Identifying and characterizing regulators of histone acylation and replication stress

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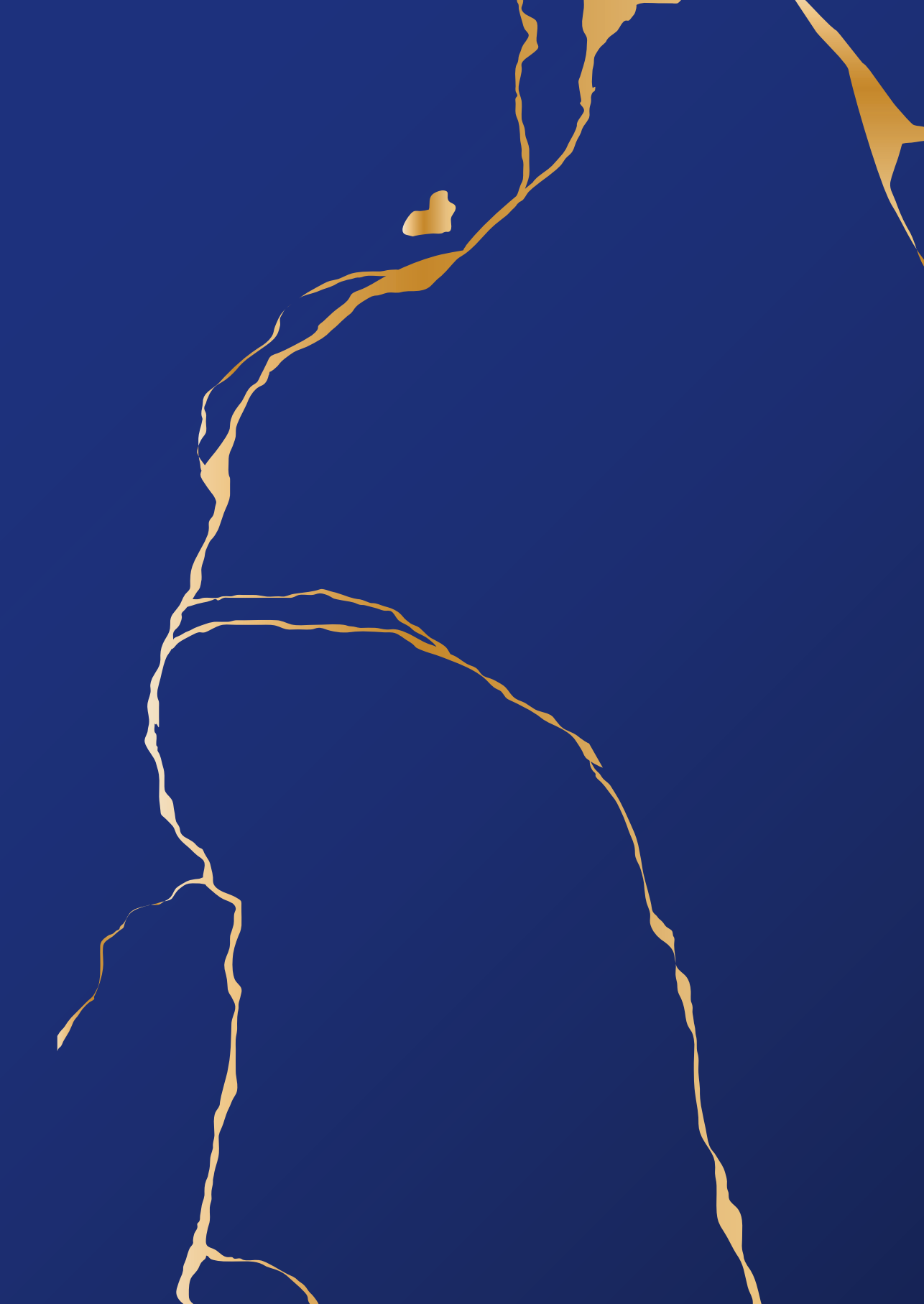

CHAPTER 6

Perspectives

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The work presented in this thesis describes various applications of genetic screening in yeast to identify regulators of chromatin, transcription and DNA replication. To gain more insight into these processes, we aimed to dissect these mechanisms by performing three distinct genetic screens in which we profiled over ~50.000 unique mutant-phenotype combinations. By employing the Epi-ID technology, we identified genes involved in the regulation of histone acylation. Moreover, in follow-up studies, we identified and characterized the ADA and NuA4 complexes as broad acylation writer proteins (**Chapter 2** and **Chapter 3**). Furthermore, we screened for novel regulators of the DNA replication stress response. To this end, we developed a novel technology termed Replication-Identifier (Repli-ID) to interrogate replication fork stability and progression in thousands of mutants simultaneously (**Chapter 4**). Lastly, through drug sensitivity screens we identified a new role for the Mediator complex in preventing replication stress by regulating R-loop formation (**Chapter 5**). From this thesis, various new insights in chromatin regulation and replication stress pathways have arisen, which will be discussed and put into perspective in this final chapter.

Yeast mutant libraries as a screening tool

The application of the yeast deletion and DAmP collections were a fundamental part in generating the data presented in this thesis. Ever since these collections were established, a plethora of genetic screens has been performed (Giaever and Nislow, 2014). Initially, deletion library screening characterized single mutant-fitness towards various environmental stresses ((Hillenmeyer et al., 2008) and **Chapter 5**). However, the application of the Synthetic Genetic Array (SGA) technology enabled genetic interaction studies en masse, revolutionizing functional genomics in yeast (Costanzo et al., 2016; Tong et al., 2004). From these studies, whole genome interaction networks were established, which not only gave insight into gene function, but also served as a great resource to ascribe functions to previously uncharacterized genes. Further building on the genetic interaction studies and gene-fitness studies, the dE-MAP technology ingeniously integrated both these approaches. Here, differential genetic interactions induced by various DNA damaging agents were uncovered, providing functional insights into genes that the neither the E-MAP nor the chemical interaction screens revealed (Bandyopadhyay et al., 2010; Guénolé et al., 2013; Srivas et al., 2013). By taking along the DAmP collection, also essential genes can be studies for their various interactions. Other approaches have combined the use of gene deletion and DAmP libraries with barcoder technologies, several of which are discussed later in this chapter ((Kollenstart et al., 2021; Verzijlbergen et al., 2011; Vlaming et al., 2016) and **chapter 4**).

While a vast wealth of data was obtained with the yeast libraries, there are a few points to take into consideration. For instance, it has not been properly characterized whether all DAmP alleles truly have a decreased mRNA expression. Secondly, proper library handling is required to limit the number of generations and prevent the accumulation of errors and mutations. Throughout the years, small errors in the yeast deletion collection have been identified. For instance, we have observed batch effects during a library cross with a *RAD52* deletion mutant. In this case, a series of adjacent gene deletions on chromosome 13, created by a single lab, where highly resistant to various forms of DNA damage, suggesting these deletions carry a founder mutation (Kollenstart et al., unpublished results). Similar observations have been made on blocks of neighboring gene deletions on chromosomes 2 and 15 (Hoffert and Strome, 2019; Lehner et al., 2007). Another inherent feature of the library is the neighboring-gene effect (NGE), whereby a single gene deletion influences the expression of a neighboring gene. NGE is estimated to affect about 10% of all genes in yeast and while the exact mechanism is unclear, often the gene deletion decreases the mRNA levels of the neighboring gene, leading to erroneous annotation of causal genes (Ben-Shitrit et al., 2012). While NGE effects have not been studied in the DAmP library, it is likely they could also have effects in these hypomorphic alleles. For identified deletions with NGEs, new mutations like, preferably, point mutations could be designed. For others an algorithm has been designed to try and identify NGE in large scale genetic screens (Ben-Shitrit et al., 2012). However, these features are difficult to circumvent in yeast deletion library screening and need to be kept into consideration when evaluating hits.

Recently, nearly all 4,732 homozygous diploid YKO strains were sequenced and various founder mutations and hyper-mutator knockout strains were identified. In addition, around 36% of the strains in this diploid YKO library contained chromosomal abnormalities or showed instability of repetitive DNA elements. However, most strains containing such signatures of defective DNA damage repair can be attributed to loss of known DNA repair genes like *RAD52* (HR) or *MSH2* (mismatch repair). This indicates that even longer cultured deletion strains are unlikely to acquire hypermutated phenotypes (Puddu et al., 2019). This systematic study not only gives insight into the effects of all gene deletions on genome architecture, but also functions as a reference to crosscheck deletion mutants of interest for unintended mutations.

During the second and last update of the yeast deletion collection in 2006, many of the initially identified errors were corrected. However, as mentioned previously, since then additional errors have been identified. With the frequent use of the yeast deletion library, the current CRISPR or gene-trapping technologies could be applied to replace some of the erroneous knockouts or even create an entirely new version of the knockout library. Regardless of the inherent features

and obstacles in using the yeast deletion library, its existence has been momentous in increasing our understanding of DNA damage repair, DNA replication and transcription, among others. Analogous to how the full genome sequence aided protein-coding gene annotation, the yeast deletion collection identified phenotypes for nearly every gene. These results could not have been achieved without these systematic and genome-wide screening methods. In conclusion, the yeast deletion collection has grown into an unparalleled resource for functional genomics.

Identifying and characterizing chromatin and replication regulators with barcode screening

Options for genome-wide screening approaches for chromatin regulators are limited in both yeast and human cells. However, the development of technologies like Epi-ID enabled the unbiased interrogation of the impact of all yeast genes on the epigenetic status of a single chromatin locus (Vlaming et al., 2016). In **chapter 2**, we applied Epi-ID to screen the yeast deletion and DaMP collections for regulators of histone acylations. This led us to characterize the crotonyltransferase and butyryltransferase activities constituted by the broad acylation writer complex ADA (**Chapter 2** and **chapter 3**). In addition, we identified several known (*GCN5, HDA1, RPD3*) as well as multiple unanticipated (*MET7* and *MTF1*) regulators of acylation (**Chapter 2**). Epi-ID informs about gain or loss in chromatin state but cannot distinguish between indirect and direct loss-of-function effects in yeast mutant. However, because factors like *MTF1*, *MET7* and *CLB3* lack HAT and HDAC activities, their effects on chromatin acylation are likely to be indirect. Exactly how these factors contribute to histone acylation is still not fully understood. However, metabolic processes can have a major effect on acylation precursor production (further discussed below). Therefore, in absence of mitochondrial factors like Mtf1 (Mitochondrial RNA polymerase specificity Factor 1) or Met7 (Methionine requiring 7) deregulated mitochondrial transcription could lead to severe consequences for chromatin regulation. Similarly, loss of Clb3 could affect metabolic processes in unknown ways, for example by affecting cell cycle progression (Krebs et al., 1999). If loss *MTF1* or *CLB3* has metabolic effects in the cell leading to a decrease in acyl-CoA, this may be a phenotype that could be rescued by external addition of acetyl-CoA to the cells, unlike when HATs would be lost. These Epi-ID results demonstrate the complex regulation of chromatin and the wide variety of processes that can contribute to this process.

Barcode screening methods are not limited to histone modifications. Coincidentally, the barcodes applied in Epi-ID were integrated into the vicinity of an origin of replication. Instead of investigating an epigenetic feature, we conceived a novel method to track replisome components on this barcoded origin of replication (**Chapter 4**). With this method, termed Repli-ID, we successfully investigated the recruitment of Pol2, the catalytic subunit of the

leading strand DNA polymerase ε, to the barcoded origin ARS404 in a pool of yeast deletion mutants (**Chapter 4**). The results obtained with Repli-ID provide a powerful resource for future studies on the regulation of replication fork progression and stability. Like Epi-ID, this method can report both on factors that directly contribute to Pol ε stability, such as for instance the Pol ε subunit Dbp4 and Pol δ subunit Pol32, as well as factors that indirectly contribute, such as for instance the Mediator subunit Cse2, which is unlikely to be present at replication forks. The clear advantage of the Epi-ID and Repli-ID approaches is their direct read-out of the barcoded locus. The presence of histone modifications, chromatin binders or replisome components can be directly monitored and their regulators identified. This read-out contrasts with most studies performed in yeast or human cells, where cell growth or survival is the sole read-out. Fortunately, results from barcoder-dependent screens are not merely limited to the barcoded regions because for most of these factors, their effects can be observed at other genomic loci. Therefore, Epi-ID and Repli-ID can report on changes that manifest throughout the genome.

Suggestions for future applications of Repli-ID screens

In **chapter 4**, we applied Repli-ID to investigate replication fork progression/integrity through the presence of polymerase ε during replication stress conditions. In addition to stressed conditions, the Repli-ID technology provides numerous other opportunities to study replication fork dynamics. First, Pol ε-based Repli-ID can be performed under different physiological conditions. The hydroxyurea treatment in **chapter 4** triggered a replication stress response through Mrc1-dependent recognition of replication fork stalling. Contrary, the MMS-induced replication stress response is mostly dependent on the recognition of post-replicative DNA damage by Rad9 (Bacal et al., 2018). Treatment with an alkylating agent can potentially reveal additional or different regulators of replication fork progression/integrity. However, Pol ε binding can also be followed without replication stress to study replication fork progression/integraty in unperturbed conditions. Second, Repli-ID can be performed with various other replisome components or DNA repair factors. For instance, following Rad18 after treatment with alkylating agents could provide further insight into the regulation of DNA damage tolerance pathways by identifying novel regulators of this pathway. A third option is to barcode a different locus. By integrating barcodes at sites of G4-quadruplexes or common fragile sites (CFSs), replication fork dynamics can be studied at endogenous loci that are a source of replication stress. Lastly, all these applications can be combined with a secondary mutation. A deletion mutant of interest can be crossed with the Repli-ID deletion library to study replisome dynamics in a double mutant background. This method can potentially unravel suppressors or synthetic sick phenotypes in relation to the query gene and provide novel insight into epistatic and non-epistatic pathways that coordinate replisome dynamics under different conditions.

Concerns about histone acylation antibody quality

Another focus of this thesis was the regulation of histone acylations, most notably crotonylation, butyrylation and succinylation. These acylation modifications show many structural similarities. For instance, butyryl and crotonyl modifications both contain a four-carbon acyl chain but are distinctive by the C-C double bond present in crotonyl groups. To better understand the regulation of these acylations, we performed high-throughput barcoder Epi-ID screens to identify modulators of various acylation modifications (**Chapter 2**). The high correlation we observed between the various histone acylation Epi-ID screens prompted further investigation of the specificity of the antibodies that were employed. Close examination and additional validation revealed severe cross-reactivity and off-target recognition of several acylation antibodies (**Chapter 2**). We explained these finding by both the low stoichiometry of the non-acetyl modifications and the decreased affinity HATs have for these acyl co-factors. Findings by us and others show antibody cross-reactivity between crotonylation and butyrylation for pan-recognizing antibodies as well as H3K9cr antibodies ((Gowans et al., 2019; Simithy et al., 2017) and **Chapter 2**). In our case, crotonylation and butyrylation were recognized by both pan-K-crotonylation and pan-K-butyrylation antibodies with equal stoichiometry (**Chapter 2**) but in the latter case, H3K9cr antibodies recognized crotonylation better than butyrylation by a factor of 10. However, we need to consider the stoichiometry of these modifications as histone butyrylation is more abundant than histone crotonylation. Specifically, butyryl-CoA is ~6-fold more abundant than crotonyl-CoA in HeLa cells (Simithy et al., 2017). In addition, the *in vitro* acylation activity of p300 towards H3K9 is 8.5 times more efficient for butyrylation than for crotonylation (Simithy et al., 2017). These results exemplify why a 10-fold increase in specificity might be ineffective in differentiating butyrylation and crotonylation *in vivo*. Therefore, it is possible that the antibody-based observations ascribed to crotonylation may be tantamount to butyrylation.

The knowledge about relative levels of histone acylation and their precursors was essential in understanding acylation stoichiometry and how this implicates antibody specificity ((Simithy et al., 2017) and **Chapter 2**). These findings are not only limited to cross-reactivity between crotonylation and butyrylation but also explain why some acylation antibodies mainly seem to recognize histone acetylation ((Bos and Muir, 2018) and **Chapter 2**). In HeLa cells, acetyl-CoA is around a 1,000-fold more abundant than crotonyl-CoA, suggesting histone crotonylation levels are also far lower than histone acetylation levels (Sabari et al., 2015). We show that the low binding of a crotonylation antibody to acetylation on a dot-blot can be fully negated *in vivo* because of the high levels of acetylation over crotonylation or butyrylation (**Chapter**

2). Increasing intracellular crotonylation levels by a large factor will decrease the chance of an antibody binding acetylation instead of crotonylation. We observed this after extracellular crotonate addition to cells, causing acetylation levels to stay equal while crotonylation levels increased, as detected on western blot (**Chapter 3**). These findings illustrate why the inclusion of acetylation and other peptide competitors is indispensable for acylation antibody validation experiments. Unlike protein recognition, histone PTM antibody specificity cannot be checked by knock-down or knock-out of a gene. Thus, peptide competitors provide essential information in the *in vivo* off-target effects, which cannot be recapitulated in dot-blot or peptide array experiments. Consequently, concerns still exist about the hitherto published data that solely relied on the use of these antibodies mostly without validation. Fortunately, most conclusions previously drawn are supported by mass spectrometry or other read-outs that complement antibody findings. Ultimately, a solution is to disregard antibodies altogether and focus on the development of chemical probes that recognize specific moieties. For instance, a crotonyl-specific probe has been developed that does not cross-react with other modifications (Bos and Muir, 2018). Currently, this probe is linked to a biotin group and detection can be performed with streptavidin antibodies. However, the application of this probe in studying crotonylation is currently very limited since it is not produced in a commercial setting. Nonetheless, the development of these types of chemical probes can be vital in studying structurally similar histone modifications and prevent misinterpretation of antibody signals.

The efficiency of HATs towards various acyl-CoAs

The affinity of HATs towards acyl-CoA often decreases concomitantly with the increasing length of the acyl-CoA carbon chain (Simithy et al., 2017). Initially, *in vitro* analysis indicated that acetyltransferase activity is inhibited in the presence of longer acyl-CoAs. For instance, butyryl-CoA can inhibit the acetyltransferase activities of p300 and Gcn5 by occluding the enzymatic pocket (Carrer et al., 2017; Montgomery et al., 2015; Ringel and Wolberger, 2016). However, both these HATs can utilize butyryl-CoA as a co-factor for histone butyrylation *in vivo*, suggesting that longer acyl-CoAs are not (solely) inhibiting acetyltransferases but are also utilized by these HATs ((Chen et al., 2007) and **Chapter 2**). These studies also called attention to the difference in transferase efficiency between acetyl-CoA and longer acyl-CoAs. For example, the crotonyltransferase activity of p300 is 64-fold less efficient than its acetyltransferase activity (Kaczmarska et al., 2016). Another factor contributing to histone crotonylation levels is the abundance of crotonyl-CoA, which is very low compared to other acyl-CoAs (Sabari et al., 2015; Simithy et al., 2017). Taking this low stoichiometry into account, the relative level of acetyl-CoA compared to crotonyl-CoA will need to decrease substantially in cells, either

locally or globally, to support histone crotonylation. Indeed, studies of the yeast metabolic cycle demonstrated that when acetylation levels are decreased, crotonylation is increased (Gowans et al., 2019). Therefore, extracellular crotonate addition drives crotonyl-CoA production on such a large scale that HATs use both acetyl-CoA and crotonyl-CoA as a substrate ((Sabari et al., 2015) and **Chapter 3**). These observations demonstrate that acetyl-CoA is the preferred co-factor for HATs and that during most conditions acetylation remains the most abundant histone acylation.

Histone acylation in transcription

Ever since histone acetylation was discovered over 50 years ago, it has become the subject of many studies that aimed at understanding its role in chromatin regulation and transcription (Allfrey et al., 1964; Kurdistani et al., 2004). Only recently, the repertoire of histone acylations was vastly extended by the identification of modifications like crotonylation and butyrylation (Chen et al., 2007; Tan et al., 2011). Like acetylation, crotonylation and butyrylation have mainly been ascribed to function in transcriptional regulation. Genome-wide localization studies indicated that crotonylation and butyrylation associate with active chromatin (Goudarzi et al., 2016; Sabari et al., 2015). In cell-free systems and human cells, increased histone crotonylation stimulates transcription (Sabari et al., 2015). This is further supported by the presence of and dependence on the transcriptional activator AF9 YEATS domain during crotonate-induced expression (Li et al., 2016). Crotonyltransferases like p300, Gcn5 and Esa1 contribute to transcription after external crotonate addition ((Li et al., 2016; Sabari et al., 2015) **Chapter 2** and **Chapter 3**). In yeast, this addition of crotonate increased crotonylation levels and resulted in both increased and reduced gene expression (**Chapter 3**). However, localization studies indicated that crotonyl writers Gcn5 and Esa1 were only found to be present at promoters of stimulated genes, suggesting these HATs promote transcription through histone crotonylation (**Chapter 3**).

The generation of a separation-of-function HDAC, incapable of histone deacetylation but proficient in histone decrotonylation, revealed that decrotonylation activity was sufficient to repress transcription (Wei et al., 2017). While these observations suggest crotonylation is solely involved in transcriptional activation, one study reported the opposite. Here, increased histone crotonylation at pro-growth genes was associated with repressed gene expression during the yeast metabolic cycle (YMC) (Gowans et al., 2019). Unlike other transcription experiments with crotonylation, no external crotonate was added during these YMC experiments and these outcomes are therefore more likely to represent the endogenous regulation of histone crotonylation levels. Interestingly, the increase in histone crotonylation is concomitant with

a decrease in histone acetylation, suggesting a distinct regulation for these two modifications. Moreover, the effects on gene repression associated with histone crotonylation seem to conflict with the existence of transcriptional activators that have specialized crotonylation reader domains. For instance, crotonylation reader protein Taf14 functions in transcriptional activation, but not repression (Andrews et al., 2016; Li et al., 2016). However, during the YMC, Taf14 is present at repressed genes, suggesting it does not function as a transcriptional activator in this context (Gowans et al., 2019). Another theory to explain gene repression is the exclusion of certain reader proteins from chromatin during high levels of crotonylation. Many bromodomain-containing transcriptional activators do not efficiently bind to longer acyl chains and some are even repelled by histone crotonylation (Flynn et al., 2015). If histone crotonylation can exclude binding of bromodomain proteins that are required for gene expression, the latter process is repressed following high histone crotonylation levels. Interestingly, bromodomain proteins Bdf1/2 and Snf2 are, together with Taf14, still found at repressed growth gene promoters that contain high levels of H3K9cr, suggesting these reader proteins are not excluded from chromatin (Gowans et al., 2019). However, additional studies of these and other bromodomains would be needed to fully support this conclusion. Additionally, the authors suggest that crotonylated chromatin can keep the bromodomain proteins in a poised state for transcription in the next phase of the YMC. However, it is unclear how such a state would repress transcription.

Interestingly, a mechanism has been described for how butyrylation may repel transcriptional regulators. During spermatogenesis, the histone acetylation levels fall below the butyrylation levels and this leads to the exclusion of bromodomain protein Brdt from chromatin (Goudarzi et al., 2016). Similarly, (P)BAF (polybromo-associated BAF) chromatin remodeling complex subunits containing bromodomains preferentially bind acetylation and propionylation but not butyrylation (Kebede et al., 2017). The exclusion of bromodomains by butyrylation and/or crotonylation demonstrates these modifications are phenotypically distinct from histone acetylation. Future research on the role and regulation of transcription by crotonylation and butyrylation can shed light on the conflicting results between transcriptional activation and repression. Including separation-of-function HATs capable of only conjugating a single acylation would greatly help in distinguishing the mode-of-action of various histone acylations. Efforts to create such a mutant resulted in a mutant form of p300, incapable of acetylation and propionylation but functional in butyrylation and crotonylation (Liu et al., 2017). This clearly demonstrated the transcription promoting function of butyrylation and crotonylation. Thus, the role of various histone acylations is context-dependent and, unlike histone acetylation, other acylations can both promote transcription as well as inhibit transcription by excluding the binding of certain transcriptional activators.

Regulation of histone acylation through metabolism

In recent years, the link between histone acylations and metabolism has become clear because acyl-CoA precursors are mainly produced through metabolic pathways. (Choudhary et al., 2014; Fellows and Varga-Weisz, 2019; Menzies et al., 2016; Sabari et al., 2017; Trefely et al., 2020). Not only are acyl-CoA produced from endogenous sources, also specialized microbiota or bacteria can affect intracellular histone acylation levels through their metabolite production (Fellows and Varga-Weisz, 2019). However, it is still largely unclear how crotonyl-CoA or butyryl-CoA production is stimulated and how they regulate transcriptional responses. In yeast, the production of acetyl-CoA depends on the availability of a carbon source. When glucose levels are high, acetate is produced which leads to high histone acetylation levels and transcription (Cai et al., 2011; Takahashi et al., 2006; Wellen et al., 2009). Similar to acetyl-CoA production, acyl-CoA synthases produce crotonyl-CoA from crotonate ((Sabari et al., 2015) and **Chapter 3**). Contrastingly, butyrate is not only converted into butyryl-CoA but can also be converted into acetyl-CoA (Donohoe et al., 2012). This crosstalk between metabolic pathways complicates research on their effects on histone acylation. However, the activity of certain metabolic pathways can affect the ratio of cellular acyl-CoAs which can account for various transcriptional responses. For instance, enzymes that promote acetyl-CoA production, like the mitochondrial citrate transporter SLC25A1, Citrate Lyase and the ATP Citrate Lyase (ACLY), negatively regulate histone crotonylation levels (Brockmann et al., 2017). This suggests an inverse correlation exists between acetyl-CoA levels and histone crotonylation. This inverse correlation was also observed during the YMC, where histone acetylation levels decrease with a concomitant increase in histone crotonylation levels (Gowans et al., 2019). However, how these ratios are regulated remains to be elucidated. Therefore, investigating the source and production of acyl-CoA could provide more insight into the regulation of histone acylations.

A role for Mediator in preventing R-loops

Genetic screening for hydroxyurea (HU) sensitive yeast mutants implicated a role for the Mediator complex in replication stress. In follow-up studies we observed several phenotypes in genes encoding the Mediator subunits Soh1 and Srb2 of which increased R-loop formation is the most likely to contribute to HU sensitivity (**Chapter 5**). Ironically, Soh1 was originally identified as a repressor of the R-loop induced hyperrecombination phenotypes observed in *hpr1Δ* mutants (Fan et al., 1996). Hpr1 is a member of the THO complex, which is required for coupling transcription to mRNA export, thereby preventing R-loop formation during a process called gene-gating (García-Benítez et al., 2017; Strässer et al., 2002). When replisome progression is blocked by an R-loop, the collision can induce fork stalling. Stalled forks in turn

often collapse and may then converted into DSBs. The emerging DSBs can induce transcription-associated recombination (Prado and Aguilera, 2005). This hyperrecombination induced by RNAP-replisome conflicts is dependent on high rates of transcription (González-Barrera et al., 2002; Prado and Aguilera, 2005). As observed by us and others, RNAPII occupancy and transcription is decreased in Mediator mutants ((Eyboulet et al., 2013; Knoll et al., 2018) and **Chapter 5**). Therefore, when THO mutants are combined with Mediator mutants, this leads to a decrease in transcription and subsequent suppression of the hyperrecombination phenotype (Jimeno et al., 2008).

Like in THO mutants, we observed increased R-loop formation in Mediator mutants. We speculate that these R-loops are the source of replication stress since Ddc2 foci formation was suppressed after overexpression of RNase H1 (**Chapter 5**). However, contrary to THO mutants, the elevated level of R-loops in *srb2Δ* did not induce hyperrecombination (Piruat and Aguilera, 1996). To trigger hyperrecombination, DSBs need to be formed at transcription-replication conflicts (González-Barrera et al., 2002). We observed that Mediator mutants experience increased levels of Ddc2 foci after HU treatment, but this does not affect their survival after recovery from HU (**Chapter 5**). Therefore, we hypothesize that increased R-loop levels in Mediator mutants induce transcription-replication conflicts that cause replication fork stalling but not replication fork collapse. Consequently, stalled replication forks can continue replication after relieving HU treatment and, therefore, Mediator mutants can perhaps fully recover from replication stress (**Chapter 5**). In addition, the decreased rate of transcription could limit the number of transcription-replication conflicts in Mediator mutants. These results will need to be confirmed for instance by investigating Rad52 foci formation in Mediator mutants, which is indicative of collapsed forks and DSBs.

How exactly Mediator prevents the accumulation of R-loops is still unclear. We formulated two different hypotheses to explain the results that have been obtained thus far. The first is based on RNAPII levels, which are increased near the TES in Mediator mutants. When R-loops are formed near the TES, this leads to increased collisions between DNA and RNA polymerases, thereby inducing fork stalling and/or collapse. Consequently, these types of R-loops are considered most detrimental and a source of genome stability (Promonet et al., 2020). However, Mediator both leads to decreased RNAPII levels overall but a shift in distribution of RNAPII towards the TES. It is still unclear if this relative increase in RNAPII levels at the TES is enough to increase R-loops in these cells. Determining the genomic location of the R-loops generated in Mediator mutants could provide more insight into the formation of R-loops and link it to the observed changes in RNAPII occupancy. Nevertheless, the results this far may suggest

that Mediator prevents the accumulation of RNAPII across the gene body, particularly near the TES. Consequently, this would decrease replication stress by preventing collisions between transcription and replication machineries (a mechanism also discussed later in this chapter).

Secondly, we formulated a gene gating hypothesis based on the physical interaction between the Mediator complex and the mRNA export complex TREX-2 subunit Sac3. Similar to THO mutants, TREX-2 mutants are defective in gene gating which results in R-loop dependent transcription-associated recombination (Gallardo et al., 2003; González-Aguilera et al., 2008). In the absence of either Sac3 or Soh1, the interaction between Mediator and TREX-2 is lost and gene gating is impaired (Schneider et al., 2015). Therefore, it is highly probable that, similar to TREX-2 mutants, impaired gene gating in Mediator mutants results in the back-hybridization of the RNA with the DNA thereby blocking replication fork progression (see model in **Chapter 5**). Another consequence of the impaired binding between Mediator and TREX-2 is also a decreased interaction between Mediator and RNAPII, resulting in decreased RNAPII occupancy across gene bodies in both *soh1Δ* and *sac3Δ* mutants ((Schneider et al., 2015) and **Chapter 5**). Therefore, the decreased loading of RNAPII we observed in Mediator mutants and the reported gene gating phenotypes are highly intertwined and may both impact R-loop dependent replication fork stalling. It would be of interest to study whether for restoring the gene gating defect in Mediator mutants relieves the observed increase in R-loop levels and replication stress (García-Benítez et al., 2017). Since Mediator is a highly conserved complex, further studies in both yeast and human will have to shed light on how Mediator mechanistically prevents replication stress.

RNA polymerase II removal from chromatin during replication stress

The removal of RNAPII from chromatin has been suggested to prevent transcription-replication conflicts, thereby reducing replication stress (Felipe-Abrio et al., 2015). It is generally thought that RNAPII needs to be evicted from chromatin to facilitate replication fork progression in S phase. This is supported by observations that the PAF1 and/or INO80 complexes facilitate the removal RNAPII from chromatin on genes during replication stress (Poli et al., 2016). The global RNAPII distribution before and after replication stress described in this thesis agrees to a certain extent with these findings. Upon replication stress, we observed that RNAPII binding was lower near the TSS but its binding was shifted towards a peak near the TES (**Chapter 5**). Decreased RNAPII binding was limited to TSS regions, suggesting that elongating RNAPII is less prone to be evicted upon replication stress. The observed shift could also indicate that the total binding of RNAPII is not decreased but its distribution is merely shifted towards the end of genes. However, this is not supported by chromatin binding assays showing that

RNAPII binding is decreased upon replication stress (Poli et al., 2016). Nevertheless, this study contains the first genome-wide results on RNAPII occupancy after replication stress, which invites further study into the regulation of RNAPII occupancy during replication stress.

Bringing yeast (barcode) screening to mammalian systems

The applications described in this thesis illustrate how budding yeast can serve as a powerful and versatile model organism for genetic studies on a variety of cellular processes, including chromatin regulation, transcription and DNA replication (Yoshida et al., 2013). Here, we for instance took advantage of the latter by tracking the progression of replication forks in thousands of yeast mutants simultaneously (**Chapter 5**). A future step is to bring this type of screens to higher eukaryotes. While the establishment of both haploid cell lines and CRISPR-Cas9 tools has revolutionized genetic screening, still many challenges are faced in bringing screens like Repli-ID, as well as Epi-ID, to mammalian systems. The basis of these two techniques is the integration of barcodes at a common genetic locus. In CRISPR-Cas9 screening, the sequences of gRNAs are regularly used as molecular identifiers. However, lentiviral-based integration of these gRNAs can take place throughout the genome. Using these barcodes as a read-out for Epi-ID will, therefore, result in position effects. A more precise application might be the combination of gRNAs with recombinases like Cre, Flp or Phi (Chi et al., 2019). The recombinase recognition sites can be integrated at a locus of choice prior to the start of the screen. With this method, gRNAs recognition and expression cassettes can be integrated at a common locus and simultaneously function as molecular identifiers and as guides for Cas9-mediated generation of gene knockouts. After knockouts are established, the barcoded locus can be interrogated similarly to Epi-ID. Unfortunately, translating Repli-ID to mammalian systems is far more difficult due to the stochastic nature of DNA replication initiation in higher eukaryotes (Rhind and Gilbert, 2013). Because origins of replication are not located at a fixed position, DNA replication progression cannot be tracked in a pool of mutant cells by the integration of barcodes at a common locus. However, the recent progress in single-cell sequencing techniques could provide a tool to bypass the integration of barcodes at a particular locus. Because barcode or gRNA integration can be detected throughout the genome, single-cell sequencing alleviates the need for a specific integration site. Currently, single-cell Repli-seq (scRepli-seq) methods investigate DNA copy numbers to differentiate between replicated and unreplicated DNA (Dileep and Gilbert, 2018; Takahashi et al., 2019). To link this method to a Repli-ID-like approach, scRepli-seq could be combined with classic CRISPR-Cas9 screening to study which gene mutants affect replication dynamics. However, the current applications of scRepli-seq are limited to the identification of large replication domains. Future studies will need to improve the resolution of these type of methods to be able to determine features such as replication fork progression, origin use or inter-origin distance.

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