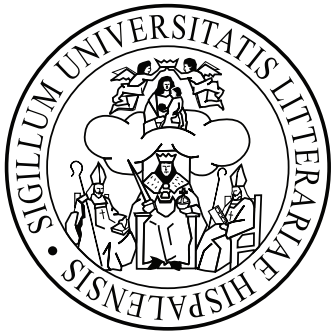

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Papel de la cromatina en la formación de R-loops

Desiré García Pichardo

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Trabajo realizado en el Departamento de Genética, Facultad de Biología (Universidad de Sevilla), y en el Departamento de Biología del Genoma, CABIMER (Universidad de Sevilla-CSIC-UPO), para optar al grado de Doctora en Biología por la licenciada Desiré García Pichardo.

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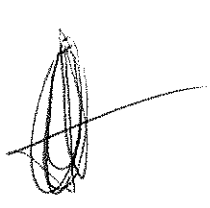

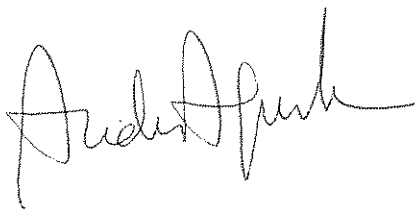

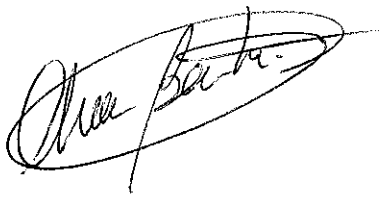

Desiré García Pichardo

Andrés Aguilera López

Ana B. García Rondón

Resumen

La inestabilidad genómica es una patología celular asociada a múltiples enfermedades genéticas, incluido el cáncer (Aguilera & García-Muse, 2013). Fallos en procesos metabólicos que ocurren en el ADN, tales como la replicación o la reparación de daños, son una fuente de inestabilidad. Otra fuente importante de inestabilidad es la transcripción, ya sea porque impida la progresión de la maquinaria de replicación o porque genere estructuras nocivas como los bucles R (R-loops) (Santos-Pereira & Aguilera, 2015). Éstos están constituidos por un híbrido de ARN-ADN y la cadena sencilla de ADN desplazada, y se forman generalmente durante la transcripción por hibridación de la cadena de ARN naciente con la cadena molde de ADN. Aunque estas estructuras son intermediarios naturales de diversos procesos biológicos, su acumulación puede ser una fuente importante de inestabilidad genómica (Santos-Pereira & Aguilera, 2015). La relevancia biológica de los fenotipos de inestabilidad genética que presentan las células que acumulan R-loops nos hace plantearnos en esta tesis el estudio de los mecanismos o elementos celulares que están implicados en la formación de dichas estructuras.

El ADN no está desnudo dentro de las células sino asociado a histonas y otras proteínas estructurales, formando la cromatina. La cromatina puede constituir una barrera para los procesos que ocurren en el ADN, por ello la célula ha desarrollado distintos mecanismos para solventar estas situaciones. Las modificaciones postraduccionales de las histonas favorecen el reclutamiento de remodeladores de la cromatina y chaperonas de histonas, facilitando el acceso al ADN (Tessarz & Kouzarides, 2014). Por lo tanto, cabe la posibilidad de que la cromatina sea un factor regulador de la formación de R-loops, controlando la accesibilidad del ARN al ADN. El objetivo principal de este trabajo es dilucidar si la cromatina tiene algún papel en la formación de R-loops y la inestabilidad genómica asociada, y si así fuera, explorarlo.

En esta tesis hemos escrutado una colección de mutantes puntuales no esenciales de las histonas H3 y H4 en el organismo modelo Saccharomyces cerevisiae, para identificar mutaciones que alteran la formación de R-loops. Hemos identificado mutantes de ambas

histonas que aumentan la inestabilidad de forma dependiente de AID y sensible a RNasa H, siendo el AID una enzima que actúa en la cadena sencilla desplazada del R-loop deaminando las citosinas y cuya acción incrementa la recombinación en levaduras con altos niveles de R-loops (Gómez-González & Aguilera, 2007). El análisis genético y molecular de estos mutantes confirma la acumulación de R-loops en los mismos, poniendo de manifiesto que la estructura de la cromatina ejerce un papel en prevención de la formación de híbridos de ARN-ADN. Los mutantes identificados no presentan defectos ni en replicación ni en transcripción, a diferencia de otros mutantes que acumulan R-loops, tales como hpr1Δ, rnh1Δ, sen1-1, etc (Huertas & Aguilera, 2003; Mischo et al., 2011). Por lo tanto, en esta tesis se describe por primera vez que los R-loops por sí mismos no suponen una amenaza para la integridad del genoma, ya que los mutantes de histonas identificados acumulan R-loops y no presentan fenotipos de inestabilidad. A nivel molecular observamos que los mutantes de histonas no acumulan histona H3 fosforilada en la serina 10 de la histona H3, una marca de cromatina condensanda, al contrario de los mutantes que acumulan R-loops y presentan inestabilidad genómica, tales como hpr1Δ o sen1-1 (Castellano-Pozo et al., 2013; Mischo et al., 2011). De hecho, los altos niveles de histona H3 fosforilada en la serina 10 y los fenotipos de inestabilidad presentes en los mutantes hpr1Δ o sen1-1 son suprimidos por las mutaciones de histonas seleccionadas, que impiden dicho incremento en fosforilación. Los datos sugieren que esta modificación postraduccional es necesaria para que el R-loop sea una fuente de roturas del ADN y, por tanto, de inestabilidad.

En conclusión, los resultados obtenidos en esta tesis nos llevan a proponer que para que los R-loops causen inestabilidad genómica se requiere un paso adicional en el que el R-loop induciría cambios en la cromatina, tales como la fosforilación de la serina 10 de la histona H3. Esta tesis, por tanto, sugiere una explicación para la diferencia entre los R-loops que se consideran "buenos" y "malos", y abre nuevas vías de investigación para comprender el papel de los R-loops y las modificaciones de la cromatina en el origen de la inestabilidad genómica.

Introduction

1 Sources of genome instability

The DNA is the molecule where genetic information is stored, as well as the transmission system of this information to the following generations. DNA is susceptible of being damage, since it is continuously exposed to many agents, both endogenous and exogenous, that could cause genome instability. Metabolic processes occurring on the DNA are important sources of endogenous DNA damage, being failures in replication or DNA damage response (DDR) the most common causes. Exogenous agents, such as chemical agents, ultraviolet (UV) light or ionizing radiation, could also induce genome instability. Genetic instability is a large and unspecific term that covers a variety of genetic alteration depending on the cellular machinery implicated, such as mutations, including point mutations and microsatellite contractions and expansions, variations in chromosome number, gross chromosomal rearrangements (GCRs), copy number variants, hyper-recombination and loss of heterozygosity (Aguilera & García-Muse, 2013). This instability is crucial to generate genetic variation, being even associated with regulated processes, such as immunoglobulin diversification. However, genome instability is a mayor threat to cell survival and the origin of multiple pathological disorders, being associated in humans with premature ageing, predisposition to various types of cancer and inherited diseases (Aguilera & Gómez-González, 2008).

Cell proliferation implicates a coordinated machinery to preserve genome integrity and faithful genome duplication. For that purpose, cells have developed multiple biological responses to DNA damage, differentiating mechanisms in DNA damage tolerance or DNA repair. Tolerance to DNA damage involves several mechanisms to prevent replication stalling by promoting the bypass of DNA lesions through specific DNA polymerases. DNA repair comprises a number of pathways, such as base-excision repair (BER), nucleotide-excision repair (NER), mismatch repair (MMR), homologous recombination (HR) or non-homologous end-joining repair (NHEJ), that restore DNA integrity. DNA repair pathways are activated after the recognition of DNA alterations, through activation of the checkpoints. Checkpoints

consist of a complex set of DNA damage sensors, signal mediators, transducers and effectors. *S. cerevisiae* have three DNA damage checkpoints: G1/S, intra-S and G2/M. Checkpoint activation promotes a transient arrest in cell cycle progression to allow repair of lesions that could originate genetic instability (Hoeijmakers, 2001).

1.1 Replication as a cause of genome instability

Replication is the crucial biological process where from a DNA molecule two identical replicas are produced. Replication could be an important source of genome instability. If the replisome encounters obstacles, such as DNA adducts, secondary structures or bound proteins, replication fork (RF) could stall. RF stalling could consist on a transient pausing or a longer delay. If the obstacle is removed the RF could restart, but if the blockage remains it can lead to replisome disassembly, ssDNA gaps and DSBs formation that could compromise genome integrity.

Due the complexity of the replication process, S-phase is the most susceptible of the cell cycle phases. DNA damage during S-phase or replication impairment activates intra S-phase checkpoint that coordinates cell cycle to DNA repair and RF restart preventing RF collapse (Aguilera & Gómez-González, 2008; Aguilera & García-Muse, 2013). In *S. cerevisiae*, S-phase checkpoint activation triggers the recruitment of the protein kinase Mec1. Mec1 is activated in response to stalled RFs and other DNA damage that causes RPA-coated ssDNA accumulation. Mec1 kinase phosphorylates effectors, such as the protein kinase Rad53, Sgs1 and Mrc1. Mrc1 forms a complex with Tof1 and Csm3, that together with the Sgs1 helicase associates to the RF and prevents replisome disassembly and collapse (Katou et al., 2003). Rad53 phosphorylation deactivates origin firing, prevents late origin firing, stabilizes the RF and prevents formation of aberrant replication intermediates (Aguilera & Gómez-González, 2008; Aguilera & García-Muse, 2013). In case of detecting a DSB, Mec1 also phosphorylates the C-terminal tail of the histone H2AX (γH2AX). This chromatin modification spreads

around the DSB, amplifying the DNA damage signal and recruiting chromatin-remodeling complexes that favors repair (Pardo et al., 2009).

Mutations in S-phase checkpoint genes compromise maintenance of the replisome and, consequently genome integrity. In the presence of replication inhibitors mutations in Rad53 cause RF collapse and accumulation of replication intermediates (Sogo et al., 2002). Moreover, mutations in yeast S-phase checkpoint, such as Rad53, Mec1 and other transducer proteins, cause a high increase in the frequency of GCRs, while mutations in G1 and G2 checkpoints have a smaller effect (Myung et al., 2001; Cobb et al., 2005). Therefore, S-phase checkpoint is essential for maintaining genome integrity.

1.2 Transcription-associated genome instability

Transcription is the initial step in gene expression where genomic information is copied to RNA molecules. Transcription not only plays a role in gene expression, it is also an important source of genome instability. Transcription of a DNA sequence enhances its frequency of mutations and recombination, processes called transcription-associated mutation (TAM) or transcription-associated recombination (TAR), respectively, and transcription-associated genome instability (TAGIN) all together. TAGIN could be caused by different sources such as DNA melting, topological changes, transcription-replication collision or R-loop formation.

Although TAR and TAM are resolved differently, the processes could be initiated by the same intermediate, the formation of ssDNA by DNA melting. During transcription the two complementary DNA strands are separated allowing the RNA polymerase (RNAP) to copy one of the strands, forming a transcription bubble inside the RNAP. The template strand (TS) is paired with the RNA while the non-transcribed strand (NTS) stays as ssDNA. TAGIN phenomena is understood by the fact that ssDNA is more vulnerable to nucleases and DNA

damaging agents than dsDNA (Gaillard et al., 2013; Gaillard & Aguilera, 2016; Aguilera & Gómez-González, 2008). Consistently, genotoxic agents such as methyl methanesulphonate causes a synergistic increase in recombination at transcription regions in yeast (García-Rubio et al., 2003).

In addition, the process of transcription triggers topological changes due to the accumulation of positive supercoils ahead of the elongating RNAP and negative supercoils behind it. This torsional stress leads to DNA opening that may induce transient ssDNA formation behind the RNAP, causing the TAGIN phenomena. These supercoils are resolved by the action of topoisomerases, that relax superhelical stress in duplex DNA (Gaillard et al., 2013). In eukaryotic cells two major topoisomerases are found: topoisomerase I (Top1) and topoisomerase II (TopII). Both topoisomerases can relax positive and negative torsion, being able in most cases to substitute for each other. However these proteins differ in their catalytic way of action, Top1 depend on a ssDNA cleavage and Top2 on a DSB (Wang, 2002). In the absence of Top1 and Top2 torsional stress are accumulated at transcribed regions (El Hage et al., 2010; Schultz et al., 1992). Yeast lacking topoisomerases (Top1 and Top2) show high rates of TAR (García-Rubio & Aguilera, 2012). Moreover, top1Δ, whose wildtype protein is involved mainly in resolving negative supercoils in transcription, is responsible for the majority of TAM events in yeast (Lippert et al., 2011).

Furthermore, TAR has also been related to RF impairment. Either transcription blockage by DNA adducts formed during replication (Scicchitano et al., 2004) or the collision of transcription and replication machineries might cause TAGIN (Wellinger et al., 2006). In *S. cerevisiae* TAR is detected only in S-phase, suggesting that a collision between RNAPII and RF is the main origin of TAR (Prado & Aguilera, 2005). RF blockage by collisions with the transcription machinery has been described in RNAPI (Takeuchi et al., 2003), RNAPIII (Deshpande & Newlon, 1996) and also in RNAPII highly transcribed genes (Prado & Aguilera, 2005). RF progression through protein obstacles is facilitated by helicase activities, being in

S. cerevisiae Rrm3 the most studied. An accumulation of Rrm3 in highly transcribed genes has been observed a genome-wide scale (Azvolinsky et al., 2009), supporting the idea that transcription is an obstacle to replication. Definitively, TAR is linked to the ability of the transcription machinery to impede RF progression by physically obstructing or promoting lesions that block DNA synthesis, and can cause genome instability (Aguilera & Gómez-González, 2008).

Finally another cause of TAGIN is the mRNA production itself. In eukaryotes, transcription is coupled to mRNA processing (5'-end capping, splicing, mRNP formation and 3′-end processing) and export to the cytoplasm. Nascent mRNA from RNAPII is bound by nucleoproteins to build an export-competent mRNA particle (mRNP) which leaves the nucleus through the nuclear pore complex. Co-transcriptional processing, in addition to increasing the efficiency of mRNA processing, prevents the accumulation of naked RNA that could re-anneal with the template DNA forming a structure known as R-loop, hence deficiencies in mRNP assembly can stimulate R-loop formation. R-loop is formed by and RNA:DNA hybrid and the displaced NTS that stays as ssDNA. Accumulation of these structures is linked to an increase of genome instability (Kim & Jinks-Robertson, 2012; Gaillard et al., 2013).

2 R-loop formation and genome instability

As previously mentioned, an R-loop is a nucleic acid structure formed by a RNA:DNA hybrid and a displaced ssDNA, identical to the RNA molecule. This structure is more stable than dsDNA, thus, R-loop removal is not an effortless process. R-loops are commonly by-products of transcription where the nascent RNA transcript synthesized by RNAP anneals back as soon as it exists to the transcribed DNA strand (Santos-Pereira & Aguilera, 2015).

R-loops formation and stabilization depend on different features, such as DNA

supercoiling, GC content, cleavage of the DNA template or the formation of G-quartets (four intra-strand Gs paired) in the ssDNA. Negative supercoiling accumulated behind the RNAP might lead to a short-term unwinding of the DNA strands, which establishes the conditions for nascent RNA hybridization to template DNA outside the polymerase. G clusters at the NTS favour R-loop initiation while the G content of the RNA is involved in stabilization and elongation of the RNA:DNA hybrid. In addition, a high G content in the ssDNA displaced might favour R-loop stabilization by promoting the formation of G-quartets. Therefore in a G-rich NTS, G-quartets leave the TS accessible to the RNA. Finally, the presence of DNA nicks increase RNA ability to hybridize to DNA probably increasing DNA accessibility (Santos-Pereira & Aguilera, 2015; Aguilera & García-Muse, 2012).

In vivo R-loops are intermediate structures in important biological processes such as immunoglobulin diversification, initiation of mitochondrial DNA replication or plasmid replication in E.coli. However, their uncontrolled formation can be a significant cause of genome instability, causing also replication stress, chromatin alterations or even gene silencing. (Aguilera & García-Muse, 2012).

2.1 Mechanisms that prevent R-loop formation

Increased formation of R-loops is a threat to genome stability. For this reason, cells have evolved several mechanisms to prevent or limit cotranscriptional R-loop formation. Dysfunction of any of these activities causes R-loop accumulation, genetic instability and might alter replication.

2.2.1 Ribonucleases and helicases

RNase H is the most studied enzyme in the context of R-loop removal (Figure

I1A). It is commonly used to verify the presence of these structures by suppressing hyper-recombination or hypermutation phenotypes. RNase H degrades specifically the RNA strand of the RNA:DNA hybrid eliminating the R-loop. There are two different types of RNase H enzymes, RNase H1 and RNase H2. Although they have different structure both remove RNA:DNA hybrids. RNase H1 has a hybrid domain conferring processivity and affinity for the substrate. RNase H2 is composed of three different proteins, the catalytic subunit and two other subunits that have yet unknown functions, but are necessary for catalysis (Cerritelli & Crouch, 2009). The activity of RNase H1 is regulated at a step after hybrid recognition and it is region-specific, whereas RNase H2 has a global function (Zimmer & Koshland, 2016).

R-loops can also be removed by RNA:DNA helicases that unwind the hybrid or limit their formation. A good example is the helicase Sen1 in *S. cerevisiae* and its human homolog senataxin (SETX), that are implicated in resolving R-loop formation (Figure I1A). The sen1-1 mutant, with the helicase activity inactivated, accumulates R-loops in highly expressed genes (Mischo et al., 2011). Moreover, a sen1 mutant presents RF blocks and accumulates R-loops at regions where replication and transcription converge (Alzu et al., 2012). SETX depletion causes an increase of R-loops at transcription pause sites present at the 3' end region of several genes, suggesting that SETX role in the dissolution of R-loops is stronger during transcription termination (Skourti-Stathaki et al., 2011). Other helicases involved in removing R-loops are the human RNA helicase aquarius (AQR) (Figure I1A) and human DNA helicase RECQ5 (Sollier et al., 2014; Li et al., 2015). The human DHX9 helicase unwinds DNA- and RNA-containing forks, DNA- and RNA-containing displacement loops (D- and R-loops), and also G-quadruplexes; acting preferentially on the R-loops and DNA-based G-quadruplexes (Chakraborty & Grosse, 2011). In *S. cerevisiae* the member of the DEAD-box RNA helicase superfamily Sub2 was suggested to function in preventing the accumulation of transcription-mediated replication obstacles, including R-loops (Gómez-González et al., 2011).

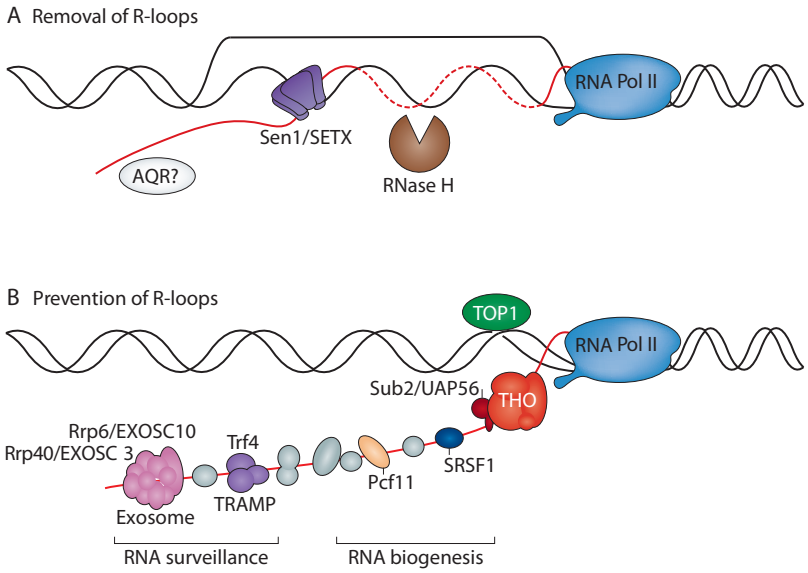

Figure I1. Mechanisms controlling R-loop accumulation.
(A) R-loops can be removed by: RNase H enzymes that degrade the RNA moiety of the RNA:DNA hybrid; helicases, such as Sen1 in yeast and senataxin (SETX) or Aquarius (AQR) in humans that unwind R-loops. **(B)** Processes preventing formation of R-loops are: Topoisomerase I (TOP1) that resolves the local negative supercoiling formed behind the elongating RNA polymerase II; RNA-binding proteins involved in RNP biogenesis (including THO complex) or in RNA surveillance (including the TRAMP complex) that limit free RNA able to hybridize with DNA. Figure adapted from (Santos-Pereira & Aguilera, 2015).

2.2.2 Accurate mRNP biogenesis

Defects in assembling nascent RNA into a functional mRNP favor RNA hybridization to template DNA and formation of R-loops. This may occur when mRNP biogenesis and processing factors are deficient, although not all mutants exhibit equally strong defects. The observations that RNA metabolic functions prevent R-loops was first described in mutants of the THO/TREX complex (Huertas & Aguilera, 2003) (Figure I1B). THO/TREX is a nuclear multiprotein complex, conserved from yeast to human, that binds to the nascent mRNA during elongation. THO complex in *S. cerevisiae* is formed by 5 subunits (Hpr1, Tho2, Mft1, Thp2 and Tex1) that interact strongly. The mRNA transport factors Sub2 and Yra1 interact with THO forming a large complex called TREX (Peña et al., 2012; Sträßer et al., 2002). Mutations in THO impair transcription elongation, and induces TAM and high levels of TAR (Chávez et al., 2000; Jimeno, 2002; Sträßer et al., 2002). TAR is partially suppressed by RNase

H1 overexpression, suggesting a direct connection between genome instability and R-loop formation (Huertas & Aguilera, 2003). These results were also confirmed in other model systems, such as human cells and C. elegans (Domínguez-Sánchez et al., 2011; Castellano-Pozo et al., 2012).

Other mRNP biogenesis factors that preclude R-loop accumulation are: transcription termination and mRNA 3'-end processing factors (Pcf11, Rna14…), the TRAMP complex; splicing factors like the human ASF/SF2 protein and Npl3 (Figure I1B). The yeast mutants in Trf4, the polyadenylation polymerase of the TRAMP complex (Trf4, Air2 and Mtr4p) involved in mRNP quality control, accumulate R-loops (Gavaldá et al., 2013). Depletion of ASF/SF2 causes genomic rearrangements suppressed by RNase H1 overexpression and a mutation profile when DNA is treated with sodium bisulfite, compatible with R-loop formation (Li & Manley, 2005). Finally, it is important the role of the mRNA export factors such as the multifunctional yeast protein Npl3, in preventing R-loop-mediated genome instability (Santos-Pereira et al., 2013).

Additional evidences indicating that the structure of the nascent mRNP is essential for R-loop prevention come from screenings for genes controlling DDR in yeast. This screening scored for several factors involved in RNA metabolism including transcription and 3'-end processing factors, that preserves the genome by inhibiting R-loop formation (Wahba et al., 2011; Stirling et al., 2012). All together these observations argue that R-loop accumulation is prevented by particular mRNA processing and transcription factors, but each one acts on different steps and has different impact on genetic stability.

2.2.3 Topoisomerases

As mentioned earlier, R-loop formation is facilitated by transcription-mediated DNA topological changes (Figure I1B). Negative supercoiling accumulated behind the RNAP

loses pairing of the DNA strands, probably facilitating the access of nascent RNA. This was suggested studying the effects that mutations in topoisomerases caused on R-loop formation. In *S. cerevisiae* R-loop accumulation is increased at the rDNA region in a top1Δ top2Δ strain, being enhanced in the absence of RNase H1 (El Hage et al., 2010). This causes RNAPI stalling and accumulation of truncated fragments of rRNA precursors (El Hage et al., 2010). In mammalian cells, TOP1-deficient cells present stalled RFs and DSBs in highly transcribed genes. These phenotypes are suppressed by RNase H1 overexpression, suggesting the role of TOP1 in avoiding R-loop accumulation (Tuduri et al., 2009).

2.2 Roles of R-loops in transcription

Different studies suggest that R-loops might have a role in transcription activation and termination. Genome-wide analyses indicate that R-loops localize preferentially to promoters and termination regions in human genes, especially at non-methylated-CpG islands (Ginno et al., 2012). CpG islands are short DNA interspersed regions rich in CpG sequences that are usually unmethylated and localized at the 5'-end of genes, functioning as promoters (Deaton & Bird, 2011). A bioinformatic analysis of these promoters shows an asymmetric distribution in the GC content with a C-rich DNA template, a characteristic that favors R-loop formation (Ginno et al., 2012). Indeed, R-loops are detected in CpG-rich promoters by the S9.6 antibody that specifically binds to RNA:DNA hybrids and by the bisulfite treatment technique. R-loop formation protects from the action of the DNA methyltransferase 3B1 (DNMT3B1) which silences genes by the novo methylation of cytosines, contributing to promoter activation (Ginno et al., 2012). Thus, CpG islands containing R-loop present chromatin modifications associated to transcription initiation or elongation, such as H3 lysine 4 trimethylation, H4 lysine 20 methylation and H3 lysine 79 dimethylation (Ginno et al., 2013).

Another mechanism of R-loop-mediated transcription activation is based in non-coding RNAs. In the human vimentin gene (Boque-Sastre et al., 2015) antisense transcription

at promoter generates an R-loop structure. These R-loops activate sense transcription by favouring chromatin opening, since they show a decreased nucleosome occupancy, and recruitment of transcription factors (Boque-Sastre et al., 2015). R-loops can also play a repressive role in transcription. At the COOLAIR antisense gene stabilization of an R-loop that covers the promoter inhibits its transcription. Antisense COOLAIR is a negative regulator of FLOWERING LOCUS C (FLC) expression. Thus reduced COOLAIR expression in turn increases FLC transcription (Sun et al., 2013). Therefore R-loops generated from antisense transcripts at promoters could play a dual role in transcription either activating or silencing the expression.

Several studies have directly related the role of R-loops in termination. R-loops form at the 3' ends of human genes with a high GC content (Ginno et al., 2013). In yeast, a genome-wide technique for mapping hybrid-prone regions showed that R-loops also peaked at the 3'-end of the ORFs even though these regions are AT rich (Wahba et al., 2016). These R-loops formed at the 3' end are required for transcription termination (Ginno et al., 2013; Skourti-Stathaki et al., 2011).

RNAPII terminates transcription by two different mechanisms: the poly(A)-dependent pathway and the Nrd1-dependent pathway (Porrua & Libri, 2015). The first one is carried out by most protein-coding genes, and it is coupled to cleavage and polyadenylation of the nascent transcript. In this process transcription of the poly(A) site is followed by RNAPII pausing and cleavage and polyadenylation of the nascent transcript. RNAPII pausing is mediated by R-loops either directly slowing down the polymerase or inducing a repressive chromatin at termination regions. Cleavage of nascent RNA generates a free 5' end that is the substrate of specific nucleases such as Rat1 in yeast or XRN2 in human cells. Degradation of the RNA still attached to RNAPII induces termination by an still ill-defined mechanism (Kim et al., 2004; West et al., 2004). Helicases like Sen1 in yeast or its homologue SETX in humans aid nucleases in this process (Mischo et al., 2011; Skourti-Stathaki et al., 2011).

They function removing secondary structures in the RNA and DNA:RNA hybrids formed at pausing regions facilitating exonucleases action (Proudfoot, 2016).

2.3 R-loops and genome instability

Although R-loops work as intermediates in important biological processes, accumulation of these structures is toxic for the cells, as they are a source of genome instability.

2.2.1 R-loop-mediated mutagenesis

The ssDNA motif of the R-loop is more susceptible to DNA damage than dsDNA, since it is more accessible to exogenous or endogenous DNA-damaging agents. In human cells, depletion of splicing factors like ASF/SF2 show a high spontaneous mutagenicity as a consequence of R-loop formation (Li & Manley, 2005). ssDNA is also the substrate of enzymes that induce mutations by modifying nucleosomes as activation-induced cytidine deaminase (AID) (Figure I2A). AID deaminates cytosines into uracil in actively transcribed DNA, with preference for cytosines present in WRC (W equals A or T and R equals A or G) motives. AID plays an important role generating immunoglobulin diversification. At immunoglobulin genes, AID acts on the ssDNA motif of the R-loops formed during transcription inducing somatic hyper-mutation (SHM) and class switch recombination (CSR) (Maizels, 2005). AID can act on R-loop formed in non-Ig genes when it is exogenously expressed (Gómez-González & Aguilera, 2007; Sohail et al., 2003). This has been used as a tool to detect R-loops in the cell. In yeast R-loop accumulating mutants, AID expression increases mutation frequency in transcribed genes following the same pattern of mutations than showed Ig genes (Gómez-González & Aguilera, 2007). Chemicals like sodium bisulfite that also deaminates cytosines present in ssDNA converting them to uracil, are used to detect R-loops in vitro too (Li & Manley, 2005) (Figure I2A). In this case since such conversion can be detected by PCR

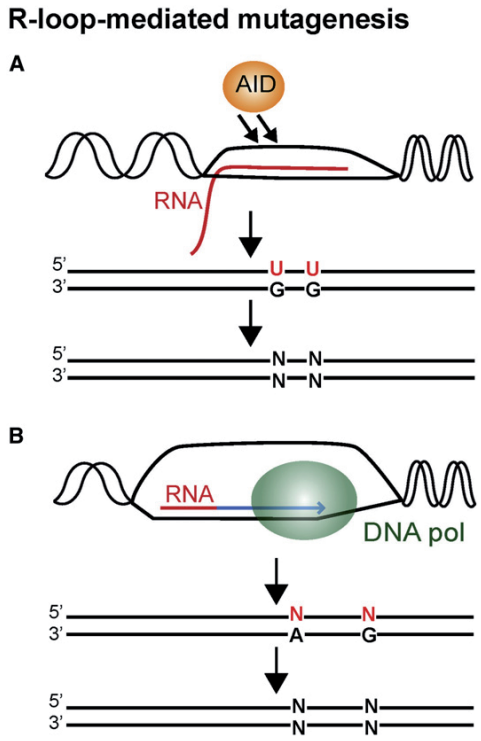

Figure I2. R-loops as a source of mutations.
(A) R-loops increase accessibility to DNA generating ssDNA that is attacked by the AID enzyme, to endogenous genotoxic metabolites or chemicals like sodium bisulfite. **(B)** R-loops can prime mutagenic DNA replication. Figure from (Aguilera & García-Muse, 2012).

amplification followed by DNA sequencing (Yu et al., 2003).

In addition, R-loops could be a source of mutations by initiating replication independently of replication origins (Figure I2B). In R-loops formed during transcription, the RNA motif hybridized to DNA can prime replication at ectopic sites in E. coli (Maduike et al., 2014) and in *S. cerevisiae* (Stuckey et al., 2015). R-loop-mediated replication increases the mutation rate in the rDNA region in top1 and rnase H mutants in yeast (Stuckey et al., 2015). Moreover, in yeast break-induced repair (BIR), a cellular process that mimics normal DNA replication but initiated at a DSB which was proposed to resolve breaks induced by R-loops, is strongly mutagenic (Deem et al., 2011).

2.2.2 R-loop-mediated DNA breaks as a source of genome instability

R-loop-mediated genome instability is originated mainly by DNA breaks (Aguilera, 2002). Genetic instability phenotypes as TAR were described in several mutants related with

RNA biogenesis or DNA metabolism, as explained before in section 2.2, that were later on described to accumulate R-loops. Although still not much is known about how the R-loops generate ssDNA gaps or DSBs, elucidating the mechanism is decisive in understanding how R-loops lead to genome instability.

The most important source of DNA breaks in the cell is replication stress (Aguilera & García-Muse, 2013). Hence, the major cause of R-loop-mediated genome instability might due to R-loop ability to stall RF progression. In yeast THO mutants the RF is blocked when the high GC content lacZ gene is transcribed. Blockage is partially restored through the cleavage of the nascent mRNA, suggesting that R-loops mediate RF stalling (Wellinger et al., 2006). Genome-wide analyses in mutants that accumulate R-loops show an over-recruitment of the Rrm3 DNA helicase in active genes. Accumulation of Rrm3 is reduced by RNase H1 overexpression. These replication defects correlate with an increased recombination and with checkpoint activation (Santos-Pereira et al., 2013; Alzu et al., 2012; Gómez-González et al., 2011).

The mechanism by which an R-loop produces the collision between transcription and replication is still unclear. First, unrepaired lesions in the displaced strand may represent an obstacle to the replisome or generate a break (Aguilera & García-Muse, 2012) (Figure I3A). Second, an R-loop itself (Figure I3B) or an RNAP that is arrested by the R-loop could be the roadblock (Figure I3C). Third, the supercoiling accumulated in front of an R-loop might lead to RF reversal, producing recombinogenic structures (Figure I3D). Finally, DNA structures formed in the ssDNA motif of an R-loop, such as G-quadruplexes or hairpins, might also hinder the progression of the replisome (Aguilera & García-Muse, 2012).

In human cells, the tumor suppressor and DNA repair genes BRCA2 and BRCA1 prevent R-loop built up (Bhatia et al., 2014; Hatchi et al., 2015). BRCA2 is a member of the Fanconi anemia pathway, a DNA repair pathway that works at lesions that block replication.

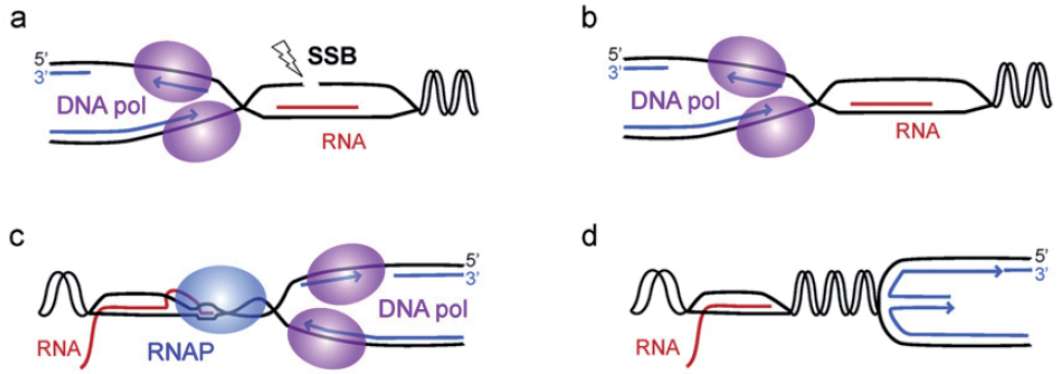

Figure I3. R-loop-mediated replication fork collision.
An R-loop might block or collapse replication fork progression by (A) favouring DNA damage by generating ssDNA, (B) acting as a roadblock, (C) retaining the RNAP at DNA (D) generating torsional stress that could cause RF reversal. Figure from (Aguilera & García-Muse, 2012).

BRCA2 and the Fanconi anemia pathway are required for restoration of RF stalled probably removing R-loops, supporting the idea that R-loop-mediated genome instability is mainly mediated by the interference with replication (García-Rubio et al., 2015; Bhatia et al., 2014).

3 Chromatin structure and modifications

DNA is not naked inside the nucleus of eukaryotic cells, it is associated with a wide variety of proteins, most of which are histones, forming the chromatin. Therefore, chromatin is the structure where the whole genome is packaged. The basic unit of chromatin is the nucleosome, consisting of approximately 146 base pairs of DNA, wrapped around a protein octamer formed by two copies of histones H3, H4, H2A and H2B (Figure I4). Each histone is composed of a highly structured globular domain and an unstructured N-terminal tail that expands from the octamer. The globular domain mediates interactions between histones and between them and DNA. The N-terminal domain is responsible for the interactions between nucleosomes and for recruiting distinct non-histone proteins such as, for example, chromatin remodelers. The nucleosomes are separated from each other by a linker DNA fragment that is variable in length (aprox. 10-90 bp), to which is attached a fifth type of histone, called

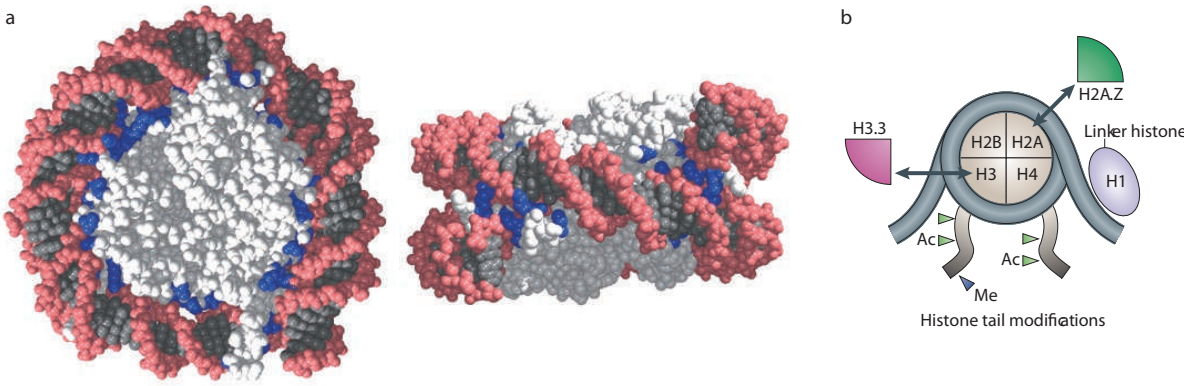

Figure I4. Nucleosome structure.
(A) Structure of a nucleosome core particle. Histones are shown in light grey, and the DNA helix is shown in dark grey with a pink backbone. Lysine and arginine residues of the DNA are shown in blue to emphasize the electrostatic contacts between the DNA phosphates and the histones. **(B)** A schematic of DNA wrapped around a nucleosome with examples of histone tail modifications. Arrows indicate the replacement of canonical histone with histone variants. Figure from (Jiang & Pugh, 2009).

histone H1, near the entry and exit site of DNA to nucleosome (Ushinsky et al., 1997) (Figure I4B). The DNA wrapped tightly around a nucleosome is less accessible than the DNA at the linker regions. Thus, the exact positioning of nucleosomes is critical for proper genome functioning. Mapping studies have shown that the majority of nucleosomes (about 80%) in *S. cerevisiae* are highly positioned or phased at the same position in virtually all the cells of population (Yuan et al., 2005; Lee et al., 2007). Nucleosome positioning is different to nucleosome occupancy, which refers to the fraction of cells from the population in which a particular region of DNA is occupied by a histone octamer

Although nucleosomes constitute the first level of chromatin compaction, chromatin is further organized into higher order structures (Woodcock & Ghosh, 2010) (Figure I5). A graded system has been proposed for classifying chromatin structure (Woodcock & Dimitrov, 2001), in which primary structure post-nucleosomal is essentially the nucleosomal array, also called "beads-on-a-string". Secondary structure is referred as the 30-nm chromatin fiber, involving inter-nucleosomal contacts and interactions with linker histones and non-histone chromosomal proteins. The tertiary structure is formed by interactions between elements of the secondary structure, consisting of long-distance contacts oR-looped chromatin domains

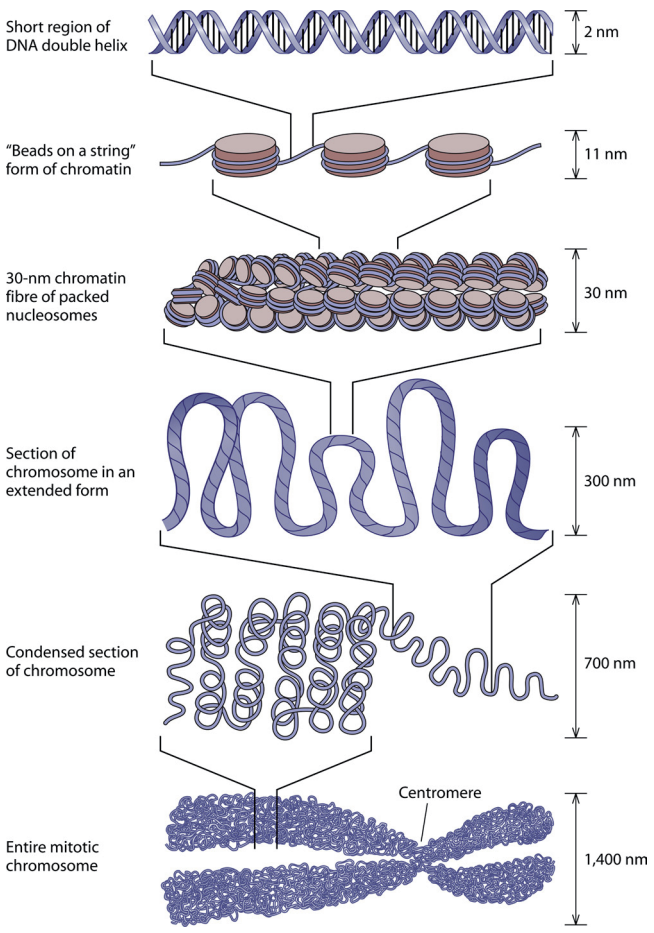

Figure I5. Chromatin structure. DNA is wrapped around a histone octamer to form nucleosomes. Nucleosomes are connected by linker DNA forming the "Beads on a string" structure. This structure is folded into a fiber-like structure of about 30 nm in diameter. These 30-nm fibers are further compacted into higher-order structures. Figure from (Jansen & Verstrepen, 2011).

that build an entire chromosome (Woodcock & Ghosh, 2010). Additionally, chromosomal regions are packaged in chromatin with different condensation levels and transcriptional potential, being classified into heterochromatin and euchromatin. Heterochromatin constitutes a condensed structure and euchromatin is less condensed and contains the transcriptionally active regions (Woodcock & Ghosh, 2010).

The study of histone function is simpler in yeast than in higher eukaryotes, since yeast genome has only two copies of each of the major histone genes, while in higher eukaryotes there are multiple copies (10 to 20) (Hentschel et al., 1981). *S. cerevisiae* genome contains four loci encoding the H3, H4, H2A and H2B histones, consisting each locus of a set of two genes that are divergently transcribed from a central promoter (Osley, 1991). Two of these loci, HHT1-HHF1 and HHT2-HHF2, correspond to genes for H3 and H4 histones, being

identical the proteins encoded in both of them. The others, HTA1-HTB1 and HTA2-HTB2, correspond to genes for H2A and H2B histones and encode slightly different isoforms. Although histone genes are essential, strains with only one set of each are viable (Smith & Stirling, 1988). This together with the easy genetic manipulation of *S. cerevisiae*, allows the study of histone mutations.

To form the chromatin, nucleosomes need to be assembled in the DNA. The majority of chromatin assembly occurs immediately following DNA replication, in the S phase where all histone are expressed (Spellman et al., 1998). The passage of the replication machinery disrupts the nucleosomes. The parental nucleosomes disassemble and histones are distributed randomly between the two new DNA duplexes, mixing with histones synthesized de novo. First histones H3 and H4 assemble followed by the incorporation of two histone H2A-H2B dimers, completing the nucleosome. This process required chromatin assembly factors and histone chaperons, that are not only important for de novo deposition of nucleosomes in replication but also for nucleosome disassembly/reassembly during transcription, recombination or DNA repair (Tyler, 2002).

Chromatin may constitute a barrier to many processes occurring on the DNA, including transcription, replication and DNA repair. Histones modulate the accessibility of the machineries carrying out the different cellular DNA-based processes. The three essential mechanisms by which chromatin structure varies are: posttranslational modifications of histones (Figure I4B), the action of chromatin remodeling complexes and the insertion of non-canonical histone variants (Figure I4B) (Kouzarides, 2007; Tessarz & Kouzarides, 2014).

3.1 Histone posttranslational modifications

Posttranslational modifications, mainly at the unfolded N-terminal tails but also at the globular domain, are detected in 60 different residues in the four different histones.

These covalent and reversible modifications influence nucleosome dynamics by disrupting intra-nucleosome or inter-nucleosome contacts or by affecting the interaction of chromatin with non-histone proteins, such as chromatin remodelers, histone chaperones and specific factors, that facilitate or restrict access to DNA. Posttranslational modifications include lysine acetylation, lysine and arginine methylation, serine, threonine and tyrosine phosphorylation, lysine ubiquitination, lysine sumoylation, glutamic acid ADP-ribosylation, arginine deimination and proline isomerization (Bannister & Kouzarides, 2011) (Figure I6).

Acetylation is one of the most well-characterized histone modifications. Although histone acetylation has related roles in DNA repair and other biological processes, its role as a transcriptional regulator is the most important. This lysine modification is regulated by the action of two families of antagonistic enzymes, histone acetyltransferases (HATs) and

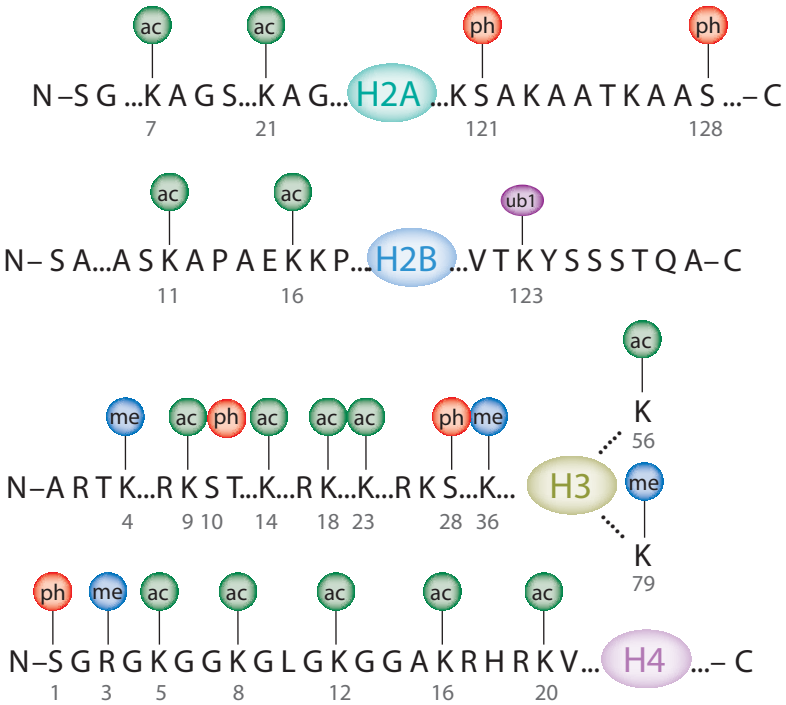

Figure I6. Histone modifications in yeast.
Summary of posttranslational modifications that could be presented in the different histones. The globular histone domains of each core histone are represented as coloured ovals. The modifications include acetylation (ac), methylation (me), phosphorylation (ph) and ubiquitination(ub1). Figure adapted from (Bhaumik et al., 2007).
vv

histone deacetylases (HDACs). The HATs remove the lysine positive charge, thereby reducing the interaction between histone and the negatively charged DNA, making the nucleosome assume an "open" structure (Strahl & Allis, 2000). Broadly, the HATs modify multiple sites in the histone N-terminal tails, such as H3K9, H3K14, H3K18, H3K23, H4K12 or H4K16, among others (Millar & Grunstein, 2006). Additional acetylation sites located in the histone globular domain, such as H3K56 alter histone –DNA interactions. H3K56 is placed at the entry-exit point of the DNA on the nucleosome and its acetylation influences nucleosome stability enhancing the unwrapping of the DNA (Xu et al., 2005). HATs are usually part of multiprotein complexes, that favours recruitment into the substrate. On the other hand, HDACs reverses lysine acetylation enhancing histones to DNA interaction. HDACs are characterized by a low specificity, being able to deacetylate multiple residues. They normally form part of multiple protein complexes, even with other HDACs, making it difficult to determine their direct targets and their particular cellular effect (Bannister & Kouzarides, 2011).

Methylation is the most frequent posttranslational histone modification in human cells, occurring at specific lysines and arginines. In yeast, this modification has only been described at lysine residues (Kouzarides, 2007). Methylation does not change the histone charge, in contrast to phosphorylation and acetylation, but can create binding sites for chromosomal proteins. This modification can have different forms, more concretely the mono-, di- or trimethyl modification in lysines; and the mono- or dimethyl modification in arginines. In *S. cerevisiae* it has been described three methyltransferase that methylates only a single histone residue: Set1 (H3K4), Set2 (H3K36) and Dot1 (H3K79) (Millar & Grunstein, 2006).

Histone phosphorylation is a highly dynamic modification. Phosphorylation, as acetylation, has the potential to alter DNA-histone or nucleosome-nucleosome contacts through electrostatic mechanisms by increasing the negative charge of histones (Kouzarides,

2007). Unlike acetylation and methylation, histone phosphorylation appears to function by serving as a platform for effector proteins, leading to a downstream cascade of events. This modification is controlled by the action of kinases and phosphatases. Most phosphorylations occur at the N-terminal tails, although it can also occur within the core of the histone (Bannister & Kouzarides, 2011). For example, mutations that mimic or inhibit phosphorylation in the H3T118 are lethal in yeast and the heteroallelic strain with one wild-type and one mutated H3 copy shows defects in transcriptional regulation and DNA repair (Hyland et al., 2005).

Histone modifications control the accessibility to DNA through two mechanisms: modificating contacts between nucleosomes and regulating recruitment of proteins to histones. As previously mentioned, acetylation and phosphorylation changes histone charge disrupting electrostatic interactions between adjacent histones and between histone and DNA. Thus acetylation in the H3 and H4 N-terminal tails is associated to transcription activation and acetylation of H4K16 modify higher-order chromatin, impeding the ability of chromatin to form cross-fiber interactions (Shogren-Knaak et al., 2006).

Posttranslationally modified histones recruit specifically chromatin-associated factors necessary to accomplish cellular processes as transcription, replication or DNA repair, by interacting with specific binding domains. In *S. cerevisiae*, acetylation is recognized by bromodomains, which are usually found in HATs and chromatin remodelling complexes (Mujtaba et al., 2007); phosphorylation is identified by the 14-3-3 protein family; and methylation is recognized by chromo-like domains of the Royal family and PHD fingers. Remarkably, methylation could be recognized by a greater variety of domains and several domains can identify the same methylation residue, indicating the importance of this modification (Bannister & Kouzarides, 2011; Kouzarides, 2007).

Besides the complexity given by the great variety of modifications existing, another factor that increases the complexity is the cross-talk between different modifications on the

same histone tail or on different ones (Bannister & Kouzarides, 2011). We can distinguish different cross-talk effects: competition between different modifications occurring on the same residue; cooperation between modifications in different residues; dependency when a modification depends on a previous one; repression when modification of a residue inhibits modification of a different one; changes in the affinity caused by modification of an proximal residue.

3.3.1 Genomic localization of histone modification

The degree of chromatin compaction/organization is not homogenous thoughout the genome. Chromatin can be divided into two different environments, heterochromatin and euchromatin. Heterochromatin is associated with highly condensed regions, such as centromeres and telomeres, contains mostly inactive genes and is not subjected to changes during the cell cycle. On the other hand, euchromatin is related to less condensed regions, contains most of the active genes and suffers cyclical changes during the cell cycle. These environments are characterized by a different set of modifications. Euchromatin is enriched in acetylation, methylation and phosphorylation at transcriptionally inactive genes (Kouzarides, 2007). Actively transcribed genes are related with high levels of acetylation, and the residues H3K4 and H3K36 are trimethylated. Heterochromatin is marked with histone modifications associated to silencing. In *S. cerevisiae*, the core histones are hypoacetylated and H3K4 and H3K79 are hypomethylated (Bi, 2014). Although there are areas that can overlap between different regions, demarcation between the environments is stablished by boundary elements. These elements recruit enzymes to modify the chromatin, playing a role in maintaining the distinction between regions (Bannister & Kouzarides, 2011).

3.2 Chromatin remodeling complexes

Chromatin remodelers are ATP-dependent multiprotein complexes that use the energy of ATP hydrolisis to remodel nucleosomes (Clapier & Cairns, 2009). These remodeling enzymes disrupt histone-DNA interactions promoting nucleosome "sliding" and partial or total nucleosome displacement. Chromatin remodelling complexes are classified attending to the ATP-ase subunit in four classes: the SWI/SNF (switch/sucrose-nonfermentable), INO80 (inositol-requiring), ISWI (imitation switch), and CHD (chromo-helicase/ATPase-DNA-binding). All remodeler catalytic subunits share a conserved ATPase subunit that provides free energy to facilitate DNA translocation, but present different remodelling activities (Clapier & Cairns, 2009). The SWI/SNF family can promote nucleosome repositioning, SWR1 insert the histone H2A variant H2A.Z, CHD remodelers slide or eject nucleosomes and the ISWI family moves nucleosomes along the DNA.

3.3 Histone variants

Histone variants differ from the canonical histone in its sequence. Some variants have profound differences, such as their time of expression and incorporation into chromatin, the structure (sequence, N- or C-terminal extensions or truncations) and their specific function in transcription, DNA repair or chromosome segregation (Malik & Henikoff, 2003). The yeast H2A is homologous to the H2A.X variant of higher eukaryote. Both contain an extension at the C-terminus which includes a serine at position 139 that, in response to DNA double-strand breaks (DSBs), is phosphorylated (Sarma & Reinberg, 2005). The variant H2A.Z substitutes the canonical H2A in extensive genomic regions, being enriched in promoter regions (Li et al., 2005). All eukaryotes present a centromere-specific H3 variant involved in chromosome segregation. In *S. cerevisiae* it is similar to the canonical H3 with the exception of their N-terminal tails (Sarma & Reinberg, 2005).

4 Role of chromatin in DNA-based processes

Chromatin remodeling and histone modifications play a key role in controling cellular processes where DNA is involved, modulating the accessibility of different machineries to DNA. Although the complexity of the modifications makes difficult to establish a code, the term "histone code" groups a series of modifications that occur in a certain biological process.

4.1 Chromatin regulates transcription

Chromatin is an obstacle to RNA polymerases that is overcome by the coordinated action of chromatin modifying and remodeling enzymes. At promoters of most yeast genes there is a nucleosome free region (NFR) flanked on both sides by two highly positioned nucleosomes, termed –1 and +1, respectively (Mavrich et al., 2008) (Figure I7). Both of them often contain the histone H2A variant H2A.Z (Raisner et al., 2005) involved together with H3.3 variant in nucleosome eviction upon activation to allow RNAPII recruitment and the expression of the genes (Venkatesh & Workman, 2015). Transcription activators also recruits HATs (SAGA and NuA4) that acetylate histone H4 (K5, K8, K12 y K16) and histone H3 (K4) introducing electrostatic repulsion that facilitates DNA access (Li et al., 2007).

Chromatin modification is not only needed for initiation but throughout elongation too. Downstream of the strongly +1 positioned nucleosome, each nucleosome is gradually less precisely localized than the previous upstream one but they still constitute a barrier for RNAPII progression, although at the 3' exists a positioned nucleosome that precede another nucleosome free region (Figure I7). Methylation in H3K4, H3K36 and H3K79 is required for efficient transcription elongation. These modifications are distributed differently across genes with H3K4me3 accumulating at transcriptional start site (TSS) and H3K36 throughout the transcribed region. This profile is established by the differential recruitment of the methyltransferases. In *S. cerevisiae*, Set1 H3K4 methyltransferase binds to the serine 5

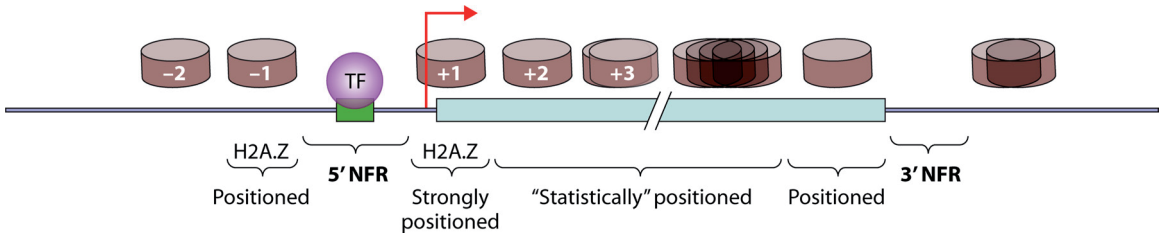

Figure I7. Nucleosome architecture of a yeast gene.
A nucleosome-depleted region (5'NFR) is surrounded by the highly localized and histone variant H2A.Z-enriched +1 and -1 nucleosomes, containing most functional cis-regulatory sequences. The +1 nucleosome is located just upstream of the transcription start site (TSS). Downstream of the +1 nucleosome, each nucleosome is gradually less precisely localized than the previous upstream nucleosome. Beyond aprox 1 kb from the TSS, consensus spacing from the TSS dissipates. At the 3' end, a positioned nucleosome precedes the 3' NFR. Figure from (Jansen & Verstrepen, 2011).

phosphorylated CTD of the RNAPII, the state of the polymerase at the 5' region of the genes (Ng et al., 2003). However, Set2 H3K36 methyltransferase binds to the serine 2 phosphorylated CTD of the RNAPII, the elongating form (Xiao et al., 2003). Via H3K4 trimethylation, H3K14 residues are hyperacetylated by NuA3 HAT. Moreover, H3K36me3 allows recruiting the Rpd3S HDAC complex, which deacetylates histones after the passage of the polymerase, avoiding the cryptic transcription (Carrozza et al., 2005). This system is an example of how antagonistic activities are complementary in biological processes. The passage of RNAPII though the body of the gene is accompanied of disruption of the chromatin structure. H2A-H2B dimer is removed by the histone chaperone complex FACT and repositioned behind RNAPII facilitating transcription progression. Loss of the H3-H4 dimer during elongation is rare in yeast but occurs in higher eukaryotes (Venkatesh & Workman, 2015).

The most relevant modifications in transcriptional regulation are acetylation and methylation, although other modifications like phosphorylation also play a prominent role. Phosphorylation of histone residues have been linked to the activation of gene expression (Baek, 2011). For instance, phosphorylation of H3S10 is recognized by the 14-3-3 family of proteins (Macdonald et al., 2005) that are present on specific genes (Pokholok et al., 2006) whose expression depend on phosphorylation.

4.2 Chromatin modifications connected to DNA replication and repair

Replication could be modulated by several chromatin modifications, at the level of origin recognition and throughout replication by chromatin assembly at the newly synthesized DNA. For instance, in human cells the HBO1 acetyltransferase binds to the MCM proteins at replication sites, and acetylate residues in N-terminal tail of H4, being this action required for S phase initiation (Kouzarides, 2007).

Posttranslational modifications have also an important role in incorporation of the newly synthesized histones during replication. Almost all organism present acetylation of H4K5 and H4K12 on newly synthesized histones (Sobel et al., 1995) performed by the histone acetyltransferase HAT1 (Parthun, 2007). These modification are not essential for histone deposition in vitro, but might be marking a transient chromatin state (Shibahara et al., 2000). In *S. cerevisiae*, H3K56 is acetylated in newly synthetized histone H3 (Masumoto et al., 2005). H3K56 is located in the DNA entry/exit point of the nucleosome contacting with DNa through the major groove. H3K56 acetylation debilitates histone-DNA interactions. Indeed, mutations that mimic an acetylated state (H3K56Q) result in increased sensitivity to micrococcal nuclease digestion (Masumoto et al., 2005). H3K56 acetylation is mediated by the HAT Rtt109 together with either ASF1 or Vps75 chaperones (Han et al., 2007). It promotes the assembly of nucleosomes during S phase (Li et al., 2008) and increases access of the repair machinery to stalled replication forks and DSBs (Han et al., 2007; Celic et al., 2008). In mammals, the role of this modification is unclear, since it is very low abundant (Xie et al., 2009). It may be present but very unstable being quickly deacetylated after deposition.

Chromatin influences DNA repair by helping to recognize the lesion and favoring recruitment of the repair factors. Phosphorylation of the histone variant γ-H2AX is the best characterized modification occurring in response to DNA damage in mammalian cells (Fillingham et al., 2006). This phosphorylation marks the injury, spreading around the

damage. DSBs repair via NHEJ involves two phosphorylation: residue S129 of H2A mediated by Mec1 (Downs et al., 2000) and H4S1 mediated by Caesin kinase II (Cheung et al., 2005). Another modification associated to DNA repair is acetylation of H3K56. As mentioned earlier, in S. cerevisae H3K56 is acetylated during S phase and deacetylated in G2 phase. In presence of DNA damage Hst3 and Hst4, the two HDACs that deacetylate H3K56, are downregulated in a checkpoint-dependent manner, maintaining the modification. Although this situation benefits DNA repair H3K56 acetyl group need to be removed as the loss of the two HDACs leads to DNA damage and replication stress (Celic et al., 2006; Celic et al., 2008).

4.3 Chromatin condensation

Chromatin condensation is an important regulated process occurring during cell cycle. During mitosis, histone modifications play an important role in condensation. H3T3 phosphorylation, which is mediated by the Haspin kinase (Dai et al., 2005), recruits the chromosomal passenger complex (CPC) that orchestrates proper chromosome segregation with cytokinesis (Figure I8). The CPC includes the kinase Aurora B in human cells and Ipl1 in yeast, that phosphorylates the H3S10 (Kelly et al., 2010; Yamagishi et al., 2010; Wilkins et al., 2014). H3S10 phosphorylation is a marker of mitosis and was linked to chromosome condensation (Hendzel et al., 1997). Aurora B mutation generates cellular polyploidy (Ke et al., 2003), an incomplete condensation of the chromosomes and lack of chromosome alignment in the metaphase plate (Goto et al., 2003). During the cell cycle, H3S10 phosphorylation levels are at their maximum degree in the G2/M phases when the chromosomes are in their most condensed state. Dephosphorylation, mediated by Glc7 in yeast and PP1 in mammals, begins during anaphase and is completed in telophase (Hendzel et al., 1997).

H3S10 phosphorylation triggers recruitment of the H4K16 deacetylase Hts2p (Prigent & Dimitrov, 2003) (Figure I8). Removal of H4K16 acetylation permits that the N-terminal tail of H4 contact the negatively charged H2A-H2B dimer interface on the neighboring

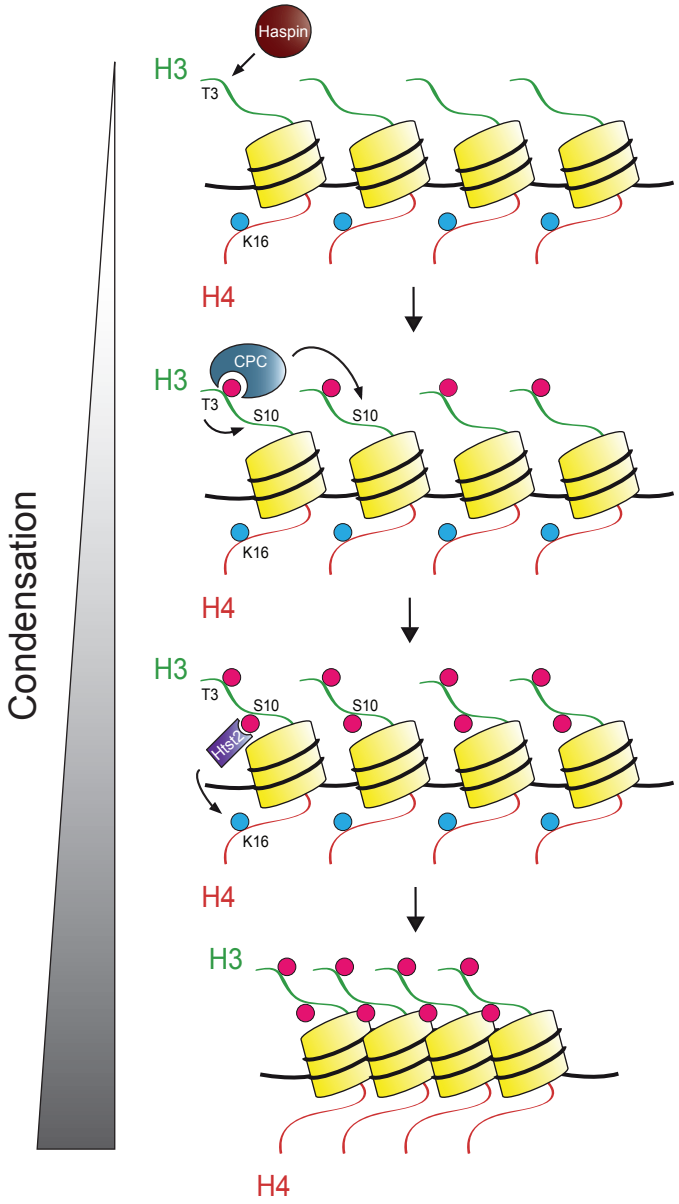

Figure I8. Cascade of histone modifications that mediate chromatin condensation.
Chromatin condensation depends on a histone modification cascade that includes H3T3-P (mediated by the Haspin kinase), H3S10-P (mediated by chromosomal passenger complex, CPC) and H4K16 deacetylation (mediated by the deacetylase Hts2p).

nucleosome (Robinson et al., 2008). This interaction is crucial for formation of higher-order chromatin structures and heterochromatin (Johnson et al., 1990). Moreover, cells with H3S10 mutated to alanine, which can not be phosphorylated, showed a relevant reduction in H2A-H4 contact, whereas a phospho-mimetic mutation had no effect (Wilkins et al., 2014).

5 Chromatin changes and R-loop formation

Numerous studies link R-loops formation to changes in the chromatin structure. On one hand, R-loops are associated with marks related with active transcription such as H3K4me1, H3K4m3, H3 acetylation and H3K36me3 (Sanz et al., 2016; Chen et al., 2015). Moreover, sequences off-targets of the AID enzyme, that acts on the ssDNA formed in R-loops, present modifications like H3K4me1, H3K27ac, H3K36me3 and high transcription rates (Chédin, 2016). The connection between R-loops and active transcription marks could be just the result of R-loop needing transcription to be formed. However R-loops correlate also with chromatin decondensation and lower nucleosome occupancy (Powell et al., 2013). In fact, R-loops prevent nucleosome wrapping in vitro (Dunn & Griffith, 1980).

A link has also been defined between the formation of R-loops and chromatin condensation or heterochromatin. This connection was established in yeast THO mutants, where defects in mRNP assembly cause genome instability and increased R-loop formation (Huertas & Aguilera, 2003), correlating this increase in R-loop formation with higher levels of H3S10 phosphorylation and a less accessible chromatin structure (Castellano-Pozo et al., 2013) (Figure I9). This link is conserved, since it was also observed in human cells and Caenorhabditis elegans (Castellano-Pozo et al., 2013). As mentioned earlier H3S10 phosphorylation is a mitosis marker involved in chromatin condensation. Although evidences link H3S10 phosphorylation with R-loop formation, it is still unknown the chromatin structure formed. R-loops has been also related to methylation of H3K9 and heterochromatin mark (Groh et al., 2014; Skourti-Stathaki et al., 2011). Therefore there are evidences in the literature that associate R-loops with either active or repressed chromatin marks.

Regardless the state of chromatin triggered by R-loops, chromatin itself can modulate formation or stability of this structure. In this sense, it is interesting that the histone chaperone complex FACT is required to preclude R-loop accumulation (Herrera-Moyano et al., 2014),

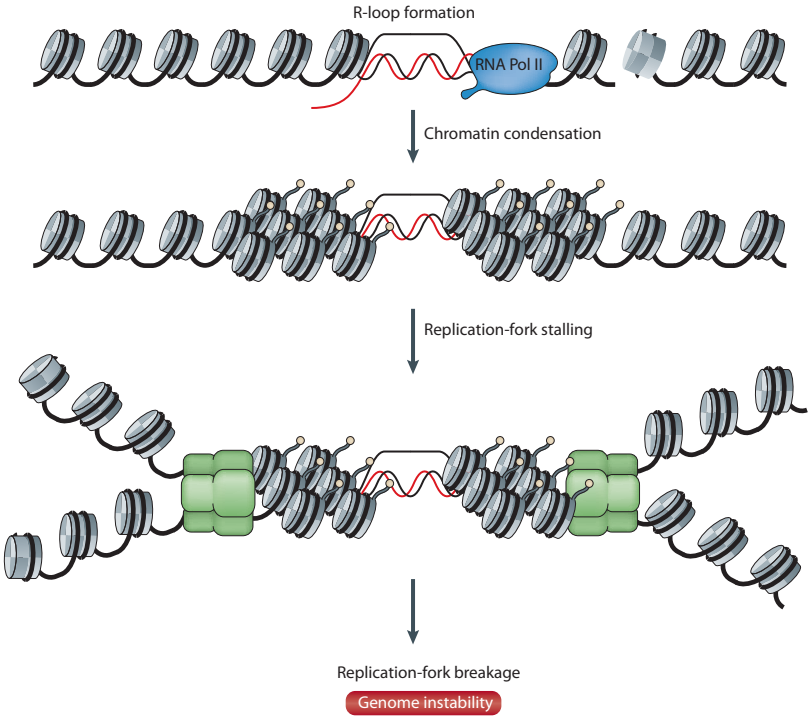

Figure I9. Replication-mediated R-loop-induced genome instability.
Co-transcriptional R-loops could trigger H3S10 phosphorylation and chromatin compaction or condensation, which would contribute to replication fork stalling and is responsible for genome instability. Figure adapted from (Santos-Pereira & Aguilera, 2015).

suggesting that R-loop formation could be prevented by an appropriate transcription-coupled nucleosome re-deposition and that chromatin structure influences R-loop formation.

Objectives

The main aim of this thesis is to understand the role of chromatin in the formation of R-loops. To this end, we have pursued the following objectives:

- To search for histone mutants that are involved in precluding R-loop formation.

- To characterize the effect of the identified residues on genome stability, as well as analysing their implication in transcription and replication

- To gain new insights into the role of chromatin structure and histone modifications in R-loop-mediated instability.

Materials and Methods

1 Growth media and conditions

1.1 Yeast culture media

The following culture media were used for yeast:

- *Rich medium YPAD:* 1% yeast extract, 2% bacto-peptone, 2% glucose, 20 mg/L adenine.
- *Minimum medium SD:* 0.17% yeast nitrogen base (YNB) without amino acids nor ammonium sulphate, 0.5% ammonium sulphate, 2% glucose.
- *Complete medium SC:* SD medium supplemented with amino acids leucine, tryptophan, histidine, lysine, methionine, aspartate and threonine and the nitrogen bases adenine and uracil at concentrations described in (Shermann et al., 1986). The absence of one or more of the requirements is specified when required.
- *Complete medium SGal*: identical to SC but containing 2% galactose instead of glucose as carbon source. Filter-sterilized galactose was added to autoclaved media without carbon source.
- *Complete medium SRaff*: identical to SC but containing 2% raffinose instead of glucose as carbon source. Filter-sterilized raffinose was added to autoclaved media without carbon source.
- *Complete medium SG/L*: identical to SC but containing 3% glycerol and 2% sodium lactate instead of glucose as carbon source. Glycerol was autoclaved separately and added to the rest of the components after they had been autoclaved.
- *Sporulation medium (SPO):* 1% potassium acid, 0.1% yeast extract, 0.005% glucose, supplemented with a quarter of the concentration of requirements described for SC medium.

Solid mediums were prepared adding 2% agar before autoclaving.

1.2 Bacteria culture media

- *Rich medium LB:* 0.5% yeast extract, 1% bacto-tryptone, 1% NaCl. LB was supplemented with 75 µg/ml ampicillin when it was necessary for plasmid selection.
- *SOB medium:* 0.5% yeast extract, 2% bacto-tryptone, 0.005% NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$.

1.3 Growth conditions

Yeast strains were incubated at 30°C, except when we used *sen1-1* strains, that were incubated at 26ºC. Bacteria strains were incubated at 37°C in all cases. Liquid cultures were incubated on horizontal orbital shakers at 200rpm. Diploid yeast strains were sporulated at 26°C in SPO medium for 4-5 days.

2 Antibiotics, drugs, inhibitors, enzymes and antibodies

2.1 Antibiotics

- *Ampicillin* (Sigma): β-lactam antibiotic that inhibits cell division in *E. coli,* preventing the cell wall synthesis. It was used for plasmid selection in *E.coli*. Concentration 100 µg/ml in liquid media and 75 µg/ml in plates.
- *Geneticin, G418* (USB): aminoglycoside antibiotic that inhibits protein synthesis by binding to the ribosome. It was used in yeast strains to select, follow and maintain the kanamycin resistance marker *KanMX* (a hybrid gene consisting of a bacterial kan^r gene under control of the strong TEF promoter from *Ashbya gossypii*). Concentration 100 µg/ml.
- *Hygromycin B* (Roche): aminoglycoside antibiotic from *Streptomyces hygroscopicus*

that inhibits protein synthesis. It was used in yeast strains to select, follow and maintain the hygromycin resistance cassette *HhpMX4 (php* gene from *K. pneumonia* under the strong TEF promoter from *Ashbya gossypii*). Concentration 250 µg/ml.

- *Nourseothricin* (Werner BioAgents): aminoglycoside antibiotic from *Streptomyces noursei*. It was used in yeast strains to select, follow and maintain the *nourseothricin* resistance *NatMX4* cassette (*nat* gene from *S. noursei* under the strong TEF promoter from *Ashbya gossypii*). Concentration 100 µg/ml.
- *Cycloheximide* (Sigma): is an antibiotic produced by *S. griseus*. Its main biological activity is translation inhibition in eukaryotes resulting in cell growth arrest and cell death. Cycloheximide (CHX) is used for selection of CHX-resistant strains of yeast

2.2 Drugs and inhibitors

- *Phenylmethanosulfonyl fluoride* (Sigma): inhibitor of serine (trypsin and chymotrypsin) and cysteine proteases. Concentration 1 mM.
- *Complete Protease Inhibitor cocktail* (Roche): mix of several protease inhibitors including serine, cysteine and metalloproteases. Used according to manufacturer's recommendations.
- *Chymostatin* (Sigma): inhibitor of many proteases, including chymotrypsins and cathepsins.
- *Diethyl pyrocarbonate* (Sigma): RNAses inhibitor.
- *Phosphatase inhibitor cocktail* (Sigma): mix of inhibitors of acid and alkaline phosphatase as well as tyrosine protein phosphatases. Contains sodium vanadate, sodium molybdate, sodium tartrate, and imidazole.
- *Hydroxyurea* (USB): inactivates ribonucleoside reductase by forming a free radical nitroxide that binds a tyrosyl free radical in the active site of the enzyme. This blocks the synthesis of deoxynucleotides, which inhibits DNA synthesis.
- *Methyl methanesulfonate* (Sigma): is an alkylating agent that acts as a mutagen by

altering DNA, adding methyl groups to DNA at 7-guanine preferentially, but also 3-adenine and 3-guanine.

2.3 Enzymes

- *Spermidine* (Sigma): polyamine involved in cell metabolism. It binds and precipitates DNA and protein-bound DNA. Concentration 0.5 mM.
- *Spermine* (Sigma): polyamine involved in cell metabolism present in all eukaryotic cells. It binds nucleic acids and contributes to stabilize the helix structure. Concentration 0.15 mM.
- *Klenow* (Roche): major fragment of the *E. coli* DNA polymerase I, with 5'-3' polymerase and 3'-5' exonuclease activities. Used for labelling radioactive probes.
- *Alkaline phosphatase* (Roche): hydrolyzes 5'-monophosphate groups from DNA ends generated after an enzymatic cut. Dephosphorylation hampers religation of cut vector, favouring insertion of the fragment of interest.
- *T4 phage DNA ligase* (Roche): it catalyzes the formation of phosphodi-ester bonds between neighbouring 3'-hydroxyl- and 5'-phosphate ends in dsDNA.
- *Expand® High-Fidelity DNA polymerase* (Roche): mix of Taq (from *Thermus aquaticus*) and Pwo (from *Pyrococcus woesei*) polymerases. It was used for high fidelity PCRs with 5'-A overhang ends.
- *Phusion® High-Fidelity DNA polymerase* (Finnzymes): a *Pyrococcus*-like polymerase fused with a processivity-enhancing domain. It was used for high fidelity PCRs with blunt ends.
- *Go-Taq® Flexi DNA polymerase* (Promega): it was used for DNA probes and checking PCRs.
- *iTaq™ Universal SYBR® Green Supermix* (Biorad): mix for quantitative PCR amplification that contains the ampliTaq Gold® DNA polymerase and the LD DNA polymerase, dNTPs with a dUTP/dTTP mixture and the ROX fluorochrome, used as

passive reference, in an optimized buffer for the qPCR reaction.

- *Pronase* (Sigma): *Streptomyces griseus* proteases.
- *Proteinase K* (Roche): serine protease from *Pichia pastoris*.
- *Restriction enzymes* (New England Biolabs and Takara): sequence-specific DNA endonucleases.
- *RNase A* (Sigma): endonuclease that degrades single-stranded RNA.
- *RNase H* (New England Biolabs): endoribonuclease that specifically hydrolyzes the phosphodiester bonds of RNA which is hybridized to DNA. This enzyme does not digest single or double-stranded DNA.
- *Zymolyase 20T* (USB): mix of enzymes from *Arthrobacter luteus* used to digest *S. cerevisiae* cell wall.
- *Lysozyme* (Sigma): enzyme from chicken egg white that hydrolyzes bacterial peptidoglycans.
- *Protein A Dynabeads* (Life Technologies): magnetic beads with recombinant Protein A coupled to its surface. Protein A binds to the Fc region of IgG, IgA and IgM immunoglobulins. It was used for immunoprecipitation assays.
- *Micrococcal nuclease* (Thermo Scientific): is an endo-exonuclease that digests single-stranded and double-stranded DNA and RNA.

2.4 Antibodies

Antibodies used in this thesis are listed in Table M1 and Table M2.

Table M1. Primary antibodies used in this thesis.

Antibody	Source	Epytope	Reference	Use
H3SS10P	Rabbit	Histone H3 Serine 10	06-570 (Millipore)	WB (1:2000) BO 0.01% Tween-20 ChIP (20 µl)
Histone H3	Rabbit	Synthetic peptide conjugated to KLH derived from within residues 1- 100 to the C-terminus of human histone H3	ab1791 (Abcam)	WB (1:2000) TBS-T 5% milk or BO 0.01% Tween-20 ChIP (5 µl)

Table M1 *(Continued)*

Antibody	Source	Epytope	Reference	Use
Anti-FLAG M2	Mouse	N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C	F3165 (SIGMA-Aldrich)	ChIP (3 µl)
Rad53 (yC-19)	Goat	C-terminal	sc-6749 (Santa Cruz Biotechnology)	WB (1:300) BO 0.01% Tween -20
Histone H4	Rabbit	Synthetic peptide conjugated to KLH derived from within residues 1-100 to the C-terminus of human histone H4	ab7311 (Abcam)	WB (1:500) TBS-T 5% milk ChIP (5 µl)
Histone H2A (phospho S129)	Rabbit	Amino acids 100-200 of S. cerevisiae Histone H2A, phosphorylated at S129	ab15083 (Abcam)	WB (1:100) BO 0.01% Tween-20
RNA Pol II (8WG16)	Mouse	C-terminal heptapeptide repeat present on the largest subunit of Pol II	MMS-126R (Covance)	ChIP (20 µl)
β-Actin	Mouse	Synthetic peptide conjugated to KLH derived from within residues 1-100 of human β-actin	ab8224	WB (1:200) BO 0.1% Tween-20
S9.6	Mouse	DNA-RNA hybrids	Hybridoma cell line HB-8730	DRIP (10 µg)

Table M2. Secondary antibodies used in this thesis

Specificity	Conjugation	Reference	Use
Rabbit	Peroxidase	A6154 (Sigma)	WB (1:2000)
Mouse	Peroxidase	A4416 (Sigma)	WB (1:2000)
Rabbit	IRDye 800CW	925-32211 (LI-COR)	WB (1:10000)
Goat	IRDye 680RD	925-68070 (LI-COR)	WB (1:5000)
Mouse	IRDye 680RD	925-68074 (LI-COR)	WB (1:10000)

3 Strains

All experiments with *E. coli* were carried out using the DH5α strain: *F- endA1 gyr96 hsdR17 ΔlacU169(f80lacZΔM15) recA1 relA1 supE44 thi-1* (Hanahan, 1983).

Histone mutant screening and other experiments were performed using the Non Essential Histone H3 & H4 Mutant Collection from Open Biosystems (Dai et al., 2008), with exceptions. Other yeast strains used are shown in Table M3 and were obtained from the referenced sources. Single, double and triple mutants were obtained by crosses and tetrad dissection (SINGER MSM 200 micromanipulator) or by PCR-mediated gene replacement using the short flanking homology (SFH) method (Wach et al., 1994).

Table M3. Yeast strains used in t his thesis

Strains	Genotype	Source
BY4741	Mat a his3Δ1 leu2Δ0 met15Δ ura3Δ0	Euroscarf
H3WT-B	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[H3]-URA3 bar1ΔHyg	This study
K9-23A-B	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 bar1ΔHyg	This study
Δ1-28-B	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3 bar1ΔHyg	This study
H4WT-B	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[H4]-URA3 bar1ΔHyg	This study
K31Q-B	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 bar1ΔHyg	This study
H3WT-W	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[H3]-URA3 GAL1pr::YLR454w (TRP1)	This study
K9-23A-W	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 GAL1pr::YLR454w (TRP1)	This study
Δ1-28-W	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3 GAL1pr::YLR454w (TRP1)	This study
H4WT-W	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[H4]-URA3 GAL1pr::YLR454w (TRP1)	This study
K31Q-W	MAT a his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 GAL1pr::YLR454w (TRP1)	This study
W303-1A	Mat a *ade2-1 can1-100 his3-11,15 leu2-3,112 rad5-535 trp1-1 ura3-1*	R. Rothstein

Table M3 *(Continued)*

Strains	Genotype	Source
WMk-1A	W303-1A mft1Δ::KanMX4	(Chávez et al., 2000)
BY4742	Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	Euroscarf
YML062C	BY4741 mft1Δ::KanMX4	Euroscarf
YCL061C	BY4741 mrc1Δ::KanMX4	Euroscarf
WP30-8D	Mat α pol30-52 trp ade his leu ura	A. Aguilera
YDR138W	BY4741 hpr1Δ::KanMX4	Euroscarf
YOL006C	BY4741 top1Δ::KanMX	Euroscarf
YML032C	BY4741 rad52Δ::KanMX4	Euroscarf
YDR007W	BY4741 trp1Δ::KanMX4	Euroscarf
YIL015W	BY4741 bar1Δ::KanMX4	Euroscarf
344115B leu+	Mat α his3-513::TRP1::his3-537 ura3-52 trp1	A. Aguilera
SEN1-R	Mat a sen1-1 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 bar1Δ	M. SanMartin
AWI2C	Mat α hpr1Δ::TRP1 ade2 leu2 ura3 trp1	A. Aguilera
YHPW-6D	MAT a his3 leu2 trp1 ura3 met15Δ0 hht1-hhf1::NatMX4 bar1ΔHyg	This study
YHPH-6B	MAT a his3 leu2 trp1ade2 ura3 lysΔ0 hht1-hhf1::NatMX4 bar1ΔHyg hpr1Δ::TRP1	This study
YHPK9-15D	MAT a his3 leu2 trp1 ura3 ade2 met15Δ0 lysΔ0 hht1-hhf1::NatMX4 bar1ΔHyg hht2-hhf2::[K9-23A]-URA3	This study
YHPK9-15B	MAT a his3 leu2 trp1 ura3 lysΔ0 hht1-hhf1::NatMX4 bar1ΔHyg hht2-hhf2::[K9-23A]-URA3 hpr1Δ::TRP1	This study
YHP28-2D	MAT a his3 leu2 trp1 ura3 ade2 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3	This study
YHP28-2C	MAT a his3 leu2 trp1 ura3 ade2 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3 hpr1Δ::TRP1	This study
YHPK31-16A	MAT a his3 leu2 trp1 ura3 ade2 hht1-hhf1::NatMX4 bar1ΔHyg hht2-hhf2::[K31Q]-URA3	This study
YHPK31-2B	MAT a his3 leu2 trp1 ura3 ade2 met15Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 hpr1Δ::TRP1	This study
YSNS-11C	MAT a his3 leu2 lys2 trp1 ura3 met15 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 bar1ΔHyg	This study
YSNK9-14C	MAT a his3 leu2 lys2 trp1 ura3 met15 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 sen1-1 bar1Δ	This study

Table M3 *(Continued)*

Strains	Genotype	Source
YSNK31-1A	MAT a his3 leu2 lys2 trp1 ura3 met15 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 sen1-1 bar1ΔHyg	This study
YHTH3-14B	MAT a leu2Δ0 lys2Δ0 ura3Δ0 hht1-hhf1::NatMX4 his3-513::TRP1::his3-537	This study
YHTK9-3B	MAT a leu2Δ0 lys2Δ0 ura3Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 his3-513::TRP1::his3-537	This study
YHT28-16D	MAT a leu2Δ0 lys2Δ0 ura3Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3 his3-513::TRP1::his3-537	This study
YHTH4-14C	MAT a leu2Δ0 lys2Δ0 ura3Δ0 hht1-hhf1::NatMX4 his3-513::TRP1::his3-537	This study
YHTK31-17A	MAT a leu2Δ0 lys2Δ0 ura3Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 his3-513::TRP1::his3-537	This study
YBYMR-1D	Mat α his3Δ1 leu2Δ0 ura3Δ0 met15Δ mrc1Δ::KanMX4	This study
YBYTP-3B	Mat α his3Δ1 leu2Δ0 ura3Δ0 met15Δ lys2Δ0 top1Δ::KanMX	This study
YRD28-3C	MAT α his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3	This study
YRDK9-13C	MAT α his3Δ200 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3	This study
YRDK31-3A	MAT α his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3	This study
YML032C	BY4741 rad52Δ::KanMX4	Euroscarf
YTPK9-18D	MAT α his3Δ leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 top1ΔKan	This study
YTP28-15D	MAT α his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3 top1ΔKan	This study
YTP31-1D	MAT α his3Δ leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 top1ΔKan	This study
YTP31-5B	MAT α his3Δ leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0 trp1Δ63 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 top1ΔKan	This study
YTP28-4B	MAT α his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hht1-hhf1::NatMX4	This study
YRDK31-17C	MAT a his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3	This study
YRDK31-4A	MAT a his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 rad52ΔKan	This study
YMRK9-1C	MAT a his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3	This study
YRD28-4D	MAT a his3Δ leu2Δ0 lys2Δ0 ura3Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3	This study

Table M3 *(Continued)*

Strains	Genotype	Source
YRDK9-5C	MAT a his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 rad52ΔKan	This study
YRDK9-3D	MAT a his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 rad52ΔKan	This study
YRD28-13D	MAT a his3Δ leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3 rad52ΔRad52	This study
YPLK31-18C	MAT a his ade leu2 trp ura3 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3 pol30-52	This study
YPL28-9C	MAT a his leu2 trp ura3 met15Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[Δ1-28]-URA3 pol30-52	This study
YPLK9-14D	MAT a his leu2 trp ura3 lys2Δ0 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 pol30-52	This study

4 Plasmids

Plasmids used in this thesis are listed in the Table M4.

Table M4. Plasmids used in this thesis.

Name	Description	Source
pLZGAID	YCp pSCH204 containing into the NaeI site the PvuII-PvuII fragment from p414GALAID (GAL::AID fusion)	This study
pRS317GALRNH1	YCp pRS317 containing into the SalI-SpeI site the SalI-SpeI fragment from pRS315GALRNH1 (GAL::RNH1 fusion)	This study
pRS315GALRNH1	YCp pRS315 containing the RNH1 gene under the GAL1 promoter	(Huertas & Aguilera, 2003)
pRS317	YCp vector based on the LYS2 marker	(Sikorski & Boeke, 1991)
pSCH204	YCp pRS314-LB containing the L-lacZ recombination system under the LEU2 promoter with the 3Kb-fragment BamHI from lacZ inserted between the two leu2 direct repeats	(Chávez & A Aguilera, 1997)
p414GALAID	YCp pRS414 containing the GAL1p::AID fusion	(Gómez-González & Aguilera, 2007)
pRS414	YCp vector based on the TRP1 marker	(Sikorski & Hieter, 1989)

Table M4 *(Continued)*

Name	Description	Source
pRS414GAL	YCp pRS414 with the GAL1 promoter and the CYC1-terminator	(Mumberg et al., 1994)
pRS413GAL	YCp pRS413 with the GAL1 promoter and the+ CYC1-terminator	(Mumberg et al., 1994)
pRS413GAL-AID	YCp pRS413 containing the GAL1p::AID fusion	(Gómez-González & Aguilera, 2007)
pWJ1344	YCp containing the RAD52::YFP fusion	(Lisby et al., 2001a)
pLY	YCp pRS313-L with the plasmid YIp5 integrated in the BglII site inserted between the direct repeats	(Prado & Aguilera, 1995a)
pRSGALlacZ	YCp pRS416 with the lacZ gene under GAL1 promoter	(Mumberg et al., 1994)
pRS-414GALAID-FLAG	YCp p414GALAID with the FLAG epitope adding in AID C-terminus protein	M. García-Rubio
pRS315	YCp vector based on the LEU2 marker	(Sikorski & Hieter, 1989)
pRS315GALAID	YCp pRS315 containing the GAL1p::AID fusion	This study
YIplac204-GAL1-YLR454W	YIplac204 containing the GAL1 promoter flanked downstream by the first 300 bp of the YLR454w coding region.	(Sträßer et al., 2002)

The pLZGAID plasmid was generated by cloning the PvuII-PvuII fragment from p414GAL:AID (Gómez-González & Aguilera, 2007), containing the GAL::AID fusion into *NaeI* digested pSCh204 (Chávez & A Aguilera, 1997). The pRS317GAL:RNH1 plasmid was generated by cloning the SalI-SpeI fragment from pRS315GAL:RNH1 (Huertas & Aguilera, 2003), containing the GAL:RNH1 fusion into SalI-SpeI digested pRS317 (Sikorski & Boeke, 1991). The pRS315GALAID plasmid was generated by cloning the PvuII-PvuII fragment from p414GALAID (Gómez-González & Aguilera, 2007) containing the GAL::AID fusion into PvuII-PvuII digested pRS315 (Sikorski & Hieter, 1989).

5 Yeast methodology

5.1 Yeast transformation

Yeast transformation was performed as previously described (Gietz et al., 1995) using the lithium acetate method.

5.2 Yeast transformation in 96-plates

Yeast transformation was performed as previously described (Gietz et al., 1995) using the lithium acetate method, but adapted to 96-well plates. Cells were inoculated in 200 µl 2X YPAD in a 96-well plate with flat bottom using a 96-pin replicator and grown for 2 days at 30ºC shaking. Cells were diluted into fresh 2X YPAD media in a 96-well plate with round bottom. Cells were incubated 3-4 h at 30ºC with shaking, centrifuged 5 min at 2000 rpm and washed with 150 µl 0.1 M LiAc/10 mM TE. Cells were resuspended in 100 µl prewarmed transformation mix (500 ng plasmid DNA, 90 µl 50% PEG, 100 mM LiAc, 1x TE, 2 µl salmon sperm DNA 6 mg/ml), treated 30 min at 30ºC and 20 min at 43ºC. Cells were washed with sterile water and resuspended in 150 µl SC-trp medium. After 2 days at 30ºC, 7 µl culture was transferred from the plate into solid selective media.

5.3 Genotoxic damage sensitivity assay

Mid-log cultures were grown in YPAD or SC medium. Drops of 3 or 7 µl of 10-fold dilutions in sterile water were plated on solid YPAD or SC medium, respectively, containing the drugs at the concentrations indicated in the figures. For UV irradiation, drops were dry before we irradiated them. Plates were incubated during 2-6 days (in the dark for UV-irradiated plates) at the indicated temperature.

6 Recombination assays

Recombination frequencies were calculated as the median value of six independent colonies. The average of three independent transformants is plotted. Recombinants were obtained by platting appropriate dilutions in applicable selective medium. To calculate total number of cells, they were plated in the same media as the original transformation used. All plates were grown for 3-5 days at 30ºC, except *sen1-1* cells that were performed at 26ºC.

6.1 Plasmid *LY* system

This system is based on *leu2Δ3'* and *leu2Δ5'* truncations of the *LEU2* gene that share 600 bp of homology. The LY system has the complete *BamHI*-digested YIp5 vector inserted. Recombinants are selected in plates without leucine (Prado & Aguilera, 1995b) (Figure M1A).

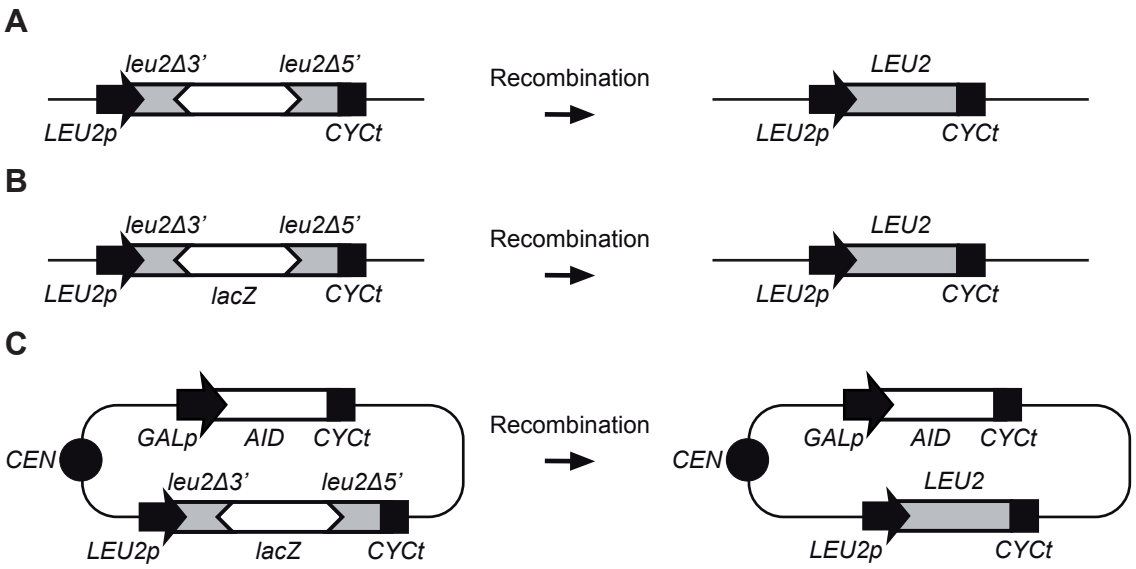

Figure M1. Recombination systems in plasmid.
Schematic representations of the LY (A), L-*LacZ* (B) and pLZGAID (C) recombination systems in plasmid with the outcome of the recombination event.

6.2 Plasmid *L-lacZ* system

Starting with an LY backbone, the sequence of the 3 kb long *lacZ* gene from *E. coli* was cloned between the direct repeats, resulting in the L-*lacZ* system. Recombinants are selected in plates without leucine (Chávez & Andrés Aguilera, 1997) (Figure M1B).

6.3 Plasmid pLZGAID

Plasmid with the direct-repeat recombination system L-lacZ (Chávez & A Aguilera, 1997) combined the AID gene under the GAL1p inducible promoter. Recombinants are selected in plates without leucine (Figure M1C).

6.4 Chromosomal *his3-513::TRP1::his3-537* system

This system is based in a duplication at the *HIS3* locus on the right arm of chromosome *XV* of a 6.1-kb *EcoRI-SalI* DNA fragment containing the *HIS3* gene. One copy of this sequence carries the allele *his3-513,* generated by mutation of the *KpnI* at the 3' site of *HIS3* coding region, and the other copy carries the allele *his3-537* generated by mutation of a *HindIII* site

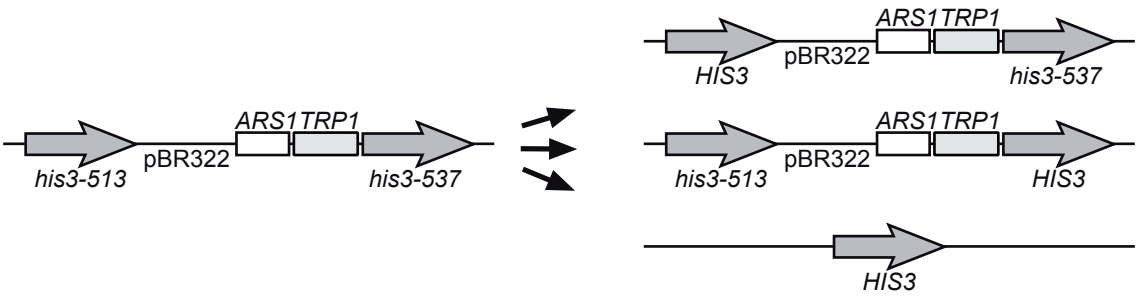

Figure M2. Recombination chromosomal system.
Schematic representations of the *his3-513::TRP1::his3-537* system with the different possible outcomes.

distal to the *KpnI* site. Between the duplications, it is located the pBR322 plasmid containing yeast ARS1-TRP1 sequence at the unique *EcoRI* site. This system allows to measure gene conversion and recombination between the repeats. Recombinants are selected in plates without histidine (Aguilera & Klein, 1988) (Figure M2).

7 Detection of Rad52-YFP foci

Spontaneous Rad52-YFP foci from mid-log growing cells carrying plasmid pWJ1344 were visualized and counted by fluorescence microscopy in a Nikon Eclipse NI-E microscope, as previously described (Lisby et al., 2001b). More than 100 S/G2 cells where inspected for each experimental replica.

8 Plasmid loss assay

Three independent transformants carrying the pRS414 (*TRP1*) plasmid were striked out in SC-trp. Then six colonies were grown overnight at 30ºC in 5 ml of non-selective rich medium until the same optical density, as previously described (Chávez & A Aguilera, 1997). Total and plasmid-maintaining cells were determined by plating the appropriate dilutions on YPAD and SC-trp plates, respectively. The data obtained represent the mean values of six independent colonies from three independent transformants.

9 Mutation analysis

Cells were grown in SC or SGAL medium plates for 3 or 5 days. Colonies of independent transformants were grown overnight in SC or SGAL medium, diluted and plated in SC with or without 3 mg/L cycloheximide. The data obtained represent the mean values of six independent colonies from three independent transformants.

10 Cell cycle synchronization and FACS analysis

For cell cycle synchronization, overnight cultures were diluted to an OD_{600nm} of 0.2 and grown until mid-log at 30ºC in rich (YPAD) or synthetic medium. Cells were synchronized in G1 adding 0.125 µg/ml of α-factor (Biomedal) for *bar1*Δ mutants and 3 µg/ml for *BAR1*. After 2.5 h, cells were released from G1 in the present or not of 20 mM HU. Samples were taken at the indicated times.

For FACS analysis, cells were processed as previously described (Moriel-Carretero et al., 2011). Briefly, 1mL of the culture was centrifuged and washed with 1 ml 1x PBS, resuspended in 1 ml 70% ethanol and stored at 4ºC. Cells were washed with 1 ml 1x PBS, resuspended in 100 µl 1x PBS-RNase A 1 mg/ml and incubated overnight at 37ºC. Next day they were washed again with 1x PBS and resuspended in 1 ml of 5 µg/mL Propidium Iodide in 1x PBS, incubated in the dark for 30 min, sonicated 5 s at 10% amplitude and scored in a FACScalibur (Becton Dickinson, CA).

11 Polymerase chain reaction (PCR)

11.1 Non-quantitative PCR

DNA amplification with temperature-stable polymerases for probe generation, strain verification or cloning were performed following standard protocols with the polymerases described in Materials and Methods 4.2.3.

11.2 Real-time quantitative PCR (qPCR)

This technique allows the measurement of the DNA quantity present in a sample during the reaction thanks to the fluorescence emitted by the SYBR® Green reactive. For this

thesis, real-time qPCRs were performed using the iTaq™ Universal SYBR® Green Supermix (Biorad). Reactions were set with 6 µl H2O, 2 µl primer mix (0.1 mM each), 2 µl template and 10 µl SYBR®. Runs were always performed with the following program: 1 cycle (10 min 95ºC), 40 cycles (15 s 95ºC and 1 min 65ºC) with a final dissociation stage (15 s 95ºC, 1 min 65ºC, 15 s 95ºC and 15 s 60ºC). Samples were run in 7500 Fast Real-Time PCR system (Applied Biosystem) Results were analyzed with 7500 System Software V2.0.6. A calibration curve with five 10-fold serial dilutions of a standard DNA sample was calculated for absolute quantification. Real-time qPCR primers were designed using Primer Express 3.0 Software (Applied Biosystems). Primers used in this thesis, for non-quantitative and quantitative PCR, are described in Table M5.

12 Chromatin immunoprecipitation (ChIP)

12.1 Chromatin extraction

Asynchronous or G1-synchronized mid-log cultures grown in YPAD or synthetic medium at 30ºC were used. Sample were processed as described (Hecht et al., 1999) with some modifications. 50 ml of the cultures were cross-linked in 1% formaldehyde shaking at low speed for 15 min at RT. Reaction was stopped adding glycine to a final concentration of 125 mM for 5 min, washed twice with cold PBS and stored at -80ºC. For cell extract preparation, pellets were resuspended in 500 µl of lysis buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1x Complete Protease Inhibitor Cocktail (Roche) and 1 mM PMSF). Next, 1 volume of glass beads were added in 2 ml tubes and cells were broken in an orbital shaker (Vibrax VXR basic, IKA) for 45 min. Samples were separated from the beads and centrifuged at 4000 rpm for 15 min to eliminate soluble proteins. The precipitate was resuspended in 1ml of lysis buffer supplemented with protease inhibitors and sonicated using Bioruptor (Diagenode) alternating 1 min high intensity and 20 s rest pulses for 15 min.

Samples were centrifuged for 15 min at 13000 rpm to eliminate cell debris. 30 µl were used as a control of total DNA (Input) and 300 µl was processed for immunoprecipitation.

12.2 Immunoprecipitation

The immunoprecipitation was performed overnight at 4ºC using Dynabeads Protein A (Life Technologies) previously incubated with the antibody for 4 h at 4ºC rotating at low speed. Samples were washed with 1 ml of lysis buffer with 275 mM NaCl, 1 ml of lysis buffer with 500 mM NaCl, 1 ml of buffer III (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 250 mM LiCl, 0.5% IGEPAL, 0.5% SDS, 0.5% sodium deoxycholate) and 1 ml TE (10 mM Tris-HCl, 1 mM EDTA pH8). Proteins were eluted in 250 µl elution buffer (50 mM Tris-HCl pH 7.4, 10 mM EDTA, 1% SDS) at 65ºC for 10 min. Samples were treated with 6 µl of 50 mg/ml pronase for 1 h at 42ºC to remove proteins and decrosslinked for 5 h at 65ºC. Quiagen DNA purification kit was used to clean DNA that was eluted in 100 µl of bidistilled water.

13 DNA:RNA Hybrid Immunoprecipitation (DRIP)

13.1 DNA extraction

For nucleic acid extraction, asynchronous, G1 or S synchronized mid-log cultures growing in YPAD were collected and washed with chilled water and resuspended in 1.4 ml spheroplasting buffer (1 M sorbitol, 10 mM EDTA pH 8, 0.1% β-mercaptoethanol, 2 mg/ml Zymoliase 20T) and incubated at 30ºC for 30 min. The pellets were rinsed with water and homogeneously resuspended in 1.65 ml of Buffer G2 (800 mM Guanidine HCl, 30 mM Tris-Cl pH 8, 30 mM EDTA pH 8, 5% Tween-20, 0.5% triton X-100) before adding 10 µl 10 mg/ml RNase A and incubated for 30 min at 37ºC. Next, samples were treated with 75 µl of 20 mg/ml proteinase K (Roche) 1 h with gentle shacking. DNA was extracted gently with chloroform:isoamyl alcohol 24:1. Precipitated DNA was spooled on a glass rod, washed

2 times with 70% EtOH, resuspended gently in TE and digested overnight with 50 U of HindIII, EcoRI, BsrGI, XbaI and SspI, 2 mM spermidine and BSA. For the negative control, half of the DNA was treated with 3 µl RNase H (New England BioLabs) overnight 37ºC.

13.2 Immunoprecipitation

DRIP was performed mainly as described (Ginno et al., 2012) with few differences. Both samples were bound to 10 µl of S9.6 antibody (1 mg/ml) in 500 µl binding buffer (10 mM NaPO4 pH 7.0, 140 mM NaCl, 0.05% triton X-100) in TE, overnight at 4ºC. Hybrid-antibody complexes were immunoprecipitated using Dynabeads Protein A (Invitrogen) during 2 h at 4ºC and washed 3 times with 1x binding buffer. DNA was elute in 100 µl elution buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS) treated 45 min with 7 µl proteinase K at 55ºC and purified with Quiagen DNA purification kit (QUIAGEN).

14 ChIP and DRIP data quantification and normalization

Sample quantifications were performed by quantitative PCR (qPCR) as described in Material and Methods Section 11.2. Means and SEMs were calculated from at least 3 independent experiments. Sample quantifications by qPCR were performed in triplicate.

- ChIP experiments: 2 µl of 1:100 and 1:10 dilutions of the Input and the IP respectively were typically used. IP/INP ratios in the different regions were calculated. In some experiments the no antibody control (NO) was included as the background control. In this case the NO signal was subtracted to IP before dividing it by INP.
- DRIP experiments: 2 µl of a 1:25 dilution for Input or the undiluted sample for IP were used for qPCR. The relative abundance of DNA:RNA hybrid immunoprecipitated in each region was normalized to the Input signal obtained.

15 Pulse-Field gel electrophoresis (PFGE)

For DNA extraction, mid-log cultures grown in YPAD were centrifuged and washed with 5 ml EDTA 50 mM pH 8. Each sample was resuspended in 180 µl CPES (40 mM citric acid pH 6, 120 mM sodium phosphate pH 6, 20 mM EDTA pH 8, 1.2 M sorbitol, 1 mg/ml zymoliase 20T), and mixed with 300 µl 2% low-melting-point agarose dissolved in CPE (40 mM citric acid pH 6, 120 mM sodium phosphate pH6, 20 mM EDTA pH 8). This mix was poured into molds and allowed to solidify for 10 min at 4ºC. Plugs were subsequently incubated as follows: overnight in 5 ml CPE with 1% v/v β-mercaptoethanol at 30ºC, then overnight in 3 ml buffer L (0.1 M EDTA pH 8, 0.01 M Tris-Cl pH 7.6, 0.02 M NaCl, 0.5 mg/ml proteinase K, 1% w/v sarkosyl) at 50ºC, and finally washed twice 1 h at 50ºC in 10 ml TE pH 7.6 containing 40 µg/ml PMSF, and 1h at RT in 10 ml TE pH 7.6.

Electrophoresis was performed at 12ºC in a Bio-Rad CHEF Mapper, using a voltage gradient of 5.5 V/cm, switch times from 5 to 30 s, switch angle of 115º, in 1% agarose gel in 0.5X TBE for 30 h, as previously described (Cha & Kleckner, 2002). Gels were treated and transferred to a Hybond XL membrane (GE Healthcare) by standard procedures.

16 Electrophoresis of DNA topoisomers

Cells were culture in SG/L medium and transferred for 1 h to glucose or galactose 2%-containing medium for analysis of transcription.

For one-dimensional electrophoresis, DNA isolation was extracted essentially as described (González-Barrera et al., 2002) with few differences. DNA was extracted gently 3 times with phenol:chloroform:isoamyl alcohol 25:24:1, 1 time with chloroform:isoamyl alcohol 24:1 and was precipitated with isopropanol. Pellet was treated with 0.5 µl 10 mg/ml RNase A and incubated for 30 min at 37ºC. Finally, DNA was cleaned with chloroform:isoamyl

alcohol 24:1, precipitated with isopropanol, washed with 70% ethanol and resuspended in TE. Electrophoresis were carried out in a 0.7% agarose gel at room temperature, loading 30 µg of DNA, in TPE buffer (50 mM tris-phosphate pH 7.2, 1 mM EDTA, 25 mM phosphoric acid) plus 4 µg/ml chloroquine at 40 V for 48 h. DNA was blot-transferred to a membrane Hybond XL (GE Healthcare) and probed with a ^{32}P-labeled DNA.

For two-dimensional electrophoresis, DNA was isolated as described previously (Roca, 2009). 50 ml cell culture were centrifuged, fixed adding 50 ml cold toluene solution (20 mM Tris-HCl pH 8, 95% ethanol, 3% toluene, 10 mM EDTA, chilled to -20ºC) and wash with TE. Pellet was resuspended in 1 ml spheroplasting solution (1 M sorbitol, 100 mM Tris-HCl pH 8.8, 20 mM EDTA, 0.1% β-mercaptoethanol, 1 mg/ml Zymoliase 20T) and incubated at 37ºC for 15 min. The spheroplasted cells were centrifuged at 2000g for 5 min and resuspended in 300 µl TE. Cells were lysed adding 30 µl of 10% SDS mixing the suspension, incubating 5 min at room temperature, and adding 200 µl of 5 M potassium acetate to the lysate. DNA was precipitated adding 1.2 ml ethanol and incubating at -20ºC for 10 min. After centrifuging at 13000 rpm 10 min, the pellet was dissolved in 100 µl TE, treated with 10 µl 10 mg/ml RNase A for 30 min at 37ºC and precipitated with 2.5 volumes of ethanol. The pellet was dissolved in 25 µl TE. Electrophoresis was carried out in a 0.6% agarose gel at RT, loading 40 µg of DNA, in TBE (0.09 M Tris-borate, 20 mM EDTA) buffer plus 1 µg/ml chloroquine at 45 V for 22h for the first dimension (top to bottom), and TBE buffer plus 5 µg/ml chloroquine at 40 V for 14 h (left to right) for the second dimension. After the first dimension, the gel was soaked in the second electrophoresis buffer for 1h shaking. DNA was blot-transferred to a membrane Hybond XL (GE Healthcare) and probed with a ^{32}P-labeled DNA.

17 MNase assay

MNase assay was performed mainly as described (Nag et al., 2008) with few differences. Mid-log cultures grown in 100 ml YPAD were centrifuged and washed with 20 ml 1 M

sorbitol. Samples was washed in 1 ml 1 M sorbitol and chaged to microcentrifuge tubes. Each sample was resuspended in 1 ml YLE buffer (10 mg/ml zymoliase 20T in 1 M sorbitol, 5 mM β-mercaptoethanol) and incubated 20 min at 30ºC. The spheroplasted cells were centrifuged at 4000 g for 3 min, washed two times with wash buffer (1 M sorbitol, 1 mM PMSF, 2 mM β-mercaptoethanol), resuspended in 1.5 ml digestion buffer (1 M sorbitol, 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, 0.5 mM spermidine, 0.075% v/v NP-40), divided into 200 µl and digested with varying concentrations of MNase (10 U/µl) for 10 min at 37ºC. The reaction was stopped adding 20 µl stop solution (5% SDS, 250 mM EDTA), mixing by inversion. DNA was treated with 10 µL RNase A 10 mg/ml for 30 min at 37ºC and with 20 µl proteinase K 20 mg/ml for 1 h at 55ºC. Samples were extracted once with phenol:chloroform:isoamyl, 25:24:1, once with chloroform:isoamyl 24:1 and isopropanol precipitated. DNA was washed with 70% ethanol and resuspended in 20 µl TE. Electrophoresis was performed on 1.2% agarose gel with ethidium bromide and the DNA signal was read using a FLA-5100 Imager Fluorescence Analyzer (Fujifilm).

18 Protein analysis

18.1 Protein extraction TCA

For protein extraction, 10 ml of mid-log yeast culture were centrifuged and kept in ice. Pellets were resuspended in 200 µl of cold 10% TCA and 200 µl of glass beads, and cells were broken by vortexing 7 times 20 seg each time at 4ºC. Supernatant was recovered and beads were washed twice with 200 µl of cold 10% TCA. Samples were centrifuged 10 min at 3000 rpm and supernatant discarded. The remaining pellet was resuspended using 100 µl of 2x Loading Buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% water-diluted Bromophenol Blue, 5% β-mercaptoethanol) supplemented with protease inhibitors (1 mM PMSF, 66 µg/ml chymostatin), 50 µl of water and 50 µl of 1M Tris (not-adjusted pH). Prior to gel loading samples were boiled for 5 min and centrifuged 10 min at 3000 rpm at RT.

18.2 Protein extraction TCA for RAD53 detection

For protein extraction from mid-log yeast culture (10^7 cell/ml) 10 mL of each strain culture were recovered and kept in ice. Proteins were extracted from pellets by adding 200 µl of cold 20% TCA and 200 µl of glass beads, then vortexing 5 min, 3 min on ice and another 3 min on the vortex at 4ºC. Supernatant was recovered from beads to a fresh tube, and beads were washed with 600 µl 5% TCA, pipetting liquid off, and recovered to the new tube (aprox. results in 600 µl in 10% TCA). Samples were centrifuged 5 min at 9000 rpm and supernatant discarded. The remaining pellet was resuspended using 100 µl of freshly prepared Laemmli buffer 1x (from 3x Laemmli buffer [150 mM Tris, 6% SDS, 30% glycerol, bromophenol Blue], fresh β-mercaptoethanol to 15% and water to adjust) and 50 µl 1M Tris Base pH 8. Prior to gel loading samples were boiled for 5 min and centrifuged 5 min at 13000 rpm at 4ºC.

18.3 SDS-PAGE

Proteins were separated in 29:1 acrylamide:bis-acrylamide gels with concentrations appropriate to the molecular size of the proteins of interest. SDS-PAGE was performed according to previously described method (Laemmli, 1970). For Rad53 detection a 4-20% gradient SDS-PAGE Criterion™ TGX ™ Precast Gels (BioRad) were used. Electrophoreses were performed in a Mini-PROTEAN 3 Cell or in a Criterion Cell (BioRad) with Running Buffer (25 mM Tris base pH 8.3, 194 mM glycine, 0.1% SDS buffer) at 100 V. Page Ruler™ (Fermentas) was used as a protein marker.

18.4 Western Blot Analysis

For Western blot, proteins were wet-transferred using Trans-Blot system (Biorad) for 2 h at 400 mA in Transfer Buffer (6 g/l Tris base, 28.8 g/l glycine and 0.5% SDS plus 20% methanol).

- **Non-fluorescent WB**: Proteins were transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare). Membranes were blocked with 1x TBS + 0.01% Tween 20 5% milk for at least 3 h. Primary antibodies were incubated during 2 to 3 hours at RT as indicated in Table M1. After 3 washes with 1x TBS + 0.01% Tween 20 of 10 min each, membranes were incubated with the corresponding secondary antibodies conjugated with the horseradish peroxidase for 2h and washed again. Finally, SuperSignalR West Pico (Pierce) or Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used for chemiluminescence detection depending on the expected strength of the signal.
- **Fluorescent WB**: A PVDF membrane with low fluorescence background (Inmobilon-FL, Millipore) was used. This membrane was first activated in methanol for 30 s and equilibrated in transfer buffer before the transference. Commercial Odyssey Blocking Buffer (LI-COR Biosciences) was used to block the membrane for at least 3 h at RT. Primary antibody was prepared to the appropriate dilution (see Table M1) in blocking buffer + 0.01% Tween 20 and incubated for 3 hours. Three washes of 10 min were performed with 1x TBS + 0.01% Tween 20 followed by incubation of 35 min with IRDye secondary antibodies. Finally, membranes where washed again 3 times, rinsed in 1x TBS and immediately scanned or left drying. Image acquisition was performed in an Odyssey CLx Imager (LI-COR Biosciences).

19 RNA analysis: Northern Blot

Yeast cell were grown in SG/L medium and transferred to galactose 2%-containing medium for *GAL1* gene activation. RNA was extracted from mid-log cultures using acid phenol (Köhrer & Domdey, 1991) and northern blot was performed following standard procedures. The DNA probes used in the hybridization experiments are listed in Table M5.

20 DNA analysis: Southern blot

Yeast genomic DNA was digested, separated in an agarose gel and transferred to Hybond-N or –XL nitrocellulose membranes (GE Healthcare), which were hybridized with ^{32}P-labelled DNA probes. The DNA probes used in the hybridization experiments are listed in Table M5.

21 Statistical analyses

Statistical tests (Student's *t*-test and Mann-Whitney *U*-test) were calculated using GraphPad Prism software. In general, a p-value<0.05 was considered as statistically significant.

22 Primers and Probes

Primers used in this thesis, both for non-quantitative and quantitative PRC, are described in Table M5.

Table M5. DNA primers used in this study.

Non-quantitative PCR primers		
Primers	**Sequence 5' to 3'**	**Use**
GAL1 Reg1 Fwd	TGCACCGGAAAGGTTTGC	GAL1 probe
GAL1 Reg2 Rev	CTCTTGTGAATTCTTCGCGAGAA	
LacZ400 Fwd	TGTACGGTACCATGGCGATTACCGTTGATG	LacZ probe
LacZ400 Rev	TAGTAGGATCCTTATTTTTGACACCAGACC	
SCR1 up	AGGCTGTAATGGCTTTCTGG	SCR1 probe
SCR1 low	GTTCAGGACACACTCCATCC	
ADE5,7-5	TACCTAAGCGTTAAGAAATCGTCTA	ADE5,7 probe
ADE5,7-3	AGAGACACCTGAGTCTGCGTATGTG	

Table M5 *(Continued)*

Non-quantitative PCR primers		
Primers	**Sequence 5' to 3'**	**Use**
GCN4 Mid Fwd	CGATGTTTCATTGGCTGATAAGG	GCN4 probe
GCN4 5' Rev	GATACATTTTCGTTGGTTGATTTAGAAC	
pdcFa	CAGCAACTGGCTTGTAACCC	PDC1probe
pdcRb	CCCCAATGGGTAAGGGTTCC	
HYG-up	TGCCTGAACTCACCGCGACG	HYG probe
HYG-down	TATTCCTTTGCCCTCGGACG	
sen1473HDIIIFw	CTCGCAACAATGGGTATTAAATTTGATACG	Primers to check sen1-1 strains
sen1-1 comp rev	CCGTCGATTGTATTGAAATCGATTGA	
GAL1P-up	CAACCATAGGATGATAATGCGATTAG	Primers to generate GAL1p:YLR454W strains
YLR454W-dw	CTGACGGTACCATCTTCTAAGG	
BAR1C	TTAGAGATGCGTTGTCCCTG	Primers to generate bar1Δ strains
BAR1D	CGTCATCCTAAACGTCCGTA	
H3 UP	TTGCTTTGAGGACGTCCCAC	Primers to check H3WT strain
H3 DOWN	CCACCTTGTCCTTAGGACAC	
K9-23A UP	TGCATGGCCTTACAGGTCTG	Primers to check H3K9-23A strain
K9-23A DOWN	CCGCAGATGAAATGTGAGCA	
Δ1-28 UP	ATAACCGAGGTGAAGTGATC	Primers to check H3Δ1-28 strain
Δ1-28 DOWN	GCCTGCACCAAATCTTGAAA	
E105Q UP	AAATTGGTCGTAATGCGTCG	Primers to check H3E105Q strain
E105Q DOWN	ACGGACCTTGTACGCGATCT	
H4 UP	TTGCTTTGAGGACGTCCCAC	Primers to check H4WT strain
H4 DOWN	CCACCTTGTCCTTAGGACAC	
K5R UP	CAGCATGTATCAAGTACGAC	Primers to check H4K5R strain
K5R DOWN	CAGCATGTAGAACGACGGAC	
K31Q UP	GCTGTAATCGCTTGTAATGG	Primers to check H4K31Q strain
K31Q DOWN	GATAGACTGATCCGCAGATG	

Table M5 *(Continued)*

Quantitative PCR primers		
Primers	**Sequence 5’ to 3’**	**Use**
GCN4 5' Fwd	CAGCCAAGTTTATTTGCTTTAAATCC	ChIP/DRIP. 5' region of the GCN4 gene
GCN4 5' Rev	GATACATTTTCGTTGGTTGATTTAGAAC	
TPD3 Fwd	GGCCAAATCCTACGCTGTGATA	ChIP/DRIP. 3' region of the TPD3 gene
TPD3 Rev	TTGCAATGAGGGTAAAATTGTGTT	
PDC1 F	GAAGGTATGAGATGGGCTGGTAA	DRIP. 5' region of the PDC1 gene
PDC1 R	CCTTGATACGAGCGTAACCATCA	
lacZ T1 Fwd	GCGCCGTGGCCTGAT	ChIP. Middle region of the lacZ gene
lacz T1 Rev	GTGCAGCGCGATCGTAATC	
GAL1 Reg2 Fwd	AAACAGGGCTTTAGTGTTGACGAT	ChIP. 3' region of the TPD3 gene
GAL1 Reg2 Rev	CTCTTGTGAATTCTTCGCGAGAA	
GAL1 Prom Fwd	CACTGCTCCGAACAATAAAGATTC	ChIP. GAL1 promoter
GAL1 Prom Rev	GGCCAGGTTACTGCCAATTTT	
YLR454W 5’ Fwd	GATGTTTCCGATTAATGTTCTACTGTACAA	ChIP. 5' region of the YLR454W gene
YLR454W 5’ Rev	GCTCCATAAGAAAGTCACTGCAAA	
YLR454W middle Fwd	TGTGGCATTGAAAATATTCCAAAC	ChIP. Middle region of the YLR454W gene
YLR454W middle Rev	GCATCGGAATCATAATCGAATATTC	
YLR454W 3’ Fwd	GGCAAAGGAAAGATGAGATTGG	ChIP. 3' region of the YLR454W gene
YLR454W 3’ Rev	AAAGTTTGGTTGGACAATCTTAAAGT	
PMA1 Fwd Prom	CGATGGTGGGTACCGCTTAT	ChIP. Promoter of the PMA1 gene
PMA1 Rev Prom	CTATTGGTGTTATAGGAAAGAAAGAGAAAA	
PMA1 Fwd 5'	AAGAAGTACGGTTTGAATCAAATGG	ChIP. 5' region of the PMA1 gene
PMA1 Rev 5'	GAATTGGACCGACGAAAAACA	
PMA1 Fwd middle	TGTTCCGACAAAACCGGTACT	ChIP. Middle region of the PMA1 gene
PMA1 Rev middle	TCGTCTGGAGAAACACCTTCAAC	
PMA1 Fwd 3'	ATCGCTATTTTCGCTGATGTTG	ChIP/RT/qPCR. 3' region of the PMA1 gene
PMA1 Rev 3'	CGGGCTTTGGAGAGTAAGGA	

Table M5 *(Continued)*

Quantitative PCR primers		
Primers	**Sequence 5' to 3'**	**Use**
PMA1 Fwd pA	GATATTAAGACGTAGTATTCGATGATTGAA	ChIP. pA region of the PMA1 gene
PMA1 Rev pA	GATGCGATTAACCGGCAAA	
V1 up	TGTTCCTTTAAGAGGTGATGGTGAT	ChIP. Non-transcribed region at positions 9716-9864 of ChrV
V1 down	GTGCGCAGTACTTGTGAAAACC	
RPB4 3' up	ACACAGCTGATGAAGCAAAGACTT	RT/qPCR. 3' region of the RPB4 gene.
RPB4 3' down	TTTCTAGGTTTGACAATTCCTTTAGTATCC	

22.1 Radioactive signal quantification

Radioactive signals were acquired using a FLA-5100 Imager Fluorescence Analyzer (Fujifilm) and were quantified using the MultiGauge 2.0 analysis software (Science Lab).

- Northern blot analysis: Signals were normalized to the *SCR1* gene, transcribed by the RNAPIII and whose transcripts are very stable. Signal was plotted as arbitrarily units (A.U.).
- PFGE analysis: The signal in each well divided by the signal in the whole lane was plotted as percentage.

23 Miscellanea

Standard molecular biology techniques were carried out following common procedures for bacterial transformation, yeast DNA and RNA extraction and other standard molecular biology techniques were carried out following common and manufacture's procedures.

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