The influence of chromatin in DNA-RNA hybrid metabolism

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The influence of chromatin in DNA-RNA hybrid metabolism

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ABBREVIATIONS

ssDNA: single strand DNA

dsDNA: double strand DNA

SSB: single strand break

DSB: double strand break

RF: replication fork

DDR: DNA damage response

HR: homologous recombination

SCE: sister chromatid exchange

HAT: histone acetytransferase

HDAC: histone deacetylase

HMT: histone methyltransferase

HDM: histone demethylase

ChIP: chromatin immunoprecipitation

DRIP: DNA-RNA hybrid immunoprecipitation

H2AP: histone H2 phosphorylation

RESUMEN

RESUMEN

El genoma, el conjunto de genes de un organismo o especie, debe copiarse de forma fidedigna de célula a célula. Sin embargo, existen situaciones que comprometen la estabilidad del genoma dando lugar a la aparición de mutaciones, roturas en el ADN, reordenamientos cromosómicos, etc...que en último lugar pueden desencadenar la muerte celular o incluso la aparición de tumores. La relevancia del mantenimiento de la estabilidad del genoma se hace patente en el hecho de que la evolución ha desarrollado complejos mecanismos de reparación y respuesta al daño en el ADN para prevenir la inestabilidad genética.

Sin embargo, el ADN no está exento al ataque de numerosos factores que pueden condicionar su estabilidad, desde agentes exógenos hasta el propio metabolismo celular. En concreto, se conoce que los procesos de transcripción y replicación del ADN son una fuente importante de inestabilidad genética. Aunque existen diversos motivos por los que estos dos mecanismos pueden generar daño en el ADN, uno de los más estudiados es la formación de R loops. Estos son estructuras formadas por un híbrido de ADN-ARN que desplaza la otra hebra del ADN dejándola en forma de cadena sencilla, más susceptible al ataque de determinados agentes como la enzima AID. Aunque los híbridos de ADN-ARN desempeñan papeles fisiológicos en las células, su acumulación puede potenciar la inestabilidad genómica. Un claro ejemplo de esto lo constituyen los mutantes del complejo THO, un complejo proteico que interviene en la formación de la ribonucleopartícula uniéndose al ARNm en formación y facilitando su exportación fuera del núcleo. En ausencia de dichos factores, la desprotección del ARNm permite su unión al ADN molde, dando lugar a una acumulación co-transcripcional de híbridos de ADN-ARN que generan altos niveles de inestabilidad genética, principalmente detectada como recombinación entre secuencias repetidas. Asimismo, existen proteínas

implicadas en la eliminación de los híbridos una vez formados, como las RNasas H y ciertas helicasas.

Es importante destacar que en el genoma de los eucariotas, el ADN no se encuentra desnudo en el núcleo de la célula sino que se asocia con determinadas proteínas dando lugar a la estructura de la cromatina cuyas unidades básicas son los nucleosomas. Cada uno de ellos está formado por un octámero proteico que contiene dos copias de cada una de las cuatro histonas H3, H4, H2A y H2B. La colas amino-terminales de las histonas sufren modificaciones post-traduccionales como metilaciones, acetilaciones, fosforilaciones o ADP-ribosilaciones que pueden tener consecuencias tanto para la estructura de la cromatina como para todos los procesos básicos del ADN, incluida la formación de híbridos de ADN-ARN. Estudios previos en el laboratorio identificaron ciertas mutaciones en las colas amino terminales de las histonas H3 y H4 que Estos híbridos a diferencia de los que se acumulan otros mutantes como los del complejo THO, no dan lugar a un incremento de la inestabilidad genómica, ni por un aumento de la recombinación entre secuencias repetidas ni tampoco mediante una acumulación de focos de Rad52.

En esta tesis profundizamos en el estudio de dichos mutantes de histonas, analizando los niveles de pérdida de heterozigosidad o pérdida de cromosomas y hemos conseguido medir la longitud y la frecuencia de los R loops gracias a la puesta a punto de una técnica basada en el uso de bisulfito sódico *in vitro*. Además, hemos utilizado una versión hiper-mutagénica de la AID con el fin de poder mapear los híbridos en todo el genoma *in vivo*. Hemos observado que los R loops que se acumulan en los mutantes de histonas tienen la misma longitud que aquellos que están presentes en cepas silvestres u otros mutantes que dan lugar a la acumulación de híbridos como los del complejo THO. Los resultados de esta tesis, por tanto, nos han llevado a concluir que la longitud de los R loops no es un factor determinante en la generación de inestabilidad genética mediada a través de estas estructuras.

Por último, para identificar nuevos factores de la cromatina que tengan un papel en el metabolismo de los R loops, hemos realizado una búsqueda para identificar mutantes en remodeladores de cromatina o modificadores de histonas. Hemos encontrado que la deleción de la acetil-transferasa Rtt109 provoca un incremento de R loops dependiente de su actividad catalítica, y gracias al análisis de distintas mutaciones en las histonas H3 y H4 hemos asociado dicho incremento con dianas concretas de entre todas las descritas para Rtt109 *in vivo* o *in vitro*. Por otra parte, dado que Rtt109 tiene un papel en la reparación de roturas de doble cadena del ADN, previamente descrito en nuestro laboratorio, hemos estudiado la relación entre los fenotipos de defecto en la reparación y la acumulación de híbridos de ADN-ARN, tanto en ausencia de Rtt109 como en los mutantes individuales en sus dianas. Los resultados de esta tesis nos indican que la acumulación de híbridos de ADN-ARN no es un impedimento para la correcta reparación de roturas de doble cadena, ni tampoco son la causa de todo el daño presente en ausencia de Rtt109. No obstante, concluimos que la persistente acumulación de roturas de doble cadena podría favorecer la formación de híbridos de ADN-ARN. De esta forma, el trabajo manifiesta la relevancia del contexto cromatínico en el que se encuentra el ADN en la formación de R loops.

INTRODUCTION

Genetic instability

The genome contains all the information needed to form from a simple bacteria to a complex human being and it is essential to preserve it from generation to generation in order to keep individuals and species alive through time. Genome can vary between distinct generations as a natural consequence of the metabolism of DNA. This process of changes in the DNA is referred as genome instability and although is needed for evolution, many factors, including exogenous and endogenous agents, can enhance it, resulting in genetic alterations which can be classified according to the type of event stimulated and the cell cycle stage: chromosomal instability, micro- and minisatellite instability, mutations, etc. This events can provoke the loss of genes, chromosome rearrangements, cancer and cell death. However, cells posses several mechanisms to preserve the integrity of the genome, starting for the machinery itself in charge of copying it, avoiding the appearing of mutations, and finishing with proteins involved in the repair of the DNA damage once is formed, such as histones and their modifications, and also non chromatin-bound factors.

DNA damage response.

DNA damage can be produced by factors outside of the cell and among the exogenous factors which can lead to genome instability we can remark the exposure to UV and ionizing radiations, environmental carcinogens and chemicals. Moreover, cellular processes also constitute endogenous sources of genome instability such as, reactive oxygen species generated by metabolism, transcription and also DNA replication **(Figure I1).** All of them differ in the type of DNA damage generated and the subsequent cellular response activated. To counteract all these lesions which could lead to genetic instability, cells have evolved specialized systems that detect the damage, signal its presence and mediate the repair in order to safeguard the stability of the genome. This is orchestrated by a highly conserved network of proteins generically termed as DNA damage response (DDR), which organize multiple cellular events that although independent, cells have to coordinate in space and time to preserve DNA integrity.

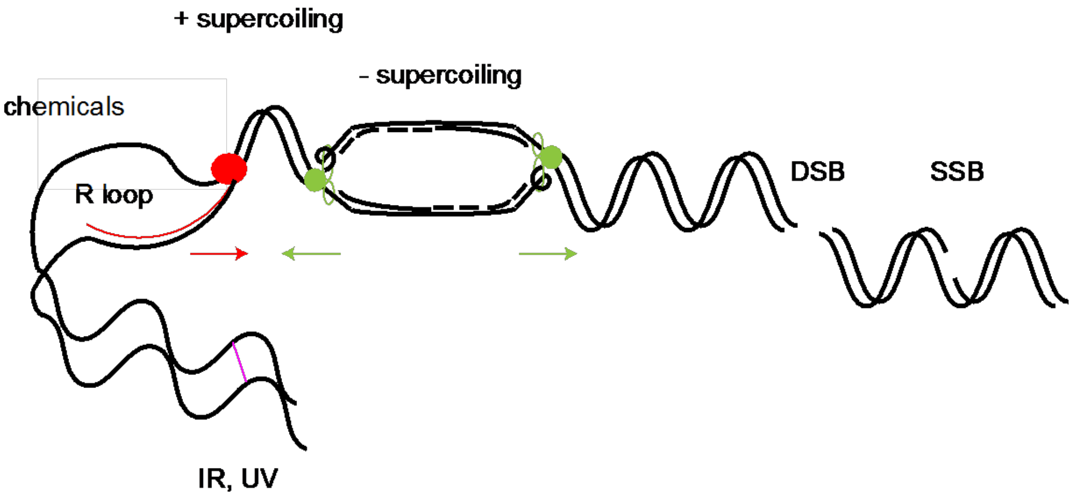


Figure I1. Factors leading to DNA damage. Positive and negative supercoiling generated during replication impedes the replication fork progression and makes DNA damage agents easy to access DNA, respectively. Hybridization of mRNA (red line) to its DNA template (R loop) exposes ssDNA, which is more sensible to the attack of DNA damage agent. Replication (green arrows and circles) and transcription (red arrow and circle) conflicts are also a source of genetic instability. Ionizing radiation (IR) and UV can provoke interstrand-crosslink (purple line). All of these DNA damage agent can lead to the formation of SSB. and DSBs.

DDR is composed of sensor, transducers and effectors. Upon the generation of a DNA lesion, the DDR coordinates the response by activating two parallel routes: 1) DNA repair pathways that deal with the restoration of the DNA lesion, and 2) DNA damage checkpoint, a cell cycle blockage that restrains mitosis until DNA damage has been fixed.

DNA damage repair

The most important repair pathways are base excision repair (BER), for oxidative damage to DNA bases and single-strand breaks (SSBs), nucleotide excision repair (NER) to remove bulky adducts, mismatch repair (MMR) for errors occurring during DNA replication, and homologous recombination (HR) or non-homologous end joining (NHEJ) to repair double-strand breaks (DSBs), which are the most deleterious lesions in the DNA, since a single DSB can lead to cell death (Rich et al., 2000). The choice of the DSB repair pathway depends on the 5' end resection needed for HR which rely on the cell cycle stage: HR is active during S/G2 phases while NHEJ is more likely to occur in G1 and also in G2 phases. However, both routes share a previous common step of recognition of the DSB by the DNA-damage checkpoint..

Recognition of DSBs prior to be repaired

When a DSB occurs, the DNA ends interact directly with the MR(X)N complex (Grenon et al., 2001), composed of Mre11, Rad50, and Xrs2 in yeast. This complex recruits Tel1 transducer kinase triggering checkpoint activation (Lee and Paull, 2005, Mantiero et al., 2007, Paull and Lee, 2005). Although the activation of Tel1 seems to be sufficient to induce a checkpoint response, the repair of persistent DSBs, through the formation of RPA-coated single-stranded DNA (ssDNA) (Zou and Elledge, 2003), require the activation of Mec1, arresting cells in G2 phase. Tel1 stimulates processing of DSB, which consists on the 5' end resection nearby a DSB. Since this resection process lead to the repair of DSBs by HR (Frank-Vaillant and Marcand, 2002), we will see it more in detail when we describe this repair pathway. After the activation of the checkpoint, it is produced an amplification of the DNA damage signal, being phosphorylation of serine 128 of the histone H2A the most important and is carried out by the checkpoint kinases Tel1 and Mec1 (Downs et al., 2000). Once the DNA damage is signaled, this can be repaired by the two main pathways: NHEJ and HR **(Figure I2).**

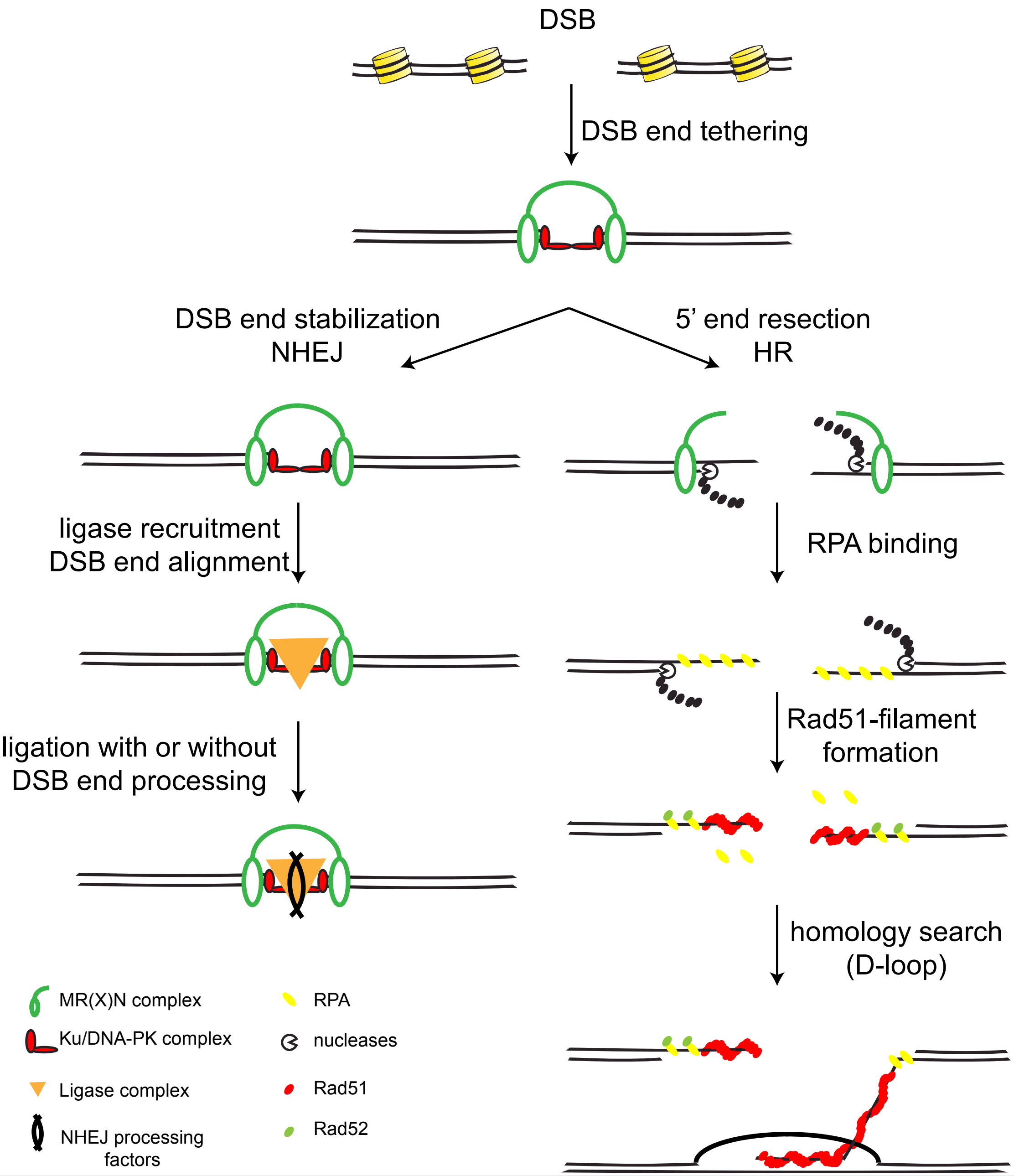

DSB
DSB end tethering
DSB end stabilization
NHEJ
5’ end resection
HR
ligase recruitment
DSB end alignment
RPA binding
ligation with or without
DSB end processing
Rad51-filament
formation
homology search
(D-loop)
MR(X)N complex
Ku/DNA-PK complex
Ligase complex
NHEJ processing
factors
RPA
nucleases
Rad51
Rad52

Figure I2. Model of DSB repair by NHEJ and HR pathways. Once a DSB is formed in the DNA (black lines), the ends of the DSB are tethered by MR(X)N and Ku/DNA-PK complexes. In NHEJ, DNA ends are stabilized and ligase complex is recruited. Ligase complex sticks the ends of the DSB if possible, and if not, many factor are recruited to process the ends following up ligation. In HR, 5'ends of DSB are digested by nucleases, generating ssDNA which is coated by RPA. Rad51 displaces Rad52-bound RPA leading to the search of homology in a DNA template giving rise to D-loop formation. Adapted from (Pardo et al., 2009)

In NHEJ repair pathway, the two ends of the DSB re-ligate independently of homologous sequences. Since it generates small insertions or deletions, it is considered to be an error-prone DSB repair pathway. NHEJ core is formed by MR(X)N, Ku and the DNA ligase complexes. Although this pathway is not well studied, it is thought that MR(X)N and Ku (consisting of Ku70 and Ku80 proteins) complexes bind DSB once it is formed, maintaining the ends proximal to each other and recruiting and stabilizing the ligase complex to the DSB, which is formed by the Lig4 ligase, Lif1 cofactor and Nej1 or Lif2 protein. Prior to ligation of DSBs by Lig4, the ends of DSBs must be processed in order to obtain a correct substrate. Finally, possible gaps formed during NHEJ are filled by DNA polymerases.

The homologous recombination pathway uses the information contained in a homologue sequence to repair a DSB. When the DNA template used for repair is the sister chromatid, the homologous recombination is termed as sister chromatid exchange. It is considered to be an error-free pathway, since it can fix a DSB without mutations always an identical homologous sequence is used. HR is characterized by three successive steps: 1) 5' end resection at both sides of DSB, 2) strand invasion into a homologous DNA duplex and strand exchange, and 3) resolution of recombination intermediates. Depending on 2) and 3) steps, there are a variety of different HR pathways which include the synthesis-dependent strand annealing (SDSA), the classical DSB repair (DSBR) model and break-induced replication (BIR).

As mention before, to channel DSB repair through HR a resection of the 5' ends of the DSBs is needed, generating a long 3' single-stranded end which searches for homologue sequences. This processing of DSBs is carried out by different proteins including Mre11, which is thought to resect modified ends such as those seen in IR- or camptothecin induced DSB, Sae2 and Exo1 nucleases, although is not discard the involvement of other nucleases in the processing of DSB ends.

As the resection occurs **(Figure I3A),** the resulting 3' single-stranded DNA end is coated by RPA, which interacts directly with Rad52 (Hays et al., 1998). Rad52 is involved in all recombination mechanisms (Symington, 2002) and form repair centers during S phase (Lisby et al., 2001). It interacts with Rad51 facilitating its loading on single-stranded DNA by displacing RPA protein (Song and Sung, 2000), and this Rad51-coated filament is ready now to the proceed to strand invasion searching for homology and forming a heteroduplex or hybrid DNA called displacement-loop (D-loop) **(Figure I3C).** At this point, the invasive 3' end of the DSB is polymerized using one strand of the D-loop to recover the information lost at DSB. The elongating DSB end can then separate from the D-loop binding to the other DSB end (SDSA) (Nassif et al., 1994) **(Figure I3D),** not leading to crossovers or the displaced strand of the D-loop can be interstrand-crosslinked between the two DSB end once they are ligated (DSBR) (Szostak et al., 1983) **(Figure I3G),** forming the holiday junctions **(Figure 3H)** whose resolution will lead or not to crossovers **(Figure I3I)** or dissolution by the action of DNA helicases and topoisomerases, not leading to crossover **(Figure I3J).** Another type of HR is BIR, which occurs when there is only one end of the DSB and is important for DNA damage presented in telomeres or in collapsed replication forks. In this pathway, once the end of the DSB forms the D-loop it initiates the DNA replication from that place until the end of the chromosome (Morrow et al., 1997). By last, resection of the DSB ends can generate the appearance of homologous sequences between the resected zones of both DSB ends which can anneal repairing the DSB (single-strand annealing, SSA) (Lin et al., 1984), but this process is error prone due to the loss of DNA sequences between DSBs.

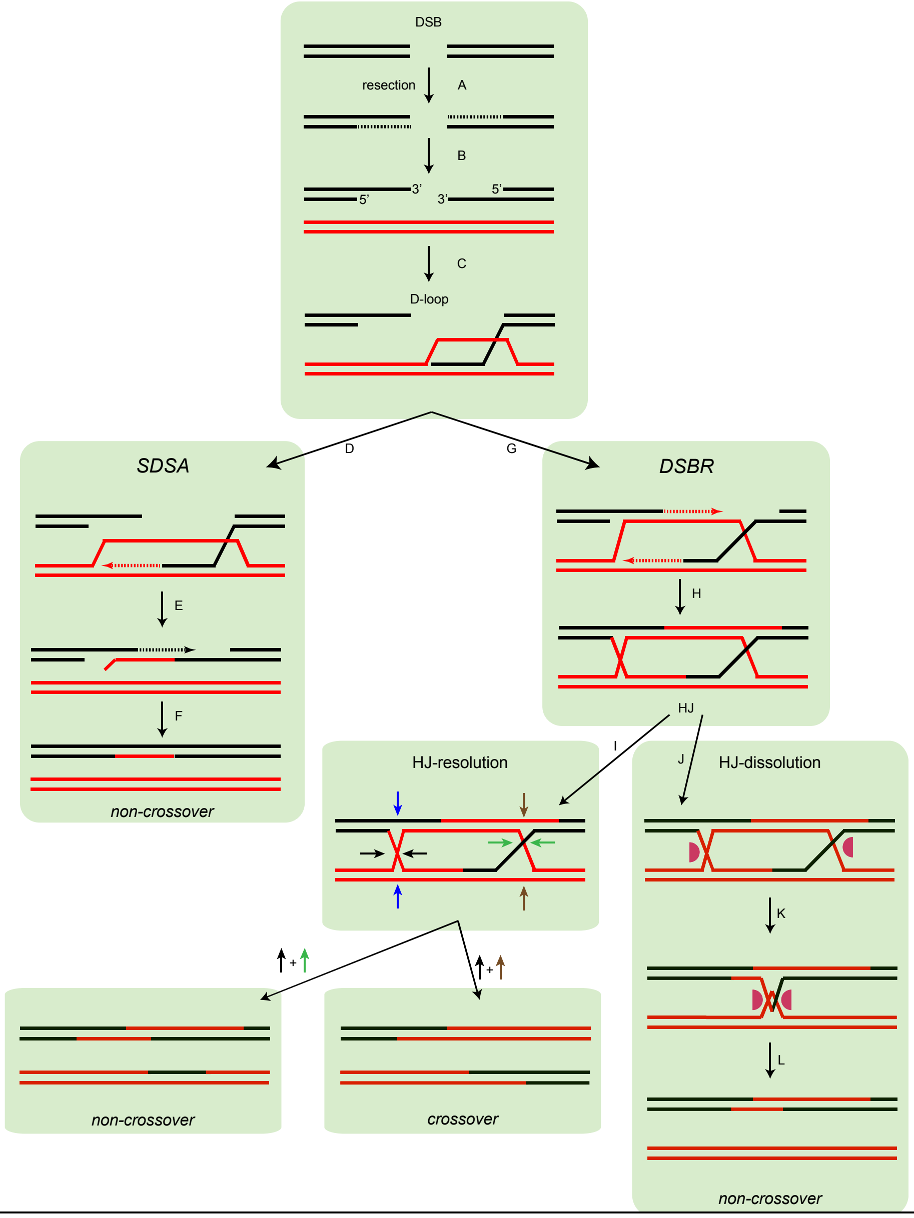

DSB
resection
A
B
3'
5'
3'
5'
C
D-loop
D
G
SDSA
E
F
non-crossover
DSBR
H
HJ
I
J
HJ-resolution
HJ-dissolution
K
L
+
+
non-crossover
crossover
non-crossover

Figure I3. Homologous recombination by SDSA and DSBR. A. Resection of 5'end at a DSB. **B.** 3' end searches homology in a homologous sequence marked in red. **C.** D-loop formation after 3' end invasion. **D.** SDSA pathway. Polymerization of 3' end. **E.** Displacement of polymerized 3' end and annealing to the other side of the DSB. **F.** Cleavage of non-homologous sequence and ligation, producing non-crossover. **G.** DSBR pathway. Both 3' end of the DSB are polymerized and ligated forming Holliday junctions. **I.** Resolution of HJ which can lead to non-crossover or crossover. **J.** HJ dissolution. **K.** HJ migration. **L.** Strand decatenation leading to non-crossover. Adapted from (Pardo et al., 2009).

DNA damage checkpoint

As mentioned before, the repair of the DNA damage once is formed does not occur independently in the cell. Checkpoint pathways consist of damage signal amplification and transduction cascades through the Mec1/Tel1 kinases that converge in the Rad53/Chk1 effector kinases to coordinate replication with DNA repair and cell cycle progression. The cell cycle is transiently arrested at different stages depending on the phase at which DNA alterations occur (G1, S and G2). Three responses have been characterized in budding yeast which are known as the G1/S, intra-S and G2/M DNA damage checkpoints. The general mechanisms underlying the DNA damage checkpoint response is the same in the different phases of the cell cycle. Focusing on the DNA damage that can appear during replication, two different pathways can operate to fix the DNA, but both of them conclude in Rad53 phosphorylation and subsequent checkpoint activation, which at last, it triggers the stabilization of (stalled) replication forks and inhibition of late origin firing. DNA replication checkpoint depends on Mrc1 protein at replication forks, while intra-S phase DNA damage checkpoint is Rad9 dependent, reacting to gaps on nascent DNA behind the replication forks exposing ssDNA on the template strand. The formation of ssDNA behind or ahead the replication forks recruits replication protein A (RPA). RPA is able to bind Mec1/Ddc2 complex, which phosphorylates Rad53 and triggers checkpoint response. Moreover, the ends of dsDNA surrounding the ssDNA are recognized by Rad24 together with the replication proteins Rfc2-5 forming a RFC-like clamp that loads PCNA-like complex formed by Rad17/Mec3/Ddc1 onto the DNA. This RFC/PCNA-like complex acts as a DNA damage sensor which participates in the Mec1 recruitment, phosphorylating again more Rad53 protein, amplifying the signal.

R loops.

During transcription, nascent mRNA can hybridize to its DNA template, forming a DNA-RNA hybrid plus a single strand DNA displaced by the mRNA **(Figure I4).** This structure is named R loop and although they are formed naturally in specific processes such as plasmid replication in *E. coli,* mitochondrial replication or class switch recombination of immunoglobulins, there are situations in which its formation is enhanced and this scenario constitutes a source of genome instability which includes mutations, recombination, chromosome loss and chromosome rearrangements. This genome instability may be link to the presence of ssDNA displaced by the mRNA of the hybrid, which could be more accessible to the attack of DNA damage agents. It is not clear whether R loops are able to cause DNA breaks, but there are some studies where the overexpression of RNase H reduces γ-H2AX signal, which is a DSB mark (Rogakou et al., 1998) in mutants with this kind of DNA damage (Paulsen et al., 2009, Chernikova et al., 2012). However, R loop formation can be favored by DSBs which provide a free 3' DNA end that would stimulate its hybridization to the nascent RNA (Sordet et al., 2009, Li et al., 2016). The relationship between R loops and DSB goes beyond, and some studies propose that R loops are involved somehow in the DNA damage repair (Ohle et al., 2016, Amon and Koshland, 2016).

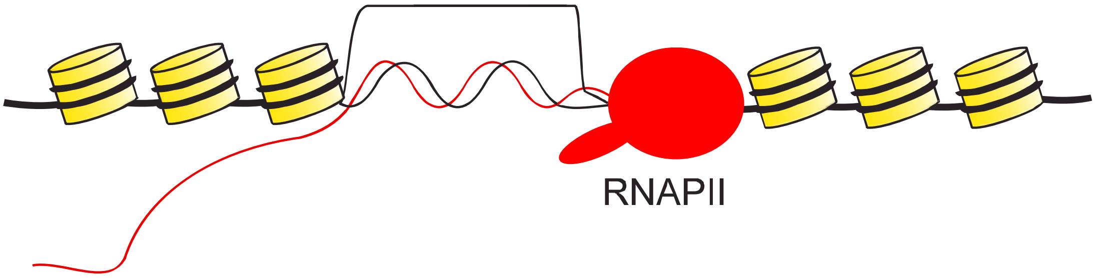


Figure I4. Model of an R loop. The nascent mRNA (red line) co-transcriptionally formed by RNA polymerase hybridizes with its DNA template, displacing the other strand of the DNA, which remains in ssDNA manner.

To avoid the deleterious effect of R loop formation over the genome stability, cells counts with several mechanisms to prevent its formation, such as specific RNA-binding proteins (RBPs) like THO complex, which bins to newly synthesized RNA to be exported from the nucleus, and also to remove them once they are formed, such as RNase H enzyme, which degrades RNAs from DNA-RNA hybrids, and the

DNA-RNA helicase Sen1, that separate RNAs from their DNA templates. Moreover, negative supercoiling during replication and transcription also favor R loop formation. In this cases, topoisomerases are very important to remove the torsional stress and thus, reducing the possibility of R loop formation.

Transcription as a source of genome instability.

Although transcription machinery or any of the related structures formed during this process can represent a blockage to the replication progression, the transcription *per se* can constitute another source of DNA damage. Sequences highly expressed are more prone to be damaged by mutations and recombination events, phenomena known as transcription-associated mutation (TAM) and transcription-associated recombination (TAR), respectively. It is thought that these genome instability phenotypes are due to a more accessibility of the non-transcribed strand (NTS) to DNA damage agents than the transcribed strand (TS) although it seems most likely that the strand asymmetric sensitivity observed is due to the NTS, but not the TS is single stranded. In this sense, R loops could explain the differences between the damage sensitivity of both strands.

Replication as a source of genome instability.

During the copy of the genome, the replication machinery can encounter with some obstacles which must overcome to ensure the correct transmission of the genomic information to daughter cells. Among all this obstacles we can remark the presence of topological stress, the structure of chromatin, different proteins bound to the DNA, the presence of DNA damage and non-B DNA structures and the transcription machinery itself.

As replication progresses, the replication forks themselves induce conformational changes in the topology of DNA, opening it behind the replication forks (negative supercoiling) and closing it ahead of the replication forks (positive supercoiling). In one hand, the negative supercoiling, makes DNA more accessible to the attack by different agents, while the positive supercoiling could impede the progression of the replication machinery and favor its stalling. However, cells count with topoisomerases (I and II) which relieve the topological stress creating a SSB or a DSB, respectively by passing of one DNA molecule through the other. The presence of DNA damage and also non-histone proteins bound to the DNA could lead to the replication fork stalling by impeding its moving ahead these obstacles. Nevertheless, there is a battery of proteins including helicases and DNA repair pathways to eliminate them from the DNA. In addition, DNA is bound to histone proteins, forming the chromatin, which can be postranslationally modified by acetylation, methylation, phosphorylation, etc, resulting in chromatin structures that can favor or hinder its replication.

Conflicts between replication and transcription machineries deserve a mention apart. When replication and transcription occur at the same time, these two machineries can collide since the replication fork cannot progress past an elongating RNA polymerase. Depending on the direction of these processes, collisions can be classified in head-on collisions, when replication forks and RNA polymerase move in a convergent opposite direction, and co-directional collisions, when both machineries move in the same direction.

Some studies in yeast revealed that head-on collision are more deleterious for the genome integrity than co-directional collisions, since only the first ones could be able to pause the progression of replication forks inducing recombination (Prado and Aguilera, 2005). Part of the different genome instability phenotypes observed in the two kind of collisions may be attributed to R loops (Garcia-Rubio et al., 2018, Hamperl et al., 2017, Lang et al., 2017). In artificial collision systems, R loops are only detected in the head-on collision (Hamperl et al., 2017, Lang et al., 2017). However, R loops can be stabilized by Yra1 overexpression and can be detected even in co-directional collisions (Garcia-Rubio et al., 2018).

It is not clear how R loops can cause the blockage of replication forks and several mechanisms have been developed to explain it. One possibility is that the retention of RNA polymerase or the recruitment of DNA-RNA binding proteins could create an obstacle to the replication fork progression. Another possibilities include the negative supercoiling generated by R loops between replication and transcription machineries or a compaction of the chromatin associated to R loops.

In order to counteract these collisions, cells posses different mechanisms to avoid or reduce their formation. In bacteria, for example, genes are co-directly oriented with the replication origins. In yeast, there are replication block carriers at rDNA locus and in mammals replication and transcription take place at different time points, since loci highly transcribed are replicated late in S phase and vice versa.

Detection of R loops among the genome.

Since the clear relation between R loop and genome instability, several strategies has evolved from the past few years to detect them. The most widely used is the S9.6 antibody, which can bind specifically to DNA-RNA hybrids. It was originally isolated by Carrico and colleagues in 1985. Mouse (BALB/c) B-cell was fused with Mouse (BALB/c) Sp2/0–Ag14 myeloma to produce monoclonal antibodies against RNA-DNA hybrids. Animals were immunized with RNA-DNA hybrids formed by using single stranded PhiX174 DNA as template for *E. coli* DNA dependent RNA polymerase (Boguslawski et al., 1986). Many labs have taken advantage of this specificity and have used it to map DNA-RNA hybrids among the genome in yeast (Chan et al., 2014, El Hage et al., 2014, Wahba et al., 2016) and mammals (Ginno et al., 2013, Ginno et al., 2012, Nadel et al., 2015, Sanz et al., 2016, Stork et al., 2016). Most of methods using S9.6 antibody capture DNA-RNA hybrids from digested DNA followed by sequencing of captured DNA (DRIP-seq) or RNA (DRIP-c-Seq (Chedin, 2016). A few authors have revealed that S9.6 antibody also presents high specificity for dsRNAs (Phillips et al., 2013, Hartono et al., 2018), which could reduce the DNA-RNA immunoprecipitation efficiency and have modified the original protocol adding an RNase III in vitro treatment prior to immunoprecipitation with S9.6 (Hartono et al., 2018, Svikovic et al., 2019), since this enzyme degrades dsRNAs. Alternative techniques to S9.6 antibody has been developed such as the used of inactive RNase H (Chen et al., 2017), which is able to bind DNA-RNA hybrids without resolving them or the use of bisulfite, which converts specifically cytosines into uraciles of the ssDNA (Ginno et al., 2012, Yu et al., 2003, Huang et al., 2006, Garcia-Pichardo et al., 2017).

Apart from S9.6 antibody and inactive RNase H enzyme, there is and additional tool to indirectly detect R loops, the Active-Induced Deaminase (AID) which is able to deaminate cytosines of ssDNA generating uracils. This enzyme is present in mammals and it is thought to trigger somatic hypermutation and class switch recombination in an R loop dependent manner (Muramatsu et al., 2000, Revy et al., 2000). Several studies have overexpressed AID *in vivo* in order to enhance a R loop-dependent genome instability phenotype (Garcia-Pichardo et al., 2017, Gomez-Gonzalez and Aguilera, 2009, Mischo et al., 2011, Garcia-Benitez et al., 2017). There is an interesting variant of this enzyme called superAID or AID* which is more mutagenic (Wang et al., 2009) and have been used in some studies to reproduce mutations found in cancers and discover regions of the genome more prone to be mutated (Taylor et al., 2013, Taylor et al., 2014), although it has never been used to map R loops among the genome.

Independently of the method used to detect R loops, RNase H is a key element in all these techniques which helps to decipher if the signal measured comes or not from DNA-RNA hybrids. RNase H can also be *per se* a good tool to detect R loops since it is able to suppress genome instability phenotypes such as growth defects (Gomez-Gonzalez et al., 2009, Feldman and Peterson, 2019).

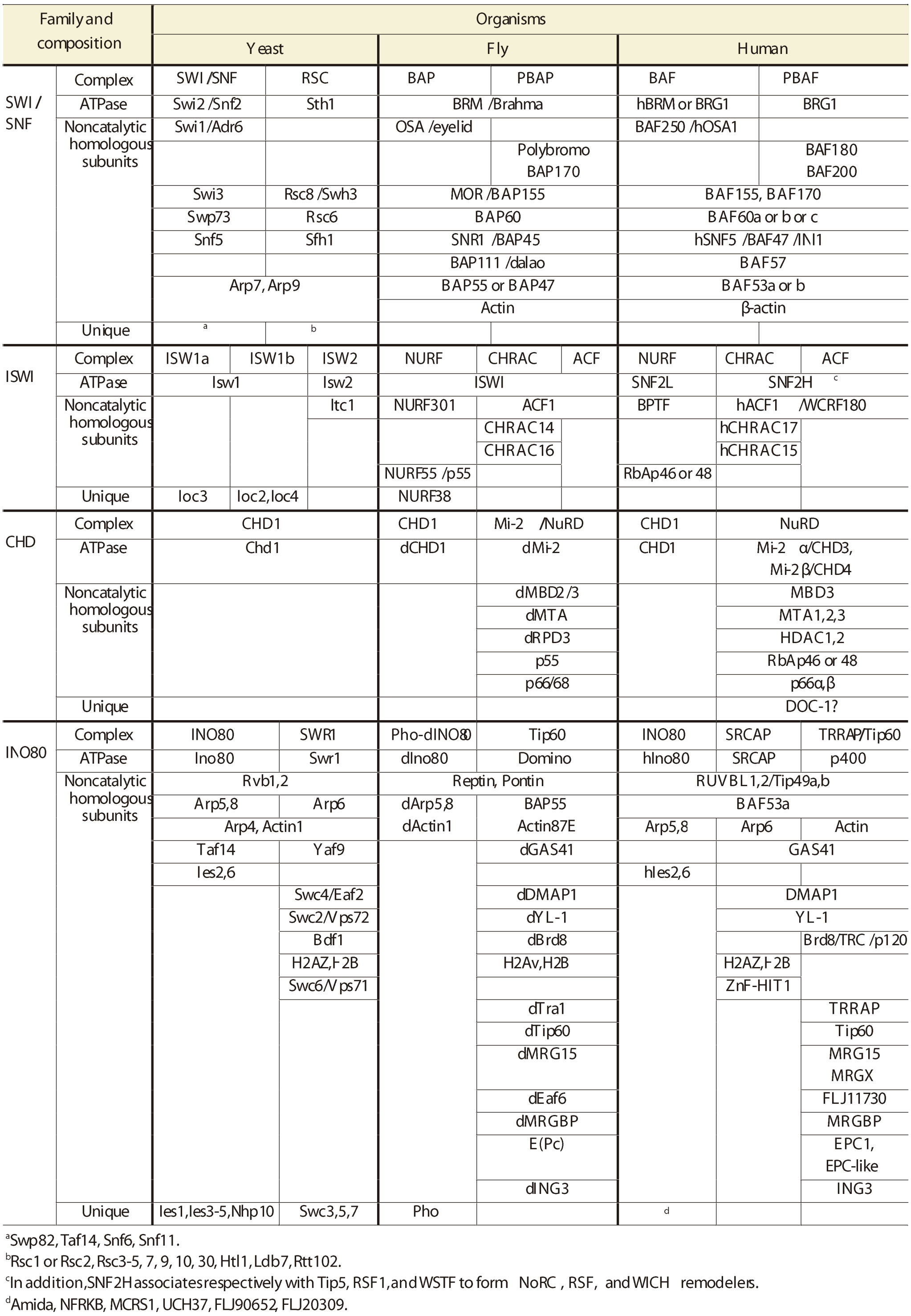
R loops and chromatin.

Although it is not clear the mechanisms surrounding the R loop-induced genome instability, many studies have related the R loop formation with changes in chromatin state. There are many observations suggesting that R loops locally open the chromatin structure, possibly by regulating nucleosome occupancy, positioning and/or turnover. DNA-RNA hybrids alter the B-structure of DNA and prevent nucleosome wrapping *in vitro* (Dunn and Griffith, 1980), and *in vivo*, loss of FACT complex, which swaps nucleosomes around the RNA polymerase during transcription elongation, causes R loop accumulation suggesting that nucleosome redeposition during transcription prevents R loop formation.

It is though that R loops can also trigger the recruitment of chromatin modifying enzymes to alter the chromatin state. After the mapping of R loops throughout the genome, a set of histone modification have been associated to R loops, finding out that their accumulation correlates with transcription active marks such as H3K3me1, H3K4me3 and H3 acetylation (H3K27ac), supporting the idea that R loops are formed in a open chromatin. However, another studies correlate R loop formation with chromatin compaction. THO mutants in yeast are characterized by a high genome instability associated by R loop formation. This increase in R loop accumulation is accompanied with higher levels of H3S10P (a mark typically associated with condensed chromosomes during mitosis) outside of mitosis, and the overexpression of RNase H suppresses H3S10P signal (Castellano-Pozo et al., 2013). In this context of R loop causing chromatin compaction, other study has shown that R loops can promote chromatin condensation at the 3' end of genes causing transcription termination (Skourti-Stathaki et al., 2014)

Chromatin

Genomic DNA is not naked inside cells, but it is wrapped 146 bp around nucleosomes which are formed by two copies of histones H2A, H2B, H3 and H4, constituting the chromatin. The consecutive nucleosomes are bound by naked DNA non-histone provided forming the chromatin fiber. A second level of organization would imply the histone H1 which binds nucleosomes one above another to form the 30 nm fiber. Finally, DNA can pack even more, generating the chromosomes observed during metaphase. According to its activity, there are two types of chromatin: heterocromatin, which is highly condensed and inactive, and the euchromatin, slightly compacted, rich in transcribed genes and for this reason active. However, what defines the state of chromatin are the basic units of nucleosomes: histones and their post-translational modifications (PTMs). In addition, chromatin can also be affected by altering whole nucleosomes through chromatin remodelers. In both processes, histone chaperons can play a role helping to chromatin modifiers and remodelers to carry out their functions.



Family and composition		Organisms								
		Yeast			Fly			Human		
SWI / SNF	Complex	SWI /SNF	RSC		BAP	PBAP		BAF	PBAF	
	ATPase	Swi2 /Snf2	Sth1		BRM /Brahma			hBRM or BRG1	BRG1	
	Noncatalytic homologous subunits	Swi1/Adr6			OSA /eyelid			BAF250 /hOSA1		
						Polybromo BAP170			BAF180 BAF200	
		Swi3	Rsc8 /Swh3		MOR /BAP155			BAF155, BAF170		
		Swp73	Rsc6		BAP60			BAF60a or b or c		
		Snf5	Sfh1		SNR1 /BAP45			hSNF5 /BAF47 /INI1		
					BAP111 /dalao			BAF57		
		Arp7, Arp9			BAP55 or BAP47			BAF53a or b		
					Actin			β-actin		
	Unique	[a]	[b]							
ISWI	Complex	ISW1a	ISW1b	ISW2	NURF	CHRAC	ACF	NURF	CHRAC	ACF
	ATPase	Isw1		Isw2	ISWI			SNF2L	SNF2H [c]	
	Noncatalytic homologous subunits			Itc1	NURF301	ACF1		BPTF	hACF1 /WCRF180	
						CHRAC14			hCHRAC17	
						CHRAC16			hCHRAC15	
					NURF55 /p55			RbAp46 or 48		
	Unique	Ioc3	Ioc2, Ioc4		NURF38					
CHD	Complex	CHD1			CHD1	Mi-2 /NuRD		CHD1	NuRD	
	ATPase	Chd1			dCHD1	dMi-2		CHD1	Mi-2 α/CHD3, Mi-2 β/CHD4	
	Noncatalytic homologous subunits					dMBD2 /3			MBD3	
						dMTA			MTA1,2,3	
						dRPD3			HDAC1,2	
						p55			RbAp46 or 48	
						p66/68			p66α,β	
	Unique								DOC-1?	
INO80	Complex	INO80	SWR1		Pho-dINO80	Tip60		INO80	SRCAP	TRRAP/Tip60
	ATPase	Ino80	Swr1		dIno80	Domino		hIno80	SRCAP	p400
	Noncatalytic homologous subunits	Rvb1,2			Reptin, Pontin			RUVBL1,2/Tip49a,b		
		Arp5,8	Arp6		dArp5,8	BAP55		BAF53a		
		Arp4, Actin1			dActin1	Actin87E		Arp5,8	Arp6	Actin
		Taf14	Yaf9			dGAS41			GAS41	
		Ies2,6						hIes2,6		
			Swc4/Eaf2			dDMAP1			DMAP1	
			Swc2/Vps72			dYL-1			YL-1	
			Bdf1			dBrd8				Brd8/TRC /p120
			H2AZ,H2B			H2Av,H2B			H2AZ,H2B	
			Swc6/Vps71						ZnF-HIT1	
						dTra1				TRRAP
						dTip60				Tip60
						dMRG15				MRG15 MRGX
						dEaf6				FLJ11730
						dMRGBP				MRGBP
						E(Pc)				EPC1, EPC-like
						dING3				ING3
	Unique	Ies1, Ies3-5, Nhp10	Swc3,5,7		Pho			[d]		

[a] Swp82, Taf14, Snf6, Snf11.

[b] Rsc1 or Rsc2, Rsc3-5, 7, 9, 10, 30, Htl1, Ldb7, Rtt102.

[c] In addition, SNF2H associates respectively with Tip5, RSF1, and WSTF to form NoRC, RSF, and WICH remodelers.

[d] Amida, NFRKB, MCRS1, UCH37, FLJ90652, FLJ20309.

Table I1. List of chromatin remodelers and their homologues in yeast, fly and humans. From (Clapier and Cairns, 2009)

Chromatin remodelers

Chromatin remodelers are tools present in cells from yeast to humans **(Table I1)** which couple the energy released from the ATP hydrolysis with changes in the nucleosomes, sliding octamers of histones across the DNA, changing the conformation of nucleosomal DNA or modifying the compositions of octamers. They can be classified in four different families: SWI/SNF, ISWI, CHD and INO80. All of these families posses and ATPase domain split in 2 parts (DExx and HELIC) where there is an insertion whose size depends on the specific family of chromatin remodeler. Moreover, they also differentiate in the domains adjacent to the ATPase domain, which confers the specificity in their functions.

SWI/SNF family.

It was the first family described in two independent genetic screenings for altered gene expression involved in regulating mating type switching (SWI) and sucrose fermentation in yeast (Sucrose Non-Fermenting). T There are two SWI/SNF ATPases in yeast: Swi2/Snf2, which is part of SWI/SNF complex, and Sth1, from the RSC complex. They all carry out their function through the bromodomain targeted to acetylation marks present in the tail of histones. Among the functions of this chromatin remodeler family, most of studies relate it with the activation of gene transcription (Armstrong and Emerson, 1998), although others works suggest they could also contribute to gene repression (Trouche et al., 1997). They can also participate in replication initiation and nucleotide excision repair (Flanagan and Peterson, 1999, Hara and Sancar, 2003, Gaillard et al., 2003)

ISWI family.

This family was name as Imitating SWItch due to was homologue to SWI/SNF family only in it ATPase domain. Members of this family are involve in nucleosome assembly and spacing and they exert their function through HANDSANT-SLIDE domains which allows to bind DNA. They are involved in transcription suppression (Vary et al., 2003, Mellor and Morillon, 2004) and also facilitates the *de novo* nucleosome assembly. In yeast there are two ISWI ATPases: Isw1 and Isw2 forming part of four different complexes whose additional members provide them specificity to their targets

CHD family.

This family contains tandem chromodomains at the N-terminal region, a DNA-binding domain at the C-terminal region that binds to A-T rich regions preferentially and helicase/ATPase like-SNF2 domains (Chromodomain - Helicase - DNA binding). This family participates in nucleosome spacing and deposition. In yeast there is only one CHD member, Chd1 which is able to bind to methylated histone H3 with unknown functions (Pray-Grant et al., 2005). It also regulates transcription elongation (Simic et al., 2003) and prevents histone exchange maintaining chromatin structure with Isw1 (Smolle et al., 2012)

INO80 family.

There are two different member in this family: Swr1 and Ino80, which gives name to the family, since it is responsible for regulation of inositol-responsive gene expression. They present a Helicase-SANT (HSA) domain essential for actin and ARP (actin related proteins) binding. The members of this family are involved in histone variant exchange, such as variant H2A.Z deposition. They can bind to the replication forks and Holliday junctions, interfere in nucleosome eviction (Krogan et al., 2003, Mizuguchi et al., 2004, Tsukuda et al., 2005, van Attikum et al., 2007) and also is believed to participate in DNA repair (Morrison et al., 2004)

Post-translational modifications of histones

Histones regulate the state of chromatin through several modifications, which in addition, can alter the function of many other factors altering the recruitment of proteins and complexes and, for so, this PTMs can have a role in multiple cellular processes such as transcription and replication of DNA as well as its repair when damaged. There are many PTMS such as, ubiquitylation, sumoylation, deimination, etc the most important for the development of this thesis are acetylation (and deacetylation), methylation (and demethylation) and phosphorylation.

Acetylation of histones occurs at their lysine residues and is catalyzed by histone acetyltransferases (HATs). These enzymes transfer an acetyl group from acetyl-CoA cofactor to the ε-amino group of the lysine side chain. This reaction has as a consequence the neutralization of charges between DNA and histones weakening their interactions. For this reason, it is though that acetylation would have a role in transcription activation, since it relaxes chromatin, making it more accessible to transcription factors (euchromatin). Moreover, this acetylation mark provides a specific bind site for bromodomain containing proteins, such as SWI/SNF chromatin remodelers. Apart from their function in transcription activation, there are another acetylation marks involved in DNA repair, such as H3K14 acetylation (Wang et al., 2012) or in replication-coupled nucleosome assembly, such as H3K56 acetylation (Masumoto et al., 2005). Acetylation of lysines is reversible, in a reaction catalyzed by histone deacetylases (HDACs). As it returns the positive charge to histones, it is thought that this reaction reinforces the interaction between DNA and histones, which leads to chromatin compaction (heterochromatin) and transcription repression since it difficulties the access to DNA by transcription factors.

Another histone PTM is methylation of lysines and arginines which can be mono-, di-, or trimethylated and mono- or dimethylated, respectively. This reaction is catalyzed by histone methyltransferases (HMTs) and did not cause the loss of histone charge. Its function is carried out by recruiting chromodomain-containing proteins which alters the chromatin structure. Depending on the interacting partners and the methylated site, can either activate or repress transcription. For example, H3K4me3 leads to transcription activation (Howe et al., 2017), whereas H3K23me3 leads to transcription repression (Boros et al., 2014).

Serines, threonines and also tyrosines are susceptible to be phosphorylated or dephosphorylated by kinases or phosphatases, respectively. It seems that histone phosphorylation may be a common feature of signal transduction pathways, in response to cell cycling or cellular stress. One of the most phosphorylated marks studied is H2A Ser129 phosphorylation by Mec1/Tel1 kinases during DSB formation. It is also known the H3S10-P mentioned before as a mark of chromatin condensation during mitosis and correlated with THO mutants which accumulate R loops.

OBJECTIVES

Objectives

The main goal of this thesis is to study the contribution of histones, chromatin remodelers and histone modifiers in the formation of DNA-RNA hybrids and its associated genome instability in the yeast *Saccharomyces cerevisiae*. For this purpose, we addressed the following specific objectives:

1. To establish new methodologies for the study of R loop length, frequency and distribution in the yeast *Saccharomyces cerevisiae* with the aim of determining the relevance of these features in genome instability.

2. To identify new factors involved in DNA-RNA hybrid formation among chromatin remodelers and histone modifiers.

CONCLUSSIONS

Conclusions

1. The DNA-RNA hybrid accumulation in*H3∆1-28, H3K9-23A* and *H4K31Q* histone mutations does not lead to LOH or plasmid loss, in contrast to previously reported DNA-RNA hybrid accumulating mutants, such as *hpr1*∆.

2. The *H3K9-23A* mutation suppresses the replication fork progression impairment of *hpr1∆.* This supports previous results that indicate that histone H3 lysines 9, 14, 18 or 23 residues are required for the genetic instability associated with DNA-RNA hybrids. In this thesis, we establish that *H3K9A*, and not *H3K14A* or *H3K23A*, is the mutation responsible of this suppression.

3. The *H3∆1-28* and *H3K9-23A* mutations alter the pattern of nucleosome positioning along the genome. This is specific for these particular substitutions, not being observed in *H3K9-23R*. Notably, we have observed no changes in nucleosome positioning for *H4K31Q*, indicating that changes in nucleosome positioning are not the cause of the DNA-RNA hybrid accumulation observed.

4. The length of R loops, as determined by the bisulfite-modification assay at the *GCN4* gene, ranges from 38 bp to 513 bp with an average of 142 bp. Strikingly, R loop length is similar in wild-type and several R-loop accumulating strains, regardless of whether they produce genome instability or not. This indicates that DNA-RNA hybrid length is not relevant–for the generation of genetic instability. Bisulfite-modification followed by qPCR can be used as a new tool, as an alternative to S9.6-based methodologies such as DRIP, to detect relative variations in DNA-RNA hybrids at specific genomics locations. Using this method we estimate that the frequency of

DNA-RNA hybrids is low, since we found no positive hits at the *GCN4* gene among 500 yeast colonies analyzed.

5. Overexpression of a hyper-mutator AID induces mutations genome-wide, preferentially in cytosines of large genes and in an R loop-dependent manner. However, the low number of induced mutations makes this tool insufficient to map R loops genome-wide.

6. The loss of the Snf2, Isw1 or Isw2 catalytic subunits of the SWI/SNF and ISWI family of chromatin remodelers does not enhance R loop accumulation or R loop-dependent genome instability.

7. The loss of the Rtt109 acetyl-transferase leads to an accumulation of DNA-RNA hybrids in all cell cycle phases.Rtt109 prevents DNA-RNA hybrid accumulation through its catalytic activity but not through its reported role of H3K56 acetylation.

8. Among all the reported substrates of Rtt109, H3K14 and H3K23 are relevant for its role preventing the accumulation of DNA-RNA hybrids, since *H3K14A* and *H3K23A* mutations increase DNA-RNA hybrids and *H3K14Q* and *H3K23Q* acetyl-mimetic mutants prevent hybrid accumulation in the absence of Rtt109.

9. We favor a model in which the persistence of unrepaired DSBs leads to the observed accumulation of DNA-RNA hybrids in *rtt109*Δ, since the previously reported repair phenotypes of defective repair in v in *rtt109*Δ are not dependent on hybrids.

MATERIALS AND METHODS

Yeast strains, plasmid and primers

Yeast strain	**Genotype**	**Source**
H3WT	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[H3]-URA3*	(Dai et al., 2008)
H3∆1-28	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[∆1-28]-URA3*	
H3K9-23A	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3*	
H4WT	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[H4]-URA3*	
H4K31Q	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3*	
H3hpr1Δ	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[H3]-URA3 hpr1Δkan*	This study
BY4743	*MATa/MATα his3∆/ his3∆ leu2∆0/ leu2∆0 met15∆/MET15 lys2∆0/LYS2 ura3∆0/ ura3∆0*	This study
YDIH-4B	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ trp1Δ63 ura3Δ0/ura3Δ0 met15Δ0/ met15Δ0 can1::MFA1pr-HIS3/CAN1 hht1-hhf1::NatMX4/ hht1-hhf1::NatMX4 hht2hhf2::[H3]-URA3/ HHT2-HHF2*	D. García-Pichardo
YDI28-11C	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ TRP1 ura3Δ0/ura3Δ0 met15Δ0/ met15Δ0 can1::MFA1pr-HIS3/ CAN1 hht1-hhf1::NatMX4/ hht1-hhf1::NatMX4 hht2hhf2::[∆1-28]-URA3/hht2-hhf2::[∆1-28]-URA3*	D. García-Pichardo

YDIK9-2D	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ TRP1 ura3Δ0/ura3Δ0 met15Δ0/ met15Δ0 can1::MFA1pr-HIS3/ can1::MFA1pr-HIS3 hht1-hhf1::NatMX4/ hht1hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3/ hht2-hhf2::[K9-23A]-URA3*	D. García-Pichardo
YDIK31-9A	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ TRP1 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 can1::MFA1pr-HIS3/ can1::MFA1pr-HIS3 hht1-hhf1::NatMX4/ hht1hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3/hht2-hhf2::[K31Q]-URA3*	D. García-Pichardo
YDIH-18B	*MATa/Matα his3/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 ura3/ura3 met15Δ0/ MET15 CAN1/ CAN1 hht1-hhf1::NatMX4/ hht1-hhf1::NatMX4 hpr1Δ::TRP1/hpr1Δ::TRP1*	D. García-Pichardo
W303-1A	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
U678-4C	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1hpr1Δ::HIS3*	Piruat and Aguilera, 1998
DD379	*W303-1A rad24Δ::TRP1*	Kanellis *et al.*, 2003
WHR24-3B	*W303-1A hpr1Δ::HIS3 rad24Δ::TRP1*	(Gomez-Gonzalez et al., 2009)
K923H	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 hpr1Δ::Hyg*	This study
K923HR	*MATa his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 can1::MFA1pr-HIS3 hht1-hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3 hpr1Δ::Hyg rad24Δ::Kan*	This study
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
Ybp249	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ RAD5*	B. Pardo

Yeast strain	**Genotype**	**Source**
WH101R5	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ RAD5 hpr1-101 (hpr1-L586P)*	This study
YMFT1	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ RAD5 mft1Δ::Kan*	This study
thp1		
WEIII-36	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ pob3-7*	(Herrera-Moyano et al., 2014)
XEI-13	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 lys2 met15 trp1-1 ura3-1 spt16-11*	(Herrera-Moyano et al., 2014)
YTOP1	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ RAD5 mft1Δ::Hyg*	This study
SEN-R	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 lys2 met15 trp1-1 ura3-1 sen1-1*	M. San Martín
RNH-R	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 lys2 met15 trp1-1 ura3-1 rnh1Δ::Kan rnh201Δ::Kan*	M. San Martín
YDIH-CAN	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ trp1Δ63 ura3Δ0/ura3Δ0 met15Δ0/ met15Δ0 CAN1/CAN1 hht1-hhf1::NatMX4/ hht1-hhf1::NatMX4 hht2hhf2::[H3]-URA3/ HHT2-HHF2*	This study
YDI28-CAN	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ TRP1 ura3Δ0/ura3Δ0 met15Δ0/ met15Δ0 CAN1/ CAN1 hht1-hhf1::NatMX4/ hht1-hhf1::NatMX4 hht2hhf2::[∆1-28]-URA3/hht2-hhf2::[∆1-28]-URA3*	This study
YDIK9-CAN	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ TRP1 ura3Δ0/ura3Δ0 met15Δ0/ met15Δ0 CAN1/CAN1 hht1-hhf1::NatMX4/ hht1hhf1::NatMX4 hht2-hhf2::[K9-23A]-URA3/ hht2-hhf2::[K9-23A]-URA3*	This study

Yeast strain	Genotype	Source
YDIK31-CAN	*MATa/Matα his3Δ200/his3 leu2Δ0/ leu2Δ0 lys2Δ0/ lys2Δ0 trp1Δ63/ TRP1 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 CAN1/CAN1 hht1-hhf1::NatMX4/ hht1hhf1::NatMX4 hht2-hhf2::[K31Q]-URA3/hht2-hhf2::[K31Q]-URA3*	This study
YSNF2	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ RAD5 snf2Δ::Kan*	This study
YISW1	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ RAD5 isw1Δ::Kan*	This study
YISW2	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1Δ RAD5 isw2Δ::Kan*	This study
YPL001W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hat1Δ::Kan*	EUROSCARF
YEL056W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hat2Δ::Kan*	EUROSCARF
YDR448W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ada2Δ::Kan*	EUROSCARF
YGR252W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn5Δ::Kan*	EUROSCARF
YBR081C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spt7Δ::Kan*	EUROSCARF
YLR055C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spt8Δ::Kan*	EUROSCARF
YLL002W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rtt109Δ::Kan*	EUROSCARF
YDR359C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eaf1Δ::Kan*	EUROSCARF
YOR023C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ahc1Δ::Kan*	EUROSCARF
YBL052C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sas3Δ::Kan*	EUROSCARF
YNL330C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpd3Δ::Kan*	EUROSCARF
YOL004W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sin3Δ::Kan*	EUROSCARF
YMR263W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sap30Δ::Kan*	EUROSCARF
YMR075W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rco1Δ::Kan*	EUROSCARF
YNL021W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hda1Δ::Kan*	EUROSCARF
YPR068C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hos1Δ::Kan*	EUROSCARF
YKR029C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 set3Δ::Kan*	EUROSCARF
YBR175W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 swd3Δ::Kan*	EUROSCARF
YJL168C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 set2Δ::Kan*	EUROSCARF

Yeast strain	**Genotype**	**Source**
YDR440W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gis1Δ::Kan*	EUROSCARF
YDR096W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dot1Δ::Kan*	EUROSCARF
YER169W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rph1Δ::Kan*	EUROSCARF
YER051W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 jhd1Δ::Kan*	EUROSCARF
YJR119C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 jhd2Δ::Kan*	EUROSCARF

Plasmid	**Description**	**Source**
pWJ1344	YCp containing Rad52::YFP fusion under its own promoter (*LEU2* marker)	(Feng et al., 2007)
pWJ1314	YCp containing Rad52::YFP fusion under its own promoter *(TRP1* marker)	(Alvaro et al., 2007)
pRS413GAL	Ycp pRS413 with the GAL1 promoter and the CYC1-terminator	(Gavalda et al., 2016)
pRS313-GAL::RNH1	YCp pRS313 containing the GALp::RNH1 fusion	(Gavalda et al., 2016)
p414GAL	Ycp pRS414 with the GAL1 promoter and the CYC1-terminator	(Garcia-Rubio et al., 2003)
p414GALAID	YCp pRS313 containing the GALp::AID fusion	(Gomez-Gonzalez and Aguilera, 2007)
pLlacZ-GALAID	YCp pSCH204 containing GALp::AID fusion	(Garcia-Pichardo et al., 2017)
pRS217GAL	Ycp pRS317 with the GAL1 promoter and the CYC1-terminator	(Garcia-Pichardo et al., 2017)
pRS317-GAL::RNH1	YCp pRS317 containing the GALp::RNH1 fusion	(Garcia-Pichardo et al., 2017)
p317RTT109wt	Ycp pRS317 with wild-type *RTT109* gene	(Han et al., 2007)
p317D89A	Ycp pRS317 with *RTT109* gene mutated at aspartate 89 to alanine	(Han et al., 2007)
p317D89N	Ycp pRS317 with *RTT109* gene mutated at aspartate 89 to asparagine	(Han et al., 2007)

pARSGLBIN-Leu2	YCp pARSGLB-IN in which the LEU2 gene was generated in vivo by homologous recombination between the *leu2* repeats	(Prado and Aguilera, 2005)
pARSGLBOUT	YCp pARSGLB-OUT in which the LEU2 gene was generated in vivo by homologous recombination between the leu2 repeats	(Prado and Aguilera, 2005)
pTINV-HO	Ycp pRS414 with TINV and GAL::HO	(Ortega et al., 2019)
pRS425AID*	YCp pRS425 with the GAL::AID* fusion	(Taylor et al., 2013)
pRS424AID*	YCp pRS424 with the GAL::AID* fusion	This study

Primer	**Sequence 5' to 3'**
	Gene disruption
rad24 pFA6a F	ATGGATAGTACGAATTTGAACAAACGGCCCTTATTACAATATAGTCTCAGTTCATTGGGCGGATCCCCGGGTTAATTAAGG
rad24 pFA6a R	TTAGAGTATTTCCAGATCTGAATCTGAAAGGGACTCACTGATAACTGGCGCTTTACGCGGATCGATGAATTCGAGCTCG
hpr1 pFA6a F	ACAATTCAAGAGGCATTAAAACTTGGGCAAAGGAGTAATACGGATCCCCGGGTTAATTAAGG
hpr1 pFA6a R	GAATTTCTTATCAGTTTAAAATTTCTATTAAGAGGATAATATCGATGAATTCGAGCTCG
top1	TAAAAAAAATCTAAAGGGAGGGCAGAGCTCGAAACTTGAAACGCGTAAAAATG
top1	GCGAACTTGATGCGTGAATGTATTTGCTTCTCCCCTATGCTGCGTTTCTTTGCG
mft1 F	GGCTGTAAAAAAGGAATCAAAGAACTAAAGCCAAAGGAGACTAACTCACAATG
mft1 R	CCTATGTGTCTATATGCCTTTTCTATTTAGTAAGAGCTATGCATTATACGTGG
snf2 F	TCGCGACTTTCTGCTATTTTCACGACTTTCGATTAATTATCTGCCCGG

	ATCCCCGGGTTAATTAA
Primer	**Sequence 5' to 3'**
	Gene disruption
snf2 R	GTCTACGTATAAACGAATAAGTACTTATATTGCTTTAGGAAGGTAGAATTCGAGCTCGTTTAAAC
isw1 F	CCATAGCATGATATTACCTGT
isw1 R	AGTAATGCTGACTCTGTCTAT
isw2 F	GCTACTCGTCGTTGCTTAGTA
isw2 R	AATTAGTTAAAGCGGCTCGAC
rtt109 F	AAGAGAGCATGATAAATCCCCGT
rtt109 R	ACTAGCCATTCTTTATCCGTCGT
	bisulfite
GCN4 N F	TACCAATTGCTATCATGTACCCGT
GCN4 N1 R	TTGGCAGTAGAAGTGGAAGCA
GCN4 C1 R	TTAACAATAAAAATAAAAACA
GCN4 N2 R	GGAGTTGAATCAGTGCTTGACG
GCN4 C2 R	AAAATTAAATCAATACTTAACA
GCN4 N3 R	GCATCTTCTAGAACAGGAGTGGG
GCN4 C3 R	ACATCTTCTAAAACAAAAATAAA
GCN4 N4 R	AATGAAATCAGCGTTCGCCA
GCN4 C4 R	AATAAAATCAACATTCACCA
GCN4 N5 R	GTGTAAAATTCTACTTAAGAA
GCN4 C5 R	ATATAAAATTCTACTTAAAAA
GCN4 N6 R	TAATCAGAAGATTATGGGTTC
GCN4 C6 R	TAATCAAAAAATTATAAATTC
GCN4 N4 F	TGGCGAACGCTGATTTCATT
GCN4 C4 F	TAACAAACACTAATTTCATT
GCN4 N7 R	TATCTAAACCTTAGCGTTTGCATTC
	qPCR
GCN4 F	TTGTGCCCGAATCCAGTGA
GCN4 R	TGGCGGCTTCAGTGTTTCTA

Primer	Sequence 5' to 3'
	qPCR
TRP F	CGGCTTGCAGAGCACAGA
TRP R	AGCAAGTCAGCATCGGAATCTAG
PRD1 F	CCGCCTAATTGGTCGTTCAC
PRD1 R	GTTGTTGATGATTTCGTTGG
PDR5 F	GTCAGAGGCTATATTTCACTGGAGA
PDR5 R	TACGTCTTGTTTCGGCCTTAATC
SPF1 F	CCCGTGGTAAACCTTTAGAAA
SPF1 R	ATATGAACGGCAAATTGAGAC
28S F	TCAACTTAGAACTGGTACGG
28S R	GCTTGGTTGAATTTCTTCAC
PDC1 F	CCTTGATACGAGCGTAACCATCA
PDC1 R	GAAGGTATGAGATGGGCTGGTAA

Media used in this study

- **YPAD**: 1% yeast extract, 2% bacto-peptone, 2% glucose, 20 mg/L adenine).
- **SD**: 2% glucose, 0.17% yeast nitrogen base (YNB) without amino acids, 0.5% ammonium sulfate.
- **SC**: SD containing aminoacids.
- **SGal**: SC without glucose containing 2% filtered-galactose.
- **SRaf**: SC without glucose containing 2% raffinose
- **SPO**: 1% potassium acid, 0.1% yeast extract, 0.005% glucose)

Experiments

Yeast transformation and disruption

Yeast strains were transformed using the lithium acetate protocol (Gietz et al., 1995). Cells at exponential growth were pelleted and resuspended in the transforming mix (0.1 % lithium acetate, PEG-50, 50 µg salmon sperm and 100 ng or 5 µg of plasmid or cassette for disruption, respectively) and incubated during 30 minutes at 30ºC. Heat shot was carried out placing cells at 42ºC during 20 minutes and then were plated in SC lacking requirements or YPD supplemented with antibiotics specific for transformant or mutant selection.

Drop assay.

Serial dilutions of exponential culture were plated in medium supplemented with different concentrations of HU, MMS and/or CPT and then incubated at 30ºC during 2 and 3 days.

Plasmid loss assay.

Cells were transformed with a plasmid and plated in a selective media. Transformants were then grown in non selective media during 3.5 hours and plated into YPD or in the selective media of transformation to count total and plasmid-containing cells.

FACS analysis.

Cells from exponential culture were fixed in ethanol, wash twice with 1 ml of cold 50 mM sodium citrate pH 7 and treated with 250 mg of RNase A during 1 hour at 50 ºC followed by 1 mg of proteinase K at 37ºC 1 hour. Propidium iodide was added at a final concentration of 16 µg/ml to stain the DNA. DNA content was then analyzed in a FACScan cytometer (Becton Dickinson). A total of 100,000 cells were counted.

Protein extraction.

Proteins were extracted from 5 ml of exponential cultures centrifugated and wash in cold water. Pellet was resuspended in 600 µl of 10 % TCA with 200 µl of glass beads and shaked at maximum speed in a multivortex for 10 minutes at 4 ºC. After centrifugation, pellets were resuspended sequentialy in 50 µl of water, 50 µl of 1 M Tris and 100 µl of 2X Laemmli buffer (60 mM Tris pH6.8, 2 % SDS, 10 % glycerol, 0.2 % bromophenol blue, 100 mM DTT)

Recombination assay.

Transformants with the recombination system were grown in plates at 30ºC during 3 or 4 days and five ten-fold serial dilutions were finally plated in media with or without leucine to count total or recombinant cells, respectively. Recombination frequencies were calculated as the median value of six independent colonies, coming from three independent transformants.

CTAB DNA extraction.

Cells were arrested in G1 with α-factor and released into fresh medium containing 40 mM HU for 30 min prior to DNA extraction. DNA extraction was performed with the cetyltrimethylammonium bromide method, and neutral-neutral 2-D gel electrophoresis was performed as described in Wellinger et al. (2003) with some modifications. 100 ml of the cultures were collected, washed with 5 ml of chilled water and carefully resuspended in 1 ml of spheroplasting buffer (1M sorbitol, 10 mMEDTA pH 8, 0.1% b-mercaptoethanol, 2 mg/ml Zimoliase 20T), and then incubated at 30 ºC 1h under soft agitation. The spheroplasts were washed with 500 µl of cold water and resuspended in 400 µl of cold water. Spheroplasts were lysed by adding 500 µl of solution I (1.4 MNaCl, 100mM Tris-Cl pH 7.6, 25mMEDTApH 8, 2%CTAB). RNAwas removed by incubating them 30 min at 5 ºC with 400 mg of RNase A. Proteins were removed by incubating them with 800 mg of Proteinase K overnight at 30 ºC under very soft agitation. After centrifugation, pellet and supernatant were treated separately. The supernatant was extracted with 500 µl (24:1) Cloroform:Isoamyl Alcohol. DNAwas precipitated with two volumes of solution II (50mM Tris-Cl pH 7.6, 10mM EDTA pH 8, 1% CTAB) and resuspended in 250 µl of solution III (1.4 M NaCl,1 mM EDTA pH 8, 10 mM Tris-Cl pH 7.6). The original pellet was resuspended in 400 µl of solution III and incubated 1h at 50 ºC. DNA was extracted with 200 µl (24:1) Cloroform:Isoamyl Alcohol and combined with the DNA obtained from the supernatant. The whole sample was precipitated then with 1 vol isopropanol at room temperature, washed with 70% ethanol and resuspended in 100 µl 10 mM Tris-Cl pH 8. DNA was prepaired to be used for 2D gel electrophoresis or bisulfite modification assay.

2D-gel electrophoresis

DNA isolated by CTAB method was digested with *Pvu*II and then charged into a 0.4 % agarose gel 1 % TBE without ethidium bromide. Electrophoresis was carried out at constant voltage (50 V). From each lane, a single piece of gel containing 3 to 10 kb fragment was cut and used as the well of the second dimension electrophoresis in a 1 % agarose gel 1 % TBE with ethidium bromide. Electrophoresis run overnight at 4 ºC and onced finished, the gel was treated with 0.25 N HCl 10 minutes, denaturation solution (0.5 M NaOH, 1.5 M NaCl) 30 minutes and neutralization solution (1M $AcNH_4$, 0.02 M NaOH).

DNA was then transferred to a Hybond N (GE Healthcare) membranes by Southern blot and analyzed using a ^{32}P-labeled 0.5-kb *SPF1* probe.

Bisulfite modification assay

DNA isolated by CTAB method was digested with NdeI, NotI and XhoI. The bisulfite modification assay was performed essentially as described(Yu et al., 2003). Genomic DNA was diluted in 42 µl of distilled water with 17.5 µl of 20 mM hydroquinone and 460.5 µl of 2.5 M sodium bisulfite (pH 5.2). The mixture was sealed with mineral oil in a 500 µl microcentrifuge tube and incubated for 16 hr at 37 ºC in the dark. Bisulfite-treated DNA was purified with the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Purified bisulfite-treated DNA was desulfonated with 0.3 M NaOH at 37 ºC for 15 min. Desulfonated DNA was recovered by ethanol precipitation and resuspended in TE (pH 8.0). Bisulfite-modified DNA was used as a template for PCR with either a pair of native primers, or a native primer paired with a 'converted' primer, the sequence of which matched the conversions anticipated owing to deamination of C to U in either the transcribed (TS) or nontranscribed (NTS) strand. PCR bands were purified from agarose gels with the Wizard SV Gel and PCR Clean-Up System (Promega) and cloned in pGEMT-easy. Independent clones were sequenced. Only molecules with more than four consecutive expected C to T changes were considered to determine the R-loop length.

DRIP assay.

Cells coming from exponential groth in YPD or SC depending of the experiment were pelleted by centrifugation and resuspended in 2.4 ml of spheroplasting buffer (1 M sorbitol, 2 mM Tris–HCl pH 8.0,100 mM EDTA pH 8.0, 0.1% v/v beta-mercapto-ethanol, 2mg/ml zymoliase). Samples were incubated at 30 ºC during 30 minutes. After centrifugation, pellet was resuspended in 1.125 ml of solution I (0.8 mM GuHCl, 30 mM Tris–HCl pH 8.0, 30 mMEDTA pH 8.0, 5% Tween 20, 0.5% Triton X-100) together with 40 µl of 10 mg/ml RNase A and incubated at 37 ºC during 30 minutes. Then, 75 µl of 20 mg/ml proteinase K were added and samples stood at 50 ºC for 1 hour. DNA was purified by chloroform-isoamyl alcohol (24:1) and precipitated with 1 volume of isopropanol. With the help of a glass pasteur pipette, dNA was transferred to a new eppendorf where resuspended in 150 µl of 1X TE (1 mM Tris–HCl pH 7.5, 0.5 mM EDTA pH 8.0) and digested overnight with 50 U of *Hind*III, *EcoR*I, *BsrG*I, *Xba*I and *Ssp*I. Half of theDNA was treated with 3 µl of RNase H (New England BioLabs) overnight 37 ºC as RNaseH control. Both samples were incubated with S9.6 antibody- Dynabeads Protein A (Invitrogen) complexes (previously incubated overnight at 4 ºC) during 2.5 hours at 4º C. Samples were then washed 3 times with 1x binding buffer (10 mM NaPO4pH 7.0, 0.14 M NaCl, 0.05% Triton X-100). DNAwas eluted in 100 mL elution buffer (50 mM Tris pH 8.0, 10 mM EDTA, 0.5 % SDS) treated 45 min with 7 µl of 20 mg/ml proteinase K at 55 ºC and purified with Quiagen DNA purification kit. Real-time quantitative PCR was performed using iTaq universal SYBR Green (Biorad) with a 7500 Real-Time PCR machine (Applied Biosystems).

Chromatin immunoprecipitation (ChIP).

ChIP was performed as described (Hecht et al., 1999) with some modifications. 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).The chromatin was sonicated alternating 1 min high intensity and 1 min rest pulses for 15 min in Bioruptor sonication equipment. Samples were centrifuged for 15 min at 13000 rpm to eliminate cell debris. 20 µl of supernatant were processed as Input and 280 µl were immunoprecipitated. The immunoprecipitation was performed overnight at 4 ºC using Dynabeads Protein A (Invitrogen) previously incubated with the antibody for 4 hr rotating at 4 ºC. Beads were washed and chromatin was eluted in 250 µl elution buffer (50 mM Tris-HCl pH 7.4, 10 mM EDTA, 1% SDS) at 65 ºC for 10 min., treated with 6 µl of 50 mg/ml pronase for 1 hr at 42 ºC and decrosslinked for 6 hr at 65 ºC. Quiagen DNA purification kit was used to clean DNA. Real-time quantitative PCR was performed using iTaq universal SYBR Green (Biorad) with a 7500 Real-Time PCR machine (Applied Biosystems).

SCE intermediates analysis.

Analysis of the SCE intermediates was performed as described in Ortega et al. (2019). Cells transformed with pTINV-HO were grown in SRaf lacking tryptophan for mantaining plasmid until exponential growth. Galactose at 2 % was added to induced HO endonuclease overexpression and samples were collected after indicated time points.

DNA was extracted by phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated in isopropanol. DNA was resuspended in 200 μl of 1X TE (1 mM Tris–HCl pH 7.5, 0.5 mM EDTA pH 8.0) and digested with *Spe*I and *Xho*I overnitgh. DNA was precipitated with isopropanol and samples were electrophoresed using a 0.8 % agarose gel. Finaly, DNA was transferred into a Hybond N (GE Healthcare) membrane and hybridized with ^{32}P-labeled 0.22-kb *LEU2* probe. Quantification was performed by calculating the signal of the bands corresponding to DSBs, SCE, or SCE + ICR fragments relative to the total DNA in each line.

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