Anti-silencing at Telomeres

by

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Gene silencing in *Saccharomyces cerevisiae* has been conclusively linked to histone deacetylation and the subsequent formation of heterochromatin. The participating histone deacetylases have also been well characterized. In contrast, the opposing histone acetyltransferases (HAT) and their mechanism of action remain elusive. In particular, very little is known about the effects of two of the nine HATs in *S. cerevisiae*, *ESA1* and *RTT109*. The focus of my research was to test if these HATs influence the silencing of genes at the subtelomeric regions of *S. cerevisiae* and to assess their mechanism of action. To address these issues I used a panel of recombinant telomeric constructs that harbor the *URA3* reporter gene. These constructs were inserted at the VIIL telomere of the mutant strains *esa1-414* and *Δrtt109*. The level of gene repression of the *URA3* reporter in each construct in both of these strains has been assessed by a routine assay measuring the sensitivity of the strain to the drug 5-fluoroorotic acid. My results indicate than none of these HATs plays a specific major role in gene repression at telomeres. Instead, they show general anti-silencing activity that cannot be linked to any specific sub-telomeric elements. Like many other HATs, Esa1 and Rtt109 seem to operate through global acetylation of histones rather than through specific recruitment. These
results provide additional insight in the function of the HATs in gene silencing and suggest that there are multiple mechanisms that we do not yet understand.

The information provided here would help in future studies to determine the mode of action of anti-silencers and subtelomeric elements involved in telomeric silencing. These issues are of fundamental significance and will contribute to the dynamic and expanding field of epigenetics.
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1. **INTRODUCTION**

Eukaryotic DNA exists in an elaborate nucleoprotein complex called chromatin. Chromatin serves two major purposes. Firstly, it packages and compresses large amounts of DNA so that it can be accommodated in the cell nucleus. For instance, each human cell has about 1.8 meters of unwound DNA which normally resides in a nucleus of about 2 μm in diameter. Secondly, it is apparent that chromatin confers an array of regulatory mechanism for gene expression, DNA replication and DNA repair and recombination (ALLIS *et al.* 2006).

The major structural unit of chromatin is the nucleosome. It is built of highly basic proteins called histones. Two copies of each of histones H2A, H2B, H3 and H4 fuse to form the core histone octamer around which 146 base pairs of DNA wrap in about 1.7 turns. About 20 base pairs of DNA serve as “linker” between nucleosomes. This stretch of DNA binds histone H1 thus producing further tightening and compaction of DNA. Several nucleosomes then wind together in a helical fashion to form a structure called a solenoid. These structures package the long DNA strands into short chromatin fibers (ANNUNZIATO 2008; LEHNINGER 2005).

The nucleosomes are not just a simple packaging device for DNA. For example, histones show remarkable conservation with very little structural and sequence dissimilarity between diverse species. Nevertheless, many histone “variants” have been discovered in different organisms (TALBERT & HENIKOFF 2010). These histone variants confer subtle alterations to the nucleosome structure and are linked to specific perturbations of chromatin such as DNA repair, or linked to long term gene silencing (TALBERT & HENIKOFF 2010). In addition, all variants of histones are subject to extensive post-translational modifications including acetylation, methylation, phosphorylation, ubiquitination and others (KOUZARIDES 2007). These modifications are usually clustered at the N-terminal portions of the histones that protrude from
the nucleosomal core (Luger et al. 1997). It is well established that such modifications control
the strength of DNA-histone interactions and ultimately regulate the access of transcription,
replication and repair factors to DNA. Even more importantly, the specific arrangements of post-
translational modifications at the N-termini of histones (the so called histone code) provide
specific domains for interaction with numerous chromatin factors or other regulatory proteins
(Kouzarides 2007). Therefore, despite the seemingly uniform structure of the nucleosome, it
contributes substantial diversity ultimately leading to specific forms of chromatin at different
genomic loci.

In general, two major forms of chromatin have been characterized. The so called
heterochromatin and euchromatin have been initially defined as differentially staining regions of
nuclear substance with heterochromatin more intensely stained than euchromatin. It has been
later demonstrated that euchromatin is the site of active transcription, while heterochromatin is
generally transcription-free (Buhler & Gasser 2007). Thus, the idea that chromatin structure
may regulate gene activity and gene expression has emerged. A considerable amount of evidence
has indicated that the maintenance and transition between heterochromatin and euchromatin
domains is critical for the correct execution of gene expression programs, DNA replication and
DNA repair (Allis et al. 2006). A hallmark of euchromatin is the significant level of histone
acetylation in the nucleosomes. In addition, euchromatin is lightly packaged and more
susceptible to nuclease degradation. In contrast, heterochromatin contains tightly packaged
DNA, regularly spaced nucleosomes and hypoacetylated histones. Importantly, eukaryotic cells
contain elaborate mechanisms for the transmission of the existing chromatin state into daughter
cells which are not very well understood. These epigenetic traits play a key role in the
continuation of gene expression programs, in gene silencing and are implicated in multiple
disorders including cancer and genetic disease (Cavalli 2006; Bredy et al. 2010; Chi et al. 2010; Ottaviani et al. 2008).

1.1. Maintenance and modification of chromatin state

The maintenance and transition of chromatin state are mediated by a complex network of activities including post translational modification of histones, nucleosome repositioning and the exchange and rebuilding of nucleosomes.

1.1.1. Post translational modifications of histones

1.1.1.1. Histone acetylation and deacetylation

The acetylation and deacetylation of the amino groups of conserved lysine residues in N-terminal histone tails is linked to transcriptional activity and is the most extensively studied histone modification (Shahbazian & Grunstein 2007). Acetylation neutralizes the basic charge on the histone tails which may reduce their affinity for DNA resulting in altering the interactions between histones and DNA and varied interactions between histones and other regulatory proteins (Allis et al. 2006). Thus, acetylation ultimately helps in furnishing an environment permissive for transcription. Besides this direct effect of histone acetylation on gene activity, indirect effector-mediated responses to histone modifications must be considered. Indeed, this acetylation or deacetylation along with other post translational modifications serve as a “binding platform” for interactions with other modifiers of histones, chromatin remodeling factors or histone chaperones (Shahbazian & Grunstein 2007; Kouzarides 2007). Eventually these histone mediated interactions will determine the overall architecture of the chromatin domain and its accessibility to regulatory proteins such as transcription, replication or repair factors. In this respect, Histone Acetyl Transferases (HATs) and the modification they introduce are most
frequently linked to gene activation; however exceptions of this rule can be attributed to the indirect effector-mediated responses (Kouzarides 2007). The opposing activities of the Histone Deacetylases (HDACs) counteract the activity of Histone Acetyl Tranferases (HATs) and normally lead to gene repression (Figure 1).

Both HATs and HDACs can act globally or focally through recruitment to specific loci. Elegant experiments on the IFN gene activation in mammalian cells (Agalioti et al. 2002) or at the PHO5 gene in S.cerevisiae (Cosma et al. 1999) have provided detailed insights on the recruitment of HATs by transcriptional activators prior to the switching on of the promoter and full activation. Under these circumstances it is believed that certain uncharacterized HDACs maintain the “off” state of the genes by global rather than focal activity (Carrozza et al. 2003). On the other hand, recruitment of the SIR2 HDAC at silenced loci plays a critical role in gene repression, while certain uncharacterized HATs counter this effect by global activity. Details on these mechanisms are provided in Section 1.2

1.1.1.2 Histone Phosphorylation and methylation.

The phosphorylation of the N-terminal tail of Histone H3 at serine 10 by cAMP-dependent kinase has emerged as a key histone modification. This modification is vital for cell-cycle progression and chromosome condensation during the M-phase of the cell cycle (Lee et al. 2010). This modification is also relevant during interphase because it enables the transcription of a significant number of genes that are triggered as a consequence of a variety of cell-signaling events (such as the stimulation of the acetyltransferase activity of Gcn5 toward the histone H3
Figure 1: Histone Acetylation and Deacetylation
Adapted from figure 10-58 (Molecular Cell Biology, 2000).
(a) Repressor-directed histone deacetylation. The DNA-binding domain of a repressor interacts with the gene it regulates. The repression domain binds Sir3 and Sir4, subunits of a multiprotein complex that includes Sir2, a histone deacetylase. Deacetylation of histone N-terminal tails on nucleosomes in the region of the repressor binding site inhibits binding of general transcription factors at the TATA box, thereby repressing gene expression.
(b) Activator-directed histone hyperacetylation. The DNA-binding domain of transcriptional activator such as Gcn4 interacts with the genes it regulates. The Gcn4 activation domain then interacts with a multiprotein histone acetylase complex that includes the Gcn5 catalytic subunit. Subsequent hyperacetylation of histone N-terminal tails on nucleosomes in the vicinity of the Gcn4-binding site facilitates access of the general transcription factors required for transcriptional initiation.
tail by prior phosphorylation of serine 10). So, it seems that the phosphorylation of H3 is involved in processes requiring opposing chromatin states (PÉREZ-CADAHIÁ et al. 2009). The mechanism by which phosphorylation contributes to transcriptional activation or chromatin condensation is not well understood. It has been found that several acetyltransferases have increased HAT activity on H3 serine 10-phosphorylated substrates facilitating the transition of heterochromatin to euchromatin (LEE et al. 2010).

Histone methylation occurs through enzymes known as Histone methyltransferases (HMTs) (CHEUNG & LAU 2005). Their function is to catalyze the transfer of methyl groups to the lysine and arginine residues of the histone proteins (particularly H3 and H4). Characteristically, these enzymes contain a SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain which is flanked by two cystine rich sequences (PRE-SET and POST-SET) fused to it. In S. cerevisiae, there are 6 proteins with SET domains and all of them possess HMT activity. The SET domain functions by transferring an S-adenosyl-L-methionine (AdoMet) methyl group to the amino group of a lysine residue on the histone protein, generating a methylated lysine residue and a cofactor by-product S-adenosyl-L-homocysteine (AdoHcy) (DILLON et al. 2005).

The HMT Dot1 has attracted significant attention. Dot1 is a non-SET domain containing enzyme that methylates H3 at Lys 79. In S. cerevisiae, Lys 79 of about ninety percent of H3 is mono-, di- or trimethylated. Methylation seems to repel silencing proteins and prevents their spreading thus promoting the euchromatic state. Thus, Dot1 appears to have an anti-silencing function (CHEUNG & LAU 2005). On the other hand, Dot1 is also required for the maintenance of the silenced state of subtelomeric genes and the genes at the constitutively silenced mating type loci (LACOSTE et al. 2002; NG et al. 2002; OSBORNE et al. 2009). In summary, the role of Dot1 in gene silencing and activation is awaiting more refined analysis (TAKAHASHI et al. 2011).
1.1.2. Chromatin remodelling and nucleosome re-positioning

The positions of nucleosomes are altered by multiprotein complexes called chromatin remodelling factors, which ultimately facilitate the action of the RNA polymerase. The SWI/SNF family and the ISWI family are the two general types of chromatin remodeling complexes (MELLOR & MORILLON 2004; SMITH et al. 2003).

These act by disrupting nucleosomes and rebuilding them at a nearby position or by destabilizing nucleosomes and sliding them along DNA (MELLOR & MORILLON 2004). This process is critically important for the preparation of promoters for subsequent transcription. However, chromatin remodelling factors are also involved in DNA replication and DNA repair. Because these factors are not critical for my thesis, they will not be described in further detail.

1.1.3. Histone chaperones

Another class of chromatin factors that is not central to this thesis is the histone chaperones (AVVAKUMOV et al. 2012). Newly synthesized histones are transported from the cytoplasm to the nucleus and subsequently targeted to the required loci to package DNA into chromatin. In addition, proper maintenance and alteration of chromatin require transient histone eviction and replacement. When histones are not in association with DNA, they are bound to escort proteins known as histone chaperones, which prevent unintended interactions of histones with other factors controlling the histone supply and its incorporation into chromatin (DE KONING et al. 2007). These chaperones are capable of recruiting specifically modified histones to a specific locus to reconstitute chromatin structure after DNA replication. Additionally, they can exchange histone variants at non-replicated loci (DE KONING et al. 2007).
1.2. **Epigenetic maintenance and gene silencing**

Epigenetic maintenance is the conservation of a chromatin domain through many cell generations. It is mediated through the joint activity of chaperones and histone modifying enzymes plus structural chromatin proteins such as cohesins and SIR (Silent Information Regulator) proteins (BIRD 2007; MALECO’ & MORRIS 2010). A specific phenomenon of epigenetic maintenance is gene silencing, meaning that DNA is inaccessible to the enzymes that catalyse transcription. Epigenetic silencing is characterized by its position dependent and promoter independent nature. The DNA sequences that mediate this process are known as silencers. Epigenetic silencing is most intensely studied in *S. cerevisiae* (REHMAN & YANKULOV 2009; RUSCHE et al. 2003).

1.2.1. **Cis-acting elements: Silencers and Proto-silencers**

In *S. cerevisiae*, gene silencing functions at the mating type loci (*HML* and *HMR*), the *rRNA* gene cluster and in the subtelomeric regions of the chromosomes (RUSCHE et al. 2003).

Silencing is regulated by dedicated *cis*-elements called silencers and proto-silencers (REHMAN & YANKULOV 2009; FOUREL 2002). Silencers are strong elements which efficiently nucleate a silenced heterochromatin domain through the recruitment of the histone deacetylase complexes Sir2/3/4 (Silent information regulators). Different elements serve as silencers at the telomeres and at the mating type loci or the *rRNA* encoding DNA cluster (RUSCHE et al. 2003). Proto-silencers are weaker elements that augment the recruitment of the Sir2/3/4 complex, but cannot work on their own (FOUREL et al. 2002) (Figure 2). Proto-silencer activity has been designated to the Autonomously Replicating Sequences (ARS) and the binding sites for Rap1 and Abf1 (BOSCHERON et al. 1996). All these individual elements are found scattered within the
**Figure 2: Proto-silencers**  
Adapted from figure 10-57 (Molecular Cell Biology, 2000).  
A master silencer can autonomously establish and maintain silencing, whereas a proto-silencer cannot do so. A proto-silencer amplifies, stabilizes, or prolongs silent chromatin propagation only in silencing-conducive environments.
highly repetitive core X and Y' elements that are positioned next to the telomeric repeats at the ends of the chromosomes (Tye 1988). Importantly, when these elements are moved to a crippled HMR mating type locus, they continue to exert proto-silencing activity (Fourel 1999). So, the activity of these proto-silencers does not seem to be restricted to the telomeres.

1.2.2. Histone deacetylation and SIR2 - Spreading of SIR complex from Silencers

The Histone deacetylase Sir2 plays a key role in the establishment and maintenance of silencing at all repressed loci. Sir2 deacetylates the histones on the nucleosome juxtaposed to the silencer or to the proto-silencer. Subsequently, Sir3 and Sir4 bind to the deacetylated histone tails and recruit a new Sir2 molecule which deacetylates the next nucleosome. In this sequential manner, the Sir proteins spread over several kilobase pairs of DNA (Rusche et al., 2003) (Figure 3). The spreading of histone deacetylation by Sir2 is counteracted by Histone Acetyl Transferases (Power et al. 2010). The mechanism of their action at the silenced loci is currently not as well understood as that of the SIR genes.

1.2.3. Discontinuous Silencing

It was found that the telomere proximal ends of the subtelomeric core X and Y' elements dampened repression of a reporter gene when it was located between the gene and the telomere (Pryde et al. 1999; Fourel et al. 1999). On the other hand, these low levels of repression were counteracted by reinforced levels of silencing displayed in the centromere proximal regions of core X and Y' elements due to proto-silencing elements located there. In this way, at natural telomeres repressed domains have been observed at positions away from the telomere and
beyond an insulated expression domain. This phenomenon was referred to as discontinuous silencing (FOUREL et al. 2001) (Figure 4).

The precise mechanisms of discontinuous silencing are not entirely understood. One model suggests that the Rap1-Sir complexes at the telomere interact with proteins bound to the core X proto-silencer elements by folding back into a structure known as the Telomere loop (T-loop) (CESARE 2008). Thus, the proto-silencers and the telomere come in proximity where the silencing activity of the Sir proteins is further relayed, augmented and stabilized by the proto-silencer. As shown in Figure 4, the looped region between the proto-silencer and the telomere remains undetermined and as a result may remain euchromatic. Activity of unidentified chromatin partitioning elements (or chromatin boundaries) between the heterochromatin and the euchromatin domains is suspected but not proven (FOUREL 2002). The alternative explanation is that not chromatin boundaries, but competition between silencing and anti-silencing factors determine the mosaic gene repression patterns at subtelomeric regions (FOUREL 2002).

Some studies also suggest that distal heterochromatin domains or even domains from different chromosomes can become associated in the larger heterochromatin clusters. Such clustering has been observed for multiple telomeres at the nuclear periphery of S.cerevisiae (BYSTICKY et al. 2005). Similar structures in higher eukaryotes are probably represented by the so called chromocenters that reside both in the nuclear periphery and in the nucleoplasm (PROBST & ALMOUZNI 2011). Higher order long range chromatin interactions are also believed to contribute to the discontinuous gene silencing along the chromosome. For example, long range interactions between the mating HMR and the telomere have been demonstrated by transmethylation assays (BOSCHERON et al. 1996) or the 3C (Chromatin Conformation Capture) (MIELLE et al. 2009).
Figure 3: Spreading of the Sir complex from Silencers
Adapted from figure 10-57 (Molecular Cell Biology, 2000).
Multiple Rap1 bind to telomere region, which lacks nucleosomes. This leads to the assembly of a multiprotein complex through protein-protein interactions between Rap1, Sir2/3/4 and the hypoacetylated amino-terminal tails of histones H3 and H4 of adjacent nucleosomes. Green color represents hyperacetylated histone amino-terminal tails.
The Rap1p-Sir complexes of the telomere interact with proteins bound to the core X element by folding back into a structure known as the Telomere loop (T-loop). Strong levels of repression are observed in the vicinity of the ARS consensus region located in the subtelomeric core X element while the area between the ARS and the telomere can remain active.

**Figure 4: Discontinuous Silencing**
Adapted from figure 10-57 (Molecular Cell Biology, 2000).
1.3. **Anti-silencing**

1.3.1. *Cis*-acting elements: anti-silencer regions (*STAR*) and *ARS* as potential anti-silencers

Anti-silencing refers to mechanisms which protect active genes from forming into silent chromatin structures and the elements involved in this process are known as anti-silencers (HILD & PARO 2003). The active gene states are usually accompanied by histone acetylation (Figure 5). Very little is known about the factors and the mechanisms that counteract gene silencing. To date, only two *cis*-elements, *STAR*s and *ACS*s, have been implicated in anti-silencing.

Subtelomeric regions contain SubTelomeric Anti-silencing Region (*STAR*) elements. These are positioned within the telomere-proximal portions of either the X or Y’ elements (FOUREL et al. 1999). Their removal increases telomeric silencing. *STAR*s are also known to counteract gene repression when moved from the telomere to the *HMR* mating type loci (FOUREL et al. 1999). It is not known exactly how *STAR*s work to counter repression. For example, it is not known if *STAR*s prevent spreading of the Sir proteins or if they facilitate the recruitment of counteracting HATs.

Subtelomeric *ACS* function as proto-silencers (FOUREL et al. 2002) which relay and enhance the silencing signals emitted by the telomere. In wild type cells *ACS* enhance silencing (REHMAN et al. 2006; RUSCHE et al. 2003) through the recruitment of Sir1 through Orc1. However, in several replication mutant strains (strains harboring mutations in their replication factor genes) the removal of *ACS* actually increases silencing indicating that in these genetic contexts *ACS*s act as anti-silencers (REHMAN et al. 2009). It has been speculated that the extension of S-phase in such mutants could aid the association of anti-silencing modules to *ACS*. It has also been proposed that Mcm5, which associates with telomeric *ACS*, could have input in
this anti-silencing activity of ACS (REHMAN et al. 2009).

1.3.2. Histone Acetyl Tranferases Sas2 and Sas3 and other HATs.

Since active gene expression is always associated with histone acetylation, it is conceivable that HATs play a critical role in anti-silencing by counteracting the Sir proteins. However, little is known about the nature of the HATs involved in this process and their mechanism of action. To date, nine HATs have been identified in S. cerevisiae (Table 1) (ALLIS et al. 2007). Sas2 is believed to be the major Sir2-counteracting HAT at the telomeres (DANG et al. 2009; KOZAK et al. 2010). Sir2 and Sas2 opposingly regulate the acetylation of histone H4 lysine 16 and the spreading of heterochromatin by functioning in concert to regulate gene expression (DANG et al. 2009). Sas3 is the catalytic HAT component of the NuA3 complex and has a role in transcriptional activation through acetylation of histone H3 (TAKECHI & NAKAYAMA 1999). Sas3 does not share the specificity for H4-K16 with Sas2 (ALLIS et al. 2007). However, the deletions of SAS2 and SAS3 have a similar effect in substantially reducing silencing (ESPINOSA et al. 2010). Thus, suggesting a more complex and elaborate mechanism of action. Nevertheless, current models assume that on natural telomeres SAS2 counteracts SIR2. It is known that HAT1 and GCN5 can have moderate effects on gene silencing; however they can function indirectly through the regulation of gene expression.
**Figure 5: Anti-silencers**
Adapted from figure 10-57 (Molecular Cell Biology, 2000).
Model of action of anti-silencers at heterochromatin boundaries. Without histone acetylation, Sir proteins tend to spread along the chromatin fibre. Anti-silencers are recruited onto and acetylate heterochromatin proximal regions and this acetylation efficiently antagonizes Sir protein dependent heterochromatin spreading.
Table 1: Histone Acetyl Transferases

<table>
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<tr>
<th>Known HATs</th>
<th>Substrate Specificity</th>
<th>Function</th>
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<tbody>
<tr>
<td>a) Rtt109</td>
<td>H3 (56)</td>
<td>Genome stability and transcription elongation</td>
</tr>
<tr>
<td>b) Esa1</td>
<td>H4 (5,8,12,16) H2A (4,7)</td>
<td>Transcription activation and DNA repair</td>
</tr>
<tr>
<td>c) Sas2</td>
<td>H4 (16)</td>
<td>Chromatin boundaries, Dosage compensation and DNA repair</td>
</tr>
<tr>
<td>d) Sas3</td>
<td>H3 (14,23)</td>
<td>Transcriptional activation and elongation, DNA replication</td>
</tr>
<tr>
<td>e) Hat1</td>
<td>H4 (5,12)</td>
<td>Histone deposition and DNA repair</td>
</tr>
<tr>
<td>f) Gcn5</td>
<td>H3 (9,14,18,23,36)/H2B</td>
<td>Transcription activation and DNA repair</td>
</tr>
<tr>
<td>g) Hap2</td>
<td>H3 (14) H4</td>
<td>Unknown</td>
</tr>
<tr>
<td>h) Taf1</td>
<td>H3 and H4</td>
<td>Transcription activation</td>
</tr>
<tr>
<td>i) Elp3</td>
<td>H3</td>
<td>Transcriptional elongation</td>
</tr>
</tbody>
</table>

The substrate specificity indicates the preferred histone substrate of the HAT (H3, Histone 3; H4, Histone 4). The lysine residues acetylated in the N-termini of the respective histones are shown in brackets.
1.3.2.1 **ESA1**

*ESA1* (Essential Sas2-related acetyltransferase) is the catalytic subunit of the HAT complex NuA4 and the Piccolo multi-protein complex (LAFON et al. 2007). *ESA1* belongs to the MYST (*MOZ, YBF2/SAS3, SAS2, TIP60*) subfamily of HAT enzymes. The NuA4 complex is the largest 1.3 MDa MYST complex in *S.cerevisiae*. It contains 13 subunits. A smaller subset of the NuA4 complex is the Piccolo complex which comprising of three subunits, Esa1, Yng2 and Epl1, has also been purified from cell extracts (BOUDREAULT et al. 2003).

*In vitro* studies have demonstrated that Esa1 has a distinctive pattern of histone acetylation. Although it shares a common catalytic acetyltransferase motif with other HATs such as Gcn5 and Hat1, it primarily acetylates histone H4 and to a lesser extent H3 and H2A (CLARKE et al. 1999). In contrast, Gcn5 is most active on H3 and acts very weakly on H4 (CLARKE et al. 1999). Even more, in the context of NuA4 Esa1 efficiently acetylates free histones. On the other hand, the Piccolo complex has significantly higher HAT activity towards histones in nucleosomes as compared to free histones (BOUDREAULT et al. 2003).

The key distinction that sets *ESA1* apart from other HATs is that it is essential for cell viability. The temperature sensitive *esa1* mutant alleles that have been shown to abolish enzymatic activity *in vitro* and show a partial loss of an acetylated isoform of histone H4 *in vivo* (CLARKE et al. 1999). At restrictive temperatures, strains with these mutant *esa1* alleles arrest in the G2/M phase of the cell cycle. They succeed in replicating their DNA but fall short of advancing through mitosis and cytokinesis (CLARKE et al. 1999). This arrest is caused by the *RAD9*-mediated DNA damage checkpoint. Importantly, the deletion of *RAD9* allows the cells to progress into the M phase of the cell cycle but does not compensate for the loss of viability. So, the essential role of *ESA1* is not necessarily related to the regulation of the G2/M→M transition.
One of the critical functions of *ESA1* seems to be in DNA repair. The acetylation of H4 by Esa1 has been indicated as vital for DNA double stranded break repairs (Bird *et al.* 2002). In addition, Esa1 has been located to the site of the breakage.

*ESA1* also participates in transcriptional regulation of ribosomal protein genes (Clarke *et al.* 2006). Surprisingly, while contributing to transcriptional activation, *ESA1* also has a role in the repression of telomeric and rDNA genes (Clarke *et al.* 2006). For example, the over-expression of *ESA1* bypasses the necessity of *SIR2* in the silencing of rDNA, suggesting that these two genes with opposing activities function in concert to achieve appropriate nucleolar structure and functioning.

As Esa1 is a HAT, it was expected that the temperature sensitive *esa1* mutants would exhibit decreased levels of H4 acetylation and consequent increased transcriptional silencing. To address the question if Esa1 functions in both transcriptional activation and silencing, reporter genes were placed at the mating type loci (*HML* and *HMR*), the rRNA gene cluster and in the subtelomeric regions of the chromosomes (Clarke *et al.* 2006). The mutants showed a modest and intermediate silencing defect compared to *sir2* deletion mutant in the *HMR* and subtelomeric regions respectively. At the rDNA the mutants showed very strong silencing defects, even more than the *sir2Δ* mutant.

In summary, *ESA1* plays a role in many unrelated processes (Clarke *et al.* 1999; Clarke *et al.* 2006; Lafon *et al.* 2007). It is no surprise that to date the essential function of *ESA1* is still not firmly established. In terms of gene activation, it has been proposed that *ESA1* counters the repression activity of a histone deacetylase called Rpd3 (Chang & Pillus 2009). Paradoxically, it has recently been shown that Rpd3 actually supports the anti-silencing Sas2 through the establishment of chromatin boundaries at the telomeres therefore acting as an anti-silencing
factor (EHRENTRAUT et al. 2010). It is fair to say that we do not completely understand the mechanism of action of ESA1 in silencing.

1.3.2.2 RTT109

RTT109 (Regulator of Ty1 Transposition 109) is another histone acetyltransferase, which associates with transcriptionally active genes and is required for proper acetylation of histone H3 at lysine 56 (HAN 2007; D’ARCY & LUGER 2011). However, recent evidence suggests that lysine 9 and lysine 27 can also be targeted by this HAT (FILLINGHAM et al. 2008; BURGESS et al. 2010). It prefers non-nucleosomal H3 as a substrate and is the only HAT that is known to be activated by histone chaperones (D’ARCY & LUGER 2011). However, it is not entirely clear if Vsp75 or Asf1 is the principle activating chaperone for Rtt109. Nevertheless, it is strongly believed that Rtt109 acetylates free H3 histones prior to their incorporation into nucleosomes and that it is intimately involved in the replication-coupled reassembly of chromatin (AVVAKUMOV et al. 2012). RTT109 is critical for cell survival in the presence of DNA damage during S phase, and is believed to support the repair of double stranded breaks through non homologous end joining (DRISCOLL et al. 2007). RTT109 is also a regulator of transposition of the yeast retrotransposons (HAN 2007). Finally, the acetylation of H3 K56 has been linked to efficient elongation by RNA polymerase II (VARV et al. 2010) and to the silencing at the mating type loci (MILLER et al. 2008), but conclusive evidence for the involvement of RTT109 in these processes is yet to be obtained. Practically nothing is known on the role of RTT109 in telomeric silencing.
1.3.3. Other anti-silencing activities

Recently, engineered telomeres built of different combinations of core X, Y′ and STARs have been analyzed in several histone acetyl transferase deletion mutants (POWER et al. 2011). These mutations displayed subtle silencing or anti-silencing effects. Some of these effects were dependent on the presence of STARs or core X. However, no strong candidate for a master anti-silencer has emerged. Remarkably, it was shown that core X and Y′ dramatically reduce variations in telomere position effect (TPE) and act as epigenetic “buffers”. Of the nine HATs in S.cerevisiae, only the deletion of RTT109 and ESA1 conditional mutants were not tested. Analyses in these mutants will complete the comprehensive study of the effects of HATs on telomeres and will indicate if any of these genes operate through specific subtelomeric elements.

1.4  Telomere Position Effect (TPE) and Variegation

Subtelomeric genes display a variegated pattern of expression, implying that the genes can be either fully silenced or fully active in any single cell from generation to generation (RUSCHE et al. 2003). The phenomenon of the meta-stable state of subtelomeric genes is known as Telomere Position Effect (TPE) and is one of the most extensively studied Position Effect Variegation (PEV) domains.

Mechanism of Telomere Position Effect:

As mentioned before, the telomere acts as a major silencer and interacts with multiple Rap1 molecules (RUSCHE et al. 2003; REHMAN et al. 2009; FOUREL et al. 2002). This leads to recruitment and subsequent assembly of the histone deacetylase complexes Sir2/3/4, which results in the formation of a silenced heterochromatin domain (XU et al. 2007). The core X element contains proto-silencers and binding sites for Orc1, Abf1 and Sir1. These proteins are not necessary for the assembly of the SIR complex but are important for spreading of silencing.
In addition, subtelomeric regions also contain sequences which harbor anti-silencing activities (FOUREL et al. 2002). These elements with opposing activities are continuously attempting to shift the balance of repression or de-repression. This interplay makes a switch in the gene expression possible, leading to variegation.

A gene inserted in the subtelomeric region is repressed due to its juxtaposition with heterochromatin, which is stabilized and amplified by proto-silencers. In the same population of cells, the same gene may be expressed due to counter activation of this silencing by anti-silencing elements such as STARs and ACSs, which leads to switching in the state of the gene. A very similar behavior has been observed in the eye of Drosophila upon translocation of the white gene next to centromeric heterochromatin block. Due to this spectacular display, the latter case is the classical textbook example for position effect variegation.
2 RATIONALE AND OBJECTIVES

It has been postulated that HATs (Histone Acetyl Transferases) counteract the activity of HDACs (Histone Deacetylases) to establish a level of acetylation and regulate transcription. Although the mechanism of action of HDACs is well elucidated, the mode of action of HATs, especially at telomeres, is not very clear. The roles of some HATs (SAS2, SAS3) in anti-silencing have been extensively studied. Others (HAT1, GCN5) have received moderate attention. Practically nothing is known about the roles of RTT109 and ESA1. The focus of my research will be to address the mode of action of these two HATs at telomeres.

2.1 Hypothesis

ESAI and RTT109 are involved in anti-silencing activities at telomeres through specific subtelomeric elements.

2.2 Research Goals:

- To analyze the effects of deletion of RTT109 on the silencing of a URA3 reporter in a set of telomeric constructs that contain different subtelomeric elements.
- To analyze the effect of a mutant ESA1 allele on the silencing of a URA3 reporter in a set of telomeric constructs that contain different subtelomeric elements.
3 MATERIALS AND METHODS

3.1 Yeast strains and growth

The yeast strains used in this study are listed in Table 2. Cells were routinely grown at 23ºC in liquid YPD medium or on YPD plates (Yeast extract 10 g/l, Peptone 20 g/l and 2% Glucose) containing 2% Agar. All components were purchased from Bioshop (Burlington, Ontario).

Table 2: Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4742</td>
<td>MATalpha leu2 ura3 his3 lys2</td>
<td>ATCC#4016568</td>
</tr>
<tr>
<td>Δrtt109</td>
<td>MATalpha leu2 ura3 his3 lys2 rtt109::Kan'</td>
<td>ATCC#4011490</td>
</tr>
<tr>
<td>Δrif1</td>
<td>MATalpha leu2 ura3 his3 lys2 rif1::Kan'</td>
<td>ATCC#4017170</td>
</tr>
<tr>
<td>LPY4679</td>
<td>MATα ura3-52 trp1Δ2 leu2-3 112 his3-11 ade2-1 can1-100 esa1-1-414</td>
<td>CLARKE et al. 2004</td>
</tr>
</tbody>
</table>

Selection of cells

Selection of cells with specific genotypes was conducted on Synthetic Complete (SC) drop-out media. It comprises of Yeast Nitrogen (YN) base1.7 g/l, 2% glucose and Amino acid mixture (AA mix contains isoleucine 20 mg/l, methionine 20 mg/l, phenylalanine 20 mg/l, tyrosine 20 mg/l, valine 80 mg/l, arginine 20 mg/l, lysine 40 mg/l, threonine 80 mg/l, aspartic acid 60 mg/l, glutamic acid 60 mg/l, serine 20 mg/l and alanine 75 mg/l). Adenine, uracil, leucine, tryptophan, and histidine were added to this mixture at concentrations of 40 mg/l, 20 mg/l, 50 mg/l, 30 mg/l and 30 mg/l respectively, from 100X concentrated solutions. For selection of auxotrophic strains
(these strains have null mutations or deletions in ADE2, URA3, LEU2, TRP1 and HIS3) one or more of these components are omitted from the culture medium.

For strains that were selected for the genomic integration of URA3, uracil was omitted to produce SC/-Ura plates. For selection against the expression of URA3, 5-fluoro-orotic acid (FOA) was added at 10 g/l to complete SC medium to produce SC/FOA plates.

### 3.2 Plasmids and production of integrating constructs.

The plasmids for the production of telomeric integrating constructs were a gift from G. Fourel (Ecole Normale Superiore, Lyon, France). Detail on their production can be found in (FOUREL et al. 1999) and in Table 3. These plasmids were produced in E.coli DH5alpha strain. E.coli cells were routinely grown in Luria-Bertani (LB) broth or LB plates (consists of sodium chloride 10 g/l, peptone 10 g/l and yeast extract 5 g/l) at 37°C supplemented ampicillin (100 µg/ml).

All integrating constructs had been subcloned in a plasmid called pUCAIV (Figure 7) (GOTTSCHLING et al. 1991). The integrating constructs were released from the plasmids by digestion with SalI and EcoRI. This digestion produces a single band from the plasmid backbone that is identical for all pUCAIV plasmids. The second band has a different length for the different constructs. The identity and the quality of the integrating construct were confirmed by analyzing an aliquot of the digest on 1% agarose - TAE gel electrophoresis. The digests were then precipitated with EtOH to get rid of any salt that can interfere with the subsequent electroporation into yeast cells.
<table>
<thead>
<tr>
<th>Construct Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF1 (URA3-tel)</td>
<td><em>URA3</em> reporter gene, flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats for targeted integration into the left telomere of chromosome <em>VII</em></td>
</tr>
<tr>
<td>GF2</td>
<td><em>URA3</em> reporter gene and <em>STARs</em> from the <em>core</em> X-<em>IIR</em>, flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats</td>
</tr>
<tr>
<td>GF3</td>
<td><em>URA3</em> reporter gene and <em>STARs</em> from the <em>Y</em>-XIII element, flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats</td>
</tr>
<tr>
<td>GF6</td>
<td><em>URA3</em> reporter gene, <em>STARs</em> from the <em>core</em> X-<em>IIR</em> and the <em>core</em> X element flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats</td>
</tr>
<tr>
<td>GF9</td>
<td><em>URA3</em> reporter gene, <em>STARs</em> from the <em>Y</em>-XIII and the <em>Y</em> element flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats</td>
</tr>
<tr>
<td>GF44</td>
<td><em>core</em> X element (distant from the telomere), <em>URA3</em> reporter gene, flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats</td>
</tr>
<tr>
<td>GF46</td>
<td><em>Y</em> element (distant from the telomere), <em>URA3</em> reporter gene, flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats</td>
</tr>
<tr>
<td>GF61</td>
<td><em>URA3</em> reporter gene (distant from the telomere) beyond two <em>STARs</em>, <em>core</em> X and <em>TRP1</em> gene, flanked by a portion of <em>ADH4</em> (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG repeats</td>
</tr>
</tbody>
</table>
3.3 Yeast transformation and selection

Integration of constructs in the VII telomere of *S. cerevisiae* is a highly reproducible, but rare event. For this reason yeast cells were transformed using the highly efficient electroporation procedure. Briefly, yeast cells were grown to stationary phase at 23°C. This starter culture was used to inoculate a fresh medium at OD$_{600}$=0.2 and allowed to grow to OD$_{600}$=1.0. Under these conditions the cells are growing exponentially. The cells were then harvested by centrifugation at 4000 rpm for 3 minutes and the pellet was resuspended in 50 ml of sterile ice-cold water. The cells were washed twice more with 15 ml of sterile ice-cold water before they were resuspended in 3 ml of sterile ice-cold 1M sorbitol. These cells were then centrifuged and resuspended in 3 times the pellet size amount of 1M sorbitol. This treatment produces a dense cell suspension (OD$_{600}$=200) in isotonic solution. An aliquot of 40 µl of this suspension was electroporated using the Biorad MicroPulser, with 2 mm cuvettes. Immediately after electroporation, 1ml of recovery medium (YPD + 1M sorbitol) was gently added to the cells. These cells were then incubated for 3 hours at room temperature. Cells were collected by centrifugation, resuspended in the residual recovery medium and plated on SC/-Ura plates. Colonies from these plates were re-streaked on Sc/-Ura plates and then single colonies were isolated and tested by FOA sensitivity assay.

3.4 5-Fluoro-orotic acid sensitivity assay

Transformed cells were selected on SC/-Ura plates. Three colonies were re-streaked on SC/-Ura plates to eliminate any untransformed cells (SC/-Ura suppresses growth of cells without *URA3*, but does not kill the cells) and then streaked on SC/FOA plates to confirm variegated expression. FOA (Fluoro-Orotic Acid) is a neutral drug, which is converted into a toxin by Orotidine-5’-Phosphate- Decarboxylase (the enzyme encoded by *URA3*). Thus, the strains
resistant to FOA reflect the absence of expression of *URA3*. In cells with confirmed presence of functional *URA3* (growth on SC/-Ura) the resistance to FOA indicates variegated expression. This procedure is very efficient in selecting *URA3* that has been inserted in the *VIL* telomere when tested by PCR.

Single colonies from the second SC/-Ura streak were transferred into 3 ml YPD and diluted 300 times in fresh YPD medium for three consecutive days to allow the establishment of equilibrium of cells expressing or repressing *URA3*. Serial 1:10 dilutions were prepared from each culture and 5μl were spotted on YPD, SC/-Ura and SC/FOA plates. Following this, the percent of FOA<sup>R</sup> cells was acquired by dividing the average number of cells on SC/FOA plates by the average number of cells on YPD plates. Average values, error measurement, and ratios between levels of silencing in different strains were calculated in Microsoft Excel® and used to build graphs and to calculate the effects of the gene mutations relative to wild type cells.
4 RESULTS

4.1 Experimental Strategy

Gene silencing is mediated by histone deacetylation by the Sir2 histone deacetylase, with minor contribution by the HDAC Rpd3 (Rusche et al. 2003). Because gene transcription is invariably associated with histone acetylation, it is conceivable that anti-silencing involves the acetylation of histones, but the participating HATs are largely unknown.

Nine HATs have been identified in S. cerevisiae (Table 1) (Allis et al. 2007). Amongst these, no strong candidate for a master anti-silencer has emerged. Recently, the effects of some of these HATs (HAT1, GCN5, SAS2, SAS3 and YNG1) have been analyzed in the lab of Yankulov (Power et al. 2010). Significant similarities in the effects caused by mutations in these genes have been observed, however a major anti-silencing HAT has not been identified. This raised the possibility that other HATs can play a more substantial role in TPE. At the same time, the role of RTT109 and ESA1 had not been tested. The focus of my research was to analyse the effects of these two genes using a mutant esa1-414 allele (strain LPY4679, Pillius 1999) and the deletion mutant of RTT109 (∆rtt109, ATCC) as it would contribute to a comprehensive and complete study of the different HATs taking part in telomeric variegation. Additionally it would indicate if ESA1 and RTT109 participate in anti-silencing activities at telomeres by interacting with specific subtelomeric elements or by “global” activity.

These strains and the appropriate control strains were transformed with a set of constructs containing different subtelomeric elements. Thus, my study was also geared towards identifying subtelomeric elements, which mediate the effects of these genes. The effect of these mutations was tested using a construct which contained URA3 as a reporter gene. For comparison, I used
the isogenic wild type (BY4742) strain and a Δrif1 deletion mutant. RIF1 encodes of a *bona fide* telomeric anti-repression factor.

### 4.2 Choice of synthetic telomeric constructs

In order to analyze the effects of *RTT109* and *ESA1* on gene silencing, I used the set of constructs shown in Figure 6. All of them contain the *URA3* reporter gene, but substantially differ in the arrangement and the contents of a variety of natural subtelomeric elements. All constructs used were flanked by a portion of *ADH4* (Alcohol dehydrogenase isoenzyme type IV) and telomeric TG13 repeats. They are designed for targeted integration into the left telomere of chromosome VII (Figure 7). The integration of this type of constructs in the VII telomere is very efficient and normally produces targeted integration in more than 90% transformants.

These constructs contained various combinations of subtelomeric *core X, Y'* and *STAR* elements derived from different telomeres (FOUREL *et al.* 1999) (Figure 6, Table 3). The *URA3*-tel (also known as GF1) construct consisted of only the aforementioned flanking sequences and the *URA3* reporter gene and is one of the most frequently used reporters in the field of telomeric silencing. It represents a telomere without any regulatory sub-telomeric sequences. The GF2 and GF3 constructs also contained *STARs* from the *IIR* or *XIIL* telomeres, respectively. GF6 and GF9 contained the same *STARs* along with *core X* and *Y'* from the same telomeres. In GF44 and GF46 the *core X* and the *Y'* were positioned beyond *URA3*, distant from the telomere. In GF61, *URA3* was away from the telomere beyond two *STARs*, *core X* and *TRP1*.

This set of telomeric constructs contained a comprehensive array of subtelomeric elements, which are normally involved in repression, anti-repression and telomeric variegation. As at natural telomeres, the telomeric repeats act as the major silencer by binding with Rap1 and
initiating the assembly of the SIR complex, while the binding sites for ORC, Abf1 and Rap1 in the core X element stabilized and augmented these repression signals serving as proto-silencers. The STARs counteracted the repression signals. Since all aspects of the silencing, anti-silencing and variegation were present in these constructs, they could be used as a comprehensive tool for the analysis of TPE. In particular, these constructs are expected to reveal any specific element that is required for the activity of any factor working in silencing or anti-silencing at the telomeres.

The integrating subtelomeric constructs were electroporated into BY4742, Δrif1, Δrtt109 and LPY4679 strains and the telomeric integration was confirmed by assays for variegated expression as described in Materials and Methods. Importantly, random integration of these constructs would strictly produce no resistance to FOA after selection of SC/-Ura plates. Indeed, random integration will not provide any variegated repression of URA3 and consequently resistance to FOA. For this reason I did not attempt to confirm the targeted integration of each construct by PCR. Instead, I resorted to the analysis of multiple transformants and statistical evaluation of the results from the FOA resistance assay.

After observation of the variegated mode of URA3 expression, three isolated colonies were grown in non-selective medium (YPD) for 30 generations to allow for the cells to acquire variegation equilibrium. Eventually, the percentage of FOA$^R$ for each of the three colonies was separately calculated as the number of colonies on SC/FOA plates divided by the number of colonies on SC plates. The average values ± standard deviations of the three independent measurements were calculated and plotted in Microsoft Excel®. Ultimately, the percent FOA$^R$ is used as a measure of the level of repression of each construct in each strain and for comparison between strains and constructs.
All calculated % FOA<sup>R</sup> values are shown in Table 5. These values were used to build the graphs in Figure 8, Figure 10, Figure 12 and Figure 14.

**Figure 6: A diagram representing the telomeric constructs used in this study**

URA3-tel (Gottschling et al. 1990), GF2, GF3, GF6, GF9, GF44, GF46 and GF61 (Fourel et al. 1999) are represented not to scale. The URA3-tel construct consists of only flanking sequences and the URA3 reporter gene. The GF2 and GF3 constructs also contain STARs from the IIR or XIIL elements, respectively. GF6 and GF9 contain the same STARs along with core X and Y' from the same telomeres. In GF44 and GF46 the core X and the Y' are positioned beyond URA3, distant from the telomere. In GF61, URA3 is away from the telomere beyond two STARs, core X and TRP1.
**Figure 7: Targeted integration in the VII telomere**
The above constructs are flanked by a portion of ADH4 (Alcohol dehydrogenase isoenzyme type IV), telomeric TG repeats and are designed for targeted integration into the left telomere of chromosome VII. GF2 is used as an example. Integrating constructs are produced by cleavage with EcoRI and SalI and transformed into target cells.
Table 5: Levels of FOA<sup>R</sup> ratio in all strains and all constructs used

The values and calculations are described in the main text.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Construct</th>
<th>%FOAR</th>
<th>STDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>GF1</td>
<td>38.50%</td>
<td>8.80%</td>
</tr>
<tr>
<td>BY4742</td>
<td>GF2</td>
<td>1.90%</td>
<td>1.10%</td>
</tr>
<tr>
<td></td>
<td>GF3</td>
<td>3.30%</td>
<td>1.80%</td>
</tr>
<tr>
<td></td>
<td>GF6</td>
<td>63.50%</td>
<td>9.30%</td>
</tr>
<tr>
<td></td>
<td>GF9</td>
<td>32.20%</td>
<td>7.30%</td>
</tr>
<tr>
<td></td>
<td>GF44</td>
<td>47.40%</td>
<td>12.80%</td>
</tr>
<tr>
<td></td>
<td>GF46</td>
<td>65.80%</td>
<td>9.60%</td>
</tr>
<tr>
<td></td>
<td>GF61</td>
<td>40.70%</td>
<td>9.20%</td>
</tr>
<tr>
<td>Conditional mutant</td>
<td>GF1</td>
<td>0.08%</td>
<td>0.04%</td>
</tr>
<tr>
<td>esa1-414</td>
<td>GF2</td>
<td>1.40%</td>
<td>0.04%</td>
</tr>
<tr>
<td></td>
<td>GF3</td>
<td>23.67%</td>
<td>2.19%</td>
</tr>
<tr>
<td></td>
<td>GF6</td>
<td>78.65%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>GF9</td>
<td>71.41%</td>
<td>15.92%</td>
</tr>
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<td></td>
<td>GF44</td>
<td>48.33%</td>
<td>0.03%</td>
</tr>
<tr>
<td></td>
<td>GF46</td>
<td>89.52%</td>
<td>2.41%</td>
</tr>
<tr>
<td></td>
<td>GF61</td>
<td>59.41%</td>
<td>5.77%</td>
</tr>
<tr>
<td>Deletion mutant</td>
<td>GF1</td>
<td>0.31%</td>
<td>0.90%</td>
</tr>
<tr>
<td>∆rtt109</td>
<td>GF2</td>
<td>0.69%</td>
<td>0.40%</td>
</tr>
<tr>
<td></td>
<td>GF3</td>
<td>21.51%</td>
<td>0.50%</td>
</tr>
<tr>
<td></td>
<td>GF6</td>
<td>61.49%</td>
<td>12.80%</td>
</tr>
<tr>
<td></td>
<td>GF9</td>
<td>20.48%</td>
<td>10.10%</td>
</tr>
<tr>
<td></td>
<td>GF44</td>
<td>23.70%</td>
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<tr>
<td></td>
<td>GF61</td>
<td>19.19%</td>
<td>18.30%</td>
</tr>
<tr>
<td>Deletion mutant</td>
<td>GF1</td>
<td>61.31%</td>
<td>12.42%</td>
</tr>
<tr>
<td>∆rif1</td>
<td>GF2</td>
<td>74.21%</td>
<td>12.58%</td>
</tr>
<tr>
<td></td>
<td>GF3</td>
<td>37.86%</td>
<td>13.25%</td>
</tr>
<tr>
<td></td>
<td>GF6</td>
<td>83.33%</td>
<td>13.44%</td>
</tr>
<tr>
<td></td>
<td>GF9</td>
<td>42.42%</td>
<td>4.50%</td>
</tr>
<tr>
<td></td>
<td>GF46</td>
<td>69.95%</td>
<td>12.99%</td>
</tr>
<tr>
<td></td>
<td>GF61</td>
<td>75.00%</td>
<td>5.04%</td>
</tr>
</tbody>
</table>
Table 6: Ratios of %FOA\(^R\) in all the strains versus the %FOA\(^R\) in the wild type (BY4742) strain for all the constructs

The values and calculations are described in the main text.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Construct</th>
<th>%FOAR Ratio</th>
<th>Error</th>
</tr>
</thead>
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4.3 Effects of the *ESA1*

As stated earlier, *ESA1* is an essential gene. Hence, a deletion mutant of *ESA1* could not be generated. For this reason I decided to analyze a conditional *esa1* mutant with a severe phenotype, but not complete loss of function that leads to lethality (Clarke and Pillus 1999).

4.3.1 Selection of the *esa1-414* mutant strain

I initiated this project by obtaining three *esa1* mutants that have been partially characterized in a previous study (Clarke et al. 1999) and tested them for feasibility in my experiments. The strains *LPY3430* (*esa1-L327S*), *LPY4679* (*esa1-414*) and *LPY5001* (*esa1-L254P*) each carry a different non-lethal mutation.

The strain *LPY3430* harbors a point mutation that changes the leucine at positions 327 to serine. This strain grew well at room temperature and at 37°C and showed little retardation relative to the wild type *BY4742* strain. This observation generated concerns about the severity of the mutation and the adequacy of analyses with it. Even more, the cells grew on SC/-Ura plates thus precluding transformation with my reporter constructs. The *LPY5001* strain harbors a point mutation that substitutes the leucine at position 254 to proline. It was extremely sick and took 8 days to grow to saturation. Given the multi-generational nature of the TPE assays, this strain would have posed major difficulties in handling, transformation and propagation. The strain *LPY4679* contains a C-terminal deletion of *ESA1* after the amino acid at position 414. It grows slowly at permissive temperature, shows severe temperature sensitivity at 37°C and does not grow on SC/-Ura. I selected this strain for my experiments. I have introduced all constructs described in the Figure 6 in the VII telomere of this strain and measured the repression of *URA3*
as described in Materials and Methods. The calculated levels of repression are plotted in Figure 8 in parallel with the level of repression in wild type cells.

As shown in Figure 8, the esa1-414 mutation caused modest overall increase in the repression in the constructs containing core X elements (GF6, GF9, GF44, GF46 and GF61). In contrast, there was a drastic increase in repression of GF3, but modest decrease in the repression of GF2. This observation is somewhat surprising as both these constructs contain STAR elements. The levels of repression of GF1 went significantly down relative to wild type cells.

In order to get a quantitative evaluation of the effects of this mutation relative to wild type cells, I used the values in Table 6 to calculate the ratios of %FOAR in the mutant esa1-414 strain versus the %FOAR in the wild type BY4742 strain (Figure 9). In these calculations values above 1 indicate increased repression while lower than 1 represents heightened levels of expression. In agreement with Figure 8, GF1 and GF3 were the constructs with the most dramatic change of expression, while GF2, GF6, GF46 and GF61 showed around the same levels of change. GF44 showed almost no change.

These calculations support the notion of modest changes in the repression of the more complex constructs which contain natural subtelomeric elements. As indicated earlier, these elements seem to have epigenetic “buffering” activity that suppresses extreme variations in TPE (POWER et al. 2011). The most significant effects in these experiments were observed with the GF1 and GF3 constructs. The increase of expression in GF2 as compared to the decrease of expression in GF3 was surprising and raises significant concerns that are addressed in DISCUSSION. Interestingly GF6 and GF9 also showed less repression than GF3 though they contain core X or Y’ elements with proto-silencer activities. Similarly GF44 and GF46 showed different levels of silencing though both contain the URA3 reporter gene immersed in between
Figure 8: Overall increase in repression levels of *esa1-414* mutant

Percentage of FOA^R^ cells in the *esa1-414* strain and in the wild type *BY4742* strain for the constructs is plotted. Data is from Table 5. Standard deviation is shown by error bars. The reporter constructs shown along the vertical axis were integrated in the *esa1-414* strain. The horizontal axis represents the % FOA^R^ . Data is from Table 5.
the core X or Y' elements and their respective STARs. GF61 showed repression similar to GF46 while URA3 is away from the telomere beyond two STARs and the core X element.

Altogether, the results support the notion of moderate effect of ESA1 on telomeric gene silencing. It seems that the severe sickness of this strain can not be attributed to deregulation of TPE and gene silencing and that this mutation probably affects some other process.

Figure 9: Levels of FOA^R ratio between esa1-414 mutant and wild type cells

Ratios of %FOA^R in the esa1-414 mutant strain versus the %FOA^R in the wild type (BY4742) strain. Data is from Table 6. The arrows underneath the exponential graph indicate increase or decrease of silencing.
4.4 Effects of Artt109

Artt109 gene is not essential for cell survival. Hence, experiments with a strain with deleted RTT109 are feasible. The Δrtt109 strain I obtained form ATCC has shown no temperature sensitivity. It grows at marginally slower rate compared to the wild type strain.

Similarly to the experiments with esa1-414 mutants, the constructs shown in Figure 6 were inserted in the VIIL telomere of the Δrtt109 cells. The experiments for the assessment of the levels of telomeric gene expression was performed as previously described. All data acquired in these studies and the calculated levels of URA3 repression in different constructs are summarized in Table 5. The calculated levels of repression are plotted in Figure 10 in parallel with the level of repression in wild type cells.

Opposite to the effects of esa1-414, my data shows that the deletion of RTT109 caused modest overall decrease of repression in most constructs (Figure 10). Hence, under the conditions of my experiment Rtt109 seems to display properties of a silencing factor. The most dramatic decrease of repression was observed in GF1 and GF2, while GF9, GF44, GF46 and GF61 displayed moderate effects. Again, GF3 was different from the rest of the constructs in showing increase in repression.

Altogether, several measurements of the repression of these constructs have shown that this HAT is also unlikely to be a major regulator of TPE.
Figure 10: Overall decrease in repression levels of \( \Delta \text{rtt109} \) mutant

Percentage of FOA\(^R\) of the reporter constructs integrated in the \( \Delta \text{rtt109} \) strain. The reporter constructs shown along the vertical axis were integrated in the \( \Delta \text{rtt109} \) strain. The horizontal axis represents the % FOA\(^R\). Data is from Table 5.
Figure 11: Levels of FOA$^R$ ratio between $\Delta rt109$ mutant and wild type cells

Ratios of $\%$FOA$^R$ in the $\Delta rt109$ mutant strain versus the $\%$FOA$^R$ in the wild type (BY4742) strain. Data is from Table 6. The arrows underneath the exponential graph indicate increase or decrease of silencing.
4.5 Effects of ∆rif1

The slight decrease in the levels of repression in ∆rtt109 cells is counterintuitive. One would expect that a HAT, even when working globally and non-specifically, would act as an anti-silencing rather than silencing factor. This observation led to doubts about the overall adequacy of the constructs I used and the adequacy of my experiments. At the same time, all these constructs have recapitulated many previously reported effects of many different genes (FOUREL et al. 1999; FOUREL et al. 2001; REHMANET et al. 2006; REHMAN et al. 2009; POWER et al. 2011).

To clarify this issue I used a deletion mutant of a bona fide anti-silencing factor (∆rif1) as a control strain. Rif1 binds to the C-terminus of Rap1 and also acts synergistically with Rif2 to regulate telomere length (HARDY et al. 1992). In S. cerevisiae the Rif1 and Rif2 proteins compete with Sir3 and Sir4 for Rap1 binding and therefore have a negative effect on subtelomeric silencing (ROSAS-HERNÁNDEZ 2008). Deletion of RIF1 has been shown to increase telomeric repression due to two reasons. Firstly, due to the absence of competition there is more of Rap1 to interact with Sir proteins and establish silencing. Secondly, the deletion of RIF1 leads to elongation of telomeres and the consequent increase in the recruitment of the Rap1-Sir silencing complexes to the telomeres.

In my hands the ∆rif1 shows no growth deficiencies or temperature sensitivity. In this respect this strain behaves similarly to ∆rtt109. I have introduced most of the constructs described in the previous section into this mutant strain, and measured the repression of URA3 as previously described. The calculated levels of repression are shown in Figure 12 in parallel with the level of repression in wild type cells.
**Figure 12: Expected increase in repression levels of Δrif1 mutant**

Percentage of FOA\(^{R}\) of the reporter constructs integrated in Δrif1 strain. The reporter constructs shown along the vertical axis were integrated in the Δrif1 strain. The horizontal axis represents the % FOA\(^{R}\). Data is from Table 5.
Figure 13: Levels of FOA<sup>R</sup> ratio between Δrif1 mutant and wild type cells:

Ratios of %FOA<sup>R</sup> in the Δrif1 mutant strain versus the %FOA<sup>R</sup> in the wild type (BY4742) strain. Data is from Table 6. The arrows underneath the exponential graph indicate increase or decrease of silencing.
As expected, the deletion of \textit{RIF1} caused an increase of silencing in URA3-tel (Figure 12). There is a more significant increase of silencing in GF2 and GF3, while GF 6, GF9, GF46 and GF61 showed mostly modest changes in the repression of \textit{URA3} relative to the wild type strain. This suggests \textit{RIF1} may work in parallel with \textit{STAR} elements to diminish silencing. More importantly, my assays have recapitulated the expected effects of the deletion of \textit{RIF1}. Hence, I validated that there is no obvious flaw in the design and the execution of my experiments and that the effects of the $\Delta rtt109$ mutation should be taken at face value.

\textbf{4.6 Comparison of the effects of \textit{esa1-414}, $\Delta rtt109$ and $\Delta rif1$}

In Figure 14, I put all the data together to compare the effects of the three mutations in all constructs. This display of my data reiterates the notion that the deletion of \textit{RTT109} produces different effects as compared to the \textit{esa1-414} mutation. The $\Delta rif1$ strain has shown the expected effects. Figure 15 recaptures the dramatic and sometimes unexpected results with the GF1, GF2 and GF3 constructs, which lack \textit{core X} elements. The construct GF3 in all of the strains examined here showed very high levels of repression on mutation or deletion of the HATs, which suggests that the anti-silencing activity of HATs may also involve interaction with the \textit{STARs} of the \textit{Y'} element. The alternative is that GF3 has some unknown “defect” that precludes analysis of TPE. I also observed that the constructs GF6, GF9, GF44, GF46 and GF61 show very modest effects of repression or de-repression compared to other constructs. This reinforces the notion that leads \textit{core X} and \textit{Y'} elements may act as “epigenetic” buffers to protect the subtelomeric regions form extreme variation in chromatin structure and levels of gene expression.
Figure 14: Compiled repression levels of all mutants and controls

The reporter constructs shown along the vertical axis were integrated in the respective strains. The horizontal axis represents the % FOA^R. Data is from Table 5.
Figure 15: Levels of FOA$^R$ ratio between all mutant strains and wild type cells

Ratios of %FOA$^R$ in the mutant strains versus the %FOA$^R$ in the wild type (BY4742) strain. Data is from Table 6. The arrows underneath the exponential graph indicate increase or decrease of silencing.
5 DISCUSSION

5.1 Role of ESA1 and RTT109 in gene silencing

My hypothesis was ESA1 and RTT109 are involved in anti-silencing activities at telomeres through specific subtelomeric elements. In order to test this hypothesis I transformed the mutant strains esa1-414 (a conditional mutant of ESA1) and Δrtt109 (a deletion mutant lacking RTT109) with an array of constructs containing a URA3 reporter gene and a variety of natural subtelomeric elements. The levels of repression were measured as per cent FOA^R cells. The comparison of these mutant strains to wild type also shed light on the relation of the HATs of interest and the subtelomeric elements.

As a whole, the data has shown that the esa1-414 mutation caused the overall increase of repression of the constructs (Fig. 14). This outcome is consistent with a role of ESA1 as a modest anti-repressor. However, the effects are generally weak and similar to the ones observed for other HATs (HAT1, GCN5, SAS2 and SAS3) (POWER et al 2010). This notion suggests that all these HATs probably act indirectly by globally counteracting the activity of focal deacetylation by Sir2. The overall involvement of each of these HATs in gene silencing should therefore be considered non-specific. One would imagine that they provide a low level of HAT activity that need to be overcome by the local strong HDAC activity achieved through the recruitment of the SIR complex.

In contrast, the deletion of RTT109 caused an increase in expression of the constructs. This is a somewhat surprising effect for an enzyme that is supposed to facilitate the formation of open chromatin. Again, I anticipate that this effect is indirect, and most likely takes place through the regulation of the expression of genes that could be involved in the finer tuning of TPE. For example, one possibility is that Rtt109 acetylates and promotes the expression of a gene which
produces a silencing factor and the deletion of *RTT109* results in repression of this gene, which brings about over all increased expression. An alternative to this explanation is that in ∆rtt109 cells the supply of histones to the advancing replication fork is reduced thus leading to perturbation of the reassembly of heterochromatin and the consequent de-repression.

In conclusion, my experiments did not produce data to suggest that these two HATs are directly involved in the regulation of telomeric gene repression.

### 5.2 The nature of the mutations used in this study

In my research the strains I used were *esa1-414* and ∆rtt109. The deletion mutant of *ESA1* could not be generated as it is essential for cell viability. Thus, I acquired a temperature sensitive mutant strain with a severe growth phenotype from Dr. Pillus (CLARKE & PILLUS 1999). Under permissive temperature this conditional mutant has partial, not complete loss of function. On the other hand, gene silencing is defined as gene repression over multiple generations. Hence, the TPE assays are multigenerational thus precluding experiments with this strain at the restrictive temperature where complete loss of function is expected. It is clear that the residual activity supports some critical function, but it is not clear to what extent it affects gene silencing. Hence, my conclusions regarding the role of *ESA1* are indicative, but cannot serve as decisive evidence for the role of this gene in TPE. For example, even if complete loss of Esa1 function causes dramatic alterations in TPE, these effects will be masked by the loss of viability. An alternative approach to address this question would be to monitor the expression of a *URA3* reporter by PCR immediately after shifting to restrictive temperature and test for dramatic decline/increase in the abundance of the *URA3* mRNA. Another alternative is to apply the single cell format assays with GFP as a reporter that have been used previously to study the repression
at the mating type loci (Osborne & Rine, 2009). For example, GFP reporter driven by the URA3 promoter can be inserted at the telomere and its expression can be monitored in single cells upon shifting the esa1-414 mutant to restrictive temperature. Any increase in the proportion of cells expressing GFP would be indicative of change in the levels of gene silencing.

In contrast to esa1-414, the deletion of RTT109 leads to complete loss of function and allows unequivocal conclusions.

5.3 Effects of the mutations in individual constructs

In my set of subtelomeric constructs, GF1 showed the most variability. It is important that this simplified construct contains no subtelomeric elements. Many studies (Kimura et al. 2002; Rehman et al. 2006; Reifsnyder et al. 1996; Shia et al. 2006; Suka et al. 2002; Xu et al. 1999) have shown that mutations that have subtle effect on natural telomeres show remarkably strong reduction of silencing with this construct. So, this construct could be an exceptionally sensitive probe for minor effects on gene silencing, but does not truly represent the situation at natural telomeres. According to the data with this construct only, both RTT109 and ESA1 act as anti-silencers and their effect is similar to that of the deletion of SAS2 and SAS3 (Power et al. 2011). SAS2 counteracts the deacetylation of H4-K16 by Sir2 and may work in imposing the formation of a chromatin boundary at this simplified telomere (Maillet et al. 1996; Roy & Runge 2000; Wiley & Zakian 1995). If so, ESA1 and RTT109 could also act to limit the indiscriminate association of silencing factors to chromatin away from the telomere as is the case with SAS2 (Kimura et al. 2002; Suka et al. 2002).

GF2 and GF3 contain STARs from the core X and Y’ elements. These elements reduce telomeric and mating type loci silencing (Fourel et al. 1999). If any of the mutations tested in
this study works through these elements, I should see this reduction diminished. This is not the case for GF2. In both *esa1-414* and *Δrtt109* mutants this construct displayed de-repression relative to GF1 (Fig. 14). Interestingly, GF3 showed the opposite effect. This leads me to conclude that there is some deficiency in this construct that I am not aware of. The experiments with GF3 should be repeated to confirm if the *STARs* in GF2 and GF3 truly have opposite activities in these mutants.

The rest of the constructs contain natural *core X* and *Y’* subtelomeric elements (Chan and Tye 1983; Walmsley et al. 1984). In my hands, TPE remains largely undisturbed at these *core X* - and *Y’*- containing telomeres (Figure 14). Earlier studies have also demonstrated that the anti-silencing caused by mutations in DNA replication factors or other HATs is reduced by *core X* - and *Y’* (Power et al. 2011; Rehman et al. 2006). Whereas the precise mechanism of the effects of each individual HAT or replication factor mutation remains unknown, it is apparent that *core X* - and *Y’* tamper most of the effects observed at the “simple” URA3-tel telomere. I also need mention that the synthetic *core X* - and *Y’*- containing telomeres display moderate deviations in TPE that compare in magnitude the effects observed at natural telomeres (Dang et al. 2009; Ehrentraut et al. 2010; Kimura et al. 2002; Kozak et al. 2010; Suka et al. 2002).

It is known that *core X* and *Y’* contribute to gene repression, and that subtelomeres contain anti-silencing modules such as the *STARs* (Fourel et al. 1999; Pryde and Louis 1999; Rehman et al. 2006; Rehman et al. 2009). The opposing signals emitted by these elements have been implicated in the variegated nature of subtelomeric gene expression (Fourel et al. 2004). An interesting feature of TPE at individual telomeres is that despite the random conversion between active and repressed state, the proportion of cells with active/repressed genes remains stable. The mechanisms that sustain this meta-stable balance are not understood. Here I provide data, which
supports the idea that subtelomeric core X and Y’ could play a significant and unexpected role in the dynamic meta-stability of telomeric expression. My data together with the rest of the data in Power et al. (2011), show that these elements can also reduce telomeric gene repression when silencing increases. Therefore I propose that these elements contain not only individual proto-silencers such as ACS and binding sites Rap1 and Abf1 (Fourel et al. 2002), but also some unidentified anti-silencers. Ultimately, the multiplicity of individual weak proto-silencers and anti-silencers in core X and Y’ build up “buffering” cis-elements, which suppress extreme variations in TPE.

6 SIGNIFICANCE OF MY RESEARCH

As mentioned above, though the mechanisms of silencing were fairly well understood, very little was known about the mode of operation of anti-silencers in comparison.

I found the HATs function indirectly rather than through specific subtelomeric elements to achieve anti-silencing at telomeres, this provided novel insight into how HATs globally counteract focal deacetylation by SIR2. This suggests there are numerous other mechanisms at work which must be taken into account. I also observed to some extent that HATs acted in concert with STAR elements. On the whole, we still need to find out if other subtelomeric elements contain some unidentified anti-silencers.

This information gives us a deeper understanding of anti-silencing and will help in future studies and in determining the mode of action of anti-silencers and subtelomeric elements involved. These issues are of fundamental significance and will contribute to the dynamic and expanding field of epigenetics.
7 FUTURE DIRECTIONS

Fluoro-orotic acid (FOA) has a selective toxicity for cells expressing \textit{URA3}. This effect is mediated by the \textit{URA3}-encoded Orotidine-5-Phosphate-Decarboxylase (O-5-PD), an enzyme involved in the synthesis of pyrimidines. FOA is a neutral substance which is converted into a powerful toxin by O-5-PD. Hence, the selection of \textit{URA3}-containing cells on FOA medium allows the growth of cells in which \textit{URA3} is repressed. The FOA\textsuperscript{R} assay is simple and very sensitive, due to its reliability it has been widely used as genetic screens. However, recently the validity and applicability of FOA-resistance assay has been contested. FOA is a drug and it has been suggested that it may have side effects on the data it generates, particularly fluctuations in the nucleotide pool and an imbalance in the levels RiboNucleotide Reductase activity induced by FOA (Takahashi \textit{et al.} 2010; Yankulov 2011). These two side effects could be mostly responsible for discrepancies in the data produced by this assay.

In the future we can design and prepare transforming constructs for a generation of strains that can be used in novel assays of gene silencing and positional variegation. We can try to produce a fusion product of \textit{URA3}-\textit{GFP} in \textit{UCAIV} by subjecting the \textit{URA3} reporter gene to polymerase chain reaction with primers which would anneal just upstream of the STOP codon of \textit{URA3} and would contain an open reading frame of GFP as a tag. As a result we could generate a fusion protein \textit{URA3}-\textit{GFP} protein which would include the properties of \textit{URA3} and \textit{GFP}. The \textit{URA3} product of the fusion protein could serve as a selectable marker while we could analyze silencing or expression by observing the fluorescence provided by the GFP portion. This alternative assay would have several advantages over the traditional FOA-resistance assay, such as GFP fluorescence can be used to monitor the variegation for a single cell. But the greatest asset would be that there could not be a discrepancy originating through modification of
biochemical pathways which can be produced if we use a drug as a rigid negative selection pressure.
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