Rap1-Mediated Chromatin And Gene Expression Changes At Senescence

Shufei Song
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Rap1-Mediated Chromatin And Gene Expression Changes At Senescence

Abstract

RAP1-MEDIATED CHROMATIN AND GENE EXPRESSION CHANGES AT SENESCENCE

The telomeric protein Rap1 has been extensively studied for its roles as a transcriptional activator and repressor. Indeed, in both yeast and mammals, Rap1 is known to bind throughout the genome to reorganize chromatin and regulate gene transcription. Previously, our lab published evidence that Rap1 plays important roles in cellular senescence. In telomerase-deficient S. cerevisiae, Rap1 relocalizes from telomeres and subtelomeres to new Rap1 target at senescence (NRTS). This leads to two types of histone loss: Rap1 lowers global histone levels by repressing histone gene transcription and it also results in local nucleosome displacement at the promoters of the activated NRTS. Here, I examine mechanisms of site-specific histone loss by presenting evidence that Rap1 can directly interact with histone tetramers H3/H4, and map this interaction to a three-amino-acid-patch within the DNA binding domain. Functional studies are performed in vivo using a mutant form of Rap1 with weakened histone interactions, and deficient promoter clearance as well as blunted gene activation is observed, indicating that direct Rap1-H3/H4 interactions are involved in nucleosome displacement. In addition, I explore histone chaperones and chromatin remodelers that may function as Rap1 co-activators at senescence, and found that the histone H3/H4 chaperone Asf1 is required for full Rap1-mediated nucleosome displacement and gene activation. The epigenetic mark H3K4me3 is similarly involved, though the exact details of how this crosstalk occur remain to be elucidated. Remarkably, blunting NRTS activation, at least in the cases of the Rap1 mutant and Asf1 deletion, do not affect the pace of senescence-related cell cycle arrest, suggesting that negative aspects of senescence-related gene expression changes can be uncoupled from the tumor-suppressive properties of cell senescence. Furthermore, as features of the yeast and mammalian Rap1 proteins are conserved, I also explore roles of hRAP1 in human fibroblast senescence. Genome-wide ChIP-seq in senescent IMR-90s and RNA-seq in fibroblasts overexpressing hRAP1 reveal important roles for hRAP1, including relocalization to and regulation of histone genes and senescence-related genes. Direct H3/H4 interactions are also conserved, though more mapping studies are needed to determine the exact interaction surface on mammalian RAP1. Together, these studies illuminate mechanisms of Rap1-mediated senescence changes.

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RAP1-MEDIATED CHROMATIN AND GENE EXPRESSION CHANGES AT SENESCENCE

Shufei Song

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in

Biochemistry and Molecular Biophysics

Presented to the Faculties of the University of Pennsylvania

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Degree of Doctor of Philosophy

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ACKNOWLEDGMENT

I recently went back to personal statements I had written when applying to graduate school, and perhaps in a moment of naivete I wrote “Not only do I want to push and test the known boundaries, I also hope to contribute something worthwhile to science and humanity as we battle with the secrets of life”. Grad school quickly disillusioned me when I realized that science, much like anything else in life, is composed of the nitty-gritty: the grand ideas yes, but also the endless troubleshooting, the frustrations of experiments not repeating, the uncertain interpretation of results and the effects of these on one’s happiness and self-worth. Looking back, there were certainly low points in my PhD that I couldn’t have moved past if not for the very special people in my life.

Therefore, I really want to thank my advisor Brad for his constant support and his optimism, for explaining science in gentle and fun ways, for always being available with helpful suggestions, from big-picture directions on where my project is headed down to details of buffer components when experiments fail. He has modeled for me and others in the lab what it means to be a scientist with his extensive grasp of literature and keen sense for hot science topics and developments, but most importantly, with his character and integrity. He has also been one of my main sources of American political news, and though I still can’t keep who’s who straight in my head, Brad’s deep concern for the world, compassion for the less fortunate, and his staunch insistence on doing what’s right instead of what’s easy or beneficial has moved me to no end.

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ABSTRACT

RAP1-MEDIATED CHROMATIN AND GENE EXPRESSION CHANGES AT SENESCENCE

Shufei Song
F. Brad Johnson

The telomeric protein Rap1 has been extensively studied for its roles as a transcriptional activator and repressor. Indeed, in both yeast and mammals, Rap1 is known to bind throughout the genome to reorganize chromatin and regulate gene transcription. Previously, our lab published evidence that Rap1 plays important roles in cellular senescence. In telomerase-deficient S. cerevisiae, Rap1 relocalizes from telomeres and subtelomeres to new Rap1 target at senescence (NRTS). This leads to two types of histone loss: Rap1 lowers global histone levels by repressing histone gene transcription and it also results in local nucleosome displacement at the promoters of the activated NRTS. Here, I examine mechanisms of site-specific histone loss by presenting evidence that Rap1 can directly interact with histone tetramers H3/H4, and map this interaction to a three-amino-acid-patch within the DNA binding domain. Functional studies are performed in vivo using a mutant form of Rap1 with weakened histone interactions, and deficient promoter clearance as well as blunted gene activation is observed, indicating that direct Rap1-H3/H4 interactions are involved in nucleosome displacement. In addition, I explore histone chaperones and chromatin remodelers that may function as Rap1 co-activators at senescence, and found that the histone H3/H4 chaperone Asf1 is required for full Rap1-mediated nucleosome displacement and gene activation. The epigenetic mark H3K4me3 is similarly involved, though the exact details of how this crosstalk occur remain to be elucidated. Remarkably, blunting NRTS activation, at least in the cases of the Rap1 mutant and Asf1 deletion, do not affect the pace of senescence-related cell cycle arrest, suggesting that negative aspects of senescence-related gene expression changes can be uncoupled from the tumor-suppressive properties of cell senescence. Furthermore, as features of the yeast and mammalian Rap1 proteins are conserved, I also explore roles of hRAP1 in human fibroblast senescence. Genome-wide ChIP-seq in
senescent IMR-90s and RNA-seq in fibroblasts overexpressing hRAP1 reveal important roles for hRAP1, including relocation of and regulation of histone genes and senescence-related genes. Direct H3/H4 interactions are also conserved, though more mapping studies are needed to determine the exact interaction surface on mammalian RAP1. Together, these studies illuminate mechanisms of Rap1-mediated senescence changes.
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CHAPTER 1 Introduction and Literature Review

*Sections of this chapter is adapted from a published review by Song et al., (Song & Johnson, 2018)

1.1 An overview of aging mechanisms

Aging is one of the major causes of death in the developed world, even though it is rarely recognized as such. It is usually some other cause that is written down on death certificates, e.g. heart failure, stroke, cancer, pneumonia or respiratory failure. However, there is no doubt that these pathologies are brought on largely by age-related biological changes.

The question of why we age is still the subject of vigorous debate. Aging occurs in most species, although the fact that some show little to no signs of aging indicates that it is not necessarily inevitable (Schaible et al., 2015). Historically, aging theories have often been divided into two seemingly opposing categories, invoking either biological programs or random damage. Programmed aging theories include those postulating that individual organisms are set on a purposeful path of deterioration and ultimately death, perhaps enhancing survival of the species by making room for new individuals. Proponents of this view point out that different species of animals, despite their similarities at cellular and molecular levels, and despite living in similar environments, nonetheless age at drastically different rates (Kirkwood & Austad, 2000). For example, mice live at most three-to-four years, twice the lifespan of the forest shrew, but only a tenth that of the naked mole rat, which has a lifespan of three decades. Therefore, the argument is that there must be some internally programmed clock dictating an organism to age and die. The other set of theories invoke the “wear and tear” concept, i.e. that aging is an accumulation of damage that causes cellular and organismal functions to decline and ultimately lead to death. A less-than-elegant way of describing this is that things simply “fall apart”, as entropy drives biological systems away from the intricate order essential for life.
There are numerous problems with these theories, at least as they are presented above. In the case of programmed aging theories, several considerations raise concerns. First, when thinking about human aging in particular, only in the past two-to-three centuries, out of the 200,000 years of human existence, did aging become a major roadblock to the survival of most individuals. Michel de Montaigne wrote in the 16th century, “To die of old age is a death rare, extraordinary, and singular, and therefore so much less natural than the others: it is the last and most extreme sort of dying... And therefore my opinion is that when once forty years old, we should consider it as an age to which very few arrive... and since we have exceeded the ordinary bounds which make the just measure of life, we ought not to expect to go much further.” With an average life expectancy of thirty years, it seems hardly likely that evolution, with its selection based on reproductive fitness, would have prioritized a program that only initiates well past expected lifespan. Second, and for the same reason (that evolution selects for reproductive fitness), a deleterious, pro-aging program that manifests after genes have already been passed to offspring is unlikely to be selected against strongly. Rather, it is likely the decline in selective pressure that occurs with each additional offspring produced allows for genetic mechanisms that contribute to aging to emerge and persist in the gene pool over evolutionary time (Kirkwood & Austad, 2000). A third, and more fundamental, problem with programmed aging theories is that they rely on circular logic: there is no need for an aging program to eliminate older individuals if there is no aging in the first place. Fourth, rare individuals in whom aging programs have been lost due to random mutation would, if anything, have greater reproductive success than their aging counterparts, and thus the program would tend to be lost gradually from the population over generations. Therefore, it is quite unlikely that aging is programmed per se, i.e. that its purpose is to ensure the decline and eventual death of individuals. Of course, this still leaves open the possibility that aging is driven by biological programs selected by evolution for other purposes. As described further below, a good example of such a program is cellular senescence, which helps prevent cancer into reproductive age, but also has the side effect of driving tissue aging. In addition, biological programs that do not drive aging, and may even protect against it, can modulate precisely how aging unfolds. Overall,
although not programmed as an end in itself, aging involves highly mechanistic, and thus understandable and malleable, biological processes.

The wear-and-tear theories also leave questions unexplained, although it is fairly clear that in some fashion molecular and cellular damage are fundamental drivers of aging. In particular, the identities of the chief sources of damage remain uncertain, with current leading candidates including the damage and mutation of DNA, both nuclear (including telomeres) and mitochondrial, oxidative damage, glycation, protein aggregation, immune dysregulation, and, the dysfunction of epigenetic mechanisms (Fulop et al., 2017; Gorbunova & Seluanov, 2016; Klaips, Jayaraj, & Hartl, 2018; Park & Larsson, 2011; Senatus & Schmidt, 2017; Song & Johnson, 2018; Srivastava, 2017; T.-L. B. Yang, Song, & Johnson, 2016). Furthermore, these mechanisms, which involve seemingly random molecular damage, may not comport with our conceptions of how biological processes are impacted by aging. On the one hand, different individuals do experience aging distinctly, consistent with roles for stochastic damage. For example, some individuals are spared wrinkled skin but are profoundly affected by Alzheimer’s disease, whereas others succumb to cancer or cardiovascular disease despite many having lived highly health-conscious lifestyles. On the other hand, the fact that aging has highly stereotyped features, particularly within a species, is inescapable: people generally have no difficulty in distinguishing old individuals from young, and there are indeed many biological changes and hallmarks that occur with regularity, some even across different organisms (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013).

There must, then, be ways in which the various types of molecular damage are summed into stereotyped biological outputs. Good examples of such integrators are the cellular stress responses, including a specialized form called cellular senescence. Cellular stress responses generally contribute to cell, and thus tissue and organismal survival in the face of various sources of damage and stress, including starvation, extremes in temperature, chemical toxins, and ionizing radiation. Examples of such stress responses are the use of metabolic flexibility to utilize available energy sources, activation of enzymes that neutralize oxidants and other toxins, induction of heat
shock proteins with chaperone refolding of denatured proteins, upregulation of autophagy and other proteolytic mechanisms which can degrade damaged or aggregated proteins as well as provide new sources of cellular fuel, and the recognition and repair of DNA damage. Remarkably, genetic, dietary, or pharmacologic manipulations that promote longevity typically also enhance stress responses generally, supporting roles for these responses in countering aging, and indeed in many cases particular stress responses are known to be required for the enhanced lifespan and healthspan provided by various experimental manipulations (Dues et al., 2016; Labunskyy et al., 2014; Postnikoff, Johnson, & Tyler, 2017). Failures of these responses to fully resist or repair damage may therefore be key drivers of aging, and thus the weakest links in the web of stress responses may correspond to the most pro-aging insults. Because aging *per se* is not selected evolutionarily, but rather is due to the decline in selective pressure after genes have been passed to offspring, it should be freer to vary than many biological mechanisms, and therefore the particular links that are weakest may be different among species and even among individuals within a species. In this way, stress responses can help unify how cells experience damage while still leaving room for some variation in aging phenotypes.

1.2 Cellular senescence and aging

Cell senescence is a programmed stress response different from those just described in that, rather than countering aging, it can contribute to it. It is activated by stresses that put cells at risk for becoming cancerous, including DNA damage, telomere dysfunction, chromatin perturbations, and mitochondrial dysfunction. Cell senescence is characterized by both a permanent arrest of cell division but maintained cellular viability, and by profound changes in cellular gene expression and physiology, including the secretion of inflammatory cytokines and proteases collectively called the SASP (senescence-associated secretory phenotype) (Rodier & Campisi, 2011). Although cell senescence protects younger individuals from cancer, the accumulation of senescent cells with age can interfere with normal tissue homeostasis. There is
strong evidence that cell senescence can drive age-related pathology in two ways: 1) by limiting stem cell replicative capacity (Krishnamurthy et al., 2006; Molofsky et al., 2006; Sharpless, A, 2009, n.d.) and 2) by disrupting the tissues in which senescent cells reside. Of particular note, Jan van Deursen's group showed that elimination of senescent cells forestalled the development of age-related pathologies in mutant mice with premature aging symptoms (Baker et al., 2011). Recently, the same group showed that targeting and clearance of senescent cells occurring with natural aging in mice delayed tumorigenesis and slowed age-related deterioration (Baker et al., 2016). Other studies have shown similarly that targeted apoptosis of senescent cells can restore fitness, hair density, and renal function (Baar et al., 2017), inhibit atherosclerosis (Childs et al., 2016), and ameliorate pulmonary fibrosis (Schafer et al., 2017).

1.3 Telomere structure and function in aging

Telomeres are the structures at chromosome ends, and comprise tandem repeats of DNA, which in vertebrates have the sequence 5’ – TTAGGG – 3’, and end with a 3’ single stranded overhang at least 100 nucleotides in length. Vertebrate telomeres are usually “capped” by shelterin complex proteins, including TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 (Palm & de Lange, 2008). For several reasons, telomere length can shorten with each cell division. This is often attributed to the “end replication problem”, which is the inability during genome replication of the RNA that primes lagging strand synthesis of the final Okazaki fragment to be replaced by DNA. During replication of most of the genome, subsequent Okazaki fragments extend in the 3’ direction to replace the RNA primers priming the previous fragment, thus replacing it with DNA. However, because no such subsequent fragment exists at the chromosome end, the removal of this RNA primer leaves a gap that is not filled by DNA synthesis. This would make sense as a cause of telomere shortening save for the fact that telomeres naturally contain long 3’ overhangs, which means that, if the RNA primer is placed at the very chromosome end, no shortening of the lagging strand need occur. The actual reasons for telomere shortening caused by normal genome
replication, at least in the human cells studied, appear to be that 1) rather than being placed at the very end of the telomeric DNA, the final RNA primer for lagging strand synthesis is placed internally and 2) after replication generates the blunt ended product of leading strand synthesis, it is exonucleolytically processed so as to develop the 3’ single stranded overhang necessary for telomere functions (Chow, Zhao, Mak, Shay, & Wright, 2012; Wu, van Overbeek, Rooney, & de Lange, 2010). On top of these considerations, direct damage to telomere DNA (e.g. oxidative damage) and occasional replication fork collapse within telomeres, which are inherently difficult to replicate at least in part due to DNA secondary structures, also degrade telomeres and can sometimes lead to sudden and dramatic shortening (Richter & Zglinicki, 2007; Serra, Zglinicki, Lorenz, & Saretzki, 2003; Sfeir et al., 2009; Suram & Herbig, 2014; Suram et al., 2012; Zglinicki, 2002; Zglinicki, Saretzki, Döcke, & Lotze, 1995). Telomere shortening can be countered by the action of telomerase, which can synthesize new repeats at telomere ends. However, in humans telomerase activity is restricted primarily to the progenitor cells of high turnover tissues, and telomeres shorten with age in most tissues (Daniali et al., 2013; Müezzinler, Zaineddin, & Brenner, 2013).

Telomere length has proven to be an important regulator of gene expression and cellular signaling. The most well-established mechanisms involve triggering of DNA damage responses (DDRs) by critically shortened, i.e. “uncapped”, telomeres, which lead not only to gene expression changes but also to cellular senescence or apoptosis. Of particular note, in mice and zebrafish the mRNA levels from the genes encoding the six shelterin subunits change with age, in a tissue specific fashion (Wagner et al., 2017), and it will be exciting to test if these changes may impact age-related pathologies.

Several lines of evidence indicate that changes at telomeres contribute significantly to human aging. Although much of this evidence has historically been indirect or correlational, recent lines of evidence are more convincing. We, and others, have reviewed this evidence in detail (Song & Johnson, 2018; T.-L. B. Yang et al., 2016), and so will only summarize it here. First, it is clear
that telomeres shorten in most human tissues with age (Müezzinler et al., 2013). Second, telomere shortening can lead to cell senescence or apoptosis in cultured cells (Bodnar et al., 1998). Third, the progeroid syndromes dyskeratosis congenita, Werner syndrome, and Hutchinson-Gilford progeria syndrome include telomere defects, and several lines of evidence indicate that these contribute to premature onset of various age-related diseases affecting these individuals (Benson, Lee, & Aaronson, 2010; Crabbe, Jauch, Naeger, Holtgreve-Grez, & Karlseder, 2007; Crabbe, Verdun, Haggblom, & Karlseder, 2004; Du et al., 2004; Ishikawa et al., 2011; Kudlow, Stanfel, Burtner, Johnston, & Kennedy, 2008; Ouellette et al., 2000; Schulz et al., 1996; Wyllie et al., 2000). Fourth, up to 15% of dermal fibroblasts in elderly baboons contain uncapped telomeres and signs of cell senescence (Herbig, Ferreira, Condel, Carey, & Sedivy, 2006; Jeyapalan, Ferreira, Sedivy, & Herbig, 2007), although similar studies have not yet been reported in humans and in other tissues. Fifth, telomere shortening in easily measured cells (peripheral leukocytes, buccal epithelial cells) is correlated in cross-sectional epidemiological studies with age related diseases, including cardiovascular diseases, diabetes mellitus, osteoporosis, pulmonary fibrosis, cirrhosis, and cancer (Alder et al., 2008; Atzmon et al., 2010; Aviv & Levy, 2012; Fyhrquist, Saijonmaa, & Strandberg, 2013; Moslehi, DePinho, & Sahin, 2012; Rudolph, Chang, Millard, Schreiber-Agus, & DePinho, 2000; Valdes et al., 2007). Sixth, within diseased tissues telomeres are shortest at sites of pathology, including the vascular smooth muscle cells of atherosclerotic plaques and fibrotic areas of lung (Matthews et al., 2006; Snetselaar et al., 2017).

None of the above observations demonstrates definitively that telomere shortening drives aging. For example it could be argued that short telomeres are only a marker of disease, being caused by the same stresses that lead to the disease itself (Jurk et al., 2014). However, two lines of evidence more strongly support a causal role for telomere shortening in natural aging. First, overexpression of telomerase can extend mean lifespan and slow aspects of aging in mice (Bernardes de Jesus et al., 2012; Tomás-Loba et al., 2008). This is true even though compared to humans, mouse aging is relatively unaffected by telomere shortening, a fact which can be seen by comparing the nearly normal health of first-generation mice fully lacking telomerase to people with
dyskeratosis congenita, who only partially lack telomerase activity but who nonetheless become
sick typically in adolescence or early adulthood. These considerations suggest that improved
telomere maintenance may even have greater effects in humans, though we caution that this likely
includes elevated cancer risk, especially in younger individuals. Second, and moreover, so-called
Mendelian randomization studies in humans argue that short telomeres drive several age-related
pathologies, including cardiovascular diseases, pulmonary fibrosis, diabetes mellitus, and even
Alzheimer’s disease while longer telomeres increases risk of cancer (Codd et al., 2013; Demanelis,
Tong, & Pierce, 2019; Kachuri et al., 2019; Kuo, Pilling, Kuchel, Ferrucci, & Melzer, 2019;
Telomeres Mendelian Randomization Collaboration et al., 2017; Zhan et al., 2015). These studies
quantify the extent to which inheritance of different alleles affecting telomere length (for example in
TERT, TERC and NAF1, genes required for full telomerase activity) impact disease risk in
proportion to their effects on telomere length. The demonstration, for example, that someone born
with alleles that lead to telomere lengthening has a lower risk for cardiovascular disease, and that
this lower risk applies to other similar alleles in proportion to their effects on telomere length, argues
clearly that the longer telomeres are responsible for the lowered disease risk. Remarkably, such
individuals are also at higher risk for various cancers, explaining why humans haven’t simply
evolved longer telomeres or higher levels of telomerase, which from a purely technical standpoint
is easily accomplished and in fact characteristic of many species. This lends weight to the
hypothesis that human telomere length is an example of antagonistic pleiotropy: it reflects a
compromise between deleterious effects later in life and protection against cancer earlier in life that
supports fitness and fecundity. Thus, the set point for human telomere length reflects a “tug-of-
war” between death from cancer and death from degenerative diseases. Together, these findings
support a causal role for telomeres in age-related diseases.

1.4 Aging-related histone losses
Loss of histone proteins appears to be a conserved feature of aging from yeast to humans, and is of functional importance in at least some of these cases. Yeast mother cell aging is accompanied by a profound loss of total cellular histones, including a ~50% decrease in nucleosome occupancy genome wide, and is accompanied by less defined positioning of remaining nucleosomes to their usual sites within chromosomes (Feser & Tyler, 2011; Hu et al., 2014). Similar histone losses occur with the replicative senescence of telomerase-deficient yeast, and this is clearly of major importance for determining the altered gene expression in senescent cells because these patterns are closely mimicked by the artificial downregulation of histones (Platt et al., 2013; Wyrick et al., 1999). Moreover, artificial overexpression of core histones promotes longevity in both yeast models of aging, indicating that histone losses may be a key driver of aging. Similar age-related histone losses have been reported in C. elegans worms (Maures, Greer, Hauswirth, & Brunet, 2011). Furthermore, genetic inactivation of the SET-26 histone methyltransferase or lithium treatment enhance both lifespan and levels of histones, or mRNAs encoding histones, respectively (McColl et al., 2008; Ni, Ebata, Alipanahiramandi, & Lee, 2012). These observations suggest that such histone losses may limit longevity in worms, although because each of these manipulations affect more than histone expression (e.g. SET-26 affects H3K9 methylation and lithium has widespread metabolic effects) it is possible that other effects of these manipulations explain the increased lifespan.

Similar decreases in histone synthesis and global histone levels have been observed in mice and humans, although the functional importance of such changes is not yet clear. A decline in histone mRNAs in quiescent (i.e. non-cycling) mouse skeletal muscle stem cells occurs with age, which might contribute to known age-related losses in function of these cells, though it is notable that these transcripts return to normal levels when the stem cells are activated by acute injury (L. Liu et al., 2013). Cultured early-passage fibroblasts from a 92-year-old were found to have 50% less synthesis of histones compared to those from a nine-year-old (O'Sullivan, Kubicek, Schreiber, & Karlseder, 2010). Furthermore, human IMR90 and WI38 fibroblasts passaged extensively in culture to drive their replicative senescence demonstrated significant changes in histone
biosynthesis and processing, including downregulation of histone H3 and H4 synthesis and extensive changes in the levels of several histone PTMs (O'Sullivan et al., 2010). In vivo histone loss in human skin was also observed in the melanocytes of benign (i.e. pre-malignant) nevi. Interestingly, depletion of histones was most marked in regions of the nevi that have been inversely linked with malignancy, suggesting that histone loss is linked to enhanced senescence-associated proliferation arrest and thus tumor suppression (Ivanov et al., 2013).

How histone levels decline, and how such declines drive aging, are poorly understood. In the case of human senescent fibroblasts, histone losses were suppressed by artificial expression of telomerase, and therefore DNA damage signals coming from shortened telomeres are likely to be involved (O'Sullivan et al., 2010). In the case of senescence in yeast telomerase mutants, DNA damage signaling is also involved, and a key role has been defined for Rap1 (Platt et al., 2013), reviewed in detail below. In both yeast mother cell aging and human cultured fibroblast, IMR90, senescence, the total synthesis of histones is downregulated, but underlying mechanisms are not known. Besides reduced synthesis, increased degradation of histones may also be involved. For example, senescent cells show an upregulation in autophagic and lysosomal activities, and inhibition of the lysosomal pathway antagonized the decrease in histone content in senescing IMR90s (Ivanov et al., 2013). Furthermore, chromatin in senescent cells is ubiquitinated and co-localized with “p62 bodies”, which recognize and target ubiquitinated proteins for autophagic degradation (Ivanov et al., 2013). Despite this evidence for autophagy driving aspects of cellular senescence, several lines of evidence indicate that autophagy actually helps promote longevity (J. T. Chang, Kumsta, Hellman, Adams, & Hansen, 2017; Eisenberg et al., 2009; Hara et al., 2006; Harrison et al., 2009; Meléndez et al., 2003; Pyo et al., 2013), and thus any role for autophagy in age-related histone losses seem unlikely to be a major determinant of its effects on aging overall.

If histone losses are an important driver of aging, how exactly they contribute is still an open question. However, it is likely that inappropriate gene expression is involved. For example, a decline in global histone levels might contribute to heterochromatin losses, which in turn could
lead to derepression of genes and aberrant gene expression. In worms, age-dependent loss of peripheral heterochromatin and changes in nuclear architecture were detected in non-neuronal cells (Haithcock et al., 2005). In *Drosophila*, decreased heterochromatin correlates with shortened lifespans and vice versa (Larson et al., 2012). In humans, cells cultured from HGPS patients showed a loss of heterochromatin along with loss of heterochromatic marks H3K27me3 and H3K9me3 (Shumaker et al., 2006). Skin fibroblasts from normally-aged individuals showed similar defects compared to HGPS patients, including nuclear aberrations, epigenetic changes and accumulation of DNA damage (Scaffidi & Misteli, 2006). ATAC-seq analyses of human peripheral blood mononuclear cells revealed age-related stochastic opening of silenced genomic regions, but along with decreased chromatin accessibility in regulatory regions associated with T cell signaling (Ucar et al., 2017). In addition, a decline in total histone levels may lead to preferential losses at particular genomic regions, and thus an imbalance in the expression of genes (transcriptional drift), which may compromise the ability of cells to perform normal functions and respond to stresses.

How the distribution of nucleosomes throughout the genome changes with age is understudied, but careful measurements in aged yeast mother cells indicate that remaining nucleosomes mostly become delocalized (Hu et al., 2014). However, this view comes from examining populations of old cells, and thus whether nucleosomes might linger at different particular loci in individual cells remains unclear. An alternative way in which preferential nucleosome losses at particular genomic regions might contribute to aging is toxicity from the overexpression of certain genes. Along these lines, there is a growing body of evidence that depression of transposable elements, which are normally silenced in healthy cells, occurs with age and may also drive age-related defects. For example, age-dependent increased expression of LTR retrotransposons have been observed in *Drosophila* brain (W. Li et al., 2013); retrotransposon elements (RTEs) and satellite sequences increased with age in both *in vivo* mouse models and cultured human diploid fibroblasts (De Cecco et al., 2013); LINE, SINE and LTR transposon derepression is commonly seen in neurodegenerative disorders in humans (Coufal et al., 2011; Douville, Liu, Rothstein, & Nath, 2011; Jeong, Lee, Carp, & Kim, 2010; Muotri et al., 2010), and repression of LINE1 with reverse
transcriptase inhibitors in SIRT6 deficient mice significantly improves health and extends lifespan (Simon et al., 2019).

The DNA damage response (DDR) is likely to be one important trigger of histone losses with age. Senescing human fibroblasts accumulate DNA damage at telomeres and elsewhere, and thus activate the DDR (Herbig, Jobling, Chen, Chen, & Sedivy, 2004), and it was proposed that DDR-mediated decreases in levels of the stem-loop binding protein might contribute to diminished expression of histones (O’Sullivan et al., 2010). Histone losses driven by Rap1 in senescing yeast require Mec1, an ATR/ATM homologue which mediates much of the DDR, including that activated in response to critical telomere shortening (Platt et al., 2013). Because critically shortened telomeres resist repair, it was proposed that, like other difficult to repair DNA breaks, the purpose of induced histone losses might be to enhance both chromatin flexibility and “openness” so as to stimulate the search for homology and invasion of intact template DNA by broken ends that underlies repair by homologous recombination (HR) (Dion, Kalck, Horigome, Towbin, & Gasser, 2012; Miné-Hattab & Rothstein, 2012; Platt et al., 2013). In other words, a mechanism that is helpful for the repair of non-telomeric DNA breaks in normal cells might also be activated in senescent cells though perhaps without benefit. Indeed, experimental depletion of histone H4 enhances HR (Prado & Aguilera, 2005), and, moreover, it has been found that cellular histone levels drop by 20-40% genome-wide in response to DNA damage (Hauer et al., 2017), a degree comparable with senescent cells. This loss is proteosome-mediated and requires both the DNA damage checkpoint and the INO80 chromatin remodeler (Hauer et al., 2017).

Several lines of evidence suggest that histone chaperones and chromatin remodelers also could be key players in reshaping the chromatin landscape during aging and senescence. Asf1, a histone H3/H4 chaperone that facilitates histone deposition, exchange and removal, and serves as a “histone buffer” to ensure that a pool of free histones is readily available to be deposited (Groth et al., 2005), has been linked to histone loss and chromosome condensation. In yeast mothers, cells lacking Asf1 are short-lived by about 20 generations compared to wildtype (Feser et al., 2010).
The mechanism by which Asf1 promotes longevity is still unclear, though it is possible that it facilitates dynamic exchange of damaged histones and increases histone deposition to repress stochastic gene transcription (Das & Tyler, 2012). An alternative mechanism that might be of particular importance in the setting of critically shortened telomeres or other sources of DDR signaling, could involve regulation of the dynamics of histone levels. Because yeast Asf1 binds soluble histones, and moreover, through direct binding inhibits the ability of the DDR checkpoint kinase Rad53 to direct the proteolytic degradation of histone proteins (Jiao et al., 2012; Singh, Kabbaj, Paik, & Gunjan, 2009), Asf1 might be important to restrain excessive histone degradation. Furthermore, histone H3 and Rad53 compete for binding to Asf1, and therefore the diminished levels of histones with age or senescence would free up Asf1 to inhibit Rad53, so that when the DDR ceases following repair of any reversible telomere or other DNA damage, Rad53 would be robustly and rapidly inhibited, leading to restoration of histone levels and resumption of the cell cycle (Tsabar et al., 2016). Consistent with this idea, in late passage IMR90 fibroblasts, ASF1a and ASF1b were substantially downregulated (O'Sullivan et al., 2010).

If losses of histones at particular genomic loci contribute to aging, then it stands to reason that ATP-dependent chromatin remodelers, which can reposition nucleosomes away from some sites and toward others, might also regulate aging. Indeed, several such enzymes have been implicated in age-associated chromatin reshaping and lifespan regulation. There are at least five evolutionarily conserved families of chromatin remodeling ATPases: SWI/SNF, ISWI, CHD/NuRD, INO80, and SWR1 (Becker & Workman, 2013). It was found that in yeast, deletion or mutation of the Isw2 enzyme complex delays yeast mother cells aging and replicative senescence (Dang et al., 2014). Further investigation suggests that deletion of Isw2 and caloric restriction, which delays aging in many organisms, show significant overlap in nucleosome positioning shifts, suggesting that they may share a common pathway in promoting lifespan extension. In C. elegans, the subfamily SWI/SNF was linked to DAF16-mediated gene transcription, stress response and longevity (Riedel et al., 2013), possibly through chromatin remodeling mechanisms.
1.5 Role of telomeric protein Rap1 in senescence and aging

Repressor Activator Protein 1 (Rap1) is a shelterin protein that is conserved from yeast to humans, and has been connected to cellular senescence and histone loss, at least in yeast. In yeast cells, Rap1 is a sequence-specific transcription factor that typically activates transcription, and does so at roughly five-to-ten percent of all yeast genes, particularly the highly expressed genes encoding ribosomal proteins and glycolytic enzymes (Chambers, Tsang, Stanway, Kingsman, & Kingsman, 1989; Graham, Haw, Spink, Halden, & Chambers, 1999; Huet et al., 1985; McNeil, Dykshoorn, Huy, & Small, 1990; Mizuno et al., 2004; Scott & Baker, 1993). The role of Rap1 in gene activation is correlated with its ability to displace nucleosomes, and our work has made new connections in how this is achieved. In certain contexts, Rap1 can instead function as a repressor, notably in conjunction with the SIR proteins at telomeres and the silent mating loci (Cockell et al., 1995; Hecht, Strahl-Bolsinger, & Grunstein, 1996; F. J. McNally & Rine, 1991; Moretti & Shore, 2001; Stavenhagen & Zakian, 1998). Rap1 also has other indispensable roles in chromosome end protection (Bonetti et al., 2010a; Bonetti, Clerici, Manfrini, Lucchini, & Longhese, 2010b; Pardo & Marcand, 2005; Runnberg, Narayanan, & Cohn, 2017; C.-W. Yang et al., 2017), telomere length regulation (Hardy, Sussel, & Shore, 1992; Kyrion, Liu, Liu, & Lustig, 1993; Marcand, Gilson, & Shore, 1997; Wotton & Shore, 1997), negative regulation of telomerase (Negrini, Ribaud, Bianchi, & Shore, 2007), chromatin barrier function (Bi & Broach, 1999; Fourel, Miyake, Defossez, Li, & Gilson, 2002), and regulation of mRNA stability (Bregman et al., 2011), making it an essential protein for yeast survival.

Mapping studies on scRap1 have identified an N-terminal region that includes the BRCA1 C-terminal (BRCT) domain, a tandem Myb DNA binding domain, and a Rap1-specific C-terminal (RCT) protein interaction domain (Fig. 1-1). The N-terminus is known to interact with transcriptional activators Gcr1 and Gcr2 to regulate glycolytic gene expression (Mizuno et al., 2004), plays a role in the regulation of yeast cell wall integrity (Azad, Singh, Baranwal, Thakare, &
Tomar, 2015), and may have certain functions in bending the DNA immediately adjacent to the Rap1 recognition site (Müller et al., 1994). However, it can be deleted with little to no effects on gene transcription and growth (Shore, 1994). The RCT domain is required for important Rap1 functions such as transcriptional silencing at telomeres and the silent mating type loci via interactions with transcriptional repressors Sir3 and Sir4 (Moretti & Shore, 2001; Strahl-Bolsinger, Hecht, Luo, & Grunstein, 1997), as well as inhibition of telomere length elongation via recruitment of regulatory proteins Rif1 and Rif2 (Kyrion et al., 1993; Marcand et al., 1997). The binding of the Sir and Rif proteins appear to be mutually exclusive (Feese & Wolberger, 2008; Palladino et al., 1993). Both the N and C terminal regions are dispensable for chromatin opening (Yu, Sabet, Chambers, & Morse, 2001). The DNA binding domain (DBD) consists of two Myb-like domains containing the characteristic helix-turn-helix motif. The first Myb domain is also a SANT domain, a motif of ~50 amino acids with protein-interacting abilities. Some SANT domains are known to interact with histone tails (e.g. in Ada2) (Boyer et al., 2002; Boyer, Latek, & Peterson, 2004). Each one of two Rap1 Myb domain can bind to one of two DNA hemi-sites separated by a three base pair linker sequence (Del Vescovo et al., 2004). The first Myb domain interacts with the more conserved 5'-hemisite of the Rap1 recognition sequence (ACACC) while the second Myb domain interacts with the more divergent 3'-hemisite, thus allowing Rap1 to recognize a plethora of diverse binding sites within the yeast genome (Taylor, O'Reilly, Leslie, & Rhodes, 2000). High affinity Rap1-DNA binding have been reported, with Kd values ranging from 10 pM up to 30 nM (Vignais, Huet, Buhler, & Sentenac, 1990; Williams, Truong, & Tyler, 2008), and the binding of one Myb domain to a DNA hemi-site results in a five- to ten-fold reduction of affinity compared to when both Myb domains are bound (Feldmann & Galletto, 2014; Feldmann, De Bona, & Galletto, 2015). Crystal structures of the DBD in complex with DNA (Konig, Giraldo, Chapman, & Rhodes, 1996; Matot et al., 2012) shows that helices 2 and 3 in the three-helix bundle of each Myb domain form a helix-turn-helix motif and helix 3 docks deep in the major groove of DNA. An additional loop (575-601) located C-terminal of the second Myb domain wraps around DNA and interacts with the first Myb domain to form a “clamp” that increases protein-DNA interaction surfaces by
25% (Matot et al., 2012). In addition, a “Tox” region which mediates toxicity when Rap1 is overexpressed (Freeman, Gwadz, & Shore, 1995) and transactivation domains (Graham et al., 1999; A. N. Johnson & Weil, 2017; Sussel & Shore, 1991) are located in between the DBD and RCT domains.

There is longstanding evidence that scRap1 can bind to nucleosomal DNA (Koerber, Rhee, Jiang, & Pugh, 2009; Rossetti et al., 2001), with enrichment around the entry/exit point of nucleosome-DNA contacts (Rhee & Pugh, 2011). Experiments crosslinking Rap1 to nucleosomes revealed that approximately 0.4% of all yeast nucleosomes in the genome significantly associate with Rap1, and roughly 43% of Rap1-bound nucleosomes are found at the -1 position between two divergent promoters (Koerber et al., 2009). Studies by the Shore lab have categorized the -1 nucleosome into two distinct categories based on their resistance to MNase digestion, and Rap1 was found to associate preferentially with MNase-sensitive (“fragile”) nucleosomes (Knight et al., 2014; Kubik et al., 2015). It has also been suggested that the distortion of the Rap1 DNA binding site as it wraps around a nucleosome may enhance Rap1 affinity, with nucleosomal Rap1 displaying higher site occupancy compared to non-nucleosomal Rap1 (Rhee & Pugh, 2011; Rossi, Lai, & Pugh, 2018). Furthermore, Rap1 inversely correlates with nucleosome occupancy, forming nucleosome depleted regions (NDRs) around the sequences of its binding motifs, suggesting that it can also help exclude nucleosomes, thus contributing to transcriptional activation (Ganapathi et al., 2011; Lickwar, Mueller, Hanlon, McNally, & Lieb, 2012; Yarragudi, Miyake, Li, & Morse, 2004; Yu & Morse, 1999). The fact that Rap1 is the principal duplex binding protein of yeast telomere repeat DNA, and that telomere chromatin is largely free of nucleosomes, also suggests that Rap1 can displace nucleosomes. Interestingly, multiple Rap1 binding sites can generate a nucleosome-sized particle that do not contain histones and is highly susceptible to MNase digestion (Yan, Chen, & Bai, 2018). This reciprocal relationship with nucleosomes is dependent on Rap1 occupancy times, with longer residency leading to higher levels of nucleosome depletion (Lickwar et al., 2012). Rap1 residency times may be facilitated in part by histone post-translational modifications such as H3K4me3 and acetylation of H3K9,
H3K14 and H4 (Lickwar et al., 2012). However, the mechanisms by which Rap1 displaces histones have not been thoroughly explored, though several lines of evidence suggest that coactivators and chromatin remodelers may be required (Kubik et al., 2015; Mizuno et al., 2004; Yu & Morse, 1999).

The ability of Rap1 to exclude nucleosomes from promoters and recruit other transcription factors is functionally similar to pioneer transcription factors (pTFs) in higher eukaryotes (Zaret & Carroll, 2011). At the promoters of ribosomal protein genes, Rap1 binding precedes that of other cofactors such as Fhl1 and Hmo1 (Hall, Wade, & Struhl, 2006; Yu Zhao et al., 2006). Mutation of nucleosomal Rap1 binding sites or rapid depletion of Rap1 results in significant decreases in cofactor recruitment, suggesting that Rap1 acts as a pioneer required for binding of all other transcription factors (Knight et al., 2014). In addition, it has been shown that Rap1 can bind to synthetically constructed chromatin fibers, though with much lower residence times compared to naked DNA. Single molecule FRET studies demonstrate that Rap1 binding to reconstituted nucleosomes do not evict or distort bound nucleosomes, but can perturb higher-order chromatin structure and allow for nucleosome eviction by recruiting the chromatin remodeling complex RSC and histone chaperone Nap1 (Mivelaz et al., 2019).

Previously, we have found that Rap1 contributes to gene expression and chromatin landscape changes at senescence in yeast telomerase deficient mutants. In senescent cells Rap1 relocalizes from shortening telomeres and subtelomeres to the promoters of hundreds of new genes (Platt et al., 2013). These include those encoding the core histone proteins, which are transcriptionally repressed by Rap1, thus contributing to diminished global histone levels. This repression is independent of the Sir proteins. However, most new targets of Rap1 in senescent cells are upregulated by Rap1, and this is accompanied by a preferential loss of histones at the promoters of these particular targets. Thus global and site-specific inhibition of histones appear to contribute to transcriptional activation by Rap1 at senescence (Fig. 1-2). Interestingly, histone loss is a conserved feature of senescence in several aging models, including replicative
senescence of cultured human fibroblasts and replicative yeast mother cell aging (reviewed above) though the exact mechanisms in each setting remain to be explored. However, our findings provide the first insight that at least in replicative senescence of yeast telomere mutants, Rap1 plays important roles. We found that in addition to regulating senescence-related gene expression Rap1 drives the rate of senescence, whereas overexpression of core histones delayed the pace of senescence. In addition, Rap1 relocalization from the telomeres at senescence is dependent on the DDR kinase Mec1 (Platt et al., 2013; Tomar, Zheng, Brunke-Reese, Wolcott, & Reese, 2008), which suggests that Rap1 relocalization at senescence may have broader manifestations in the larger context of DNA damage signaling and repair.

Rap1 is the only conserved shelterin protein from yeast to humans. Though no significant sequence similarities are observed between scRap1 and hRAP1 from a simple BLAST search, they contain about 25% identical residues (B. Li, Oestreich, & de Lange, 2000) and share similar domain structures with an N-terminal BRCT domain, a central Myb domain, and a Rap1-specific C-terminal (RCT) protein interaction domain (Fig. 1-1). However, in contrast to the high affinity DNA binding seen in yeast Rap1, mammalian RAP1 has little to no sequence-specific DNA binding ability, and is recruited to telomeres via interactions with TRF2 through its BRCT domain. On the other hand, one study has suggested that mammalian RAP1 may be able to recognize specific DNA structures, such as single-strand double-strand DNA junctions, independent of DNA sequence (Arat & Griffith, 2012). In addition, RAP1 is able to alter TRF2:DNA interactions by improving TRF2 binding specificity to telomeric DNA and affect subsequent telomeric loop processing by helicases (Gaulier et al., 2016; Janoušková et al., 2015; Nečasová, Janoušková, Klumpler, & Hofr, 2017). Unlike yeast Rap1, which is essential for organismal survival, mammalian RAP1 can be deleted with little to no effect on organismal viability and fertility (Sfeir, Kabir, van Overbeek, Celli, & de Lange, 2010), though some gender-specific metabolic differences do occur (Martínez et al., 2013; Yeung et al., 2013). However, despite these differences, several striking features are conserved from yeast to mammals. For one, mammalian RAP1 is found in both telomeric and extratelomeric regions, and is involved in gene silencing and
transcriptional regulation genome-wide (Kabir, Hockemeyer, & de Lange, 2014; Martínez et al., 2013; 2010; D. Yang et al., 2011; Yeung et al., 2013). Loss of RAP1 from telomeres resulted in significant impacts in the expression of telomere-proximal genes, compared with genes randomly localized in the mouse genome (Martínez et al., 2010). In addition, RAP1 was found to be associated with (TTAGGG)$_2$ consensus motifs outside of telomere regions, and loss of RAP1 deregulated 30% of genes located proximal to (TTAGGG)$_2$ repeats (Martínez et al., 2010). However, it is unlikely that RAP1 directly binds to these consensus motifs and is probably recruited via other factors (e.g. TRF2), though the exact details remain to be studied. Two, mammalian RAP1 protects telomeres from being recognized as double-strand breaks through repression of homology-directed-repair (HDR) (Sfeir et al., 2010). Three, although the presence of telomeric RAP1 is not necessary for telomeric maintenance and protection, in the absence of telomerase in mice, loss of RAP1 results in earlier onset of DNA damage and degenerative pathologies. In immortalized mouse embryonic fibroblasts (MEFs) lacking telomerase, RAP1 was gradually lost from increasingly shortened telomeres and relocalized to new genomic sites, preferentially affecting gene expression in the metabolic pathway (Martínez, Gómez-López, Pisano, Flores, & Blasco, 2016). However, because these experiments were performed in immortalized MEFs, it is unknown whether RAP1 redistribution in mammals drives histone losses and other aspects of cellular senescence as it does in yeast, though several lines of evidence suggest that it may play similar roles: 1) genome-wide split-YFP complementation assays in human cells have suggested that hRAP1 can interact with histones, hinting at possibilities that hRAP1 may contribute to chromatin remodeling and nucleosome displacement; 2) overexpression of TRF2, which recruits hRAP1, can alter nucleosome spacing at telomeres (Galati et al., 2012), suggesting that the hRAP1:TRF2 complex can modify chromatin; 3) connections between RAP1 and NF-κB signaling (Teo et al., 2010), a pathway involved in the transcriptional regulation of cellular senescence, imply that it may potentially be involved in senescence-related gene expression changes. Together, these findings leave open the possibility that hRAP1 may also play important roles in mammalian cellular senescence.
1.6 References


1.7 Figure Legends

Figure 1-1. Schematic of functional domains in yeast and human Rap1 proteins, with amino acid positions indicated.

Figure 1-2. Rap1 relocalization at senescence in telomerase-deficient yeast leads to global and site-specific histone losses. Shown are the relative levels of Rap1 and histone proteins at telomeres, nontelomeric genomic regions, and in non-chromatin-bound histone pools. In normal cells, Rap1 is clustered at telomeres and is present at the promoters of natural Rap1 target genes. When cells become senescent due to critically short telomeres, Rap1 relocalizes to the promoters of natural and new Rap1 targets at senescence (NRTS). This relocalization depends on the Mec1 checkpoint kinase, which is activated by uncapped telomeres. At NRTS promoters, Rap1 binding displaces histones, resulting in site-specific histone losses. Rap1 also relocalizes to the promoters of histone genes and inhibits their transcription. Thus, the increased availability of Rap1 together with a decline in histone levels contribute to increased Rap1 occupancy at NRTS at senescence. Global histone levels also decrease in response to non-telomeric DNA damage, mediated by histone degradation driven by the Rad53 checkpoint kinase, but whether such degradation occurs at senescence, and whether Rap1 is involved in generic DNA damage responses have not been tested (see text).
Chapter 2 Rap1-mediated nucleosome displacement can regulate gene expression in senescent cells without impacting the pace of senescence.

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2.1 Summary

Cell senescence is accompanied, and in part mediated, by changes in chromatin, including histone losses, but underlying mechanisms are not well understood. We reported previously that during yeast cell senescence driven by telomere shortening, the telomeric protein Rap1 plays a major role in reprogramming gene expression by relocalizing to the promoters of hundreds of new target genes (called NRTS, for new Rap1 targets at senescence). This leads to two types of histone loss: Rap1 lowers histone level globally by repressing histone gene expression, and it also causes local nucleosome displacement at the promoters of upregulated NRTS. Here, we present evidence of direct binding between Rap1 and histone H3/H4 heterotetramers, and map amino acids involved in the interaction within the Rap1 SANT domain to amino acids 392-394 (SHY). Introduction of a point mutation within the native RAP1 locus that converts these residues to alanines (RAP1SHY), and thus disrupts Rap1-H3/H4 interaction, does not interfere with Rap1 relocalization to NRTS at senescence, but prevents full nucleosome displacement and gene upregulation, indicating direct Rap1-H3/H4 contacts are involved in nucleosome displacement. Consistent with this, the histone H3/H4 chaperone Asf1 is similarly unnecessary for Rap1 localization to NRTS but is required for full Rap1-mediated nucleosome displacement and gene activation. Remarkably, RAP1SHY does not affect the pace of senescence-related cell cycle arrest, indicating that some changes in gene expression at senescence are not coupled to this arrest.
2.2 Introduction

Cellular senescence is a programmed response to stresses that put cells at risk for becoming cancerous, through events such as DNA damage, telomere and mitochondrial dysfunction, and oxidative stress. It is characterized by stable cell cycle arrest, but importantly also by profound alterations in chromatin structure leading to changes in gene expression that impact cell metabolism and the secretion of factors that influence the function of tissues in which senescent cells reside (Campisi 2013; van Deursen 2014; Ritschka et al. 2017; Sapieha & Mallette 2018). Although cell senescence plays beneficial roles early in life by contributing to tumor suppression, wound healing, and immunity, several lines of evidence suggest that it can also drive age-related pathologies through stem cell depletion (Molofsky et al. 2006; Krishnamurthy et al. 2006) or by disruption of tissue structure and function, apparently via secretion of factors such as proteases and inflammatory cytokines (Baar et al. 2017; Baker et al. 2011; Baker et al. 2016; Childs et al. 2016; Schafer et al. 2017). Understanding the mechanisms underlying cell senescence, particularly those regulating altered gene expression, is thus of substantial interest.

One important driver of human cellular senescence is telomere shortening. Critically short (i.e. “uncapped”) telomeres are recognized by the DNA damage response (DDR) machinery, leading to arrest and gene expression changes. Senescence driven by telomere shortening can be modeled in *S. cerevisiae*. Yeast naturally expresses telomerase to maintain telomere length, but if telomerase is inactivated genetically, cells gradually lose telomere DNA through rounds of division and eventually arrest - although rare survivors, which maintain telomeres via homologous recombination, eventually emerge from senescent populations. Many factors known to influence senescence in human cells have similar roles in telomerase-deficient yeast, including exonucleases, helicases, and DDR proteins (Herbig et al. 2004; Schaetzlein et al. 2007; IJpma & Greider 2003; Johnson et al. 2001; Ritchie et al. 1999).
A key and conserved feature of senescence, and other types of aging-related biology, from yeast to humans is histone loss (Platt et al. 2013; O'Sullivan & Karlseder 2012; Ivanov et al. 2013; Song & Johnson 2018; Liu et al. 2013). Histone gene expression, global levels of all core histones, and nucleosome occupancy at particular genomic sites are all decreased in senescent telomerase-deficient yeast (Platt et al. 2013), and similar observations have been made in aged yeast mother cells (Hu et al. 2014; Feser et al. 2010). This loss is apparently closely linked to the altered gene expression observed in senescent cells, as highly similar gene expression patterns are seen when histone levels are artificially downregulated (Platt et al. 2013). In both yeast models, artificial overexpression of core histones promotes longevity. However, little is known about the mechanisms underlying histone-related changes in senescent cells.

Previously we found that the telomeric protein Rap1 plays a major role in replicative senescence in telomerase-deficient yeast, including regulation of histone gene expression and site-specific nucleosome occupancy (Platt et al. 2013). Rap1 is conserved between yeast and humans, and the yeast protein binds directly to telomere repeat DNA in a sequence-specific fashion via two tandemly-arranged Myb domains, where it plays roles in regulating telomere length, transcriptional silencing, and capping (Marcand et al. 1997; Martínez et al. 2016; Kyrion et al. 1993; Moretti & Shore 2001; Pardo & Marcand 2005; Vodenicharov et al. 2010; Rai et al. 2016; Yang et al. 2017). It also functions to regulate transcription throughout the genome, in particular repressing expression of the silent mating type loci, and activating expression of approximately ten percent of all yeast genes, particularly the highly expressed ribosomal protein and certain glycolytic enzyme genes. During replicative senescence, Rap1 relocalizes from shortened telomeres and subtelomeres to the promoters of hundreds of new genes, named NRTS (new Rap1 targets at senescence), which have lower affinity Rap1 binding sites than natural Rap1 targets. Among the NRTS are the genes that encode the core histone proteins, which are transcriptionally repressed by Rap1, thus contributing to the loss of histone proteins observed at senescence. In contrast to the histone genes, the majority of NRTS become activated by Rap1. This activation is associated with the displacement by Rap1 of nucleosomes
from the promoters of these NRTS, but it is not known if nucleosome displacement causes NRTS activation (Platt et al. 2013). Furthermore, Rap1 drives the overall pace of senescence, because it is delayed by experimental diminishment of Rap1 levels. However, whether the function of Rap1 to repress global histone levels, or its function to locally displace nucleosomes and upregulate NRTS, might underlie its effect on the rate of senescence has not been tested.

It has long been known that Rap1 can bind nucleosomal DNA, and its ability to exclude nucleosomes from promoters is functionally similar to pioneer transcription factors (pTFs) in higher eukaryotes (Rhee & Pugh 2011; Koerber et al. 2009; Lickwar et al. 2012; Ganapathi et al. 2011; Yarragudi et al. 2004; Yu et al. 2001; Knight et al. 2014; Kubik et al. 2015; Zaret & Carroll 2011; Yan et al. 2018). However, the mechanisms by which Rap1 displaces histones have not been thoroughly explored. It is possible that direct contacts between Rap1 and histones are involved, because proteome-wide interaction screens in yeast and genome-wide split-YFP complementation assays in human cells suggest that Rap1 proteins may bind histones (Gilmore et al. 2012; Lee et al. 2011), although this has not been studied in detail. This possibility is of general interest because it has been so-far described for only two other pTFs, FoxO1 and FoxA (Hatta & Cirillo 2007; Cirillo et al. 2002). In addition, we reasoned that if Rap1-histone contacts are involved in histone displacement by locally bound Rap1, but not in other Rap1 functions including histone gene repression, then a Rap1 mutant selectively deficient in histone contact would provide a tool to not only address the role of nucleosome displacement in NRTS activation, but also test the importance of NRTS upregulation in driving the rate of senescence.

Here we describe the creation of such a Rap1 mutant, identified based on its disruption of a direct interaction established between Rap1 and histone H3/H4 heterotetramers, as well as the effects of the mutation on the functions of Rap1 at senescence. We also describe a role for a histone H3/H4 chaperone, Asf1, in nucleosome displacement and NRTS upregulation by Rap1.

2.3 Results
2.3.1 Rap1 binds H3/H4 histone tetramers

As reviewed above, Rap1 is a nucleosome-displacing factor, functionally similar to pioneer transcription factors in higher eukaryotes. Consistent with this function of Rap1, we previously reported that Rap1 displaces nucleosomes from the promoters of activated NRTS at senescence. In contemplating underlying mechanisms, we considered evidence indicating potentially direct binding between histones and the yeast and human Rap1 proteins. This evidence comes from a proteome-wide screen in yeast and a genome-wide split-YFP fluorescence complementation screen in human cells (Gilmore et al. 2012; Lee et al. 2011), but the apparent Rap1-histone interactions have not been investigated in any detail. Such an interaction, if mapped, could provide us with tools to manipulate Rap1 functions at senescence. Therefore, we decided to explore the possibility that nucleosome displacement by Rap1 might involve direct interactions with histone proteins.

To verify Rap1-histone binding and begin to map the Rap1 regions involved, we fused a GST-tag to the N-terminus of full-length Rap1 and to various fragments of the protein (Fig. 2-1A). These comprise an N-terminal fragment (amino acids 1-358, Rap1N), which contains a BRCT domain, and can be deleted with little to no effect on cell growth and gene transcription (Shore 1994; Mizuno et al. 2004); the DNA-binding domain (amino acids 359-600, Rap1DBD), which consists of a two tandem Myb domains, the first of which is also a SANT domain; and a C-terminal fragment (amino acids 601-827, Rap1C) containing the RCT domain, which interacts with various Rap1 binding partners including Rif1, Rif2, Sir3 and Sir4 (Strahl-Bolsinger et al. 1997; Moretti & Shore 2001; Shi et al. 2013).

We performed the histone binding assay by incubating 0.5 μM of GST-tagged Rap1 proteins bound to glutathione beads with 2 μM H2A/H2B dimers or H3/H4 tetramers. The beads were then washed under stringent conditions, and retained histones were examined by SDS-PAGE. We found that Rap1 bound to H3/H4 tetramers, but not H2A/H2B dimers, including under...
conditions where the H3/H4 and H2A/H2B proteins were mixed with one another prior to binding (Fig. 2-1B). Significant binding was observed in salt concentrations ranging from 150 mM to 750 mM. Rap1N did not bind to H3/H4, whereas the Rap1DBD showed similar binding strength compared to full-length Rap1 (Fig. 2-1C). When increased to levels equimolar to the histones (2 µM each), full-length Rap1 and Rap1CA showed more robust binding to H3/H4 tetramers, whereas the C terminal fragment also displayed weak binding (Figs. 2-1D & F). However, even in 7.5-fold molar excess, Rap1N failed to bind histones (Fig. S2-1). Taken together, our findings indicate Rap1 interacts directly with the H3/H4 histone tetramer, which involves relatively strong vs. weak binding interactions between histones and the Rap1 DBD vs. C-terminus.

The Rap1 DBD consists of two tandem Myb domains, the first of which is also classified as a SANT domain (amino acids 360-410). The SANT domain is a stretch of approximately 50 amino acids containing the helix-turn-helix motif and is typically involved in protein-protein interactions. Some SANT domains can bind to histone tails and have been proposed to function as histone interaction modules important for nucleosome remodeling (Grüne et al. 2003; Boyer et al. 2002; Boyer et al. 2004). Therefore, we predicted that the H3/H4 interaction seen in the DBD involves interaction surfaces within the SANT domain. To test whether it might be sufficient for binding, we fused GST to the Rap1 SANT domain and confirmed that it binds the H3/H4 tetramers under equimolar concentrations (2 µM each), with approximately half the affinity of the full-length protein (Figs. 2-1E & F). These findings indicate that the SANT domain contributes substantially to the capacity of Rap1 to bind histone H3/H4 tetramers.

2.3.2 Amino acids 392-394 (SHY) facilitates Rap1-histone interactions

To identify residues within the Rap1 SANT domain required for binding H3/H4 tetramers, we generated GST-tagged triple alanine mutants in which consecutive blocks of three amino acids in the SANT domain are mutated to alanines (Fig. 2-2A). Each of the mutants was
separately purified and incubated with equimolar concentration H3/H4 tetramers (0.5 µM). Elutions from the glutathione beads were analyzed by western blotting using H3 antibodies. A significant loss of H3 signal was observed when amino acids 392 (serine), 393 (histidine), and 394 (tyrosine) were mutated (Fig. 2-2B & C and Fig. S2-2). Based on X-ray crystal structures of the Rap1 DBD bound to DNA (Konig et al. 1996; Matot et al. 2012), amino acids 392-394 are located in the turn between helix two and three. This turn faces away from the Rap1-DNA interaction surface (Fig. 2-2D), consistent with potential involvement of the SHY patch in interactions between DNA-bound Rap1 and other proteins.

We next tested to see if replacement of SHY by AAA in longer stretches of Rap1 yielded similar loss of histone binding. Full-length Rap1 containing the replacement (Rap1\(^{SHY}\)) showed a 50% loss of H3/H4 binding signal (Figs. 2-2E & F). Similar losses were observed when the SHY to AAA replacement was introduced into Rap1 lacking the entire C-terminal fragment (Rap1\(^{C-}\)), or a portion of it (Rap1\(^{643-c}\)) (Figs. 2-2G & H; see Fig. 2-1A for map). This is consistent with an only minor role for the C-terminus in stabilizing Rap1-histone interactions.

To address whether the SHY patch impacts binding of Rap1 to soluble histones in vivo, we immunoprecipitated HA-tagged Rap1 and Rap1\(^{SHY}\) from whole cell extracts and immunoblotted for H3. The extracts were treated with benzonase to prevent indirect DNA-mediated interactions between the proteins. Rap1\(^{SHY}\) showed a significant loss of H3 binding compared to WT Rap1 (Figs. 2-2I & J). Together with the in vitro pull-down data, this strongly supports a physical interaction between Rap1 and H3/H4 that involves amino acids in the SHY patch of the DNA binding domain.

### 2.3.3 Rap1\(^{SHY}\) is deficient in NRTS activation and histone displacement

Given the physical interactions observed between Rap1 and H3/H4 tetramers, we proceeded to investigate the functional effects of Rap1\(^{SHY}\) in vivo, in particular its effects on the
different functions of Rap1 at senescence. We hypothesized that the compromised ability of Rap1<sup>SHY</sup> to interact with the H3/H4 tetrameric core of nucleosomes would interfere with its roles in NRTS promoter clearance and gene activation. As the SHY to AAA mutation is within the DNA binding domain, we first confirmed that Rap1<sup>SHY</sup> did not compromise the ability of Rap1 to bind DNA. Electrophoretic mobility shift assays (EMSA) using a telomeric sequence, a natural Rap1 binding site within the TEF2 promoter, and a representative NRTS promoter, demonstrated Rap1 and Rap1<sup>SHY</sup> bound DNA with similar affinities (Fig. S2-3A). We next used a system of Rap1 overexpression in wild type cells, which we showed previously recapitulates the selective binding of Rap1 to NRTS promoters, from which nucleosomes are displaced and gene expression is upregulated. Wild type cells were transformed with two-micron based plasmids from which either HA-tagged Rap1 or Rap1<sup>SHY</sup> expression is driven by the GAL1 promoter. Expression was induced with galactose for 130 min, which we reported previously is sufficient for local histone displacement at promoters by Rap1 but avoids potential secondary effects from toxicity manifesting as growth inhibition after eight hours of induction (Platt et al. 2013). Rap1 localization to NRTS promoters and histone displacement were measured by ChIP-qPCR, using antibodies against the HA-tag and H3, respectively. Total cellular levels (Fig. 2-3A) and localization to NRTS promoters (Fig. 2-3B) were similar for both proteins, consistent with their similar DNA binding abilities. However, Rap1<sup>SHY</sup> did not displace nucleosomes as efficiently compared to WT (Fig. 2-3C; see also Fig S2-3D, demonstrating greater histone H3 losses from the ChIPed promoters following induction of Rap1 vs. Rap1<sup>SHY</sup>). To test whether compromised nucleosome displacement resulted in changes in gene expression, we constitutively expressed full-length Rap1 and a C-terminally truncated version of Rap1 (Rap1<sup>643∆</sup>) and their respective SHY to AAA mutants, from a two-micron plasmid driven by the NOP1 promoter, a non-toxic Rap1 overexpression system which has been previously shown to be sufficient for elevated NRTS expression. Consistent with the reduced levels of H3 displacement seen by ChIP, Rap1<sup>SHY</sup> does not activate NRTS mRNA expression as strongly as WT (Fig. 2-3F). Rap1<sup>643∆</sup> can also upregulate NRTS expression, though to a slightly lower level compared to full-length Rap1, consistent with a
role for both the SANT and C-terminus in histone interactions (Fig. 2-3F). Much like Rap1 and Rap1<sup>SHY</sup>, a similar decrease in NRTS expression was observed in Rap1<sup>643Δ</sup> <sub>SHY</sub> compared to Rap1<sup>643Δ</sup> (Fig. 2-3F). However, no changes in expression were observed for representative natural Rap1 target genes, including the glycolytic gene ENO2 and the ribosomal protein gene RPS5, nor for non-Rap1 targets, when comparing strains overexpressing the WT and mutant proteins (Fig. S2-3E &F). These findings suggest that Rap1-histone interactions involving the SHY patch and C-terminal region are not required for binding to NRTS promoters but contribute to Rap1-mediated nucleosome displacement and NRTS activation.

2.3.4 Rap1<sup>SHY</sup> confers diminished NRTS activation at senescence without affecting the rate of senescence

To examine the effects of Rap1<sup>SHY</sup> in the context of senescence, we introduced the SHY to AAA mutation within one of the endogenous RAP1 loci in a TLC1/tlc1Δ diploid. Upon sporulation and dissection of tetrads, the Rap1<sup>SHY</sup> haploid spore products formed smaller colonies (Fig. S2-4A). Southern blot analysis using probes for Y' telomeric fragments showed similar telomeric lengths in RAP1 and RAP1<sup>SHY</sup> strains, as well as in their respective telomerase deletion (tlc1Δ) strains at 50 population doublings after spore germination (Fig. S2-4B), suggesting a normal level of telomere capping and maintenance by Rap1<sup>SHY</sup>. This is consistent with the similar colony sizes observed for RAP1<sup>SHY</sup> tlc1Δ double mutants and tlc1Δ strains, at least for the ~20-25 divisions needed for colony formation from the germinated spores (Fig. S2-4A). Furthermore, the colony forming efficiency of isolated RAP1<sup>SHY</sup> cells is similar to WT (Fig. S2-4C), implying that slow growth is not due to increased cell death. Expression of the natural Rap1 target genes ENO2 and RPS5, encoding glycolytic and ribosomal proteins, respectively, are not diminished by the SHY mutation (Figs. S2-4G, and S2-3D&E), and so we do not yet have an explanation for the slow growth of the mutants. In addition, Rap1<sup>SHY</sup> functions normally to silence subtelomeres (Fig. S2-4D & E) and the silent mating type loci (Fig. S2-4F).
We passaged both tlc1Δ and Rap1SHY tlc1Δ cells to senescence by performing serial dilutions into new liquid cultures every 22 hours (see Methods). Taking senescence as the nadir of the growth curve before survivor formation, Rap1SHY had no effect on the rate of senescence compared to WT Rap1 (Fig. 2-4A). However, given the reduced NRTS activation observed when Rap1SHY is overexpressed, we predicted that a similarly blunted NRTS profile would also be seen in Rap1SHY at senescence. Indeed, this was confirmed by comparing relative mRNA expression in senescent and proliferating cells (Fig. 2-4B). Interestingly, this suggests that the tested gene expression changes do not correlate with the rate of senescence. Previously we have reported that Rap1 relocalization at senescence represses histone gene expression, and that artificial overexpression of all core histones will delay the rate of senescence (Platt et al. 2013), suggesting that the rate of senescence may be related to global histone levels. Consistent with this, the degree to which expression of all eight core histone genes was repressed at senescence was similar for cells expressing Rap1SHY vs. normal Rap1 (Fig. 2-4C).

2.3.5 Asf1 contributes to Rap1-dependent NRTS activation and histone displacement

Given the interaction of Rap1 with H3/H4 histone tetramers, we reasoned that H3/H4 histone chaperones might cooperate with Rap1 to displace nucleosomes at senescence. We tested the H3/H4 histone chaperone Asf1 because it is involved in both nucleosome assembly and disassembly during replication, transcription, and DNA repair, and because it is upregulated in senescent cells (Nautiyal et al. 2002; Adkins et al. 2004; Zabaronick & Tyler 2005). Deletion of ASF1 results in histone gene misregulation (Sutton et al. 2001; Zabaronick & Tyler 2005) and genome-wide transcriptional changes, but does not affect the global level nor the stability of histone proteins (Gunjan & Verreault 2003). We found that although asf1Δ tlc1Δ mutants did not senesce at a rate significantly different from tlc1Δ (Fig. 2-5F), deletion of Asf1 significantly blunts the upregulation of activated NRTS at senescence (Fig. 2-5A). To test if this reduced NRTS activation is related to regulation by Rap1, we tested the Asf1-dependence of NRTS activation by
Rap1 overexpressed in wild type cells. Similar to its effects at senescence, deletion of ASF1 reduced Rap1-driven NRTS activation (Fig. 2-5B) and displacement of nucleosomes from NRTS promoters (Fig. 2-5D). However, Rap1 localization to the promoters did not depend on Asf1 (Fig. 2-5C), indicating that facilitation of histone displacement by Asf1 occurs after the binding of Rap1 to promoters, apparently similar to the role of the Rap1 SHY patch described above.

Asf1 partners with different complexes to chaperone histones in different contexts. Asf1 interacts with the HIR complex, comprising Hir1, Hir2, Hir3, and Hpc2 proteins, to regulate nucleosome assembly and disassembly during transcription or DNA repair. In contrast, during DNA replication, Asf1 interacts with the second subunit of the CAF-I complex, comprising Cac1, Cac2 and Cac3, to promote nucleosome assembly. Deletion of any member of the HIR complex blunted NRTS activation caused by overexpressed Rap1 (Fig. 2-5E), but deletion of cac1Δ, cac2Δ, cac3Δ had no effect (Fig. S2-5). This is consistent with the fact that cells arrest in G2/M at senescence and therefore would not be expected to utilize the replication-dependent pathway. Furthermore, similar to ASF1 deletion, HIR1 deletion did not affect the rate of senescence of tlc1Δ cells (Fig. 2-5G). Therefore, deletion of ASF1 or HIR1, or mutation of the Rap1 SHY patch, each prevent normal NRTS upregulation by Rap1 without impacting the rate senescence, suggesting that upregulation of NRTS, at least those tested, are not main drivers of this rate.

2.4 Discussion

2.4.1 Direct Rap1-histone interactions are involved in Rap1-mediated chromatin opening

There is longstanding evidence that Rap1 can bind to nucleosomal DNA both in vivo and in vitro (Koerber et al. 2009; Rossetti et al. 2001; Lickwar et al. 2012). Notably, single-nucleotide resolution ChIP-exo shows that not only does histone occupancy not interfere with Rap1 binding, but high affinity Rap1 binding sites and Rap1 occupancy are in fact more common in nucleosomal than non-nucleosomal regions of the genome (Rhee & Pugh 2011). Rap1 nonetheless
encourages nucleosome displacement, as Rap1-bound regions have generally low nucleosome occupancy that depends on the binding of Rap1 (Platt et al. 2013; Rhee & Pugh 2011; Yarragudi et al. 2004; Lieb et al. 2001; Ganapathi et al. 2011). The fact that telomeric chromatin, which includes one Rap1 monomer bound to approximately every 18 bp of the telomere repeat sequence, is largely nucleosome-free provides another apparent example of the nucleosome-displacing activity of Rap1 (Wright et al. 1992; Gilson et al. 1993; Williams et al. 2010).

Here we report that in addition to high-affinity Rap1-DNA interactions, Rap1 can also interact directly with H3/H4 histone tetramers. The SANT domain is necessary for such binding, and is facilitated by amino acids 392-394 (SHY) within the domain, although other points of contact apparently also exist, including within the C-terminal region. Mutation of amino acids SHY to AAA results in deficiencies in Rap1-histone interactions \textit{in vitro} and \textit{in vivo}, as well as blunted histone displacement and gene activation. These findings are consistent with previous mapping studies demonstrating that the N-terminal and C-terminal regions of Rap1 are dispensable for interaction with nucleosomal binding sites (Rossetti et al. 2001) and chromatin opening (Yu et al. 2001).

The SHY patch is located within the turn immediately C-terminal to the second helix in the three-helix bundle of the SANT domain. Crystal structures of Rap1 bound to DNA (Matot et al. 2012; Konig et al. 1996) show that helices 2 and 3 form a helix-turn-helix motif that docks deep in the major groove of DNA, and that the side chains of the SHY patch point away from the Rap1-DNA interaction surface. Of note, the C-terminal region of the second Myb domain (amino acids 592-601) forms a long loop which wraps around the DNA helix, contacts both DNA strands, and ends with a “clamp” formed though interaction with SANT domain residues that partially overlap the SHY patch. Specifically, Y592 interacts with G400 and Q401, whereas K597 interacts with S392 and P396. Mutation of Y592 and K597 in the full-length protein results in no changes in the migration profile of DNA-protein complexes visualized by EMSA, though DNA affinity measured by ITC is reduced by a factor of two (Matot et al. 2012). Similarly, no changes in EMSA profiles
for telomeres, natural Rap1 binding sites, or representative NRTS promoters were observed with S392 mutation, though its ITC profile has not been tested directly. However, our ChIP-qPCR measurements at NRTS promoters reveal no apparent decrease in Rap1\textsuperscript{SHY} occupancy, and so the reduced ability of Rap1\textsuperscript{SHY} to displace nucleosomes and activate transcription is apparently not a consequence of reduced levels of Rap1 at promoters (Fig. 2-3B). Whether interference with the “clamp” per se contributes to the compromised nucleosome displacement by Rap1\textsuperscript{SHY} will require additional studies.

That direct Rap1-histone interactions contribute to nucleosome displacement might seem counterintuitive at face value, as a simple energetic consequence of histone binding by Rap1 bound to a DNA target site should be to tether a nucleosome to the site. However, it is noteworthy that Rap1 interacts specifically with the H3/H4 histone tetramers, and not the H2A/H2B histone dimers. Given the sequential assembly and disassembly of nucleosomes – H3/H4 at the core and H2A/H2B on the periphery – this suggests that Rap1 may interact preferentially with a partially disassembled nucleosome. Nucleosomes naturally undergo transient unwrapping under physiological conditions, resulting in DNA partially wrapped around a hexasome or tetrasome through loss of one or both of H2A/H2B dimers (Li & Widom 2004; Chen et al. 2017). Such “breathing” not only allows exposure of DNA sequences for transcription factor binding, but also bares the tetrameric core for protein-protein interactions. Therefore, Rap1 binding to H3/H4 tetramers may drive the dynamic equilibrium of wrapped and partially unwrapped nucleosomes towards the unwrapped state, possibly by preventing H2A/H2B reassembly. Alternatively, or in addition, it may also alter the conformation of the tetrasome in a fashion that facilitates full nucleosome disassembly by H3/H4 histone chaperones such as Asf1. This is similar to the functions of some histone PTMs such as H3K56ac, which destabilize nucleosomes and enable them to be more easily disassembled (Williams et al. 2008).

Rap1 is functionally similar to pioneer transcription factors (pTFs) in eukaryotes. pTFs are the first to bind to target sites in compact chromatin and initiate the sequential binding of other
factors, possibly through opening up local chromatin. Well-known pTFs such as FoxA have DNA binding domains consisting of helix-turn-helix motifs flanked by “wings” of polypeptides, allowing the motif to bind alongside one side of DNA without interfering with the binding of histones on the other side (Soufi et al. 2015; Zaret & Carroll 2011). Such a DBD secondary structure and its orientation on DNA is very similar to those observed in Rap1. In addition, FoxA has a C-terminal domain that interacts directly with core histones H3 and H4 (Cirillo et al. 2002). Interestingly, while the C-terminal region of Rap1 also contributes to histone binding abilities, the Rap1 DBD alone is able to open local chromatin (Yu et al. 2001), consistent with our observations that direct histone interactions in the SANT domain, via amino acids SHY, are important for Rap1’s functions as a pTF.

2.4.2 Gene expression changes can be uncoupled from the rate of senescence

Previously we reported several functions of Rap1 at senescence: first, it represses histone gene expression and contributes to global downregulation of histones; second, it contributes to local nucleosome losses at promoters of upregulated NRTS (Platt et al. 2013); third, Rap1 drives the rate of senescence, probably through regulation of histone dynamics, as diminishment of Rap1 levels via destabilization of the Rap1 mRNA (DAmP allele) or artificial overexpression of core histones can each delay the rate of senescence.

Here, we explore mechanisms of Rap1-mediated local nucleosome losses. We characterize an amino acid patch – residues SHY in the SANT domain – that contains one or more residues required for direct binding of Rap1 to histones. Mutation of SHY results in deficient nucleosome clearance at NRTS promoters and subsequent reduced activation of NRTS. However, RAP1<sub>SHY</sub> does not affect the rate of senescence, nor affect histone gene repression. This is consistent with our previous finding that histone gene repression at senescence does not involve nucleosome losses from the histone gene promoters (Platt et al. 2013).
Similarly, we found Rap1-mediated gene expression changes at senescence require the histone H3/H4 chaperone Asf1 and the HIR complex. Deletion of *ASF1* results in blunted NRTS upregulation at senescence. Similar loss of activation was seen when *ASF1* or genes encoding members of the HIR complex were deleted under settings of Rap1 overexpression, which we previously found was sufficient for selective NRTS upregulation. However, much like the *RAP1*SHY mutation, despite causing substantial losses in NRTS activation, deletion of *ASF1* or *HIR1* also do not affect the rate of senescence.

This begs the question of how gene expression changes – in particular wide-spread gene upregulation that have been observed in multiple senescent models – relate to the rate of senescence. As has been reported extensively, senescent cells are accompanied by changes in chromatin organization and gene expression (Sedivy et al. 2008; Feser & Tyler 2011; De Cecco et al. 2013; Lackner et al. 2014; Shah et al. 2013). Given the large numbers of genes affected at senescence, it has been difficult previously to study the relationship between gene expression changes and the rate of senescence without perturbing fundamental cellular processes. Here, by exploring the mechanisms of Rap1-mediated gene expression changes at senescence, we were able to generate a separation-of-function Rap1 mutant that affected in particular Rap1’s functions at activated NRTS. However, despite diminished NRTS gene activation, the rate of senescence was not changed, showing that their expression can be uncoupled from the rate of senescence. This may also be true for mammalian factors controlling gene expression changes in senescent cells, because several can be manipulated to blunt the altered expression without bringing senescent cells out of cell cycle arrest (Georgilis et al. 2018; Tasdemir et al. 2016; Nacarelli et al. 2019; Correia-Melo et al. 2016). This is encouraging, as it indicates that negative aspects of cell senescence can be blocked without compromising its tumor-suppressive properties.

### 2.5 Experimental Procedures

**Yeast strains and plasmids**
All experiments are performed using BY4741/4742 background, and deletion strains are from the haploid Yeast Knockout Collection or were constructed using standard gene replacement techniques. Plasmids were made using Gateway cloning methods. Site-directed mutagenesis to generate Rap1 *E. coli* expression plasmids with AAA mutations in the SANT domain was performed using QuickChange primer design (Agilent) and primer extension using Phusion HF to introduce the changes into pGST-SANT-6xHis (BSS48); all mutations were verified by sequencing. All strains and plasmids used are listed in Supplementary Table 2-1, and primers used for mutagenesis are listed in Supplementary Table 2-2.

**Expression and purification of Rap1 and Rap1 derivatives**

All Rap1 proteins were N-terminally tagged with GST, and some were also C-terminally tagged with a 6x-His tag, as indicated in the text.

For Ni-NTA purifications, 200ml of BL21(DE3) cells containing expression plasmids GST-Rap1-6X-His or GST-SANT-6X-His were grown at 37°C to a cell concentration of OD(600) 0.4, then induced with 1mM IPTG overnight at room temperature. Protein was purified according to Ni-NTA Purification System protocol by Novex with the following modifications. Cell pellets were resuspended in 20 ml of native binding buffer (50 mM NaH$_2$PO$_4$, pH 8.0, 500 mM NaCl, 1% Triton, 1 mg/ml lysozyme, benzonase 1000 Units). Protein lysate, treated with benzonase, and clarified by centrifugation as recommended, was loaded onto Ni-NTA resin. After incubation and wash, to ensure removal of any contamination by residual DNA, an additional on-column benzonase digestion was performed (10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1.5 mM MgCl$_2$, benzonase 500 Units) at room temperature for 15 min. High sensitivity measurements of DNA by Qubit showed minimal amounts of DNA (40 Rap1 molecules for every base pair of DNA). Protein was eluted using 10 ml elution buffer (50 mM NaH$_2$PO$_4$, pH 8.0, 500 mM NaCl, 250 mM
imidazole). Protein of interest was quantified using SDS-PAGE gel electrophoresis and BSA standards and then flash frozen for storage.

For GST-only protein purifications, 200 ml of BL21(DE3) cells containing the various Rap1 expression plasmids were grown and induced as described above. Cell pellets were flash frozen and stored at -80°C. The GST-Rap1 was purified using Methods described in Schäfer et al., 2015 (Schäfer et al. 2015) with modifications. Clarified protein lysate treated with benzonase was loaded onto 2.5 ml of glutathione resin (GE Healthcare). An on-column benzonase digestion as described above was performed to remove residual DNA. Protein concentration was determined by boiling 10 µl of resin in 1X SDS-PAGE buffer and SDS-PAGE gel electrophoresis using BSA as standards.

**GST histone pull-down assay**

Protein purified by Ni-NTA were thawed and diluted using 1 volume of binding buffer (50 mM Tris-Cl, pH 7.5, 1 mM BME, and 150 mM – 750 mM NaCl, as indicated in text), and incubated overnight with equilibrated glutathione resin (50 µl). GST-6X-His proteins were used as negative controls. After supernatant is removed, resin was washed twice in 500 µl of binding buffer. Proteins purified using GST protein purifications were used directly. Histones were purified as described in Ricketts et al. 2015. The pull-down was performed by incubating 2 µM purified histones and desired concentration of GST-tagged protein for 90 min in 500 µl of binding buffer with rotation at 4°C. Resin was then washed 4X with 500 µl of binding buffer before elution of proteins by boiling in 2X SDS-PAGE buffer. 10% of the pull-down were then analyzed by SDS-PAGE and Coomassie staining using Coomassie Brilliant Blue G-250 or by western blot (anti-H3, Abcam ab 1791, 1:2000).
Coimmunoprecipitation from whole cell extracts

BY4741 cells containing NOP1 driven HA-tagged Rap1 or Rap1\textsuperscript{SHY} expression plasmids were grown in SC-His to a concentration of 2x10\textsuperscript{7} cells/ml. Cells were suspended in lysis buffer (50 mM Hepes – KOH, pH 8, 5% glycerol, 300 mM KCl, 0.1% NP-40, 0.1 mM DTT, 1X EDTA-free protease inhibitor cocktail (Roche), 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 5 mM NaF), and subjected to mechanical disruption with bead-beating at 4°C (60 seconds, 4X). After removal of beads, MgCl\textsubscript{2} and benzonase were added to a final concentration of 2 mM and 25 U/ml, respectively, and the whole cell extract (WCE) was incubated while rotating at room temperature for 30 min, followed by addition of 4 mM of EDTA to quench benzonase digestion. Protein concentration in the clarified WCE was determined by Bradford quantitation, and 500 µg of protein in 150 µl of lysis buffer was used for each coIP. WCE were diluted in equal volume of 50 mM Tris, 5% glycerol, 1 mM EDTA containing 2X protease inhibitors and incubated with HA-antibody (Abcam ab 9110) bound Dynabeads for 2 hrs at 4°C. Beads were then washed in lysis buffer, and bound proteins were solubilized with 2X SDS sample buffer and analyzed by standard SDS-PAGE and western blotting.

Chromatin immunoprecipitation

BY4741 cells containing GAL1 driven expression plasmids were grown in SC-Ura-raffinose to a concentration of 0.5×10\textsuperscript{7} cells/ml, then induced with galactose (final concentration 2%) for 130 min. Cells were crosslinked with 1% formaldehyde solution (w/v), methanol free (Thermo Scientific Ref 28908) for 30 min at room temperature and quenched with 125 mM (final concentration) glycine. Cell pellets were frozen and stored at -80°C. For ChIP, cells were lysed in FA-lysis buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.1% Triton, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 1X protease inhibitor cocktail) and subjected to mechanical disruption with bead beating (60s, 6X). Lysate was sonicated with
Covaris S220, peak power 240.0, duty factor 20.0, cycles/burst 200, time: 420s and protein concentration in the whole cell extract (WCE) was determined using Bradford quantitation. 1.5 mg of WCE was used for pull-down of HA-tagged proteins, and 0.5 mg of WCE was used for H3 pull-down. 200 µl of Protein G Dynabeads (Invitrogen) were blocked with Block Solution (0.5% BSA) and incubated with the appropriate amount of antibodies (anti-HA, Abcam ab 9110, 7.5 µg, anti-H3 Abcam ab 1791, 5ug, and rabbit IgG, ImmunoPure 31207, 7.5 µg and 5 µg, respectively) in 500 µl of Block Solution for a minimum of 6 hours. WCE were incubated with respective antibodies or IgG overnight at 4°C with rotation. Beads were washed 2X with FA-Lysis Buffer, 1X with FA-Lysis Buffer/500 mM NaCl, 2X with LiCl solution (10 mM Tris-Cl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40), 2X with TE+0.1% NP-40, then eluted with TES (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% SDS) 3 times for 15min at 65°C. Eluted DNA was reverse crosslinked with 200 mM NaCl at 65°C overnight and subjected to 1 hour each of RNase A (0.4 mg/mg) and Proteinase K (0.3 mg/ml) incubations at 37°C and purified using QIAGen MinElute Spin Columns. qPCR was performed as described in Platt et al., 2013. Statistics were performed using two-tailed unpaired t-tests.

**Quantitation of mRNA analysis**

mRNA expressions were quantified using methods described in Platt et al., 2013. All mRNA analysis for Rap1 or mutant overexpression were performed using protein expression driven by the NOP1 promoter. Signals were calculated using standard curves of pooled cDNA samples and normalized to ACT1. Error bars indicate standard error of mean. P-values were calculated using two-tailed unpaired t-tests.

**Electrophoretic mobility shift assays**
Proteins were purified using Ni-NTA and GST resin and quantified using Coomassie blue staining with BSA standards. DNA probes were generated using polynucleotide kinase to $^{32}\text{P}$-end label oligos, followed by annealing to their unlabeled complementary strands (Table S6). The fraction of active protein was similar for Rap1 and Rap1$^{SHY}$ preparations (~85%), and was quantified by incubating 10 nM protein as measured by Coomassie blue staining with increasing amounts of TEF2 probe (0 – 100 nM), taking the fraction of protein-DNA complex formation at saturated DNA concentrations as a measure of active protein. For EMSAs, 0.5 nM $^{32}\text{P}$-labeled duplexes were incubated with increasing concentrations of active protein in binding buffer (20 mM Hepes-KOH, pH 8, 100 mM KCl, 10 µg/ml BSA, 1 mM EDTA, 2 mM MgCl$_2$, 5% glycerol) for 30 min at room temperature. Reactions were loaded on 6% DNA retardation gel (Invitrogen) and electrophoresis was conducted at 100V at 4°C. Radioactive signals were visualized using Typhoon FLA 7000.

**Integration of SHY to AAA mutation in the RAP1 locus**

Genome-editing was performed using the 50:50 method for PCR-based seamless editing in yeast (Horecka & Davis 2014). Forward and reverse primers encompassing SHY->AAA mutation and homologous to URA3 were used for amplification of URA3 from pRS306 by PCR. As RAP1 is an essential gene in S. cerevisiae, the mutation was introduced into diploid cells (TLC1/tlc1$^{\Delta}$, which were then sporulated and dissected. Haploids containing the mutation were confirmed via sequencing.

**Southern blotting**

Telomere lengths were determined as described (Johnson et al. 2001), using Xhol digested DNA run on a 1% agarose gel, transferred to a Hybond-XL membrane, and probed using a radio-labeled telomere Y' fragment.
Senescence assays

Senescence assays were performed as described in Platt et al., 2013. In short, cells from the Yeast Knockout Library were mated with early generation tlc1::LEU2 and the diploids were grown for 60 doublings to allow for equilibration of telomere lengths. Diploids were sporulated, dissected and genotyped. All comparisons between different genotypes were derived from the same tetrad heterozygous from tlc1Δ deletion and other mutations of interest (e.g., RAP1/RAP1SYN) to ensure inheritance of similar telomere length. Spore products were grown in YPAD liquid media and passaged every 22 hours. For each passage, cells were counted by the Coulter counter, and diluted to 10^6 cells/ml in 5ml of liquid media. Cell counts were used to determine population doublings, and the point of senescence was determined from the PD displaying the lowest level of growth. Cells for mRNA expression at senescence were obtained ~5PDs prior to the nadir to avoid formation of survivors, and grown 2-3 more doublings to a density of 1×10^7 cells/ml in fresh medium before harvest.
2.6 References


the Saccharomyces cerevisiae Rap1 protein is determined by its binding to DNA. *Nucleic Acids Res.* 40, 3197–3207.


2.7 Figure Legends

**Fig. 2-1 Rap1 binds histone H3/H4 tetramers.**

A. Schematic of Rap1 fragments and domains, with amino acid positions indicated. B. GST-Rap1-6X-His protein was expressed in *E. coli*, purified using the His tag and then subjected to a GST pull-down histone binding assay (300 mM NaCl; note that similar results were obtained up to at least 750 mM NaCl). Rap1 (0.5 µM) binds to H3/H4 tetramers (2 µM), but not H2A/H2B (2 µM) dimers. When H2A/H2B dimers and H3/H4 tetramers are mixed in 2:1 ratio as in the octameric histone core (4 µM H3/H4 and 2 µM H2A/H2B), Rap1 interacts specifically with H3/H4 tetramers. C. Panels C-E use Rap1 protein purified using only the GST tag. GST pull-down histone binding assay with truncated regions of Rap1 (400 mM NaCl). Rap1\(^C\Delta\) (0.5 µM) binds H3/H4 tetramers (2 µM) with similar strength as full-length Rap1. Rap1\(^N\) and Rap1\(^C\) do not bind histones under these conditions. Note that when Rap1 constructs are not purified via a C-terminal 6X-His tag, proteins near the size of GST are detected, presumably due to translational termination or proteolytic degradation near the GST-Rap1 junction. D. GST pull-down histone assay with equimolar Rap1 and histones (300 mM NaCl). Full-length Rap1 (2 µM) and Rap1\(^C\Delta\) (2 µM) each binds H3/H4 (2 µM) robustly (~1:1). Rap1\(^C\) (2 µM) displays detectable H3/H4 binding under these conditions. Similar interaction strengths are observed from salt concentrations ranging from 150 mM to 750 mM NaCl. E. GST pull-down histone binding assay with SANT domain (300 mM NaCl). Top panel: Coomassie stain of SANT domain (2 µM) interacting with H3/H4 (2 µM). Bottom panel: immunoblot against histone H3. F. Quantitation of Rap1 fragments binding to H3/H4 under equimolar conditions. Error bars indicate the standard error of the mean (N=4).

**Fig. 2-2 Amino acids 392-394 (SHY) facilitate Rap1-histone interactions.**

A. Location of alpha helices within the SANT domain, redrawn from Konig, P. *et al.*, 1996. Triple alanine mutants were generated from amino acids 359-410. B. Immunoblot analysis of *in vitro* GST pull-down of
histones showing representative triple alanine mutants. Pull-down was performed with equimolar GST-SANT (0.5 µM) and histones (0.5 µM) at 400 mM NaCl. Bottom panel is the blot stained with Ponceau S as a loading control. C. Quantitation of triple alanine mutants binding to H3/H4, normalized to Ponceau stain signal, and with WT SANT set to 1.0. Error bars for all quantitations indicate standard error of the mean (N=2). Only mutant 12 (amino acids 392-394, SHY) showed a significant loss of H3 signal. D. Two views of the SANT domain bound to DNA. Amino acids SHY side chains are colored in magenta. SHY is located immediately C-terminal to helix 2, with side chains facing away from the Rap1-DNA interaction surface. Image generated using Pymol (PDB ID: 3UKG). E. Representative immunoblot analysis of GST pull-down histone binding assay with full-length Rap1 and Rap1^{SHY}. Pull-down was performed with 0.5 µM each Rap1 and H3/H4 at 400 mM NaCl. Rap1^{SHY} displays a ~50% loss of histone binding. Bottom panel is a loading control gel stained with Coomassie blue. F. Quantitation of full-length Rap1 and Rap1^{SHY} binding to H3/H4 (N=3). G. Representative immunoblot analysis of GST pull-down histone assay using two truncated versions of Rap1 lacking the C terminus, Rap1^{CΔ} and Rap1^{643Δ}. Pull-down was performed with 2 µM Rap1 truncated constructs and 2 µM H3/H4 at 400 mM NaCl. Both truncated forms show a significant and similar loss of histone signal when amino acids SHY is mutated to AAA (rightmost two lanes). Bottom panel is Coomassie loading control. H. Quantitation of G (N=3). I. Representative coimmunoprecipitation of HA-Rap1 and HA-Rap1^{SHY} with histone H3. Input is 5% of the whole cell extract. Rap1^{SHY} shows a significant loss of histone binding in vivo. J. Quantitation of I (N=3).

**Fig. 2-3 Rap1^{SHY} is deficient in NRTS activation and histone displacement.** A. Immunoblot analysis of TCA extracts of GAL1 driven HA-Rap1 and HA-Rap1^{SHY} accumulation after 130 min of induction with galactose, conditions also used for panels B and C. B. Rap1 levels at the promoters of the upregulated NRTS, measured by qPCR of ChIP samples from cells overexpressing HA-Rap1 or HA-Rap1^{SHY}, and normalized to input. IgG is control immunoglobulin
from non-immunized rabbit, and *MDP1* is a non-Rap1 target. **C.** Loss of H3 levels at the promoters of the upregulated NRTS. The fold H3 ChIP enrichment is the ratio of H3 levels at the promoters of the activated NRTS in induced vs. uninduced cells, normalized to their levels at the promoter of the non-Rap1 target gene *MDP1* (*p*<0.04). **D.** Accumulation of HA-Rap1 and HA-Rap1<sup>SHY</sup> driven by the *NOP1* promoter. **E.** Accumulation of HA-Rap1<sup>CΔ</sup> and HA-Rap1<sup>CΔ,SHY</sup> driven by the *NOP1* promoter. **F.** mRNA levels of activated NRTS induced by Rap1 overexpression, measured by qPCR and normalized to *ACT1* and vector control. Rap1<sup>SHY</sup> and Rap1<sup>643Δ,SHY</sup> are similarly compromised in NRTS activation (*p*<0.03). All error bars indicate the standard error of the mean.

**Figure 2-4.** Rap1<sup>SHY</sup> prevents upregulation of activated NRTS at senescence without affecting histone gene expression and the rate of senescence. **A.** Rap1<sup>SHY</sup> does not alter the rate of senescence. Senescence assay of *RAP1* (n=3), *RAP1<sup>SHY</sup>* (n=3), *tlc1Δ RAP1* (n=7), *tlc1Δ RAP1<sup>SHY</sup>* (n=7) spore products. **B.** Rap1<sup>SHY</sup> confers less NRTS activation at senescence. Relative expression of activated NRTS, measured by qPCR and normalized to non-senescent strains (*p*<0.05). **C.** Histone gene repression is not affected by Rap1<sup>SHY</sup>. Relative histone gene expression was measured by qPCR and normalized to non-senescent strains. All error bars indicate the standard error of the mean.

**Fig 2-5.** Asf1 is required for NRTS activation and histone displacement. **A.** NRTS mRNA levels at senescence, measured by qPCR, normalized to *ACT1* and non-senescent strains. *asf1Δ tlc1Δ* double mutants have reduced NRTS activation compared to *tlc1Δ* strains (N=5, *p*<0.025). **B.** Asf1 is required for NRTS activation in response to *NOP1*-driven Rap1 overexpression (Rap1 OE) (*p*<0.02). **C.** ChIP-qPCR of Rap1 in WT and *asf1Δ* strains with Rap1 OE driven by *GAL1*. Rap1 localization to promoters of activated NRTS is not affected by *ASF1* deletion (*p* values
insignificant). ChIP signals are normalized to non-induced cells. D. ChIP-qPCR of histone H3 in WT and asf1Δ strains with Rap1 OE driven by GAL1. Histone displacement is diminished in asf1Δ strains (p<0.05). ChIP signals are normalized to non-induced cells. E. NRTS activation by Rap1 OE is blunted upon deletion of members of the HIR complex (p<0.02). F. asf1Δ does not affect the rate of senescence. Senescence assay with WT (n=2), asf1Δ (n=2), tlc1Δ (n=5), tlc1Δ asf1Δ (n=5). G. hir1Δ does not affect the rate of senescence. Senescence assay with WT (n=2), hir1Δ (n=2), tlc1Δ (n=5), tlc1Δ hir1Δ (n=5). All Error bars indicate the standard error of mean.

Figure 2-6. Model of Rap1 interacting with histones at activated NRTS promoters. Rap1 interacts directly with histones to displace nucleosomes at the promoters of upregulated genes at senescence. A mutation that perturbs Rap1-histone interactions leads to compromised nucleosome loss and blunted gene activation, but no change is observed in the rate of senescence. Our findings shed light on mechanisms of Rap1-mediated gene expression changes at senescence and show that these can be uncoupled from the pace of senescence.

Fig. S2-1. The Rap1 N terminal fragment does not bind H3/H4. GST pull-down histone binding assay with full-length Rap1 and Rap1N (400 mM NaCl). Full-length Rap1 (2 µM) binds H3/H4 tetramers (2 µM) at equal-molar concentrations, but Rap1N does not bind H3/H4 even when in vast excess (15 µM).

Fig. S2-2. Triple alanine screen. Immunoblot analysis of GST pull-down of histones. Pull-down was performed with equimolar GST-SANT (2 µM) and histones (2 µM) at 400 mM NaCl. Bottom panel is the blot stained with Ponceau S as a loading control. Mutants 5 and 13 did not express well in E. coli and so were not tested.
Fig. S2-3. The SHY mutation does not compromise Rap1 binding to natural and NRTS target DNA, and it does not affect expression of tested natural Rap1 target and non-target genes. A. EMSA of Rap1 and Rap1\(^{SHY}\) binding to telomeric sequence TeloA. Proteins were titrated from 0.5 nM to 100 nM. Quantifications were performed by measuring the fraction of probe bound to protein at a given protein concentration. Rap1\(^{SHY}\) exhibited higher affinity for the telomeric sequence compared to Rap1. B. EMSA of Rap1 and Rap1\(^{SHY}\) binding to the TEF2 promoter sequence, a canonical Rap1 binding site. Proteins were titrated from 5 nM to 50 nM. Rap1 and Rap1\(^{SHY}\) showed similar binding affinities. C. EMSA of Rap1 and Rap1\(^{SHY}\) binding to a NRTS promoter sequence of GAC1. Proteins were titrated from 5-500 nM. Rap1 and Rap1\(^{SHY}\) displayed similar affinities, and has a Kd approximately 10-fold lower than that observed in B. D. H3 levels at the promoters of the upregulated NRTS in induced and uninduced cells, normalized to MDP1. No significant differences were observed in uninduced Rap1 and Rap1\(^{SHY}\) strains, but Rap1\(^{SHY}\) had less nucleosome displacement compared to WT after induction (*p<0.05). E-F. mRNA levels of glycolytic gene ENO2, ribosomal gene RPS5, and non-Rap1 target gene SPC42 in cells overexpressing the indicated Rap1 proteins, measured by qPCR and normalized to vector control and ACT1. Rap1\(^{SHY}\) and Rap1\(^{643\Delta, SHY}\) do not significantly compromise expression of natural Rap1 targets or non-Rap1 target genes.

Fig. S2-4. Characterization of Rap1\(^{SHY}\). A. RAP1 and RAP1\(^{SHY}\) haploid spore products from tetrad dissections of sporulated RAP1\(^{+/SHY}\) TLC1/tlc1\(^\Delta\) diploids. RAP1\(^{SHY}\) in the endogenous locus has a slower growth phenotype. However, RAP1\(^{SHY}\) TLC1 (square) does not have a significant growth difference from RAP1\(^{SHY}\) tlc1\(^\Delta\) (circle). Triangles indicate RAP1 tlc1\(^\Delta\), and unmarked colonies are RAP1 TLC1. B. Southern blot of telomere lengths using the \(^{32}\)P-labeled Y' telomeric probe. No significant differences were observed between Rap1 and Rap1\(^{SHY}\) strains or in their respective tlc1\(^\Delta\) strains (50 PDs after spore germination). Note that an inadvertently unloaded
lane between the last two lanes was cropped from the figure. C. Colony-forming efficiency. 200 RAP1 and RAP1SHY cells were plated and colonies counted. RAP1SHY forms a similar percentage of colonies compared to WT. D-E. Telomeric silencing. Cells bearing the URA3 marker in the VII-L telomere were pre-grown in either complete medium (D) or SC-Ura (E). 200 (D) and 400,000 (E) cells were plated on 5-FOA plates to select for silencing of the URA3 gene. RAP1SHY had no defects in telomere silencing compared to WT. F. mRNA expression of the silent mating type normalized to ACT1. mRNA was harvested from RAP1 and RAP1SHY strains and no differences in HML and HMR expression were observed by qPCR. G. mRNA expression measured by qPCR of natural Rap1 targets glycolytic gene ENO2, ribosomal gene RPS5, and non-Rap1 target SPC42, normalized to ACT1. RAP1SHY has higher glycolytic gene expression compared to RAP1. All error bars are standard error of the mean (**p<0.01, N=3).

Fig. S2-5. The CAF complex does not contribute to Rap1-mediated NRTS activation. mRNA levels of activated NRTS, measured by qPCR and normalized to ACT1 and vector control. Rap1 overexpression is driven by NOP1.
Figure 2-1

A. Rap1

B. Rap1

C. H3/H4

D. H3/H4

E. H3/H4

F. Graph
Figure 2-2

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Figure 2-3

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Figure 2-4

A. Graph showing cell density (cells/ml) vs. population doubling. The graph compares different strains labeled as TLC1, RAP1, tlc1Δ, RAP1, tlc1Δ, RAP1SNY.

B. Bar graph showing relative expression vs. non-senescence. The bars represent strains RAP1 and RAP1SNY.

C. Bar graph showing relative expression vs. non-senescence for genes HHT1, HHT2, HHF1, HHF2, HTA1, HTA2, HTB1, HTB2.
Figure 2-5

A. Senescence

B. Rap1 OE

C. Rap1 OE

D. Rap1 OE

E. Rap1 OE

F. Cell density (cells/ml)

G. Cell density (cells/ml)
Figure 2-6

Telomere shortening leads to nucleosome displacement by Asf1, which in turn upregulates genes associated with senescence. Therap1 protein plays a role in this process, with different forms (Rap1\(^N\), Rap1\(^{DBD}\), and Rap1\(^C\)) affecting the displacement and subsequent gene expression.
Figure S2-1

Figure S2-2
Figure S2-3

A. TeloA

B. TEF2

C. GAC1
Figure S2-5

Rap1 OE

XBP1  GAC1  NCA3  GPG1  IDH2

WT  cac1Δ  cac2Δ  cac3Δ
**Table S2-1**

**Supplementary Table 2-1. Yeast strains**

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</table>

*All KanMX-replaced alleles (in ASF1, HIR1/2/3, HPC2, and CAC1/2/3), are derived from the yeast haploid KO collection in BY4741*
### Table S2-2

#### Supplementary Table 2-2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>BJP201</td>
<td>pAG423-NOP1p::ccdb</td>
<td>Platt et al., 2013</td>
</tr>
<tr>
<td>BJP69</td>
<td>pAG423-NOP1p::RAP1</td>
<td>Platt et al., 2013</td>
</tr>
<tr>
<td>BSS20</td>
<td>pGST-His</td>
<td>This study; constructed using Gateway destination vector pDEST15</td>
</tr>
<tr>
<td>BSS18</td>
<td>pGST-RAP1</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS104</td>
<td>pGST-RAP1-6xHis</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS48</td>
<td>pGST-SANT-6xHis</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS54-BSS70</td>
<td>pGST-SANT*-6x-His</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS122</td>
<td>pGST-RAP1N</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS124</td>
<td>pGST-RAP1C</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS143</td>
<td>pGST-RAP1CA</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS163</td>
<td>pGST-RAP1CASHY-6xHis</td>
<td>&quot;</td>
</tr>
<tr>
<td>BSS165</td>
<td>pGST-RAP1&lt;sup&gt;643SHY&lt;/sup&gt;-6xHis</td>
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</tr>
<tr>
<td>BSS167</td>
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<tr>
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<td>This study; constructed BJP201</td>
</tr>
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<td>BSS209</td>
<td>pAG426-GAL1::HA-RAP1</td>
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<td>BSS211</td>
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<td>This study; via site directed mutagenesis of BSS209</td>
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*Triple alanine screen: every three consecutive amino acids in the SANT domain (amino acids 360-410) was mutated to AAA
**Table S2-3**

**Supplementary Table 2-3. Triple alanine mutation primers**

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<tr>
<td>359-361</td>
<td>a46g_a47c_a49g_a50c_t54a_</td>
</tr>
<tr>
<td></td>
<td>5'-catcttcctcactgtgtaaaagagtgcagcgtggagggcaagccagccagcc -3'</td>
</tr>
<tr>
<td></td>
<td>5'-ggctggccgtttgcctccccaacgctgcagcactcftttacagatgaggaagatg-3'</td>
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<tr>
<td>362-364</td>
<td>t55g_t58g_t59c_a61g_</td>
</tr>
<tr>
<td></td>
<td>5'-aaaataaactcatcctctctcatgcagcagcagttttatgtggagggcaagccagc -3'</td>
</tr>
<tr>
<td></td>
<td>5'-cactgcttgccctccccacacatgaggaagatgagtttattttttggaag -3'</td>
</tr>
<tr>
<td>365-367</td>
<td>a65c_a68c_a71c_</td>
</tr>
</tbody>
</table>
|          | 5'-ttcccaataaaagctcctttttacagctgcagcatctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
| 392-394 | t145g_c148g_a149c_t151g_a152c_  
| 5'-cccgtgtgttgtagcacaacacgcgctttatctcgtgtaaagagtaagttatgtgtgtgtgca-3'  
| 5'-ccaggcgtacaacacacacactccttttagcataaagctgctatgctgctgatacagggg-3' |
| 395-397 | t155c_c157g_a160g_a161c_  
| 5'-taatagaattacccgtaggtgagcgcgacatagctgtaaagagatgtgtgtgtgca-3'  
| 5'-cacatactctttacgcgaaatatccattatgtgcgctcgcgccacacagggaattcttt-3' |
| 398-400 | a163g_a164c_a166g_g170c_  
| 5'-ctaaatcggtgcttaatagataaggcccgccggttaggcacataatgggatatttc-3'  
| 5'-gaaatatccattatgtgcctaaacgcgctgtaatcattaggccgcggtaggtgca-3' |
| 401-403 | a172g_a173c_t175g_a178g_t179c_  
| 5'-aaagatagactcttaatatctgctgctgtaaagagatgtgtgtgtgca-3'  
| 5'-cccattagctgctaaacacacggtgtgtctgctgctacaggcgcacattgctcttttt-3' |
| 404-406 | a181g_g182c_c184g_a185c_c187g_g188c_  
| 5'-tagctctcttttaggaaagatagactcttaatatctgctgctgtaaagagatgtgtgtgca-3'  
| 5'-tatgctgctaaacacacggtgtcattttagcgtacatgctcttttccaaagactaca-3' |
| 407-409 | t190g_t191c_a193g_g194c_t197c_  
| 5'-acgactcctcttgagagaagatagactcttaatatctgctgctgtaaagagatgtgtgtgca-3'  
| 5'-cacaggggtaatcttattaggccgcggtagcgtgagcctctttctttttccaaagactaga-3' |
Table S2-4

Supplementary Table 2-4. 50:50 Primers for SHY->AAA in the endogenous *RAP1*

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<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>SHY Check F 21mer</th>
<th>WT Check F 21mer</th>
<th><em>RAP1</em> Internal R</th>
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<tr>
<td>50 forward primer</td>
<td>GTGAGAAAAATCCAACCAGGCTACAACACATACTCTTTTACGATGAAATAGCCGCTGCTGTGCCCTAACCACACGGGTAAATTCTATTAGGCACCGATTTAGAGTCTATCTGCTGCTTAACTATGCGGCATCAGA</td>
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<td>TTACGATGAAATAGCCGCTGCT</td>
<td>TTACGATGAAATATCCCATTA</td>
<td>CGTCCCCCTACGGCTTTGGGT</td>
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<tr>
<td>50 reverse primer</td>
<td>AGATAGACTCTAAATCGGTGCCTAATAGAATTACCCGTGTGGTTAGGCACGTGCGGTATTTCAACACCAGG</td>
<td>GTGAGAAAAATCCAACCAGGCTACAACACATACTCTCTTTTACGATGAAATAGCCGCTGCTGTGCCCTAACCACACGGGTAAATTCTATTAGGCACCGATTTAGAGTCTATCTGCTGCTTAACTATGCGGCATCAGA</td>
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### Table S2-5

**Supplementary Table 2-5. Primers used for RT-qPCR**

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>ENO2</td>
<td>ACAACGTCATTGCTGCTGCT</td>
<td>TCAGCGGGTGTGAATGTTTGG</td>
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<td>RPS5</td>
<td>TGACTGACAAAACCAATCCA</td>
<td>CACCGACTGTTGGTGTTCTTCT</td>
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<td>SPC42</td>
<td>AAGAGCTGCAAGCATGATGGAC</td>
<td>GACTGGATTGGAAGAAATGACGA</td>
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For a full list of RT-qPCR and ChIP-qPCR primers, see Platt et al., 2013.

### Table S2-6

**Supplementary Table 2-6. Oligonucleotides used for EMSA**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
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<td>TeloA-5'</td>
<td>GCCGCACACCCACACACAGTG</td>
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<tr>
<td>TeloA-3'</td>
<td>CACTGGTGTTGGGTGGC</td>
</tr>
<tr>
<td>TEF2-5'</td>
<td>TGTTCACCCACACATTA</td>
</tr>
<tr>
<td>TEF2-3'</td>
<td>TAAATGTGTGGGGTCACA</td>
</tr>
<tr>
<td>GAC1-5'</td>
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<td>GAC1-3'</td>
<td>GTACCAGCCTTATAGACAGTTAATGTGTATTATT</td>
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Chapter 3 Conclusions and Future Directions

3.1 Conclusions

*Saccharomyces cerevisiae* repressor activator protein 1 (Rap1) is an evolutionarily conserved protein that has been studied for more than three decades. It has well established roles in various aspects of cell activities, including, among others, transcriptional activation and repression, telomere length regulation, chromosome end protection, chromatin barrier formation, chromatin landscape remodeling and mRNA stability control (see Chapter 1 and reviews Azad & Tomar, 2016; Morse, 2000; Song & Johnson, 2018 for details). Previously our lab has published the first evidence that Rap1 also plays important roles in cellular senescence, contributing to senescence-related gene expression changes and histone loss. Rap1-controlled histone loss at senescence comes in two flavors: 1) it contributes to global histone loss by repressing histone gene expression and 2) it contributes to site-specific histone loss by nucleosome displacement at the promoters of the activated genes (Platt et al., 2013). I have built on this work and others and defined an amino acid patch within the Rap1 DNA binding domain that is involved in Rap1-mediated nucleosome displacement and gene activation via interactions with the histone H3/H4 heterotetramer. This has allowed us to generate a separation-of-function mutant that specifically affects site-specific histone losses and Rap1-regulated gene activations at senescence without perturbing histone gene repression or the pace of senescence. This is exciting, as it suggests that negative aspects of senescence can be blocked without changing its tumor suppressive properties.

In addition, it has long been known that Rap1 can displace nucleosomes and it has been proposed that Rap1 can function similarly to pioneer transcription factors (pTFs) described in higher eukaryotes, though the mechanisms of how Rap1 invades closed chromatin and displaces nucleosomes are poorly understood. Recent work by the Zaret lab characterizing ~2000 transcription factors has identified several features of potentially strong nucleosome
binders, including 1) DNA binding affinities within the nanomolar range; 2) high nucleosome binding affinities that preferentially stabilize a certain rotational frame within the nucleosome; 3) DNA binding domains containing basic helix-turn-helix motifs, homeodomains or zinc fingers (Fernandez Garcia et al., 2019). Rap1 fits within all descriptions with a high DNA binding affinity (reported Kd ranges from 10 pM to 10 nM), high nucleosome binding affinity (nanomolar range) and two helix-turn-helix motifs within the tandem Myb domains that dock deep in the major groove of DNA. However, whether strong nucleosome binders rely only on strong protein-DNA interactions that distort the nucleosome, or have other points of contact, e.g. interactions with histone proteins, is only beginning to be explored. In addition, how pTFs remodel chromatin after binding is also unknown, though this may be transcription-factor specific. To date, direct histone interactions have only been described for two other pTFs, FoxO1 and FoxA (Cirillo et al., 2002; Hatta & Cirillo, 2007). Our work adds to this literature by characterizing direct Rap1-histone interactions in vitro as well as in vivo. We also show functional consequences on transcription associated with perturbations Rap1-histone contacts in vivo. Interestingly, the mammalian RAP1, which has little to no sequence-specific DNA binding ability, can also interact directly with the H3/H4 tetramer, though the Myb domain appear to be uninvolved (Appendix, Section C). The mammalian RAP1 has been shown to localize at both telomeric and extratelomeric sites, and contribute to the regulation of gene expression (Martínez et al., 2013; Martínez, Gómez-López, Pisano, Flores, & Blasco, 2016; Martínez et al., 2010; Yang et al., 2011; Yeung et al., 2013), though little is known about how this is achieved, as RAP1 itself probably does not directly bind to DNA. Therefore, the fact that RAP1 can interact with histones provide the first insight into how mammalian RAP1 might similarly remodel chromatin, likely recruited to sequence-specific sites by association with factors. In addition, several other roles at senescence seem to be conserved between yeast and human Rap1 proteins (Appendix, Section C). I have performed preliminary genome-wide ChIP-seq in proliferating and senescent human fibroblasts (IMR90s) and found that hRAP1 also relocalizes to new target sites during senescence. Of note, hRAP1 occupancy increases at certain histone promoters and senescence-related genes. In addition, RNA-seq of
hRAP1 overexpression via lentiviral delivery in fibroblasts showed that hRAP1 regulates the expression of many senescence-related genes, and remarkably, preferentially affect histone gene expression. However, histone genes tend to be upregulated rather than downregulated, suggesting a possible role for hRAP1 in compensating for histone losses in senescent cells. These activation mechanisms may be related to direct hRAP1 and histone interactions, similar to scRap1, though more studies need to be done.

Epigenetic regulators also appear to play important roles in Rap1-mediated nucleosome displacement and gene activation. Of note, histone -H3- lysine -4 trimethylation (H3K4me3), an important epigenetic marker that is often associated with active transcription, is necessary for Rap1-mediated full histone losses from NRTS (new Rap1 targets at senescence) promoters and subsequent gene activation, but seem to be uninvolved in Rap1-mediated histone gene repression (Appendix, Section A). Loss of H3K4me3, either through deletion of the histone H3K4 methylase subunit SWD3 (swd3Δ), or a point mutation changing K4 to arginine (H3K4R), delays the pace of senescence, maybe due to a more compact chromatin and changes in the gene expression profile (Appendix, Section A).

H3K4me3 and several other epigenetic regulators also play some part in mediating Rap1 toxicity. It has long been known that Rap1 overexpression is toxic, though the mechanisms of how this manifests remains to be explored. While Rap1 toxicity may not be the most exciting scientific question in and of itself, we have several reasons to believe that it may be linked to other important functions of Rap1, such as regulation of histone levels, senescence phenotypes and organismal lifespan. For example, while manipulations of histone gene dosage had no apparent effect on Rap1 toxicity at first, some preliminary data suggest that cumulative effects of histone shortage through many population doublings (>50 PDs) may drastically increase Rap1 toxicity (Appendix, Section B). In addition, Rap1\textsuperscript{SHY}, a mutant form of Rap1 that has decreased histone binding and nucleosome displacement abilities, is less toxic when overexpressed compared to wildtype Rap1, and provides resistance against DNA damaging reagents (Appendix,
Section B), suggesting that direct Rap1-histone contacts and subsequent consequences of this also affects Rap1 toxicity. Along these lines, I investigated whether histone marks known to affect histone protein levels had any effects on Rap1 toxicity, and found that H3K4me3 protects against Rap1 toxicity, while histone acetylation is necessary for its manifestation (Appendix, Section B). H3K4me3 promotes normal cell proliferation through regulation and maintenance of histone levels, and it has recently been reported that set1Δ (H3K4 methyltransferase) and H3K4R mutants have lower levels of histone gene expression due to the spreading of the repressive complex Asf1/HIR/Rtt106 into histone gene coding regions (Mei et al., 2019), which may contribute to the synthetic sickness observed when Rap1 is overexpressed in the H3K4R background. However, the explanation for the synthetic sickness observed when Rap1 is overexpressed in yeast strains lacking H3K4me3 may not as simple as a simple loss of histones, as deletion of histone acetyltransferase GCN5 rescues Rap1 toxicity, even though it has been reported that gcn5Δ also causes reduced levels of core histones (Petty, Lafon, Tomlinson, Mendelsohn, & Pillus, 2016) and a spectrum of other defects in gene activation and chromatin structure, particularly at stress-regulated genes (Gregory et al., 1998; Pérez-Martín & Johnson, 1998). Remarkably, both set1Δ and H3K4R delays replicative senescence and promotes longevity (Appendix, Section A), while gcn5Δ was found to strongly reduce yeast replicative mother cell and chronological lifespan in several studies (Kim, Ohkuni, Couplan, & Jazwinski, 2004; Laschober et al., 2010; Matecic et al., 2010). These interesting correlations suggest that factors that regulate longevity, maybe more than histone levels, are involved in regulating the toxic phenotypes of Rap1 overexpression.

3.2 Future Directions

3.2.1 Further characterization of both yeast and mammalian Rap1-histone interactions
The focus of my thesis has been to address how the ability of Rap1 to displace nucleosomes connects with its other functions in senescent cells, including upregulating senescence-specific target genes (i.e. upregulated NRTS), downregulating histone gene expression, and driving the rate of senescence. To accomplish this, I set out to identify a Rap1 separation-of-function mutant that selectively perturbs site-specific histone losses. I found that the SHY patch within the DNA binding domain of Rap1 is involved in Rap1-histone H3/H4 heterotetramer interactions, and functional studies of a SHY -> AAA mutant, which proved to be deficient in histone displacement, demonstrated that Rap1-mediated nucleosome displacement is correlated with NRTS upregulation by Rap1. Furthermore, such nucleosome displacement and NRTS upregulation can be uncoupled from the ability of Rap1 to repress histone gene expression and to drive the pace of senescence. In the process of identifying this unique mutant, I characterized to some extent the nature of Rap1-histone interactions. Structural details of this interaction were not explored due to my concentration on functional analysis. However, this interaction is nonetheless interesting in and of itself, as it can provide mechanistic insights into how pTFs in general decompact and disassemble nucleosomes to allow for recruitment of other transcription factors and subsequent gene activation. Therefore, further characterizations of Rap1-histone contacts are of general interest and can be approached from several directions. For example, we have not yet tested the relative importance of each residue within the SHY patch for histone binding. Furthermore, mutation of the SHY patch to alanines (Rap1SHY) results in ~50% loss of Rap1-histone binding affinity in vitro and in co-immunoprecipitation studies using cell extracts, indicating additional points of contact between the proteins. Therefore, it will be interesting to map such points of histone contact within Rap1, as well as points of Rap1 contact with histones, and to examine the in vivo effects of the full loss of Rap1-histone interactions. Histone pull-down assays with truncated versions of the full-length protein suggest that other interaction surfaces may exist in the second Rap1 Myb domain, and also within the C-terminal region (Chapter 2). However, comparison of the first and second Myb domains show no conservation of the SHY patch.
The human RAP1 protein also interacts directly with H3/H4 tetramers, though the Myb domain containing the helix-turn-helix motif appears to be uninvolved (Appendix, Section C). It is likely that this interaction is occurring through the C-terminal region of hRAP1, which contains the RCT domain, is highly acidic (pI 3.8) and is known to interact with other proteins, including TRF2. This would not be completely unexpected on two accounts: 1) the C-terminal region of scRap1 can interact with histones H3/H4, albeit weakly, compared to the DBD, and it could be that in hRAP1, the C-terminal region has taken over as the primary mediator of histone binding, 2) FoxA, a pTF known to interact with histones H3/H4, does so through its C-terminal protein-interacting and transactivation domain, independent of its DNA binding domain (Cirillo et al., 2002). This suggests that pTF-histone contacts do not have to be within the same protein domains that interact with nucleosomal DNA, and scRap1 may be the exception rather than the norm in this case.

In addition, it will also be interesting to map Rap1-histone contacts on the histone H3/H4 surfaces, as the exact locations may shed light on how exactly nucleosome structures are perturbed. Studies by Cirillo et al. suggest that histone tails are probably not involved, which is consistent with yeast two hybrid screens that I have performed using scRap1 and histone tails (data not shown). Therefore, the contacts most likely exist within the nucleosomal core, which lends weight to our hypothesis that Rap1 binding can distort nucleosomes and shift the equilibrium towards nucleosome disassembly. We did not observe inhibition of Rap1-H3/H4 binding by histones H2A/H2B in vitro, suggesting that Rap1 may not function by disrupting binding of H2A/H2B to partial nucleosomes containing H3/H4. Further investigations as to whether these points of contact are near the entry/exit point of nucleosome-DNA contacts or more centered towards the dyad of the nucleosome will provide a clearer picture of how this is achieved.

Proteins can interact with nucleosomes via various binding modes (Horn, Van Ingen Epigenetics, 2018, n.d.; Kale, Gonçarenclo, Markov, Landsman, & Panchenko, 2019; Zhou,
Gaullier, & Luger, 2019), including, but not limited to 1) binding to nucleosomal DNA (Zhu et al., 2018); 2) binding to multiple components of the nucleosome core particle, including a combination of nucleosomal DNA, histone tails and/or histone octameric core (Xiao et al., 2017); 3) binding to modified histones or histone variants (Weaver, Morrison, & Musselman, 2018); 4) binding to the histone core surface (Armache, Garlick, Canzio, Narlikar, & Kingston, 2011; Wilson et al., 2016). Given the high DNA binding affinity of Rap1, it is plausible that Rap1 is first recruited to NRTS promoters via tight interactions with nucleosomal DNA, and can further interact with histones H3/H4 while being held in close proximity to the histone core (Fig. 3-1). However, this has yet to be explicitly shown by structural studies, and it would be highly exciting to use a combination of biophysical methods such as X-ray crystallography, single-particle cryo-electron microscopy (cryo-EM) and nuclear magnetic resonance (NMR) to obtain such information. Also of note, our co-IP studies indicate that Rap1 can bind histones outside of chromatin in cell extracts (consistent with the submicromolar apparent Kd we estimated from our in vitro studies using purified proteins), and so Rap1 might bind histones within cells even when not also bound to nucleosomal DNA. Comparison of Rap1-histone binding modes on and off nucleosomes might provide clues to the mechanism by which Rap1 contributes to nucleosome displacement.

Recent advances have been made in crystallizing proteins with nucleosomes, and the list of protein-nucleosome complexes deposited in the Protein Data Bank (PDB) show an exponential increase (Zhou et al., 2019). Most of the nucleosomes in these structures are assembled using the Widom 601 DNA sequence, including the two most recent spectacular structures on INO80 bound to nucleosomes (Ayala et al., 2018; Eustermann et al., 2018). This well-established DNA sequence has the advantage of forming relatively stable nucleosomes that easily pack into crystals. However, pTF binding is highly DNA sequence-specific, and detailed mechanisms of how pTFs interact with and disassemble nucleosomes may require a local environment that involves more accessible DNA sequences, pTF – histone contacts, and weaker histone – DNA associations that can only be achieved through the assembly of nucleosomes with native DNA sequences. This is especially true for Rap1 binding to NRTS promoters, where the nucleosomal
DNA is known to be enriched in G/C–capped poly T tracts, which have weak nucleosome destabilizing activities. Such nucleosomes are more unstable by nature, and may resist crystal-packing. However, cryo-EM might be able to avoid such restrictions.

As of date, no structure of any pTF with nucleosomes is available, although in 2018, a cryo-EM structure of a nucleosome containing the \textit{ALB1} enhancer DNA sequence, to which the pTF FoxA binds, was published (Takizawa et al., 2018). This structure reveals that the \textit{ALB1} enhancer DNA forms a nucleosome which is more accessible to DNaseI compared to the Widom 601 sequence, and is more weakly associated with histones. Unfortunately the resolution of this structure (4.0 – 4.5 Å) was not high enough to visualize detailed histone-DNA interactions, and further structural studies will be needed to clarify how the \textit{ALB1} enhancer DNA sequence becomes accessible to FoxA. The study of Rap1 bound to nucleosomes may face similar challenges.

\textbf{3.2.2 Additional mechanisms of nucleosome disassembly by pTFs}

I have shown that amino acids SHY within the DBD of scRap1, histone chaperone Asf1, and the histone mark H3K4me3 to be important for nucleosome displacement at NRTS promoters. However, more detailed mechanisms of how nucleosome disassembly takes place can be further explored, and may be important for the study of chromatin invasion and decompaction mechanisms of other pTFs. Of note, the Shore lab has designed a single-molecule fluorescence resonance energy transfer (FRET) system to study the effects of Rap1 binding to reconstituted nucleosomes that contain Rap1 binding sites of various affinities within the context of the 601 DNA template. Interestingly, they found that Rap1 binding does not distort or evict single nucleosomes, but can decompact an array of nucleosomes that is similar to compact chromatin fibers. In addition, they found that nucleosome disassembly requires the chromatin remodeling complex RSC and histone chaperone Nap1 (Mivelaz et al., 2019). It is worth noting
that when testing the RSC complex and Nap1 findings by the Shore lab *in vivo* with deletion of *NAP1*, I found that Nap1 affects NRTS gene activation in a gene-specific manner (data not shown), suggesting that local factors may dictate the specific remodelers/chaperones recruited for nucleosome disassembly.

This FRET system by the Shore lab might be tailored to address the action of Rap1 at NRTS promoters by reconstituting nucleosomes using native NRTS DNA sequences (Fig. 3-2). These DNA sequences are enriched for a sub-optimal Rap1 binding motif which binds to one of the two DNA-binding hemi-sites, and a G/C-capped poly-T tract with weakly nucleosome destabilizing activities. The intrinsic instability of such nucleosomes may offer further insight into how Rap1 binding changes nucleosome conformations, the details of which may be elusive when tight nucleosome-associating DNA sequences such as the 601 DNA template is used. Such relatively unstable nucleosomes may also better reflect the highly dynamic nature of NRTS nucleosomes in both conformation and composition, as it is known that nucleosomes undergo rapid transient wrapping and unwrapping, also called “DNA breathing” (Chen et al., 2017; Li & Widom, 2004). With this system, it will be interesting to study 1) how Rap1 and Rap1\(^{SHY}\) binding distorts single nucleosomes; 2) the residency times (on/off rates) of Rap1\(^{SHY}\) binding to nucleosomes compared to WT Rap1; 3) effects of H3K4me3 on nucleosome disassembly in single nucleosomes and nucleosome arrays; 4) when and how Asf1 is needed for Rap1-mediated nucleosome displacement.

In addition, while single reconstituted nucleosomes may not become distorted *in vitro*, *in vivo* factors may recognize such nucleosomes as being less stable. Therefore, *in vivo* studies will also be of considerable interest, though may be more challenging. This can be approached from several different directions. For one, genome-wide MNase-seq using varying concentrations of MNase or ATAC-seq can provide clues to the changing chromatin landscapes upon binding of Rap1 and Rap1\(^{SHY}\). In particular this may allow identification of nucleosomes that are sensitive to MNase concentration changes, as these may have become “fragile” and therefore are more
readily targeted for disassembly. This can be a powerful tool coupled with recent advances in ChIP-exo-seq, in which transcription factor footprints are mapped to base-pair resolution (Rhee & Pugh, 2011; Rossi, Lai, & Pugh, 2018). It will also be interesting to see how the nucleosomal binding profile of Rap1SHY changes relative to WT Rap1. For another, mass genome-wide screens using commercially available yeast libraries, for example, the yeast knockout library (Giaever et al., 2002) and histone mutant libraries (Jiang et al., 2017), can yield more chromatin remodelers, histone chaperones, and epigenetic marks that are important for Rap1-mediated nucleosome disassembly in vivo.

Furthermore, studies in yeast using an inducible tagged histone expression system can decipher the genomic locations and rates of histone exchange (Dion et al., 2007; Rufiange, Jacques, Bhat, Robert, & Nourani, 2007). In short, yeast cells were engineered to have a constitutively expressed Myc-tagged histone H3 and an inducible FLAG-tagged H3. FLAG-H3 can be induced for variable amounts of time, and ChIP-chip using antibodies against both tags could allow visualization of both locations of histone replacement and turnover rates (number of H3 replacement events per unit of time). Using this system, it was found that median H3 turnover rates at promoters, about once per cell cycle, were almost two orders of magnitude higher than that of protein coding regions. This rate is probably even higher at promoters with unstable nucleosomes, such as that of the NRTS. It is worth noting that the yeast cells used for this experiment were arrested in G1 before induction of FLAG-H3, suggesting that a full cell cycle, including passage through the S phase, is not necessary for nucleosome turnover and displacement. These findings were further validated in unsynchronized cells, which had well-correlated but even faster turnover rates, as expected given the global deposition of H3 during replication. Such a system can be used to test how Rap1 and Rap1SHY overexpression impact nucleosome displacement and histone turnover at NRTS promoters. Rapid loss and/or turnover is expected given the ability of Rap1 to bind to nucleosomal DNA and histones and the relative instability of nucleosomes at NRTS promoters due to both its native DNA sequence and Rap1 binding. However, there are certain limitations to this system: for one, inducible expression of H3
is limited to a short time window which restricts the temporal resolution of histone exchange; for another, reducing the number of histone copies to one (vs. two in wildtype) and the subsequent overexpression of tagged histone proteins could result in superficial phenotypes that do not reflect the true state of histone replacement and turnover.

Another interesting question that remains to be addressed is whether Rap1-mediated remodeling of nucleosomal landscape at NRTS promoters involves nucleosome eviction or sliding, though the fact that Asf1, a histone chaperone, is necessary for NRTS activation, suggests that nucleosome disassembly is more likely than sliding. One way to approach this question more directly is to test the level of chromatin-bound histones vs. the free histone pool upon Rap1 or Rap1\textsuperscript{SHY} overexpression. Subcellular fractionation techniques that separate chromatin from other nucleosomal fractions are fairly mature. Given that there are ~500 NRTS at senescence, about 100 of which are upregulated by Rap1 (~40 NRTS are downregulated by Rap1, and the remaining gene expressions are unaffected despite Rap1 localization), and a total of ~5000 genes in the yeast genome, a total of 2 – 10% loss of chromatin-bound histones is expected. Whether such a change can be detected accurately with immunoblotting or mass spectrometry-based measurement of histone H3 remains to be tested. A more sensitive way to study the number of free histones would be to assess the level of histone H3 bound by Asf1. Co-immunoprecipitation experiments using tagged Asf1 and blotting for histone H3 may reveal a significant increase of H3 bound to Asf1 upon Rap1 overexpression. However, this is only achievable if the level of Asf1 is not limiting.

3.2.3 Determine mechanisms of Rap1-mediated histone repression

We, and others, have explored to some detail mechanisms of Rap1-mediated gene activation, and nucleosome interacting regions (with both nucleosomal DNA and histones), transactivation domains, as well as coactivators for Rap1 have been identified to some extent.
Mechanisms of Rap1-mediated repression and silencing have also been studied, though mainly at telomeres and the silent mating type loci, and corepressors such as Sir3 and Sir4 (Moretti & Shore, 2001; Moretti, Freeman, Coodly, & Shore, 1994), and Rif1 and Rif2 have been identified (Hardy, Sussel, & Shore, 1992; Shi et al., 2013; Wotton & Shore, 1997). During replicative senescence, Rap1 was found to relocalize to the promoters of all core histone genes and downregulate histone gene expression, leading to a global loss of histones, but little is known about the repression mechanisms, except that the Sir proteins are apparently uninvolved, and no histone H3 losses were observed at histone gene promoters (in contrast to H3 losses at upregulated NRTS) (Platt et al., 2013). These repression mechanisms may be of considerable interest, as loss of histones is a conserved feature of senescence across several models, and the overexpression of core histones, both in yeast mother cells (Feser et al., 2010) and in telomerase-deletion yeast, extends lifespan and delays the pace of senescence. In addition, Rap1SHY does not affect Rap1-mediated histone repression at senescence, in contrast to its diminished capacity in mediating local nucleosome disassembly and gene activation compared to WT Rap1, and also does not change the pace of senescence, leaving open the possibility that the Rap1-dependence of the rate of senescence may be driven by histone expression levels.

Various mechanisms could be involved in Rap1-mediated histone repression, the simplest of which involve corepressors. Therefore, genome-wide screens using yeast knockout libraries for corepressors may prove fruitful. Previously I have tried to identify novel co- of Rap1-dependent histone repression via a reporter synthetic genetic array (R-SGA) (Fig. 3-3). The R-SGA crosses a query manipulation, in this case, Rap1 overexpression (OE), with a collection of deletion mutations (~4,800 non-essential genes), so that mutations that prevent histone gene repression by Rap1 can be identified. Rap1 OE is achieved by integrating GAL1-RAP1 into the genome. Rather than using growth as an output as in standard SGA, R-SGA uses changes in fluorescence intensity from GFP under control of a test reporter (the HTA1 promoter) compared to tdTomato driven from control reporter (the non-NRTS ACT1). It is worth mentioning that this approach is based on the original use of this system (without Rap1 overexpression) by the
Andrews lab to identify novel transcriptional regulators of histone gene expression (Fillingham et al., 2009). However, my efforts to reproduce the published screen were unsuccessful, mainly due to inconsistent readouts of ACT1-driven tdTomato fluorescence signal. If these problems could be overcome (e.g. by testing other control loci and/or fluorescent proteins), this system could prove to be a powerful tool to screen for Rap1-dependent histone gene regulators.

Rap1, and other general regulatory factors (GRFs), have also been proposed to act as roadblocks in transcription, with RNAPII pausing immediately upstream of GRF binding sites and subsequently releasing nascent mRNA to be cleaved and polyadenylated (Candelli et al., 2018; Colin et al., 2014). These GRFs serve as a transcription quality control to limit pervasive transcription events. However, in the case of yeast histone genes, this is unlikely to be the case, as histone gene pairs share divergent promoters, reducing the likelihood that “upstream” transcription events are involved.

It has recently been reported that Rap1 can repress divergent non-coding transcription by binding asymmetrically to nucleosome depleted regions (NDRs) within divergent promoters (Wu et al., 2018). Interestingly, this asymmetric binding is also a conserved feature of mammalian pTFs (Sherwood et al., 2014), suggesting that repression mechanisms among pTFs may be broadly conserved and therefore understanding Rap1 repression mechanisms of histone genes may have more extensive impacts. No other Rap1 cofactors appear to be involved in this repression mechanism. In addition, Wu et al. proposed that it is likely that Rap1 represses divergent transcription through steric hindrance. In the absence of Rap1, the RSC complex carves out a narrow NDR from which cryptic transcription is observed (Kubik et al., 2018; Wu et al., 2018). It is therefore likely that Rap1 competes locally for binding with RSC and the basal transcriptional machinery, thus suppressing transcription. Similar steric hindrances could be at play at the histone promoters, and identifying truncation or point mutants that disrupt this blockage could prove this concept. This is made easier by the fact that Wu et al. have narrowed
down the patch of amino acids involved in this repression mechanism to amino acids 631-696 within the C-terminal region.

*S. cerevisiae* contains two copies of each core histone gene, with each gene pair sharing a promoter in divergent directions. In addition, the arrangement of the yeast histone genes leaves ~650 bp between the divergent promoters, so this should enable assembly of a short array of nucleosomes (~4) between the transcription start sites which allows for *in vitro* studies. Although we previously found that Rap1 binding to histone gene promoters does not result in loss of histone H3 (in contrast to nucleosomal loss at activated NRTS), a *rap1-DAmP* allele which accumulated less Rap1 protein at histone promoters correlated with slightly enriched levels of H3 (Platt et al., 2013). This suggests that Rap1 may still be able to alter the chromatin environment at the histone loci. This slight alteration, coupled with steric hindrance by Rap1 proposed by Wu et al. described above, could mean that Rap1 binding results in small but significant changes within the nucleosome structure that now exposes repressor binding sites. The effect of Rap1 binding on local chromatin structure can be assessed *via* FRET, much like the system designed by the Shore lab, described above (Mivelaz et al., 2019), or by electron microscopy. However, as it is not yet known whether Rap1 alone can alter histone promoter landscapes and repress histone gene expression, or whether other co-regulators are needed, it is difficult to accurately assess whether a simple *in vitro* system involving only reconstituted nucleosomal arrays built with native histone promoter sequences and purified Rap1 proteins will yield anything meaningful. If the screen for coregulators proposed above prove successful, targets from the screen should also be included in any *in vitro* tests.

In addition, along the lines of barrier-formation, Mei et al. (Mei et al., 2019) proposed that H3K4me3 may form a barrier at the histone gene promoters to prevent the spreading of Asf1/HIR/Rtt106 repression complex into the coding regions. It is conceivable that these barriers may be removed by Rap1-mediated mechanisms during senescence. If so, connections between Rap1 and H3K4me3 demethylases that impact histone gene expression may come to light.
### 3.2.4 Determine how mammalian Rap1 contributes to cellular senescence, in particular to changes in histone expression

Mammalian RAP1 is a distant ortholog of scRap1, and its importance in telomere biology and transcriptional regulation has varied somewhat in studies from different labs. However, there are several encouraging pieces of evidence that suggest hRAP1 may have interesting roles during senescence (see Appendix, Section C), including published evidence from the Blasco and Sfeir labs that it binds throughout the genome to regulate gene expression, and that with telomere shortening it localizes to new target genes, similar to what we’ve described for yeast. Given our preliminary findings on hRAP1 relocalization and in particular, its effects on histone gene expression, future work assessing the effects of hRAP1 knockdown (with shRNA) or overexpression (*via* lentiviral delivery) on the rate of fibroblast senescence may yield interesting results. In addition, genome-wide MNase-seq coupled with hRAP1 ChIP-seq in cell lines lacking or overexpressing hRAP1 could provide clues as to how hRAP1 impacts the chromatin landscape in mammalian cells.
3.3 References


3.4 Figure Legends

**Figure 3-1. Schematic of Rap1 binding to nucleosomes.** Rap1 binds to nucleosomes via contacts with nucleosomal DNA and histone H3/H4 tetramer. Grey: nucleosomal DNA; light blue: histone H2A/H2B dimers; orange: histone H3/H4 dimers; dark blue: Rap1 DNA binding domain.

**Figure 3-2. Schematic of FRET approach to probe nucleosome structural changes as a function of bound Rap1.** A. Native NRTS promoter DNA sequence with fluorescent probes attached. B. Rap1 binding to nucleosomes without distorting nucleosome structure. Readout is high FRET signal. C. Rap1 binding interfering with nucleosome structure. Readout is low FRET signal.

**Figure 3-3. Genome-wide R-SGA screen.** Constructed yeast strain with GAL1-Rap1 and ACT1-tdTomato integrated into the genome. GFP expression is driven by HTA1 promoter. Under conditions of Rap1 overexpression, gene XXX represses core histone genes, seen as lower GFP fluorescence intensity (pale green). Deletion of XXX (∆xxx) results in increased GFP fluorescence intensity (dark green). XXX is identified as a repressor of histone genes that cooperates with Rap1 to repress histone gene expression.
Figure 3-1

Figure 3-2

A. 

B. Rap1

high FRET

C. Rap1

low FRET
Figure 3-3
Appendix

Section A: H3K4me3 is important for Rap1-mediated chromatin and gene expression changes at senescence

A.1 Introduction

The histone -H3- lysine -4 trimethylation (H3K4me3) modification, often thought to be a marker of active gene expression, is intimately involved in aging across model organisms (McCauley & Dang, 2014; Sen, Shah, Nativio, & Berger, 2016). In C. elegans, deletion of any of the three Trithorax group proteins (WDR-5, SET-2 and ASH-2) that facilitates H3K4 trimethylation results in decreased global levels of H3K4me3 and increased lifespan (E. L. Greer et al., 2010). Similarly, in flies, deletion or RNAi knockdown of the histone demethylase Lid resulted in increased levels of H3K4me3 and 15-24% reduced lifespan (L. Li, Greer, Eisenman, & Secombe, 2010). Remarkably, in the context of neurodegeneration driven by artificial expression in flies of the TDP-43 protein, which in humans is a key driver of amyotrophic lateral sclerosis and frontotemporal dementia, H3K4me3 appears to instead play a salutary role, promoting the recruitment of the Chd1 chromatin remodeler (Berson et al., 2017). In addition, H3K4me3 is seen to redistribute within genomic chromatin of cultured human fibroblasts with senescence or following DNA damage (Shah et al., 2013). H3K4me3 likely contributes to the transcriptional changes observed with aging, senescence and damage, although the exact mechanisms by which it modulates lifespan are still unclear.

There is increasing evidence that Rap1-mediated chromatin and gene expression changes may involve H3K4me3. For example, synthetic genetic interactions impacting growth rate exist between the hypomorphic rap1-DAmP allele and deletions of genes encoding chromatin-modifying enzymes, such as H2Bub ubiquitylating enzymes (BRE1 and LGE1) and members of the COMPASS complex (SWD1, SWD3, BRE2, SDC1 and SPP1) (Collins et al.,
H2BK123 ubiquitylation has been shown to be involved in a number of chromatin-regulated pathways in yeast, and is necessary for the trimethylation of H3K4. In addition, unpublished work from our lab reveal that H3K4me3 and H2BK123 ubiquitylation (H2Bub) change globally and also at the NRTS promoters at senescence. Specifically, H3K4me3 ChIP experiments show increased levels of H3K4me3 at the promoters of the activated NRTS (Platt, 2014). Furthermore, we have found that inactivation of the H2BK123 E3 ubiquitin ligase components Bre1 and Lge1, as well as the Set1-containing COMPASS complex, not only selectively inhibits Rap1-dependent transcriptional regulation of NRTS, but their inactivation also delays senescence. This is interesting because although it has long been known that H3K4me3 is a “marker” of active transcription, its specific roles in transcriptional activation remains to be elucidated. Although no Rap1 domains have been identified to interact directly with H3K4me3 itself, it is possible that other “readers” of H3K4me3 may link Rap1 to the mark and thereby activate transcription.

In addition, like Rap1, H3K4me3 is also involved in telomere maintenance and the DNA damage response. A genetic screen aimed at finding potential new regulators of telomere homeostasis has identified bre1Δ, lge1Δ, set1Δ and H3K4R mutants as particularly sensitive to DNA damage when combined with deficiency in the members of the MRX complex (Faucher & Wellinger, 2010), which is involved in DNA repair. Specifically, in yeast cells, the Set1 methyltransferase and H3K4me3 is detected at newly created DSBs. The recruitment of Set1 is dependent on the chromatin remodeling complex RSC, which is known to associate with DSBs at very early time points of DSB repair (Chai, Huang, Cairns, & Laurent, 2005; B. Liang, Qiu, Ratnakumar, & Laurent, 2007). Deletions of Set1 or H3K4me3 display a defect in DSB repair by non-homologous end joining (NHEJ), accumulate damage, and are more prone to lose viability (Walter, Matter, & Fahrenkrog, 2014). Similar accumulations of H2Bub and H3K4me3 at DSBs are also seen in HeLa cells after treatment with ionizing radiation (Nakamura et al., 2011). However, inconsistently, a comprehensive study of histone modifications at DSBs in human cells have found that H2Bub decreases and changes to acetylation, leading to subsequent decreases
These findings suggest that, among other roles, the chromatin marks H2Bub and H3K4me3 are important players in the DNA damage response (see reviews Yongcan Chen & Zhu, 2016; Fahrenkrog, 2015).

Furthermore, work by Addinall et al. in cdc13-1 temperature sensitive mutants (Addinall et al., 2008) suggest that H3K4me3 may also be involved in telomere protection. Cdc13 binds telomeric DNA to recruit telomerase and to "cap" chromosome ends. Addinall et al. found that Lge1 and members of the COMPASS complex contribute to the viability of these mutants at non-permissive temperatures. Together, these results suggest that H2Bub and H3K4me3 help protect telomeres. Superficially, this appears to contradict our findings that loss of H2Bub and H3K4me3 delays senescence, and suggests that the delay may occur despite compromised telomere capping and through a different mechanism (e.g. regulation of histone gene expression).

Interestingly, a similar scenario is observed with the rap1-DAmP allele, which may have compromised telomere protection due to loss of Rap1 from telomeres, but nonetheless have an extended lifespan (Platt et al., 2013).

To address whether these effects of H3K4me3 also extends to senescent cells, I investigated its role in Rap1-mediated gene expression changes at senescence.

A.2 Results and Discussion

A.2.1 Loss of H3K4me3 delays the rate of senescence

Previously, we have shown that decreases in Rap1 levels using the rap1-DAmP allele delay senescence and extend lifespan (Platt et al., 2013). In addition, deletion of BRE1 and LGE1 also slow the pace of senescence (Platt, 2014). Given the genetic interactions observed between Rap1 and H3K4me3, the increase of H3K4me3 at the promoters of the activated NRTS, and that
Bre1 and Lge1 are upstream effectors of H3K4me3, we speculated that H3K4me3 may also affect the pace of senescence.

To test this, we generated two mutant strains: deletion of an essential subunit of the COMPASS complex, swd3Δ, and a point mutant changing lysine 4 to arginine in the single copy of an integrated H3 gene in a strain lacking the endogenous H3 sequences (H3K4R). Cells were mated with strains deleted for tlc1Δ and grown for 60 population doublings (PDs) to allow for equilibration of telomere length, then sporulated, dissected and genotyped. All comparisons between different haploid genotypes were derived from the same tetrad heterozygous for tlc1Δ deletion and other mutations of interest (swd3Δ or H3K4R).

Senescence assays were then performed by making serial dilutions into new liquid cultures every 22 hrs (see Experimental Procedures). Taking senescence as the nadir of the growth curve before survivor formation, we found that both swd3Δ and H3K4R delayed senescence by approximately 7-8 PDs (Fig. A-1A, A-1B). This difference is comparable with the delay in senescence seen in lge1Δ (~7 PDs), and somewhat smaller than that of bre1Δ (~20 PDs) (Platt, 2014). Bre1 and Lge1 form a complex with Rad6p to ubiquitinate H2BK123, which is required for the subsequent methylation of H3K4 and H3K79 (Song & Ahn, 2010; Wood et al., 2003). However, deletion of Lge1 only partially compromises H2BK123ub and downstream H3K4me3 (Hwang et al., 2003). In addition, Bre1 also interacts with the RNA binding protein Npl3 to regulate mRNA splicing (Moehle, Ryan, Krogan, Kress, & Guthrie, 2012), and plays important roles in telomere end resection (Wu et al., 2017). Therefore, the stronger effect in the rate of senescence seen in bre1Δ strains may be due to other additional effects independent of H2BK123ub.

Interestingly, apart from H3K4me3, the histone 3 lysine 4 can apparently also be acetylated (Guillemette et al., 2011). H3K4ac is enriched at promoters of actively transcribed genes, upstream of H3K4me3. Even though methylation and acetylation occur on the same lysine, their interactions are perhaps more complicated than competitive antagonism. Specifically, while deletion of SET1, a gene that encodes for a subunit of the COMPASS complex, results in a global and in particular a
promoter-concentrated increase in H3K4ac, deletion of acetyltransferases (gcn5∆ rtt109∆) did not result in a reciprocal increase in H3K4me3. These findings suggest that H3K4me3 may regulate H3K4ac, perhaps by forming a barrier at actively transcribed gene promoters to prevent spreading of H3K4ac into gene bodies. However, it is worth noting that experimentation with the H3K4ac antibody by Guillemette et al. did not seem to be H3K4ac specific in my hands, and instead had some cross reactions with H3K4A (data not shown).

Along these lines, deletion of SWD3 abolished H3K4me3, but probably served to increase H3K4ac, whereas mutation of H3K4 to H3K4R abolished both methylation and acetylation. However, both strains displayed remarkably similar delays in the rate of senescence, suggesting that this effect may be largely due to loss of H3K4me3, and may have little, if any, links with H3K4ac.

A.2.2 H3K4me3 is necessary for full NRTS upregulation

Given the delay in senescence, we wondered if H3K4me3 may have effects on NRTS expression. Because H3K4me3 has been extensively associated with active transcription, we hypothesized that this delay in senescence observed in tlc1∆ H3K4R strains may be due to compromised NRTS activation. To test this, we harvested mRNA from cells ~5 PDs preceding the nadir of the senescence, and assayed for NRTS gene expression normalized to control gene ACT1. Removal of H3K4me3 resulted in decreased NRTS activation at senescence (Fig. A-2A). This is consistent with observations that mutations of the COMPASS complex cause defects in aging-related gene expression (Cruz et al., 2018). This is unsurprising, as the H3K4me3 is known to interact with chromatin remodelers such as Chd1 to clear promoters (Y. Lee, Park, & Iyer, 2017), and can interact directly with the transcription machinery, such as the TAF3 subunit of the transcription initiation factor TFIID (van Ingen et al., 2008; Vermeulen et al., 2007; Zhao et al., 2017). However, it is worth noting that in tlc1∆ H3K4R strains, while NRTS expression is several-
fold lower than \( tlc1\) strains, they are nonetheless still activated. Interestingly, global histone gene repression was largely unaffected (Fig. A-2B). The slight decrease in histone mRNA levels seen in proliferating WT and \( H3K4R \) strains is consistent with reports by Mei et al. (Mei et al., 2019). Mei et al. proposed that H3K4me3 may form a barrier at the histone gene promoters to prevent the spreading of Asf1/HIR/Rtt106 repression complex into the coding regions. It is conceivable that during senescence, specific programs are activated to remove H3K4me3 from histone gene promoters, thus leading to the little difference observed in histone gene repression in senescent WT and H3K4R strains.

Along these lines, we wondered if removal of H3K4me3 demethylase \( JHD2 \) would affect NRTS expression. We performed NRTS mRNA analysis in a Rap1 overexpression system (described in Chapter 2). Upon normalization to \( ACT1, jhd2\) did not have any significant impacts on NRTS expression (Fig. SA-1). This is unsurprising, as deletion of \( JHD2 \) does not result in a major increase in H3K4me3 in bulk histones (Ingvarsdottir et al., 2007; Tu et al., 2007), suggesting that Jhd2 may not be the only demethylase at play, or that under steady-state conditions, H3K4me3 accrues to abundant levels that are unable to be completely demethylated by Jhd2.

Next, we asked if H3K4me3 is involved in Rap1 recruitment and promoter clearance. To test this, we performed ChIP-qPCR using senescent cells approximately ~5 PDs from the nadir of senescence. Compared to WT, \( H3K4R \) strains accumulated more Rap1 at the promoters of the activated NRTS (Fig. A-2C). However, despite the presence of increased Rap1, histone loss was not as pronounced compared to WT (Fig. A-2D). This is consistent with the blunted NRTS expression profile, and perhaps subtle amounts of histone level variations could be associated with much larger differences in transcriptional outcomes. Interestingly, the increased accumulation of Rap1 at H3K4R strains could form higher stoichiometric complexes, which was proposed by Feldmann et al. to contribute to Rap1 toxicity (Feldmann & Galletto, 2014), resulting
in slower growth rate observed in \textit{H3K4R} strains when Rap1 is overexpressed (see details in Appendix, Section B, Fig. B-4D).

It is unclear why H3K4R strains accumulated more Rap1 at NRTS promoters compared to WT, though it is apparent that H3K4me3 is not necessary for the localization of Rap1 to NRTS promoters. Speculatively, a feedback mechanism may regulate Rap1 localization, histone displacement and gene transcription. In the circumstances when gene expression is not sufficiently activated, this feedback loop may recruit more Rap1 to ensure gene expression levels are closer to WT. This suggests that the presence of Rap1 alone is not sufficient to evict nucleosomes and completely clear promoters, which is consistent with our previous observations (described in Chapter 2), and may require cofactors to work in concert with H3K4me3. Along these lines, in \textit{Drosophila}, ASF1 has been shown to selectively remove H3K4me3 by interaction with the silencing complex LAF (Moshkin et al., 2009). In \textit{S. cerevisiae}, Asf1 is involved in maintaining H3K4me3 at specific genomic sites along with other chaperones such as FACT and Spt6 (Jeronimo, Poitras, & Robert, 2019), and is also required for NRTS activation. Therefore, it may be of interest to explore how Rap1-Asf1-H3K4me3 crosstalk is facilitated at senescence.

We hypothesized previously that Rap1 relocalization may be part of the DNA damage response (DDR), possibly by contributing to global and local histone losses to enhance the search for repair templates by broken ends. In support of this, it is known that dissociation of Rap1 from telomeres is dependent on the ATM/ATR kinase Mec1 (Platt et al., 2013), and Rap1 is recruited to the RNR3 in response to DNA damage (Tomar, Zheng, Brunke-Reese, Wolcott, & Reese, 2008). Indeed, this was confirmed by work in the Gasser lab showing that DNA damage is accompanied by wide-spread histone loss from chromatin, resulting in increased chromatin fiber flexibility and enhanced DNA repair rates (Hauer et al., 2017). H3K4me3 also plays pivotal roles in the DDR, where it is detected at newly formed double strand breaks (DSBs) (Faucher & Wellinger, 2010). While H3K4me3 seems to have little effects on Rap1 recruitment, it possibly works in concert with Rap1 at DSBs to facilitate chromatin opening.
A.3 Experimental Procedures

Strains and plasmids used in this chapter can be found in supplemental Table SA-1. Details of senescence assays, quantitation of mRNA, and chromatin immunoprecipitation can be found in Chapter 2.
A.4 Figure Legends

Figure A-1. Loss of H3K4me3 delays the pace of senescence. A) SWD3 drives the rate of senescence. Senescence assay with WT (n=3), tlc1Δ (n=5), swd3Δ tlc1Δ (n=5). Deletion of SWD3, a member of the COMPASS complex, delays senescence by ~ 7 PDs. B) Senescence assay with WT (n=3), H3K4R (n=3), tlc1Δ (n=7), tlc1Δ H3K4R (N=7). Mutation of lysine -4 of H3 to arginine delays the pace of senescence by ~ 8 PDs.

Figure A-2. H3K4me3 drives gene expression and full nucleosome displacement. A-B) Relative expression of activated NRTS and histone genes at senescence. Gene expression was measured using qPCR and normalized to the ACT1. H3K4me3 is required for full NRTS activation, but is not involved in histone repression at senescence. C-D) Rap1 and H3 levels at activated NRTS promoters. Rap1 is recruited to NRTS promoters regardless of the presence of H3K4me3. Loss of H3K4me3 resulted in more Rap1 detection (p<0.05). Loss of H3 at NRTS promoters was comparable, with a slight trend towards more histone loss with H3K4me3.

Figure SA-1. mRNA expression analysis of activated NRTS under Rap1 overexpression driven by NOP1. A) Deletion of JHD2 does not affect NRTS activation. B) Deletion of CHD1 affect NRTS in a gene-specific manner.
Figure SA-1

A.

B.
Table SA-1

Supplemental Table A-1. List of strains.

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Section B: Rap1 Toxicity

B.1 Introduction

It has long been known that overexpression of Rap1 is toxic to the growth of *S. cerevisiae*, though the exact mechanisms of how this toxicity manifests is unknown. The Rap1 DNA binding domain (DBD) and a short stretch of amino acids directly adjacent to it (amino acids 598-616, aka “Tox” domain) have been identified as being required for toxicity upon Rap1 overexpression. Deletion of the Tox region results in a complete nontoxic phenotype without compromising essential Rap1 functions (Freeman, Gwadz, & Shore, 1995). A screen for suppressors of Rap1 toxicity revealed that increased dosage of the gene *SKO1* could alleviate the growth inhibition observed in cells overexpressing high levels of Rap1 (Freeman et al., 1995). *SKO1* encodes a basic leucine zipper transcription factor of the ATF/CREB family (Nehlin, Carlberg, & Ronne, 1992) and has been identified to interact with histone proteins H3 and H4 in a genome-wide mass affinity capture study (Krogan et al., 2006).

Two consecutive studies by Feldmann and Galletto et al. speculate that the Rap1 Tox domain may contribute to growth inhibition by modulating different Rap1 binding modes (Feldmann & Galletto, 2014; Feldmann, De Bona, & Galletto, 2015). The Tox domain is immediately adjacent to the DBD, which consists of two tandem Myb domains and a C-terminal wrapping loop. Crystal structures of the DBD with DNA show that the C-terminal wrapping loop interacts with the N-terminal Myb domain to form a closed "ring" around DNA (Matot et al., 2012). Feldmann et al. reported that both the DBD alone and full-length Rap1 can bind to DNA with multiple stoichiometries. Specifically, at high affinity DNA binding sites such as telomeric sequences, Rap1 binds to DNA with 1:1 stoichiometry *in vivo*. This is probably due to both Myb domains of the DBD binding to hemi-sites within the Rap1 DNA recognition sequence. However, interestingly, higher stoichiometric DNA: Rap1 complexes (1:2 and 1:3) are observed on lower affinity DNA recognition sites. This may be the result of only one Myb domain binding DNA. This
switch in binding modes is mediated by the C-terminal wrapping loop and the Tox region. Deletion of the C-terminal wrapping loop results in reduction in the stability of 1:1 complexes, and complexes of higher stoichiometries are favored. In contrast, deletion of the Tox region stabilizes the 1:1 complex, thus disfavoring the transition to higher stoichiometry (Feldmann et al., 2015). Though it has not been shown, it is interesting to speculate that Rap1 toxicity under overexpression conditions could be partly due to formation of higher stoichiometric complexes.

Previous studies from our lab and others indicate that the yeast Rap1 is involved in regulation of histone levels and aspects of the DNA damage response (DDR) (see Chapter 1 for details). In short, Rap1 is recruited to ribonucleotide reductase genes upon activation of the DDR, possibly to facilitate DNA repair and telomere expansion (Tomar et al., 2008). Relocalization of Rap1 to hundreds of new target genes at senescence depend on the yeast ATM/ATR homologue Mec1 (Platt et al., 2013). Wide-spread histone loss is observed upon DNA damage and is required for optimal repair (Hauer et al., 2017) and we have speculated that this may be mediated in part by Rap1, as Rap1 can contribute to both universal and site-specific histone loss at senescence or upon overexpression. In turn, tight connections have been drawn between histone levels, DNA replication and genome stability: 1) It is well established that excess histones is deleterious to cell growth by contributing to genomic instability, enhanced sensitivity to DNA damaging agents, and cytotoxicity (Maya Miles et al., 2018; Singh et al., 2010); 2) modest loss of histones that keep chromatin intact but reduces free histone levels leads to increased efficiency of homologous recombination and higher resistance to DNA damage (D. Liang, Burkhart, Singh, Kabbaj, & Gunjan, 2012); 3) more drastic loss of histones such as partial depletion of histone H4 results in delays during the S and G2/M phases, increased recombination rates, elevated replication fork collapse and greater genomic instability (Prado & Aguilera, 2005), and 4) complete loss of histones H2B, H3 or H4 results in massive gene de-regulation and nucleosome landscape changes (Gossett & Lieb, 2012; Han & Grunstein, 1988; Han, Kim, Kayne, & Grunstein, 1988; U. J. Kim, Han, Kayne, & Grunstein, 1988; Wyrick et al., 1999). Therefore, it is possible that Rap1 toxicity may manifest through perturbations of histone dosage, for example,
either by downregulating histone gene expression, or by contributing to local histone losses from chromatin.

In this chapter, I investigate the mechanisms of Rap1 toxicity as related to Rap1 binding modes and histone levels.

B.2 Results and Discussion

B.2.1 Histone gene deletions or overexpressions do not affect Rap1 toxicity

In order to address whether changes in histone dosage would affect Rap1 toxicity, we overexpressed Rap1 under conditions where histone genes are either reduced or elevated. For reduced levels of histones, we used strains that had either one gene pair encoding histone H2A/H2B (_hta2Δ·htbΔ_) or H3/H4 (_hht2Δ·hhf2Δ_) deleted. Deletions of either gene pairs in haploid cells had no deleterious effects on cell growth rates or temperature sensitivity.

Rap1 and Rap1<sup>SHY</sup> overexpression were driven by the _GAL1_ promoter in a 2-micron plasmid. This level of both Rap1 and Rap1<sup>SHY</sup> are toxic, though Rap1<sup>SHY</sup> to a lesser extent (Fig. B-1A, left panel, Fig. B-2B). Overexpression of Rap1 or Rap1<sup>SHY</sup> in histone deletion backgrounds did not increase Rap1 toxicity compared to their respective overexpression strains in the wildtype strains (Fig. B-1A, middle and right panels). It is worth noting that this result is metastable, and severely increased toxicity is sometimes observed (as seen by no growth even in the highest spot assay concentrations, Fig. SB-1), especially after cells have been through multiple passages (>50 PDs). This may be due to accumulation of deleterious effects of non-stoichiometrically matched histone proteins. However, repeat experiments replicating this cumulative effect need to be performed.

Next, we used a histone overexpression strain in which all four core histones are integrated into the genome driven by the _GAL1/10_ promoter. Histone overexpression causes slow
growth in cells. When plasmids containing high expression levels of Rap1 were induced (GAL1-driven) in this strain background, no rescue of Rap1 toxicity was observed by simultaneous histone OE (Fig. B-1B). Similarly, modest levels of Rap1 and Rap1SHY OE also did not rescue the slow growth rate exhibited by the histone OE strain (Fig. B-1C).

These negative results, while discouraging, may not be unexpected. As histone proteins in the correct ratios are essential for cell viability, yeast and mammalian cells have many compensatory mechanisms that regulate histone transcript and protein levels beyond gene copy number. For example, the HTA1-HTB1 gene pair is able to fully compensate for loss of HTA2-HTB2, therefore, hta2∆-htb∆ is phenotypically indistinguishable from WT cells (Norris, Dunn, & Osley, 1988). Deletion of HHT2-HHF2 apparently does not affect cell growth or histone protein levels from whole cell extracts, though more detailed partitioning of chromatin vs. free histone levels suggest that the free histone pool may be reduced (D. Liang et al., 2012). An extra copy of genes encoding histone H2A and H2B has no detectable effects on cell growth and steady-state H2A H2B transcript levels are unchanged (Osley & Hereford, 1981). Therefore, in order to investigate whether Rap1 toxicity manifests through changes in histone protein levels, we may need to use strains that harbor histone DAmp or mutant alleles that compensation mechanisms cannot overcome.

B.2.2 Rap1SHY overexpression is less toxic compared to WT, and is more resistant to MMS

Previously, we have characterized the SHY patch, amino acids 392-394 within the Rap1 DNA binding domain, as important for direct Rap1-histone interactions in vitro and in vivo, and ~50% loss in the affinity of Rap1 for histone H3/H4 heterotetramers is observed when the SHY patch is mutated to AAA (Rap1SHY). Upon overexpression at modest levels (Fig. SB-2A), Rap1SHY has reduced levels of histone H3 loss at the NRTS promoters compared to WT, but has no additional effects on histone gene repression (see Chapter 2). Given the interplay between
Rap1-histone dynamics, and our hypothesis that this may relate to Rap1 toxicity, we tested to see if Rap1\textsuperscript{SHY} displayed similar levels of toxicity when overexpressed.

BY4741 cells were transformed with two-micron based plasmids from which either HA-tagged Rap1 (WT) or Rap1\textsuperscript{SHY} expression is driven by the GAL1 promoter and then plated onto glucose or galactose plates. Their expression levels and accumulation were confirmed to be similar by western blot (Fig. B-2A). Rap1\textsuperscript{SHY} showed a slight growth advantage compared to WT upon galactose induction (Fig. B-2B), as seen in the first and second rows of the spot assays. Similarly, when exponentially growing WT or mutant cells were induced with galactose in liquid culture, Rap1\textsuperscript{SHY} began to overtake WT 8 hours after induction (Fig. B-2C), and by 20 hours, have accrued one additional population doubling compared to WT. The average doubling time for Rap1\textsuperscript{SHY} is 6.2 hrs compared to the 7.2 hrs by WT. Therefore, Rap1\textsuperscript{SHY} overexpression is slightly less toxic compared to WT.

Next, we tested whether Rap1 overexpression (OE) affected cell sensitivity to DNA damaging agents. Cells overexpressing non-toxic levels of Rap1 (Rap1 OE confirmed by western blot, Fig. B-3A) were streaked out on increasing concentrations of methyl methanesulfonate (MMS). After two days of growth at 30°C, cells overexpressing Rap1 displayed an obvious growth advantage compared to vector control (Fig. B-3B). Interestingly, when the same experiment was repeated with N-terminally HA-tagged Rap1, driven by the same promoter, and with similar levels of Rap1 accumulation as confirmed by western blot, the increased resistance to MMS was lost (data not shown). This suggests that the N-terminal region may be involved in the mechanisms of Rap1-mediated MMS resistance. Overexpression of Rap1\textsuperscript{SHY} to similar levels as WT Rap1 (Fig. B-3C) did not significantly decrease or increase cells’ resistance to MMS compared to WT (Fig. B-3D). Interestingly, when the SHY to AAA mutation was introduced within the endogenous locus of RAP1 (RAP1\textsuperscript{SHY}) and cells were plated on 0.02% MMS, RAP1\textsuperscript{SHY} showed a significant growth advantage compared to WT (Fig. B-3E).
Taken together, these lines of evidence suggest that the SHY patch plays a role in regulating Rap1 toxicity and resistance to DNA damage. The exact mechanisms need to be investigated in further detail. However, given the direct Rap1-histone contacts that we have characterized involving the SHY patch, it is plausible that mutation of the SHY to AAA alleviates Rap1-mediated site-specific histone losses, which provides Rap1\(^{\text{SHY}}\) with a slight growth advantage.

Along these lines, we wondered if the Tox region may also have similar functions to the SHY patch, i.e. regulate toxicity by mediating Rap1-histone contacts. To address this, we performed in vitro histone binding assays with full-length Rap1 protein and Rap1 without the Tox region (Rap1\(^{\text{Tox} \Delta}\)). No loss of binding signal was detected with Rap1\(^{\text{Tox} \Delta}\) (Fig. S5-1C). However, this does not necessarily mean that the Tox region and the SHY patch contribute to Rap1 toxicity through separate and independent mechanisms. As was proposed by Feldmann et al. (Feldmann et al., 2015; Feldmann & Galletto, 2014), deletion of the Tox region stabilizes 1:1 Rap1-DNA complexes, particularly on high affinity DNA binding sites. Therefore, Rap1\(^{\text{Tox} \Delta}\) could result in different Rap1 binding modes in which higher stoichiometries are favored. Such conformational changes could result in less histone contacts and therefore also serve to stabilize chromatin, similar to functions of the SHY to AAA mutation, but in vivo experiments would be needed to test this.

### B.2.3 Epigenetic marks regulate Rap1 toxicity

It has recently been reported that set1\(^{\Delta}\) (H3K4 methyltransferase) and H3K4R mutants have lower levels of histone gene expression due to the spreading of the repressive complex Asf1/HIR/Rtt106 into histone gene coding regions (Mei et al., 2019). This loss of histones may be contributing to the slow growth phenotype of both set1\(^{\Delta}\) (Fig. B-4A) and H3K4R strains (Fig. B-4B, blue and gray). When Rap1 is overexpressed (NOP1-RAP1) in the set1\(^{\Delta}\) background, an
increased toxicity phenotype is observed compared to WT (Fig. B-4A). Similarly, when Rap1 is overexpressed (NOP1-RAP1) in the H3K4R strain, a dramatic increase in Rap1 toxicity is seen (Fig. B-4B, gray and yellow). Of note, ChIP experiments of Rap1 when Rap1 is overexpressed in the H3K4R background shows increased Rap1 accumulation at NRTS promoters (Figure A-2C), lending weight to the idea that higher stoichiometries of Rap1 binding may be involved in toxicity mechanisms (Fig. B-4D).

Interestingly, under these circumstances, Set1 and H3K4me3 seem to counteract the toxic phenotypes of Rap1. However, H3K4me3 was also observed to work in concert with Rap1 to drive the pace of senescence (see Appendix, Section A). This suggests that Rap1 toxicity may manifest through completely different mechanisms compared to the mechanisms by which Rap1 drives the rate of senescence. In addition, H3K4me3 has been shown to play important roles in the DNA damage response. Set1 and H3K4me3 are detected at newly created double-strand breaks (DSB) in yeast, and deletions of Set1 and H3K4me3 displays defects in DSB repair by non-homologous-end-joining and are more prone to lose viability after damage (Faucher & Wellinger, 2010). Therefore, this exacerbated toxicity phenotype could be due to additive defects in the DNA damage response triggered by both loss of H3K4me3 and overexpression of Rap1.

In contrast, deletion of histone acetyltransferase GCN5 rescues Rap1 toxicity (Fig. B-4C), while deletion of RTT109 has no effect. This is surprising, as gcn5Δ also causes reduced levels of core histones (Petty, Lafon, Tomlinson, Mendelsohn, & Pillus, 2016) and a spectrum of defects in gene activation and chromatin structure, particularly at stress-regulated genes (Gregory et al., 1998; Pérez-Martín & Johnson, 1998). However, remarkably, both set1Δ and H3K4R delays replicative senescence and promotes longevity (Appendix, Section A), while gcn5Δ was found to strongly reduce yeast replicative and chronological lifespan in several studies (S. Kim, Ohkuni, Couplan, & Jazwinski, 2004; Laschober et al., 2010; Matecic et al., 2010). These interesting correlations suggest that factors that regulate longevity, maybe more than histone levels, are involved in regulating the toxic phenotypes of Rap1 overexpression.
B.3 Experimental Procedures

Strains and plasmids

For yeast strains, see Table SB-1. All yeast strains were in BY4741 background and grown under standard conditions in either complete or selective media. For a list of plasmids, see Table SB-2.

Spot assays

Single yeast colonies were inoculated in the necessary medium and grown overnight to a concentration of $1 \times 10^7$ cells/ml as determined by the Coulter counter. Serial dilutions of $1 \times 10^6$ cells/ml, $1 \times 10^5$ cells/ml, $1 \times 10^4$ cells/ml, $1 \times 10^3$ cells/ml were made, and 10 µl of each dilution was spotted on the respective plates. Cells were grown at 30°C and imaged every 24 hours.
B.4 Figure Legends

**Figure B-1.** Histone gene deletions or overexpression do not affect Rap1 toxicity. **A.** Spot assays of Rap1 and Rap1<sup>SHY</sup> overexpression driven by the GAL1 promoter. Deletions of a gene pairs encoding H2A/H2B (middle panel) or H3/H4 (right panel) do not affect Rap1 toxicity compared to WT (left panel). **B.** Spot assays of Rap1 overexpression driven by the GAL1 promoter in wildtype (top panel) and histone overexpression strain backgrounds (bottom panel). Histone overexpression does not affect Rap1 toxicity. **C.** Spot assays of NOP1 driven vector control, Rap1 and Rap1<sup>SHY</sup> overexpression in histone overexpression strains. Rap1 or Rap1<sup>SHY</sup> overexpression does not rescue the slow growth phenotype of histone overexpression strains.

**Figure B-2.** Rap1<sup>SHY</sup> is less toxic compared to WT. **A.** Accumulation of Rap1 and Rap1<sup>SHY</sup> under galactose induction. Rap1 and Rap1<sup>SHY</sup> expressions are similar. **B.** Spot assays of Rap1 and Rap1<sup>SHY</sup> overexpression driven by the GAL1 promoter. Rap1<sup>SHY</sup> is less toxic compared to WT. **C.** Population doublings for a given time after galactose induction of Rap1 or Rap1<sup>SHY</sup> overexpression in liquid cultures. Rap1<sup>SHY</sup> shows a significant growth advantage from 8 hrs onwards (*p<0.05). Rap1 average PD: 7.2 hrs; Rap1<sup>SHY</sup> average PD: 6.2 hrs.

**Figure B-3.** Rap1 overexpression provides increased resistance to DNA damaging agent MMS. **A.** Western blot of Rap1 overexpression (Rap1 OE) driven by the NOP1 promoter, compared to vector control (V.C.). **B.** Restreak for single colonies on plates with increasing amounts of MMS. Cells overexpressing Rap1 (Rap1 OE) show a significant growth advantage compared to vector control (V.C.) when MMS concentration is above 0.01%. **C.** Western blot of Rap1 and Rap1<sup>SHY</sup> overexpression driven by the NOP1 promoter. No differences in accumulation is detected. Tubulin is used as an internal control. **D.** Spot assay of cells overexpressing vector control, Rap1, and Rap1<sup>SHY</sup> on 0 and 0.02% MMS. Rap1 and Rap1<sup>SHY</sup> both confer increased resistance to DNA damage.
resistance to MMS, and no distinguishable differences between them are observed. E. Spot assay of WT and $RAP1^{SHY}$ strains on 0 and 0.02% MMS. Mutation of the endogenous $RAP1$ locus to $RAP1^{SHY}$ results in increased resistance to MMS.

**Figure B-4. Epigenetic marks affect Rap1 toxicity.** A. Spot assays of WT or set1Δ cells overexpressing vector control or Rap1. Deletion of SET1 aggravates Rap1 toxicity. B. Growth curves of WT or H3K4R strains overexpressing vector control or Rap1. Overexpression of Rap1 in the H3K4R background results in dramatically decreased growth rates. C. Spot assays of WT, rtt109Δ, and gcn5Δ strains overexpressing vector control or Rap1. Rtt109 has no effects on Rap1 toxicity, while Gcn5 rescues Rap1 toxicity. D. Schematic of possible mechanism of Rap1 toxicity in H3K4R background. H3K4me3 favors non-toxic 1:1 Rap1-DNA complexes, while H3K4R favors higher stoichiometric complexes that may result in toxicity.

**Figure SB-1. Histone deletions exacerbate Rap1 toxicity.** Spot assays of Rap1 and Rap1$^{SHY}$ overexpression driven by the GAL1 promoter in WT (left panel) and histone deletion strains (middle and right panels). Histone deletions exacerbate Rap1 toxicity after numerous passages (>50 PDs). Glucose plates are used as plating controls.

**Figure SB-2. The SHY patch within the DNA binding domain is also involved in regulating Rap1 toxicity.** A. 2 levels of Rap1 overexpression (OE). 2-fold Rap1 OE driven by the NOP1 promoter (top panel) is slightly toxic, but high levels of Rap1 OE driven by the GAL1 promoter (bottom panel) is extremely toxic. B. Deletion of the Tox domain does not result in compromised Rap1-histone interactions in vitro. 0.5 μM or 1 μM of purified GST-Rap1 or GST-Rap1$^{ToxΔ}$ attached to GST-resin were incubated with equal-molar histone H3/H4. Proteins bound to the
resin were analyzed by western blot. Top panel is H3 signals as detected by western blot. Bottom panel is loading control stained by Coomassie blue. C. Quantitation of B (N=2).
Figure B-1

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C. Histone OE

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Figure B-2

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B. Rap1 OE

Rap1<sup>ShY</sup> OE

C.

![Graph showing population doublings over time for Rap1 and Rap1<sup>ShY</sup>](image-url)
Figure B-3
Figure SB-1

Figure SB-2

A.

NOP1 driven RAP1 OE

NOP1-ccdb

NOP1-RAP1

GAL1 driven RAP1 OE

GAL1-ccdb

GAL1-RAP1

B.

Rap1

Rap1\textsuperscript{Tone}

-H3

-Rap1

C.

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1.2

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2
3
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Table SB-1

Supplemental Table SB-1. Yeast strains listed by order of appearance.

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## Table SB-2

Supplementary Table SB-2 Plasmids used listed by order of appearance.

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Section C: Human Rap1 and Its Roles in Senescence

C.1 Introduction

Rap1 is unique among the shelterin proteins because it is the most conserved telomere binding protein from yeast to humans (B. Li, Oestreich, & de Lange, 2000). Initially identified in a two-hybrid screen with human shelterin protein TRF2 as bait (B. Li et al., 2000), mammalian RAP1 was found to be an ortholog of yeast Rap1 (scRap1). Despite low sequence similarities (too low for a simple BLAST search), yeast and mammalian Rap1 share similar domain structures with an N-terminal BRCT domain, a Myb domain, and a C-terminal RCT domain (Rap1-specific protein interaction domain). However, unlike scRap1 that binds to DNA with high affinity via its two tandem Myb domains, the Myb domain of mammalian RAP1 has little to no intrinsic DNA binding abilities, consistent with a lack of positive charges on its binding surface (Hanaoka et al., 2001). In addition, mammalian RAP1 can be deleted without major perturbations to organismal viability and fertility (Sfeir, Kabir, van Overbeek, Celli, & de Lange, 2010) while deletion of the scRap1 is lethal.

Nevertheless, despite differences in essentiality and DNA binding, striking functional similarities are found between yeast and mammalian Rap1. To list a few: 1) both yeast and mammalian Rap1 protect telomeres from being recognized as double-strand breaks, though subtle differences still exist. For example, while scRap1 works through multiple mechanisms to repress non-homologous-end-joining (NHEJ) at telomeres (Marcand, Pardo, Gratias, Cahun, & Callebaut, 2008), its importance in repressing telomere fusion events in mammalian cells have varied somewhat in studies from different labs (Martínez et al., 2010; Rai, Chen, Lei, & Chang, 2016; Sfeir et al., 2010). However, it is worth noting that in vitro studies using telomeric oligos or mammalian RAP1 artificially tethered to telomeres were able to inhibit telomeric fusions in a dominant negative TRF2 allele (Bae & Baumann, 2007; Bombarde et al., 2010; Sarthy, Bae,
Scraftord, & Baumann, 2009), suggesting that mammalian RAP1 have the conserved the ability to block NHEJ, but whether it does so in vivo is unclear. Instead, mammalian RAP1 is crucial for the inhibition of homology-directed repair (HDR) (Sfeir et al., 2010); 2) similar to scRap1, which binds 5-10% of all S. cerevisiae genes, mammalian RAP1 also binds and regulates the expression of genes throughout the genome (Kabir, Hockemeyer, & de Lange, 2014; Martínez et al., 2010; 2013; Yang et al., 2011; Yeung et al., 2013); 3) extratelomeric roles have been demonstrated for both scRap1 and mammalian RAP1, e.g. in mouse embryonic fibroblasts (MEFs) depleted of mammalian RAP1, genes with (TTAGGG)$_2$ RAP1 binding consensus motifs, and in particular subtelomeric genes, become deregulated (Martínez et al., 2010); in addition, mammalian RAP1 has been shown to regulate NF-$\kappa$B signaling, a pathway involved in driving and regulating cellular senescence, by interacting with I$\kappa$B kinases (IKKs) (Teo et al., 2010); 4) in mice deficient for both RAP1 and telomerase, telomere shortening is more pronounced and an earlier onset of telomere-induced DNA damage and degenerative pathologies are observed (Martínez, Gómez-López, Pisano, Flores, & Blasco, 2016); 5) deletion of telomerase from immortalized MEFs results in RAP1 relocalization to subtelomeric and extratelomeric regions of the genome (Martínez et al., 2016), much like the relocalization of scRap1 at senescence in telomerase deficient yeast (Platt et al., 2013). Together, these lines of evidence support the idea that, similar to the yeast model, human telomeres serve as a “RAP1 depot” that stores RAP1 under normal conditions, and releases it to regulate gene transcription in cases of telomere shortening. Therefore, we wished to address whether mammalian RAP1, similar to scRap1, plays any roles during cellular senescence.

C.2 Results and Discussion

Caveat: due to the fact that we experimented with human fibroblasts in the early senescent stage (PD 72), and that sequencing reads were not of high quality, we only performed crude analysis of
ChIP-seq datasets. These experiments are very preliminary and need to be repeated with more replicates as well as with cells deeper in senescence. Nevertheless, they do offer hints to several interesting trends, presented below, which may be of interest.

C.2.1 hRAP1 relocates to new target sites at senescence

Previously, RAP1 and TRF2 ChIP have been performed in human and mouse cells and extratelomeric RAP1 binding sites have been identified. Given that 1) telomeres in human cells shorten with replication similar to telomerase knockout yeast and 2) RAP1 was found to relocalize in telomerase-deleted MEFs, we wished to address whether human RAP1 (hRAP1) relocates to new target sites at senescence. We performed ChIP-seq using IMR-90 fibroblasts in proliferating (PD 28) and early senescent (PD 72) cells, and a commercially available hRAP1 antibody (Bethyl, A300-306A). Roughly ~50 M reads were obtained for each sample (N=2) after sequencing. Data alignment was performed using bowtie, and ~17% of reads were lost from each lane during the alignment when reads that map to more than one region in the genome were eliminated (possibly telomeric repeats). Of note, very few reads were lost when PCR duplicate filters are applied, indicating that the quality of the IP was good. Differences between proliferating and senescent cells were observed at approximately 20 loci.

In addition, by crude visual inspection of alignment tracks, some senescence-related changes are observed. Of particular interest, increased hRAP1 binding at senescence is seen at the promoters of gene ISG15 (Figure C-1A), the expression of which goes up with shortened telomeres (Lou et al., 2009), suggesting that hRAP1 might play a role in their activation. In addition, reduced hRAP1 binding at senescence is seen at PPARGC1A (encoding PGC1α) (Fig. C-1B). This may have interesting implications due to 1) expression of PGC1α is decreased in livers of mRap1 knockout mice (Yeung et al., 2013), 2) expression of PGC1α goes down in senescent IMR-90 fibroblasts (Shah et al., 2013), and 3) PGC1α appears to promote healthy
biogenesis of mitochondria in human endothelial cells (Davinelli, Sapere, Visentini, Zella, & Scapagnini, 2013). This raises the possibility that hRAP1 may potentially activate PGC1α expression, and loss of RAP1 binding at senescence results in its downregulation.

In addition, when comparing the top 1% senescence gain regions (regions of the chromosome that have increased hRAP1 mapping at senescence), increased hRAP1 binding at histone promoters, particularly at H2A H2B promoters were observed (Table C-1).

**C.2.2 hRAP1 overexpression results in senescence-related gene expression changes**

In addition to ChIP-seq, we wished to investigate whether hRAP1 is involved in the regulation of senescence-related genes. To address this, we overexpressed hRAP1 via lentiviral delivery in mid-passage human fibroblasts (IMR-90, PD 38), and performed RNA-seq (N=2). hRAP1 overexpression was confirmed via western blotting (~4 fold) (Fig.3-2A) and RNA-seq (~12 fold) (Fig. 3-2B). We obtained about 100 M reads per sample and 80% of the reads uniquely mapped within regions of the genome. Differential gene analysis was processed using DeSeq2.

Out of 4,896 genes that met a minimum read count threshold of at least 10 reads per million in at least one sample, 23 significantly differentially expressed genes were found using a local fit (p<0.0002). Among these, the matrix metalloproteinase MMP1 and the SASP cytokine CCL2 are upregulated. Other senescence-related genes impacted by hRAP1 overexpression include COL27A1, SFRP1, SIX1, ROMO1, COL27A1 and FOXE1 (Fig. C-2C and Table C-2). Perhaps most stringingly, for the top 22 genes that are upregulated at senescence, six encode core histone proteins H2A and H2B, and one encodes H1 (Fig. C-2C, Table C-2). All of these histone genes are standard polyA- replication dependent histone genes except for HIST2H2BE, which is a replication-dependent histone gene but gets a polyA detail in differentiated and senescent cells. It should be pointed out that it is surprising that these polyA- histone genes are
detectable after polyA selection. However, some recent studies have shown that replication-dependent histone genes can be poly-adenylated under a variety of cellular conditions (Kari et al., 2013; Lyons et al., 2016), suggesting that whether a gene has polyA+ tail or not is likely to be more dynamic and complicated than was imagined. Interestingly, HIST2H2BE is also the only overlapping histone gene that has detected increased hRAP1 binding at senescence by ChIP-seq (Table C-1) and upregulated expression in RNA-seq (Fig. C-2C, Table C-2).

Due to some variability within the two hRAP1 overexpression samples, we selected the one with deeper sequencing and more reads for more extensive analysis. Table SC-1 shows genes whose expressions change more than 2-fold upon hRAP1 overexpression. Again, senescence-related gene expression changes are observed. For example, DIRAS3, a small GTPase whose silencing has been linked to cellular senescence (Ejaz, Mattesich, & Zwerschke, 2017), is significantly downregulated when hRAP1 is overexpressed (Table SC-1). UPK1B, a cell-surface protein involved in the NF-κB pathway and which is significantly downregulated in a progeroid aged mice model (Tilstra et al., 2012), was also found to be downregulated upon hRAP1 overexpression (Table S5-1). In addition, out of the 62 genes that have more than 2-fold changes in expression levels (0.3% of the human genome), 25 are histone genes (16% of all histone genes), suggesting that hRAP1 preferentially targets histones ($p = 1 \times 10^{-282}$).

Previously, no connections have been drawn between hRAP1 and the regulation of histone expression. ChIP-seq experiments have shown that hRAP1 binding increases at the promoters of histone genes, particularly H2A and H2B (Table C-2), consistent with the RNA expression changes. However, in contrast to scRap1, hRAP1 upregulates histone gene expression. This is surprising, as histone biosynthesis is reported to decrease with age in cultured human fibroblasts (O'Sullivan, Kubicek, Schreiber, & Karlseder, 2010). This may suggest at other histone regulation mechanisms at play in human cell senescence, or if hRAP1 is involved, different hRAP1 cofactors may be activated by senescence programs that function with hRAP1 to repress histone expression, and is not present upon hRAP1 overexpression. We have hitherto
presented evidence that scRap1 negatively regulates histone expression at senescence and upon overexpression, though the mechanisms of how this is achieved, as well as corepressors involved, are still unknown. However, these findings suggest at connections of Rap1 functions in yeast and human settings, but the exact details remain to be studied.

C.2.3 hRAP1 binds to histones in vitro

scRap1 is functionally similar to eukaryotic pioneer transcription factors, and we have mapped direct scRap1-histone H3/H4 interactions to within the DNA binding domain (see Chapter 2). We have postulated that high DNA binding affinity along with histone contacts allow scRap1 to invade closed chromatin and perturb nucleosome structures. hRAP1, though an orthologue of scRap1, is recruited to telomeres via TRF2 and does not appear to have sequence-specific DNA binding abilities. However, there have been some evidence suggesting that hRAP1 may be able to bind DNA structures at single-strand double-strand junctions irrespective of DNA sequence (Arat & Griffith, 2012) and also alter TRF2 specificity and binding to telomeric DNA (Gaullier et al., 2016; Janoušková et al., 2015; Nečasová, Janoušková, Klumpler, & Hofr, 2017). In addition, overexpression of TRF2, which recruits hRAP1, can alter nucleosome spacing at telomeres, suggesting that the hRAP1:TRF2 complex can modify chromatin (Galati et al., 2012). Along these lines, TRF2 was found to interact with histones through an N-terminal Gly/Arg-rich domain (Konishi, Izumi, & Shimizu, 2016). However, genome-wide split-YFP complementation assays in human cells have suggested that hRAP1 can also interact with histones (O.-H. Lee et al., 2011) (Fig. C-3A). Therefore, given our findings in scRap1, we wished to test if hRAP1 may have conserved some histone-binding abilities. If so, hRAP1 may contribute to perturbations of local chromatin structure and alter TRF2 binding when recruited to specific DNA sequences via TRF2.

To test this, we fused an N-terminal GST tag to hRAP1 and using purified GST-hRAP1 and H3/H4 tetramers, performed the histone pull-down assay. 0.5 μM GST-hRAP1 attached to
glutathione resin were incubated with 0.5 μM H3/H4 tetramers in binding buffer containing 300 or 400 mM NaCl. After stringent washes, proteins bound to the resin was boiled and visualized by western blot. Remarkably, GST-hRAP1 bound to H3/H4 with sub-micromolar affinity (Fig. C-3B). We next tried to determine if, similar to hRAP1, the Myb domain of hRAP1 was involved in histone contacts. Though the three-helix bundle structure of the Myb domain is conserved compared to scRap1, a simple protein blast of the scMyb domain and hMyb domain yielded no significant similarities. In scRap1, direct H3/H4 interactions involve the SHY patch, amino acids 392-394, located just behind helix 2 in the three-helix bundle. Comparisons of amino acids similarly located showed only slight conservation of one basic residue (Fig. C-3C). Not unexpectedly, histone pull-down assays using equal-molar GST-hMyb and H3/H4 yielded no significant interactions (Fig. 3-3D).

In scRap1, most of the histone-interacting abilities are contained within the DNA binding domain (DBD) alone, while the C-terminal regions may have some weak contacts (~10% of histone binding affinity). Amino acids SHY in the first Myb domain account for ~50% in the affinity of Rap1 for histone H3/H4 heterotetramers, implying that major interaction surfaces probably also exist in the second Myb domain (see Chapter 2). However, other pTFs known to interact with histones, for example FoxA, do so independent of its Myb domain, and instead utilize its protein-interacting C-terminal domain (Cirillo et al., 2002). The C-terminal region of hRAP1, which contains the RCT domain, is highly acidic (pI 3.8) and is known to interact with other proteins (B. Li et al., 2000), including TRF2. Therefore, it is possible that in hRAP1, the C-terminal region has taken over the conserved features of histone binding, though this still remains to be tested.

C.3 Experimental Procedures

Cell lines and strains
See Table SC-3.

**ChIP-seq**

Proliferating (PD 28) and senescent (PD 72) cells on 10 cm plates were cross-linked with 1% formaldehyde for 5 min and quenched with 125 mM glycine. Cells were scraped off the plates and flash frozen for later use. For ChIP, Protein A magnetic beads (Dynabeads) were prepared according to instructions, and were either incubated with 4 µg of hRAP1 antibody (Bethyl, A300-306A) or rabbit IgG (Pierce). Cross-linked cells were resuspended and incubated in cold lysis buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton-X-100) for 10 min and then precipitated. The resulting pellet was subjected to a 10 min wash in 10 ml of wash buffer (10 mM Tris-HCl, pH 8.1, 200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0), centrifuged, and resuspended in 1 ml of shearing buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris, pH 8.1). Sonication was performed with Covaris S220 (average intensity value: 35W; peak incident power: 175W; duty cycle: 20%; cycles per burst: 200) for 10 min. Protein concentration was measured using standard Bradford assay and 500 µg of protein was used for each immunoprecipitation experiment. Elutions and reverse-crosslinking was performed using standard ChIP techniques. Libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina (E7370S).

**hRAP1 overexpression via lentiviral delivery and RNA-seq**

The hRAP1 sequence was amplified from plasmid pLPC-hRAP1-fl (Addgene 12542) with primers containing BamHI and XbaI restriction sites, and inserted into pLU-EF1-MCS-iPuro to create pLU-EF1-hRAP1-iPuro. For primers, see Table S3-2. Transfections of pLU-EF1-hRAP1-iPuro into mid-passage (PD 38) IMR90s were performed using Lipofectamine 2000 (Invitrogen), according
to manufacturer’s guidelines. 1.5 µg/ml puromycin were used for selection of successfully transfected IMR90s. hRAP1 overexpression was verified via western blot. 1X10^7 cells for RAP1 overexpression or empty vector control were then harvested and then extracted for RNA using RNeasy Mini Kit (Qiagen, cat no. 74104). The mRNA direct kit was used for polyA selection and rRNA depletion (ambion 61011). RNA-seq library was prepared using the ScriptSeq v2 RNA-Seq LPK kit (Illumina SSV21106).

**Histone pull-down assays**

See chapter 2 for details
C.4 Figure Legends

Figure C-1. Examples of senescence related hRAP1 occupancy changes detected by ChIP-seq. A. At senescence, increased hRAP1 binding peaks are detected at the promoters of gene ISG15, which are known to be upregulated with shortened telomeres, suggesting a role for hRAP1 in their activation. B. At senescence, apparent decreases of hRAP1 binding is seen in the PPARGC1A gene, which encodes PGC1α. Expression of this gene has been shown to decrease in livers of mRap1 knockout mice and senescent IMR-90 fibroblasts, suggesting a role for hRAP1 in activating its expression. Scales are relative significance of hRAP1 signal compared to IgG control using MACS (-log10(p-value)).

Figure C-2. RNA-seq of hRAP1 overexpression in IMR90s. A. Confirmation of hRAP1 overexpression via immunoblotting. Negative control (N.C.) are IMR-90 cells transfected with control plasmid bearing the puromycin resistance gene (pLU-EF1-iPuro). hRAP1 are IMR-90s transfected with pLU-EF1-hRAP1-iPuro. B. Confirmation of hRAP1 overexpression via RNA-seq. TERF2IP (gene encoding hRAP1) is elevated more than 10 fold compared to negative control. C. Top 20 genes affected by hRAP1 overexpression. Of note are histone genes and genes involved in senescence and aging.

Figure C-3. hRAP1 interacts directly with histones H3/H4 tetramers in vitro. A. hRAP1 is predicted to interact with histone proteins H1, H2A and H4 in a genome-wide split YFP complementation assay (adapted from Lee et al., 2011). B. Western blot of histone pull-down assay. 0.5 μM of GST-hRAP1 was incubated with 0.5 μM H3/H4 tetramers. Bound fractions were analyzed via immunoblotting against histone H3. hRAP1 binds H3/H4 tetramers with strong affinity. GST tag is used as negative control. PD: pulldown; FT: flowthrough. C. Comparison of scMyb and hMyb (adapted from Hanaoka et al., 2001). Conserved basic residues are in blue,
conserved acidic residues in red. Amino acids SHY involved in histone interactions are underlined. D. Coomassie stain of histone pull-down assay. 2 \mu M hMyb was incubated with 2 \mu M H3/H4. No interactions are observed.
Figure C-1

A.

Relative significance (log10(p-value))

B.

Relative significance (log10(p-value))

Figure C-2

A.

kDa

47

kDa

40

N.C.

hRAP1

GAPDH

B.

log2(p-value)

N.C.

hRAP1

OE
Figure C-3
**Table C-1**

ChIP-seq of proliferating and senescent cells at histone promoters. hRAP1 occupancy increases at the promoters of a set of histone genes at senescence. The scores are scalar normalized to account for differences in total tags in each barcoded sample.

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Table C-2

RNA-seq of hRAP1 overexpression in IMR90s. Top 20 genes affected by hRAP1 overexpression are shown.

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<td>ROMO1</td>
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<td>1.63E-07</td>
<td>Homo sapiens secreted frizzled-related protein 1 (SFRP1), mRNA.</td>
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<td>ABCF2</td>
<td>-0.3885287</td>
<td>2.75E-05</td>
<td>Homo sapiens ATP-binding cassette, sub-family F (GCN20), member 2 (ABCF2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.</td>
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RNA-seq of hRAP1 overexpression in IMR90s. A subset of samples are used to eliminate outliers. Shown are genes with more than 2-fold changes upon hRAP1 overexpression.

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### Table SC-2

**List of primers**

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### Table SC-3

**List of strains and cell lines**

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Section D: References


