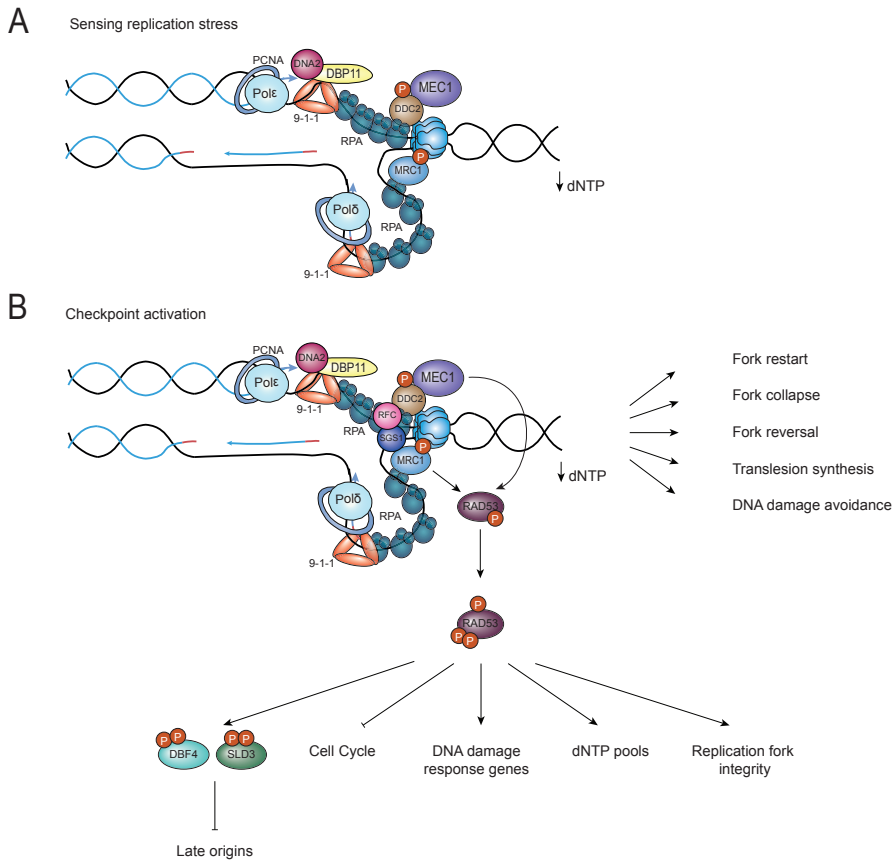

Figure 3. Overview of the replication stress response.
(A) Replication fork stalling due to lesions or low dNTP levels leads to accumulation of ssDNA which is recognized by RPA. This recruits Ddc2-Mec1 which, along with Dna2 and Dbp11 help activate Mec1 to trigger the replication stress response.
(B) Once Mec1 is activated, it phosphorylates multiple replisome targets and Rad53. This enables the checkpoint activation and amplification of the damage signaling. Rad53 activation is also stimulated through Mrc1, Sgs1 and RFC. After activation, Rad53 elicits various responses to enable the repair and resolution of the replication stress.

Transcription-replication conflicts

A major source of endogenous replication stress consists of conflicts between transcription and DNA replication. DNA replication and transcription operate on the same template during S-phase and when these two processes meet, the replisome can collide with the transcription machinery. Depending on the orientation of genes relative to that of origins of replication, replisomes and synthesizing RNA polymerase II (RNAPII) can meet in in a head-on collision or a co-directional

collision (Figure 4). Even though both types of collisions affect replisome progression, stalled replication forks are more likely to collapse during head-on collisions, leading to DSB formation and highly deleterious transcription-associated recombination events (García-Muse and Aguilera, 2016; Prado and Aguilera, 2005). To prevent fork collapse and facilitate replication fork progression, elongating RNAPII will usually be unloaded from chromatin following a transcription-replication collision (Felipe-Abrio et al., 2015; Poli et al., 2016).

Another contribution to transcription-replication collisions and genome instability stems from unscheduled R-loop formation (Costantino and Koshland, 2018; García-Rubio et al., 2018).

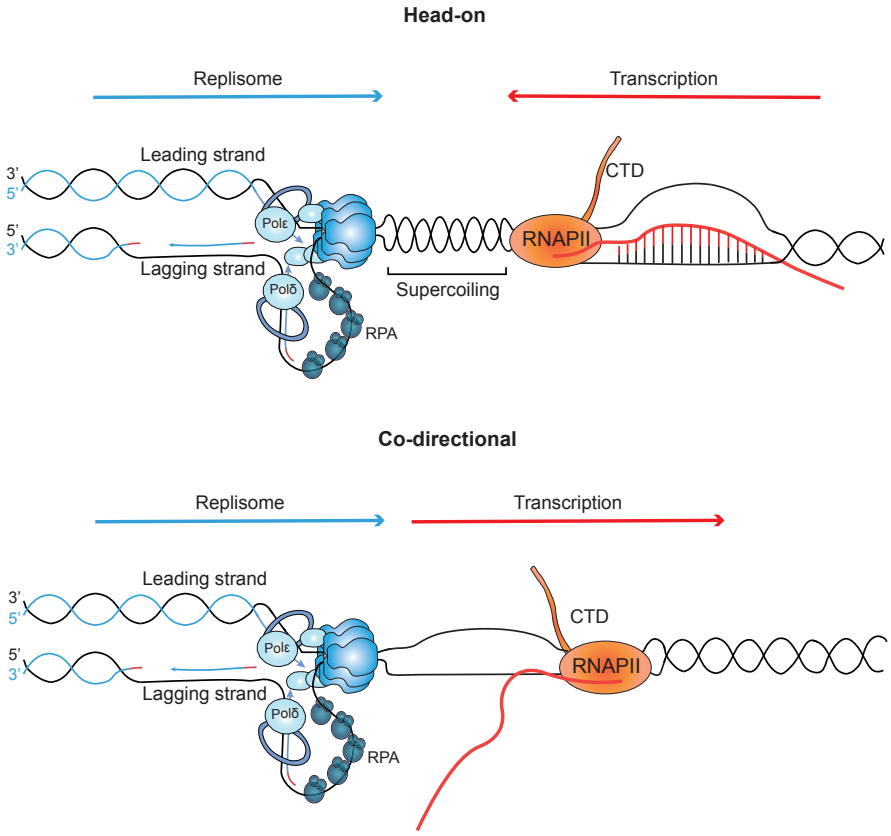

Figure 4. Head-on collisions and co-directional collisions between the replisome and the transcription machinery.
The top depicts the replisome and the transcription machinery heading towards each other and the threat of these two processes meeting in a head-on configuration. The bottom depicts the replisome and the transcription machinery heading in the same direction, potentially causing the replisome to meet the back of the transcription machinery.

R-loops form when the nascent RNA can hybridize with the DNA template while simultaneously displacing the non-templated ssDNA. These unscheduled R-loops typically form at highly transcribed genes with high GC content (Aguilera and Garcia-Muse, 2012). Various mechanisms are in place to prevent, avoid and deal with the formation of R-loops. For instance, during a process called gene gating, factors such as the THO complex contribute to proper processing and export of nascent mRNA, thereby preventing the hybridization of mRNA with the DNA (García-Benítez et al., 2017; Huertas and Aguilera, 2003). Also, preventing torsional stress associated with transcription limits the formation of R-loops (El Hage et al., 2010; Hazelbaker et al., 2013; Pfeiffer et al., 2013). Lastly, R-loop accumulation is prevented when transcription is properly terminated by the helicase Sen1 (Mischo et al., 2011). Once formed, R-loops can be removed by RNase H enzyme RNH1 that specifically targets RNA:DNA hybrids and prevents genome instability (Wahba et al., 2011). Taken together, a biological process like transcription can impede DNA replication and cause replication stress. Prevention of transcription-replication collisions is, therefore, a vital step in protecting cells from genome instability.

Screening for regulators of DNA replication stress

The yeast deletion collection has been employed in a wide variety of screens to investigate the sensitivity of cells to agents that cause replication stress. Many of these screens studied the fitness of mutants after growth on drug-containing plates or pooled growth followed by barcode identification. Robotically pinning the deletion library on plates containing various drugs like HU and/or MMS established the first chemical-genetic interaction profiles for DNA damage (Chang et al., 2002; Hartman and Tippery, 2004). This revealed how the MMS sensitivity in checkpoint mutants is due to DNA damage-induced fork collapse. In addition, genes like *MUS81* and *SLX4* were found to process DNA damage (Chang et al., 2002). In addition, pathways like threonine synthesis and retrograde signaling were proposed to respond to HU through the effects the treatment has on mitochondrial function (Hartman and Tippery, 2004). Treating the yeast deletion collection with numerous DNA damaging agents also created extensive chemical-gene-interaction profiles (Hillenmeyer et al., 2008; Lee et al., 2005; Parsons et al., 2004; Parsons et al., 2006). This profiling revealed that functionally similar drugs induce clearly distinct pathways with respect to the activated repair pathways. In particular, the gene-gene interactions in DNA repair and replication stress pathways may be condition-dependent, explaining why they had not been identified in earlier E-MAP studies in which cells were cultured in absence of DNA damaging agents. Initially, the data generated from gene-environment screens was combined with independently obtained gene-gene interaction data. Large-scale synthetic lethality screens combined with single mutant drug sensitivity defined functional modules of interacting genes. For instance, 74 query genes related to DNA integrity

generated ~5000 genetic interactions and revealed multiple genes that were previously unknown to function in DNA replication or checkpoint signaling (Pan et al., 2006). However, gene-gene and gene-drug interactions were later integrated into a differential E-MAP screening technology (Figure 1C) (Bandyopadhyay et al., 2010). The pairwise combination of 418 deletion mutants revealed an additional 1221 genetic interactions dependent on MMS treatment. A total of 379 interactions indicated DNA damage–induced sensitivity of which the majority was not present in untreated conditions (Bandyopadhyay et al., 2010). Other dE-MAPS have been performed that focused on DNA damage inflicted by UV, DNA intercalating agent zeocin (ZEO), DNA alkylating agent methylmethane sulfonate (MMS), or topoisomerase I inhibitor camptothecin (CPT) (Guénolé et al., 2013; Srivas et al., 2013), revealing that these agents do not induce a common DDR, but that each of them induces a rather distinct DDR. There are no published dE-MAPs that specifically focus mapping differential genetic networks related to HU-induced replication stress. Finally, the options for screens with read-outs other than viability are limited. A high-throughput microscopic approach investigated protein localization and abundance during replication stress (Tkach et al., 2012). However, while direct monitoring of replication stress, via for instance Rad53 activation or fork collapse, is difficult in high-throughput, we illustrate in this thesis the development of a genome-wide screen whereby we directly measure DNA polymerase occupancy at origins of replication as a measure for fork progression/stability.

In conclusion, the development of the yeast deletion library and its application in various genetic screens brought forward an enormous wealth of genetic interaction data. From this data, multiple genes and various pathways were discovered that are involved in the DDR and the control of cell fitness/viability, not only in yeast but also in higher eukaryotes. This illustrating how these types of genetic screens are a powerful tool and greatly aid in a better mechanistic understanding of the DDR.

OUTLINE OF THIS THESIS

The aim of this thesis is to improve our understanding of regulators involved in histone acylation and the response to DNA replication stress. To reach this goal, we use various forms of genetic screening and follow-up assays in yeast.

In **chapter 2**, we describe how we used an approach called Epi-ID that interrogates a common chromatin locus in the yeast deletion and DaMP collection. This approach successfully revealed novel histone acetylation and acylation regulators. In addition, extensive antibody validation experiments indicated severe aspecificity of non-acetyl pan-lysine specific acylation antibodies.

In **chapter 3**, we describe the crotonyltransferase activities of the ADA and NuA4 complexes. Increased crotonylation levels also stimulated gene expression dependent on the activity of the HATs Gcn5 and Esa1, suggesting a role for these enzymes in the crotonylation-dependent regulation of transcription.

In **chapter 4**, we describe the set-up a new approach termed Replication-Identifier or Repli-ID, that we have used to investigate the accumulation of replicative polymerase ε near a barcoded origin of replication in the yeast deletion and DaMP collection. We demonstrate that this approach is extremely powerful to study DNA polymerase occupancy directly on chromatin and identify novel regulators of DNA replication fork progression/stability.

In **chapter 5**, we show the identification and characterization of the Mediator complex in the replication stress response. We demonstrate that Mediator limits replication stress by preventing R-loop formation.

In **chapter 6**, we discuss the implications of our findings. We put into perspective the effect of histone acylations on transcription, the application of the yeast deletion library in screening for novel regulators involved in histone acylations and replication stress, the quality of acylation antibodies in epigenetic research, and the application of genetic screens performed in this thesis to higher eukaryotes.

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