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Enhancer function driving cellular senescence, DNA damage repair, differentiation, and
nuclear organization

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

Thomas Barton Suter

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2017

The Dissertation of Thomas Barton Suter is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2017

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ABSTRACT OF THE DISSERTATION

Enhancer function driving cellular senescence, DNA damage repair, differentiation, and nuclear organization

by

Thomas Barton Suter

Doctor of Philosophy in Biology

University of California, San Diego, 2017

Professor Michael G. Rosenfeld, Chair

This dissertation, by Thomas Barton Suter, discusses enhancer function driving cellular senescence, DNA damage repair, differentiation, and nuclear organization. Enhancers are a major regulatory feature of epigenomic regulation, and play a diverse array of functions. In the first chapter, I discuss the background of cellular senescence and the epigenomic modifications that are known to play a role in this process, including marks associated with enhancer function. In the second chapter, I provide primary evidence of the key role enhancers play in driving the senescence phenotype. In the third chapter, I discuss the role that DNA damage and repair plays in nuclear receptor activity at enhancers. In the fourth

chapter, I discuss the mechanisms by which regions in embryonic stem cells can be marked for later activation in future cell fates. In the fifth chapter, I present a study showing the role enhancers can play in nucleating nuclear organization.

INTRODUCTION

Regulation of the epigenome involves of interworking of multiple levels of factors and structures, all with huge implications for human pathology. This dissertation dissects many levels of such regulation.

In the first chapter, I provide a comprehensive review of the epigenetics underlying human cellular senescence, a condition that corresponds closely with human aging related disease. Senescence is characterized not only by proliferation arrest and a toxic influence on surrounding cells, but by the immutability of this state. Such an entrenchment suggests the key role of epigenetic changes in senescence, and a preponderance of literature backs this claim. Many key epigenetic factors have been shown to correlate significantly with senescence and aging across multiple organisms, and modification of these elements has been shown to have major effect upon the senescent phenotype. This chapter discusses these findings in detail, and provides an overview to the complex mechanisms enforcing senescence.

In my second chapter, I present my findings regarding specific epigenetic features of senescence, namely a novel characterization of the enhancer programs of cellular senescence, include their delineation of specific programs of senescence that correspond separately to proliferative repression and inflammatory phenotypes. We show that 2 sets of transcription factors, NFI family members and NFkB, correspond distinctly with either the proliferative or inflammatory phenotype, respectively. We additionally identify a set of histone marks as being dramatically altered in replicative senescence, providing novel evidence of a histone code that marks this condition.

In my third chapter, I present a completed manuscript that provides evidence of topoisomerase I's recruitment to and nickase activity at androgen receptor bound and activated enhancers. This study highlights the role that single stranded DNA breaks play in regulated transcription, and that major components of the DNA damage repair machinery plays a key

role in the process of regulated transcription. In this study, I performed screening and functional analysis of various DNA damage and repair factors that could potentially be involved in the nicking process described in this manuscript.

In my third chapter, I present a completed manuscript that shows how enhancers whose activity is largely restricted to specific cell types are actually pre-marked by the binding of embryonic stem cell (ESC) factors. Furthermore, pre-marked enhancers are distinct in ESCs from ESC active enhancers, as the former are marked by generally a single ESC factor, in stark contrast to the multiple factors typically bound to ESC active enhancers. A major question proposed by our model, however, is how such pre-marked enhancers have this pre-marking maintained as cells differentiate. We proposed that DNA CpG hydroxy-methylation, which is enriched at a pre-marked enhancers and is maintained throughout DNA replication and cell division, provides the molecular memory needed for the activation of these pre-marked enhancers. In this study, I performed all genome wide methylation and hydroxy-methylation experiments that were critical to the formulation of our model.

In my final chapter, I present a completed manuscript identify key enhancers as playing an organizing role in chromosomal architecture, and that this level of organization is key to our model of estrogen receptor regulated transcription. This study also shows that sub-nuclear structures play a key role in the enhancer based organization structures. In this study, I again performed genome wide analyses of DNA methylation and hydroxy methylation, which helped to better characterize the epigenetic state of both organizing and associated enhancers. I also performed analysis of the genome wide associations of the SC35 sub-nuclear structure, which helped to clarify our understanding of its association with organizing enhancers.

CHAPTER 1 THE EPIGENOMIC LANDSCAPE OF REPLICATIVE SENESENCE

Abstract

The chromosomal landscape serves as something of a foundation of cellular function, operating through a complex array of histone proteins, their post-translational modification, co-regulators, and three dimensional architecture to not only protect the integrity of the genome but also regulating its transcription and thus cellular activity. When this epigenomic integrity becomes compromised through any of a variety of chromosomal stressors, including telomeric, oxidative, or replicative stress; cellular senescence can occur. Cellular senescence can be generally defined as a cell's inability to divide even in a context which would otherwise result in its proliferation, and is both one hallmark of aging as well a means of resisting malignant transformation. Furthermore, the induction of senescence results in further alterations to the chromosomal landscape that bolster this cell state. In this review, we survey the current literature surrounding these chromosomal features that induce and reinforce cellular senescence, and look into the future of this field.

Introduction

Multicellular organisms experience aging as a result of progressive tissue dysfunction (Bishop et al., 2010; Cynthia J Kenyon, 2010; Sahin and Depinho, 2010; Stewart and Weinberg, 2006). Cellular senescence is one of many contributors to organismal aging, and is defined as a cell's persistent resistance to proliferation (Campisi and d'Adda di Fagagna, 2007; Sulli et al., 2012). As such, a senescent cell fails to proliferate when in circumstances that would normally result in proliferation, e.g. the cell receives a growth signal or is of a cell type that typically divides independent of any signal. Additionally, the senescent cell fate is typically irreversible, as the senescent state will typically persist even after the disappearance of the stressor that induced a cell's senescent state (Beauséjour et al., 2003). While the induction and maintenance of senescence involves the integrated function of many levels of cellular machinery

(Sulli et al., 2012), this review will focus upon and provide a comprehensive examination of chromatin and its role in senescence.

As mentioned, senescence is defined not only as a cell's lack of proliferation, but by its resistance to proliferation when in a circumstance or given a signal that would otherwise result in its proliferation (Campisi and d'Adda di Fagagna, 2007). As such, cellular senescence is characterized by surface level features, such as increased population doubling time, positive staining by beta-galactosidase due to the increased lysosomal content of senescent cells (Kurz et al., 2000), increased cell size and altered morphology (Campisi, 2012), and also increased activity of tumor suppressors p16INK4a/pRb (Kim and Sharpless, 2006) and p53/p21 and their associated pathways. Other hallmarks of senescence include persistent foci of DNA damage known as DNA-SCARS (Rodier et al., 2011), senescence associated heterochromatic foci (SAHF) which are visualized via staining for heterochromatin associated proteins as well as DAPI (Kosar et al., 2011), and a senescence associated secretory phenotype (SASP) wherein senescent cells secrete proteases as well as growth and inflammation associated signaling molecules (Coppé et al., 2010). While senescence is induced via the activation of tumor suppressor pathways, such as telomeric shortening, direct DNA damage, or oncogene induction, the senescent state persists even after the senescence-inducing signal is removed (Beauséjour et al., 2003). While the persistence of this signal is contributed to in part by a self-perpetuated cell signaling response, the senescent cell's chromatin state is essential to the maintenance of cellular senescence.

Senescence is initiated through three general mechanisms, all of which are linked through similar damage sensing pathways. In each case, chromatin damage results in the activation of cyclin dependent kinase inhibitors, including the p53/p21 and p16/Rb pathways. The activation of these pathways results in the repression of the E2F transcriptional program and a halt of cell cycle in the G1 phase (Campisi, 2012). This silencing of the E2F program

occurs through the recruitment of repressive chromatin modifying enzymes to E2F target promoters by Rb bound E2F transcription factors (Ferreira et al., 2001; Macaluso et al., 2006).

Oncogene induced senescence results from the over-activation of proliferative signals, which can include overexpression of oncogenes such as HRAS (Serrano et al., 1997) or E2F (Lazzerini Denchi et al., 2005), or depletion of tumor suppressors such as PTEN (Chen et al., 2005). These proliferative signals result in rapid progression of cell cycle, a dramatic increase in replication that results in stalled replication forks and increases in metabolism and associated oxidative stress, and ultimately activation of a persistent DNA damage response that results in senescence (Lee et al., 2011).

Direct damage to DNA has been shown to induce senescence through the activation of a maintained DNA damage response. This response can be activated through the direct generation of double stranded DNA breaks through sources including ionizing radiation or chemical agents (Robles and Adami, 1998). Furthermore, chemical lesions can be generated in DNA through elements including ionizing radiation, oxidative stress such as metabolism associated ROS, and a variety of chemical elements (Parrinello et al., 2003; Sedelnikova et al., 2010).

Replicative senescence is the result of the progressive shortening of telomeres that occurs during continued replication as a result of replication machinery's inability to replicate chromosomal ends completely. Telomeres are situated at the ends of linear chromosomes and utilize highly organized structures to prevent chromosomal ends from being recognized as a double stranded DNA break (Blasco, 2007). The recognition of chromosomal ends as double stranded DNA breaks would result in chromosomal ends being either inappropriately repaired, which would result in genomic instability due to inter-chromosomal fusions; or the activation of the p53 associated DNA damage machinery to induce an apoptotic or senescence response (Rodier et al., 2005). However, as telomeres become critically shortened through repeated cell

division and DNA replication, they become recognized by the DNA damage machinery, and activate the p53/p21 signaling pathway. p53/p21 activation in turn results in senescence, and this cessation of proliferation prevents further shortening of telomeres which would result in catastrophic genomic instability due to aforementioned chromosomal fusions (d'Adda di Fagagna et al., 2003). While critically shortened telomeres do not directly activate the p16 pathway, substantial cross-talk between these two pathways does exist (Rayess et al., 2012), resulting in p16 being frequently being activated during replicative senescence (Di Micco et al., 2011b; Herbig et al., 2004).

As such, senescence is an aging associated cell state that is induced through the presence of multiple types of stressors of genomic integrity. As genomic integrity is thus at the core of senescence, the various epigenomic factors regulating the genome are key to both the induction and maintenance of the senescent state. In this review, we will survey the literature surrounding epigenomic features corresponding with cellular senescence, including DNA methylation, histone levels, histone modifications, chromatin structure, and the enzymes regulating these features.

DNA Methylation

DNA cytosine methylation is fundamental epigenetic feature, and undergoes changes during senescence. More broadly, DNA methylation can occur at CG, CHG, and CHH context, with the bulk of mammalian methylation occurring at CGs (Lister et al., 2009). Clusters of CG sequences, known as CG islands, frequently occur at gene promoter regions, while the remainder of the genome is statistically depleted of the CG context. DNA methylation has been shown to play a wide variety of roles in chromatin regulation, functioning in promoter and elongation regulation, imprinting, X-inactivation, and the silencing of transposable elements (Jones, 2012). DNA methylation is catalyzed by the DNA methyltransferases DNMT1, which methylates hemi-methylated DNA and is thus responsible

for preserving methylation during DNA replication, DNMT3a and DNMT3b, which can methylated non-methylated DNA and are thus responsible for de novo methylation, and DNMT3L, which is thought to be catalytically inactive but function in the function of DNMT3A and B (Denis et al., 2011).

During senescence, progressive hypo-methylation has been observed (Wilson and Jones, 1983) (Gentilini et al., 2012), which corresponds with a decrease in levels of DNMT1 and DNMT3a (Casillas et al., 2003). This association of lower levels of DNA methylation and senescence is further supported by a centenarian study showed increased levels of global DNA methylation in the offspring of centenarians versus the offspring of non long-lived parent (Gentilini et al., 2012). Consistent with DNMT1 decline playing an active role in senescence, DNMT1 knockdown in human fibroblasts lead to the increased ubiquitylation and degradation of G9a and GLP H3K9 methyltransferases in a p21-dependent manner. This result corresponded with decreased H3K9me2 on IL-6 and IL-8 promoters, leading to their increased transcription (Takahashi et al., 2012). These results thus present a mechanism linking senescence associated changes in DNMT1 with the p21 DDR pathway as well as changes in the silencing associated H3K9 methylation, and also provides insight into the epigenomic regulation of the aging associated SASP. While conditional knockout of DNMT3a in mice also lead to premature tissue degeneration, it is undetermined whether this effect was at all related to senescence (Nguyen et al., 2007). Furthermore, accelerated senescence was observed in mesenchymal stem cells from humans suffering from RETT syndrome, a largely neural-associated disease which results from mutations in the DNA methylation binding protein MECP2. These mutations in MECP2, which is largely thought in normal cells to function in gene silencing, results in induction of senescence through impairments in DNA damage repair and activation of the p53 pathway (Squillaro et al., 2010). Interestingly, DNMT3b has been shown to increase during senescence (Casillas et al., 2003).

As hyper-methylation of specific promoters has been observed during aging, senescence up-regulated DNMT3b may function at specific regions. Interestingly, several promoters hypermethylated in senescence included the tumor suppressors LOX, RUNX3, and TIG1, suggesting senescence associated promoter hyper-methylation may function to protect against cancer resulting from aging associated genomic instability (Fraga and Esteller, 2007). Furthermore, it was shown that age associated hyper-methylation tended to occur in CG islands of promoters by bivalent domains, which contain the typically transcription activation associated H3K4me3 and the repression associated H3K27e3 (Rakyan et al., 2010).

Given the effects of DNA methyltransferase knockouts and Rett syndrome, DNA methylation clearly plays a role in genomic maintenance. However, the role of DNA methylation in the induction and maintenance of senescence is more complex. While the general hypomethylation observed in senescence is consistent with the decrease of the maintenance methyltransferase DNMT1, the fact that hypomethylation is not globally uniform indicates this mechanism needs to be further resolved. Given the association of DNMT1 with replication machinery through interaction with PCNA (Chuang et al., 1997), replication dynamics and topography may play a role in explaining senescence associated hypomethylation. The differential regulation of DNMT1 and DNMT3a versus DNMT3b may further explain why certain regions are particularly hyper- and hypo-methylated, a question that can be further explored with global analyses of these methyltransferases. The association of aging associated hyper-methylation with bivalent domains also provides some insight into the mechanism of age associated hyper-methylation, linking age associated methylation with H3K4me3, H3K27me3, and their respective readers, writers, and erasers. Philosophical questions regarding the role of DNA methylation in senescence also remain, such as whether the changes in DNA methylation play a causative role in senescence, and whether DNA methylation's role in senescence occurs at a large scale (e.g. general hypomethylation results

in global chromatin relaxation) or a locus specific level (e.g. repression or activation of specific genes such as those leading to the increase in ubiquitylation of H3K9 methyltransferases).

Histone Levels, variants, and marks

Central to the chromatin characteristics is the status of histone proteins. Broadly, histones function to package eukaryotic chromosomal DNA, and the typical octameric histone subunit consists of two tetramers, each consisting of H2A, H2B, H3 and H4 subunits. Each octamer packages 147 base pairs of chromosomal DNA into a 10nm filament. Separating each octamer is a linker region, which is typically bound by histone H1 family protein involved in the compaction of chromatin into a 30nm structure. The N and C terminal tails of histones H3 and H4 are generally exposed, allowing for their chemical modification (Hamiche and Shuaib, 2012). Given their close association with chromosomal DNA, the regulation of histone proteins plays a major role in regulating the accessibility of chromosomal DNA, which in turn regulates associated cellular functions such as transcription, DNA damage repair, and replication. This regulation can occur through a variety of mechanisms, such as the formation of higher order chromatin structures such as heterochromatin, the chemical modification of histone tails, the replacement of histone subunits with different variants, and the spacing of histone octamers along a particular chromosomal region (Kooistra and Helin, 2012).

Complicating the determination of epigenomic changes during senescence is the observation that total histone levels have been shown to be altered during senescence. In yeast, protein levels of H2A and H3 were shown to decrease with age, while overexpression of H3 and H4 resulted in increased lifespan (Feser et al., 2010a, p. 20). Protein levels of histones H3 and H4 were also shown to be reduced in aged human cells, with particular nucleosome reduction at telomeres (Roderick J O'Sullivan et al., 2010).

The mechanism underlying this reduction in histone levels has been partially explored. In yeast, H3 family histones are acetylated at H3K56 soon after translation, and this mark has been implicated in the association of histones with histone chaperone CAF-1 and thus proper nucleosome assembly (Li et al., 2008). Consistent with the observation that reduced histone levels promotes cellular aging, reduction of H3K56ac in yeast, as shown through mutants of H3K56ac regulators ASF1 and RTT109, results in reduced lifespan. Similarly, deletion of the histone transcriptional repressor HIR results in increased histone transcript levels and increased lifespan (Feser et al., 2010a). Histone transcript levels also showed considerable increase during yeast aging, and, together with the observation that yeast histone gene deletion resulted in increased transcription from the remaining histone loci, suggests the presence of compensatory transcription increases in response to the reduced histone protein levels observed during senescence (Feser et al., 2010a). In humans, stem loop binding protein (SLBP) is necessary for transcript processing of replication-dependent histones, and is considerably down-regulated by the activity of ATR, which is activated during the DDR and thus senescence (Kaygun and Marzluff, 2005). Besides SLBP, histone chaperones CAF1, ASF1a, and to a lesser extent Asf1b also show decrease during senescence in human cell lines. Similar to yeast, human H3K56ac is dependent upon ASF1a and ASF1b, functions to facilitate nucleosome assembly, and is depleted during senescence (Roderick J O'Sullivan et al., 2010). As such, the down-regulation of histone processing components and chaperones during senescence may play a considerable role in the consequent histone protein level decrease during senescence.

The functional consequence of senescence associated histone depletion also remains unclear. In yeast, nucleosome reduction through H4 depletion resulted in the altered regulation of 25% of genes, with 15% of genes being up-regulated (including genes in sub-telomeric regions) while 10% of genes were down-regulated (Wyrick et al., 1999). In human

fibroblasts, subtelomeric regions were found to be particularly depleted of histones (Roderick J O'Sullivan et al., 2010), but there is currently no data characterizing global changes in transcription upon histone depletion. As such, the mechanism determining whether a gene is up-regulated, down-regulated, or unchanged during global histone protein reduction remains unclear. Perhaps certain loci or regions have their nucleosome occupancy more drastically affected than others during global nucleosome depletion. Perhaps certain loci's transcriptional levels respond differently to equivalent changes in histone occupancy. These mechanisms, as well as the genetic and epigenomic features governing them, will require global transcriptional and epigenomic data to determine.

As mentioned above, the replacement of components of histone components with variant subunits has substantial effects upon processes including DNA replication, damage signaling and repair, and transcription. H1 has 11 variants, differing in their cell type expression, protein interactions, propensity for specific post translational modifications, and associations with heterochromatic regions (Izzo et al., 2008). While little is currently known about the role of H1 variants in senescence, the association of these variants with cell division (Hergeth et al., 2011), DNA damage response (Hashimoto et al., 2007), and heterochromatin formation (Weiss et al., 2010); indicate that further study of H1 variants in relation to senescence may be a fruitful endeavor.

Histone H2A has a multitude of variants, with H2A.Z and H2A.X being the most prominently conserved. While H2A.Z has been shown to play a major role in a wide range of biological processes, including transcription activation and repression, chromatin remodeling, and damage repair (Bönisch and Hake, 2012). H2A.Z is distributed in a non-uniform manner throughout the genome, with particular enrichment at gene regulatory regions. H2A.Z has been shown to be enriched in the promoter region of p21, functioning to inhibit p21's activation by p53 and counteract the p53 associated senescent state (Gévry et al., 2007).

H2A.Z was further shown to be depleted with age in the p21 promoter of senescent fibroblasts (Lee et al., 2012). It remains to be seen, however, whether H2A.Z plays a more global role in the onset or maintenance of senescence.

H2A.X has been long implicated in the DNA damage response, being phosphorylated at serine 139 by ATM/ATR upon the formation of a DNA double stranded break.

Phosphorylated H2A.X, referred to as γ H2A.X, demarcates DNA damage foci, where components of the DDR machinery assemble for break repair (Kinner et al., 2008). H2A.X has been thought to facilitate repair by functioning in recruitment of repair machinery, as well as playing a role in the chromatin remodeling at the site of the break (Bönisch and Hake, 2012). γ H2A.X is typically observed at increased levels in senescence, consistent with persistent DDR activation as a hallmark of senescence. Particularly, γ H2A.X foci have been observed at shortened telomeres, consistent with the activation of the p53/p21 pathway in replicative senescence (d'Adda di Fagagna et al., 2003). γ H2A.X foci were also shown to be increased upon UV radiation induced senescence in an ATR dependent manner (Hovest et al., 2006), further implicating H2A.X in senescence associated p53 activation.

MacroH2A is an H2A variant with relatively low homology to canonical H2A, and has been generally associated with transcriptional repression such as that seen during X chromosome inactivation (Costanzi and Pehrson, 1998). Its mechanism of transcriptional repression is multipartite, and has been shown to impair nucleosome remodeling, transcription factor binding, and histone acetylation (Bönisch and Hake, 2012). MacroH2A isoform MacroH2A.1.1 has also been shown to be a substrate of PARP1, and its recruitment and PARylation at damage sites has been shown to modulate the DNA damage response by compacting chromatin, affecting the recruitment of Ku proteins, and altering the γ H2A.X topography of the damage site (Timinszky et al., 2009). MacroH2A has also been noted as a component of SAHF (Zhang et al., 2005). While SAHFs have been associated with both

transcriptional repression of senescence down-regulated genes, such as E2F target genes; as well as the suppression of the DNA damage response, the specific role of MacroH2A in either of these processes in SAHFs has not been fully characterized.

H3 variants H3.1 and H3.2 are DNA replication dependent, being introduced into chromatin largely by the CAF-1 histone chaperone complex during S-phase (De Koning et al., 2007). Alternatively, H3 variant H3.3 is introduced into chromatin in a replication independent manner predominantly by the HIRA and ATRX/DAXX histone chaperone complexes. HIRA complex associated H3.3 deposition has been observed in the bodies of actively transcribed genes, as well as in regulatory regions of both active and repressed genes (Anderson et al., 2010; Mito et al., 2005). ATRX/DAXX associated H3.3 deposition was shown at transcriptional regulatory regions but also heterochromatic regions such as telomeres, pericentromeric regions, and the inactivated X chromosome (Rai et al., 2011). While the exact role of H3.3 is quite diverse and remains to be fully characterized, H3.3 deposition into the histone octamer has been shown to have considerable effect upon histone stability, such as the looser configuration observed in octamers composed of both H2A.z and H3.3 (Jin and Felsenfeld, 2007). Both HIRA complex activity and H3.3 levels in chromatin have been shown to be elevated in senescent cells (Jeyapalan et al., 2007; Rogakou and Sekeri-Pataryas, 1999). In senescent cells, the HIRA complex has also been shown to interact with PML bodies (Banumathy et al., 2009; Rai et al., 2011; Ye et al., 2007). PML bodies are subnuclear organelles that have been shown to co-localization with the ATRX/DAXX H3.3 chaperone complex independent of senescence (Ishov et al., 2004) but whose exact function remains unclear. This association of the HIRA complex with PML bodies has been shown to be necessary for SAHF formation (Ye et al., 2007). Furthermore, in senescent fibroblasts p53 activated Rb, E2F factors, and PML bodies were shown to co-localize, with E2F target promoters showing increased binding of E2F factors upon induction of senescence (Vernier et

al., 2011, p. 20). Given the aforementioned heterochromatinization of E2F target promoters that has been observed in senescence, and the association of H3.3 with PML bodies, senescence, heterochromatin, and gene silencing at promoters, H3.3 could conceivably play a role in senescence associated transcriptional regulation. It is also worth noting that ATRX/DAXX has been implicated as a negative regulator of MacroH2A incorporation into telomeres and sub-telomeric regions, with ATRX mutant lines showing increased MacroH2A as well as increased expression of genes in sub-telomeric regions (Ratnakumar et al., 2012). While this finding is intuitively contradictory, given the association of both MacroH2A and ATRX/DAXX with telomeres and the SAHF associated PML bodies, the co-localization may represent a complex dynamic of repression and repair present at SAHFs. Additionally, H3.3 was recently identified in mice as having an exceptionally long half-life, with a proportion of the proteins, which were radiolabelled embryonically, remaining even one year after birth (Savas et al., 2012, p. 20). This indicates, perhaps counter-intuitively, that even though H3.3 deposition is replication independent, at least some portion of H3.3 is remarkably stable. However, given the damage or tail cleavage that a long lived protein may experience, perhaps protein aging may also play a role in the maintenance of chromosomal integrity and the process of senescence.

Histone marks, co-activators, and co-repressors

Histone modifications are essential to the definition and function of many key regulatory features of chromatin, affecting both nucleosome structure directly as well as the association of many auxiliary factors. Histone acetylation occurs on histone lysine residues, and is typically associated with gene activation due to the negative charge of the acetyl group leading to the de-compaction of local chromatin through repulsion of the negatively charged DNA. However, acetylated lysines can be recognized by a variety of activating and repressive transcriptional regulators, and thus histone acetylation can be both activating and repressive

depending upon the residue, genomic location, and each cell's particular cohort of expressed transcriptional regulators (Verdone et al., 2006). Histone acetylation is removed by histone de-acetylases (HDACs), several of which have been implicated in senescence. Importantly, HDACs are also able to de-acetylate lysine residues on non-histone proteins, an activity that can have major effects upon cell behavior and should thus be considered alongside HDAC's histone targeted functions (Choudhary et al., 2009). HDAC1 functions as a subunit in several protein complexes, including the Sin3, NuRD, and CoREST complexes (Reichert et al., 2012). HDAC1's activities have linked to cell cycle and proliferation regulation, such as HDAC1's promoter binding and subsequent transcriptional repression of p21 in embryonic stem cells (ESCs) (Lagger et al., 2002). In senescence, drug inhibition of HDAC1 lead to accelerated onset of senescence in both fibroblasts and mesenchymal stem cells (MSCs), though it was not determined whether this effect upon senescence was due to HDAC1's histone or non-histone activities (Di Bernardo et al., 2009; Ogryzko et al., 1996). Conversely, the overexpression of HDAC1 resulted in accelerated senescence in human melanocytes. As part of this study, HDAC1 was shown to associate with Rb, and its overexpression lead to an increase in Rb associated, H3K9me3 and HP1 positive, heterochromatic foci (Bandyopadhyay et al., 2007).

The Sir2 HDAC in yeast was shown to play a major role in senescence, as Sir2 knockouts showed accelerated aging while Sir2's overexpression resulted in increased yeast lifespan (M Kaeberlein et al., 1999). Sir2 was shown to be involved in the establishment of heterochromatin at yeast telomeres, and also function in the silencing of yeast rDNA, which decreases rDNA's susceptibility to inappropriate recombination and genomic instability (Sandmeier et al., 2002). Sir2 was also shown to play dual roles in both transcriptional repression and DNA damage repair. Interestingly, the recruitment of Sir2 to sites of DNA damage lead to the de-repression of regions previously bound by Sir2, though overexpression of Sir2 mitigated this damage induced de-repression (Oberdoerffer et al., 2008). The role of

Sir2 homologs in senescence in higher organisms, however, remains uncertain, as a recent study (68) has called into question two earlier studies which indicated that Sir2 homolog overexpression resulted in increased longevity in worms (H A Tissenbaum and Guarente, 2001) and flies (Rogina and Helfand, 2004a). The two mammalian homologs for yeast Sir2 are SIRT1 and SIRT6, both of which have been shown to protect against age associated changes in mice. SIRT1 has been shown to have histone de-acetylase activity upon H1K26, H3K9, and H4K16, function in gene and repeat silencing, and be recruited to sites of DNA damage in a ATM and H2AX dependent manner (Oberdoerffer et al., 2008; Vaquero et al., 2004). As in yeast, recruitment of SIRT1 to damage sites results in de-repression of SIRT1 mediated gene silencing. This damage-induced pattern of deregulation corresponds with patterns of expression observed in aging tissue, and is abolished by SIRT1 overexpression (Oberdoerffer et al., 2008). Additionally, SIRT1 has been shown to de-acetylate many non-histone components involved in regulating transcription (including NFkB, E2F1), chromatin (including p300 and NcoR/SMRT), and the DNA damage response (including p53 and Ku80) (Vaquero et al., 2007). SIRT1 deficient mice showed developmental defects and hyper-acetylation of p53, which corresponds to increased p53 activity and thus increased DDR (Cheng et al., 2003). SIRT1 overexpressing mice were not shown to have an increased lifespan over wild type mice in unstressed conditions (Herranz et al., 2010), indicating SIRT1 may not play a role in normal replicative senescence. However, SIRT1 overexpressing mice show absence of various age associated pathologies, such as Alzheimer's disease (Kim et al., 2007) and cancer (R.-H. Wang et al., 2008); and a growing body of evidence has implicated SIRT1 in calorie restriction and IGF1 associated de-acceleration of senescence (Herranz and Serrano, 2010a). As such, the role of SIRT1 in these cell stress associated diseases indicates a possible link between SIRT1 and a subset of senescence inducing stressors (Herranz and Serrano, 2010a). Interestingly, SIRT1 was shown to negatively regulate HDAC1, which

illustrates the complexity of these pathways, the difficulty in delineating the role of each in senescence, and the need for comprehensive global datasets to determine the specific roles of these chromatin modifying complexes (Binda et al., 2008, p. 200).

Similarly, SIRT6 has been shown to de-acetylate H3K9 as well as other non-histone targets. SIRT6 has been shown to have roles in transcriptional repression of genes, particularly in association with NFkB (Kawahara et al., 2009); the DDR, through the activation of PARP1 (Mao et al., 2011, p. 20) and acetylation of the non-histone protein CtIP (Kaidi et al., 2010), and has been implicated in maintaining telomeric structure through the de-acetylation of telomeric H3K9 (Michishita et al., 2008). SIRT6 knockouts displayed reduced lifespan, but this phenotype may have resulted from the increased genomic instability observed in these mice, rather than the direct involvement of SIRT6 in senescence (Mostoslavsky et al., 2006). Interestingly, an increase in lifespan was observed in SIRT6 overexpressing male, but not female, mice, which was corresponded to changes in the lifespan-associated IGF1 signaling pathway (Kanfi et al., 2012a).

Histone acetyl-transferases have also been implicated in senescence. p300 and CBP, a pair of closely related HATs, are key and broad epigenomic regulators of transcription, with HAT activity on a wide array of histone substrates (L. Wang et al., 2008), non-histone acetyl-transferase activity, and can mediate a diverse array of interactions between transcriptional regulators (Chen 2011). p300 and CBP levels decreased during replicative senescence, and both drug and dominant negative inhibition of p300 results in accelerated onset of senescence in melanocytes (Bandyopadhyay et al., 2002). Repression of cell cycle regulator cyclin E was observed, though a global analysis of the effects of p300/CBP would be needed to fully understand its role in senescence.

Polycomb mediated gene repression occurs through the concerted functions of Polycomb Repressive Complex 1 (PRC1) and PRC2, and the actions of these repressive

complexes have been shown to play a major role in the process of cellular senescence. PRC2, which typically consists of the subunits EED, SUZ12, and EZH1 or 2; establishes both the H3K27me3 and H1K26me3 marks through the lysine methyltransferase activity of the PRC2 subunit EZH2 (Cao et al., 2002; Kuzmichev et al., 2004). Human PRC1, which is composed of subunits that include BMI1, RING1A, RING1B, a PCGF family member, and CBX7; recognizes the H3K27me3 mark through its CBX7 subunit, which results in ubiquitylation of H2AK119, chromatin compaction, and transcriptional repression (Gao et al., 2012). The Polycomb machinery has been shown to regulate p16INK4 expression, with PcG depletion corresponding to increased p16INK4 expression in senescence and subsequent activation of the p16/pRB pathway (Agherbi et al., 2009). PRC2 component EZH2 was shown to be downregulated in senescence, resulting in the depletion of H3K27me3 at p16INK4 promoter, reduced PRC1 recruitment, and increased expression of p16INK4 (Adrian P Bracken et al., 2007). Furthermore, JMJD3, a lysine demethylase acting upon H3K27me3, was shown to be increased during oncogene induced senescence where it recruit to and de-repress p16INK4 (Agger et al., 2009)(Agger et al., 2009, p. 2)(Agger et al., 2009).

Contributing to this model of Polycomb complexes functioning to inhibit senescence associated pathways, knockdown of Polycomb subunits CBX8 (Dietrich et al., 2007), BMI1 (Jacobs et al., 1999), and CBX7 in fibroblasts (Gil et al., 2004) all resulted in accelerated senescence, while overexpression of these factors in these studies resulted in opposite effects upon senescence. Additionally, PRC2 component EZH2 was shown to interaction with the replication associated DNA methyltransferase DNMT1 in conjunction with PCNA (Viré et al., 2006). Given the aforementioned decrease of DNMT1 and global DNA methylation during senescence, changes in concerted function of Polycomb, DNA methylation, and replication machinery may play a role in the chromatin changes observed during senescence.

In contrast to these results, CBX7 knockout MEFs exhibited delayed senescence as well as reduced activation of the p53 and p16 pathways (Forzati et al., 2012). These findings suggest that Polycomb mediated repression in senescence may play a more nuanced role than one of uniform suppression of senescence. More generally, PRC repression and PRC2 established H3K27me3 have been shown to apply to a diverse and developmentally regulated set of genes, showing unique functions dependent upon its distribution relative to these genes (Young et al., 2011). As such, Polycomb mediated repression's role in senescence very likely extends beyond its well characterized role in the p16INK4 loci. Global analysis of PRC activity during senescence across cell types may thus explain these apparent discrepancies in PRC function, and may provide a deeper understanding of the role of PRC mediated repression in the induction and maintenance of the senescent state.

Heterochromatization and SAHF

The role of heterochromatin in cellular senescence has been given considerable attention due to senescent cells' hallmark SAHF. However, the exact function of these bodies in the process of senescence has been recently scrutinized to generate ambiguity behind the nature of these structures. SAHF are visible through DAPI staining along, and also stain positive for H3K9me3, HP1, macroH2A, and HMGA (Narita et al., 2006). SAHF were initially associated with the silencing of E2F target genes. As previously mentioned, activation of p53 and p16 pathways results in the Rb's association with E2F. This complex then binds to the promoters of E2F target genes where it mediates the formation of heterochromatin, as seen by the increase of H3K9me3 and HP1y at E2F target gene promoters and the decrease in E2F target expression in senescent cells. SAHF were originally implicated in this silencing process given their co-localization with E2F factors as well as the heterochromatin marks that were shown to increase upon E2F target genes during senescence (Narita et al., 2003; Ye et al., 2007). Additionally, SAHF have been shown to co-localize with

centromeres (Kreiling et al., 2011), raising the possibility for a role of SAHF in regulation of centromere function or the expression of pericentric repeats. However, a recent study has shown that the inactivation of the p53 pathway components ATM or p53 in OIS fibroblasts resulted in the persistence of SAHF but also the restoration of proliferation. Citing the general association with heterochromatin functioning to repress DDR signaling, the authors suggested that SAHFs may instead function to suppress the DNA damage response in senescence (Di Micco et al., 2011b). This finding suggests that, even though they are frequently present during senescence, SAHF are unnecessary for the silencing of E2F target genes and thus the senescence state.

Regardless of the function of SAHF, heterochromatin may play a major role in the maintenance of the senescent state. As mentioned in the discussion of the function of SAHFs, heterochromatin associated factors are observed to be increased upon senescent silenced E2F target genes, a cadre of genes whose repression is likely to play a major role in the proliferation stoppage observed during senescence. H4K20me₃, a mark associated with constitutive heterochromatin, is established by lysine methyltransferases KMT5B and KMT5C, which have been shown to interact with Rb (Gonzalo et al., 2005). Consistent with H4K20me₃'s dependent upon the senescence activated Rb, H4K20me₃ has been shown to increase during senescence (Sarg et al., 2002) and was shown to be enriched on E2F target genes, presumably through KMT5B/C-Rb interaction (Gonzalo et al., 2005). However, the nature of heterochromatin in senescence remains unclear. Changes in H3K9me₃ levels vary between cell type and means of induction of senescence, the role of this key heterochromatin mark in senescence uncertain (Kosar et al., 2011; Larson et al., 2012; Roderick J O'Sullivan et al., 2010; Sarg et al., 2002). Further confusing the role of H3K9me₃ in senescence, SUV39H1, a writer of the H3K9me₃ mark, was shown to be necessary for oncogene induced senescence (Braig et al., 2005; Reimann et al., 2010). Conversely, the drug inhibition of

SUV39H1 in human microglial cells lead to the release of p21 silencing and the halting of proliferation (Cherrier et al., 2009). Polycomb mediated repression has been linked to the spread of facultative heterochromatin (Beck et al., 2010), and, as mentioned above, its levels have been shown to decrease during senescence.

In all, these studies present a complicated and heterogeneous role for heterochromatin in senescence. The patterns of heterochromatic marks such as SAHFs, H3K9me3, and H4K20me3 have been shown to vary depending upon cell type and stress condition, and the details of the changes, the conservation between cell type and stressor, etc. remain completely undetermined. As another example, facultative heterochromatin associated polycomb machinery has been discussed above as being generally depleted during senescence. However, the observation that H3K27me3 levels have been shown to increase on the promoters of E2F target genes indicates that changes in H3K27me3 and its associated Polycomb complex are not entirely unidirectional (Benhamed et al., 2012). As such, an understanding of the role of heterochromatin in senescence may require comprehensive genome-wide mapping of these aforementioned heterochromatin associated factors.

Epigenome of premature senescence

The role of genome structure and organization in senescence is emphasized through studies of HGPS, which is a disease characterized by accelerated cellular senescence and abnormal nuclear structure and function (Goldman et al., 2004). HGPS is caused by a point mutation in Lamin A, leading to the generation of a cryptic splice site that results in the production of a truncated form of Lamin A referred to as progerin (Eriksson et al., 2003). Mechanistically, the Lamin A domain absent from its truncated form was shown to be required for Lamin A's association with the NuRD complex, which is responsible for chromatin re-modelling as well as histone deacetylation through the activity of its HDAC subunit (Pegoraro et al., 2009). NuRD has been shown to de-acetylate, among others,

both H3K9ac and H3K27ac, with de-acetylation allowing for their tri-methylation into the heterochromatin associated H3K9me3 and H3K27me3, respectively (Pegoraro et al., 2009; Reynolds et al., 2012). Consistently, HGPS is likewise characterized by increased histone acetylation, as well as global reduction in the levels of H3K9me3 and H3K27me3, as well as the also heterochromatin associated HP1 (Scaffidi and Misteli, 2006; Shumaker et al., 2006). The role of NuRD in the HGPS pathology is further confirmed by knockdown of NuRD subunits RBBP4 and RBBP7, closely resembling the HGPS phenotype (Pegoraro et al., 2009). Increased progerin levels are observed with age in genotypically normal humans (Scaffidi and Misteli, 2006), implicating this disease as well as NuRD activity in normal human cell senescence. Furthermore, the NuRD complex associates with DNA methylation through its 2 subunits (Ramírez et al., 2012), creating a plausible link between NuRD mediated chromatin remodeling and histone de-acetylation and the aforementioned changes in DNA methylation observed during senescence.

Recent studies provided new insight into HGPS associated senescence, showing progerin expression corresponds to the dysregulation of Lamin A associated of genes (Kubben et al., 2012; McCord et al., 2012). It was further shown that these regions showed differential H3K27me3 binding, as well as loss of Hi-C determined global interaction compartments in later passage HGPS fibroblasts (McCord et al., 2012). As the Hi-C technique provides information on global chromosomal interactions, and thus genomic organization, this publication provides some of the first insight into the changes of chromosomal organization that occur during senescence. This type of direct organization information will be able to further the understanding of the senescence associated role of heterochromatin, which is itself a structural and organizational chromosomal feature. However, as HGPS is a highly deleterious disease caused by a mutation to a major nuclear structural component, it will be

key to determine whether the changes in genomic organization detected in HGPS fibroblasts are consistent with those observed during other more “normal” routes of senescence.

Several other aspects of chromatin have been implicated in the process of senescence. Repeat sequences, which are typically silenced due to their threat to genomic instability, are also shown to be de-repressed during senescence in both yeast and HGPS patients (Kim et al., 1996; Shumaker et al., 2006). The non-coding RNA ANRIL has been shown to regulate the p15/p16 INK4 locus, functioning particularly in the polycomb mediated repression of the tumor suppressor p15 (Kotake et al., 2011). While ANRIL has yet to be studied in rigorous association with senescence, it or similarly functioning non-coding RNAs may serve major roles in regulating the senescence associated state. Similarly, telomerase RNA TERC was shown to have non-telomeric trans-binding to regions of the genome (Chu et al., 2011).

Repetitive Elements

Repetitive DNA elements constitute around 45% of the human genome, and include a wide array of element size, function, and copy number, including short elements such as microsatellite repeats and transposable elements such as Alus, SINEs, and LINEs (Alzohairy et al., 2013; Sharp et al., 2006). Given that the expression of transposable elements results in genomic instability and damage signaling (Belgnaoui et al., 2006; Konkel and Batzer, 2010), the expression of these elements is a potential inducer of senescence. This potential role of repetitive retrotransposable elements has been further suggested by recent studies.

Differential methylation of certain repetitive elements have been observed during human aging, with Alu and HERV-K elements showing decreased methylation with age, while LINE-1 methylation showed no correlation with age (Jintaridth and Mutirangura, 2010). It was also shown in a centenarian study that Alu methylation was increased in the offspring of centenarian versus non long-lived parents (Gentilini et al., 2012). As methylation of Alu sequences plays a major role in Alu repression (Liu et al., 1994; Liu and Schmid, 1993), the

decreased Alu methylation observed in these studies suggests increased Alu transcription in aging and senescent cells. Consistently, a study has shown increased transcription of pericentric Alu repeats upon the replicative senescence of human adipose stem cells. This increased transcription also corresponded to the observation of γ H2AX and p53bp1 defined damage foci at these specific pericentric regions, and that these centromeres were impaired in their capacity to recruit the division associated cohesin and condensing complexes.

Interestingly, this study then showed that lentiviral depletion of Alu repeats restored the proliferative capacity in these cells (Wang et al., 2011). While the association of a subset of centromeric regions with senescence associated damage foci has been confirmed in mouse lung and liver tissue (Kreiling et al., 2011), the full impact of these findings has yet to be determined. Regardless, current literature strongly suggests that the expression of retrotransposable elements may play a role in senescence. While genome wide studies on repetitive elements are often complicated due to the difficulty of mapping deep sequencing reads to repeat sequencing, the increased accessibility of long read and paired end sequencing should greatly improve our ability to understand the expression, chromatin level regulation, and thus role in senescence of these factors.

Perspective

Senescence as a whole is characterized by complex and often seemingly contradictory features and purpose. In vivo, replicative senescence functions both as a major checkpoint against the unchecked growth of cells bearing cancerous mutations, while also being responsible for organismal aging through the reduction in proliferation of many tissues. SASP have a similarly dual function, as such inflammatory secretions have been theorized to lead to both an anti-cancer immune response as well as the degeneration of tissue associated with organismal aging.

The diverse set of findings covered in this review is an indication that the complexities of senescence extend to the role of chromatin in senescence. At a broadly conceptual level, chromatin can facilitate both the induction and maintenance of senescence, playing a key role in the damage response and tumor suppression pathways that maintain the delicate middle ground between apoptosis and cancer. The complexity of senescence further results from the wide array of features that have been implicated in its regulation, and the apparent contradictions that may result from the lack of comprehensive data on these elements. These contradictions become further compounded by the final layer of complexity of senescence, which is the disparate nature of the various cell types and cell stressors that follow often divergent mechanisms to ultimately achieve a senescent phenotype. Luckily, the recent advent of accessible deep-sequencing technology will allow for comprehensive mapping of all relevant epigenomic marks and features, in a wide array of cell types and stressors. Ideally, this critical mass of data, combined with sufficient computing tools and infrastructure to be made accessible, would allow for the nuanced understanding that is almost certain to characterize an omnipresent phenotype as diversely unified as that of senescence.

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CHAPTER 2 THE DELINEATION OF PROLIFERATIVE AND TOXIC ENHANCER PROGRAMS OF REPLICATIVE SENESCENCE

Introduction

Cellular senescence is a state brought about by various stressors, leading to cells entering into a dramatically altered state that includes persistent proliferative arrest, altered morphology, and secretion of cytokines and other abnormal signals in a condition known as the senescence associated secretory phenotype (SASP) (Muñoz-Espín and Serrano, 2014; Rai and Adams, 2012; Rodier and Campisi, 2011). Senescence can occur as a result of acute factors, including oxidative damage or oncogene introduction leading to replicative stress; as well chronic stressors, such as replicative senescence wherein telomeres become critically shortened upon progressive cycles of cell replication. These stressors result in activation of the p53-p21 and/or p16/INK4 pathways, leading to the complex senescent phenotype. Senescence is highly associated with aging and aging related pathologies, with senescent cells having been shown to increase in aged and deteriorated tissues across mammalian species (van Deursen, 2014). The proliferative repression associated with senescence has been implicated in aging diseases, as the BubR1 progeria mouse model has shown exhaustion of proliferative potential of progenitor stem cells (Baker et al., 2011). However, the cell non-autonomous SASP feature of senescence has been shown to play a key role in senescence and aging pathologies, as clearance of senescent cells has been shown to dramatically improve both life and healthspan in mice (Baker et al., 2016; Chang et al., 2016).

Central to senescence and its associated pathologies is the persistence of the state. While studies have shown the ability to rejuvenate aged mice through parabiosis of young and aged mouse (Conboy et al., 2005), or through treatment with GDF11 (Sinha et al., 2014), they have failed to show rejuvenation of senescent cells to a non-senescent phenotype. This degree of entrenchment of the senescent state is consistent with a multitude of causative epigenetic

features that have been observed in senescence (Booth and Brunet, 2016; Sen et al., 2016). Alterations in chromosomal architecture have been observed in senescence, and includes alteration in lamin associated domains (LADs) (Sadaie et al., 2013; Shah et al., 2013), as well as deterioration of chromosomal organization observed in HiC experiments (Chandra et al., 2015). While global DNA CpG hypomethylation has been observed in senescence, increases in DNA methylation are observed at CpG islands at the promoters of specific genes that include many associated with senescence bypass and cancer (Cruickshanks et al., 2013). While global loss of overall histone levels has been observed in senescence (Roderick J. O'Sullivan et al., 2010), changes to the levels of specific modifications have been shown to be more complicated. Senescence associated heterochromatic foci (SAHFs) have also been observed, which are regions enriched for multiple heterochromatic markers, including HP1 γ , H3K9me³, and macro H2A; that form during senescence, and may either play a protective or causative role in senescence (Di Micco et al., 2011a; Zhang et al., 2007). While overall levels of the repressive mark H3K27me³ have been shown to fluctuate during senescence (Adrian P. Bracken et al., 2007), genome wide analyses have shown both increases and decreases in the mark that are gene and region specific and correspond with transcriptional changes that occur during senescence (Shah et al., 2013). Gene and region specific changes in the transcriptional activating mark H3K4me³ have been observed in senescence as well (Shah et al., 2013), with the H3K4 methyltransferase MLL1 being necessary for SASP (Capell et al., 2016).

Consistent with the gene specific regulation of the epigenetic features of senescence, activity of several transcription factors has been shown to play a key role in the senescent phenotype. FOXO/DAF-16 is a transcription factor, which has been shown to bind to promoter and enhancer regions and have pioneering capability (Webb et al., 2013; Zaret and Carroll, 2011), has been shown to promote longevity in worms, flies, and mice (Cynthia J. Kenyon, 2010) and has been implicated in suppressing senescence in human cells in a manner

dependent on the histone deacetylase *SIRT1* (Ido et al., 2015). NFkB is a key regulator of inflammation and has been shown to play a major role in acquisition of SASP (Chien et al., 2011; Salminen et al., 2012). More recently, *GATA4* was shown to play a key role in both the proliferative and SASP features of senescence through autophagy related signaling pathways (Kang et al., 2015). The relevance of transcription factor and enhancer function in senescence was further confirmed broadly by the observation of changes in enhancers during oncogene induced senescence (Tasdemir et al., 2016). While this body of evidence suggests a major role of enhancers and transcription factors in the onset of senescence, the dynamics of enhancer activation during senescence and the impact of such a program upon the senescent phenotype remain poorly understood, as does the relationship of transcription factors with the senescence enhancer program. Here perform a comprehensive study of the enhancer dynamics of senescence, relate this program to distinct programs of cellular proliferation and SASP, associate these programs with NFI family members and NFkB respectively, and finally identify a novel change in histone dynamics relating to H3K79me3, H4R3me2sym, and H4R3me2as related to senescence.

Results

In order to examine the epigenetic landscape associated with the process of replicative senescence, we first sought to characterize the difference in enhancer profiles between early passage proliferating and late passage, non-proliferating, senescent fibroblasts. We performed these studies in BJ fibroblasts, which are a normal human foreskin cell line that reaches cellular senescence approximately at population doubling 64. To identify enhancers, we performed ChIP-seq in duplicate against H3K4me² and H3K27ac histone marks, in PD30 (proliferating) and PD64 (senescent) cells, and identified non promoter regions containing peaks of both marks. We determined that 2169 enhancers were gained in replicative senescence, based on a 2-fold gain in H3K27ac signal intensity, and a 1.5-fold gain in

H3K4me² (Fig.1). Conversely, we found that 1565 enhancers were lost based on 2-fold H3K27ac and 1.5-fold H3K4me² decreases during senescence, as well as 51,293 enhancers that did not change based on the above criteria. To determine any potential function of these gained enhancers, we performed RNA-seq and GRO-seq in PD30 and PD64 BJ fibroblasts to identify any changes in the transcriptional program between proliferating and senescent BJ fibroblasts. We identified 639 genes that were highly up-regulated during replicative senescence, and 598 down-regulated, with the criterion of >1.5-fold change in mRNA transcripts, standard error >1, and an average expression >9 tags per million (Fig.2,3). The GO terms of these genes were broadly consistent with those observed in replicative senescence, with the up-regulated genes enriching for activation of the p53 signaling pathway, regulation of growth, signal secretion and release, while the down-regulated genes enriched for genes associated with cell cycle and DNA replication (Fig.4,5). Furthermore, analysis of the GRO-seq showed considerable pause release regulation of both the up and down-regulated gene sets during replicative senescence (Fig.6).

To determine the relationship between the changed enhancer and transcription profiles in replicative senescence, we identified the nearest expressed gene for each gained enhancer, and found that genes nearby gained enhancers showed a gain of transcription during replicative senescence (Fig.7). Furthermore, by targeting shRNA to eRNAs identified by GRO-seq to be induced in replicative senescence, we observed a significant reduction on the nearby senescence-induced target genes (Fig.8). Therefore, the gained enhancers, at least in part, appear to functionally alter the transcriptome during the senescence process.

In order to explore the plasticity of the onset of senescence in BJ fibroblasts, and the role that the gained enhancer program may play in the amelioration of aspects of the senescent phenotype, we treated BJ fibroblasts with rapamycin, a potent inhibitor of mTOR and one of the most potent drugs in promoting longevity in mice and delaying the onset of replicative

senescence. Long term treatment of BJ fibroblasts with 500nM rapamycin results in approximately 25% increase in the maximum population doubling, before reaching growth arrest (Fig.9). ChIP-seq of H3K27ac and H3K4me² in rapamycin treated BJ fibroblasts showed reduction in the increase in the enhancers gained in replicative senescence (Fig.10,11). Interestingly, that rapamycin treated PD 80 BJ fibroblasts showed the morphology of early passage proliferating BJs, as well as very low levels of β -galactosidase staining when compared with senescent BJs, regardless of the fact that these rapamycin-treated cells were growth arrested and had undergone >15 additional population doublings from non-rapamycin treated BJs (Fig12). Given that rapamycin treatment seemed to delay the secretory and morphological aspects of senescence, we were curious whether the withdrawal of rapamycin would result in the activation of this aspect of senescence. To our surprise, within just 2 weeks of withdrawal of rapamycin, we observed dramatic onset of senescent morphology and increase of β -gal staining (Fig.12). Additionally, while RNA-seq of late passage BJ fibroblasts under rapamycin-maintained versus rapamycin-withdrawal conditions showed a general correlation between the genes induced with rapamycin withdrawal conditions and the genes induced in replicative senescence (Fig.13), GO analysis of the withdrawal induced genes showed striking enrichment of genes associated with the inflammatory and secretory pathways (Fig.14). We next determined whether these transcriptional and phenotype changes associated with rapamycin withdrawal correlated to the enhancer program identified in replicative senescence. ChIP-seq of H3K27ac and H3K4me² in late passage rapamycin-maintained versus withdrawal BJ fibroblasts showed a clear increase in the enhancers gained during replicative senescence (Fig.15,16). Furthermore, examination of the 1512 enhancers gained in rapamycin withdrawal not only showed a correlation with the enhancers gained in replicative senescence, but also examination of the nearest expressed genes to the enhancers gained on withdrawal

showed a strong correlation of the withdrawal-induced enhancer and transcription programs. (Fig.17,18).

As several recent publications have used HiC techniques to show dramatic changes in chromosomal architecture occurring in the onset of senescence, we performed HiC in BJ fibroblasts that were early passage (PD30), late passage senescent (PD64), late passage rapamycin treated (PD80) cells, and late passage BJ fibroblasts (PD80) withdrawn from rapamycin for 2 weeks. Consistent with literature (Chandra et al., 2015; McCord et al., 2013), we noticed many clear regions of dramatic change in interactions leading to altered in A/B compartments, even in very late passages, when comparing proliferating and senescent cells. Interestingly, we observed that these changes in A/B domains seemed to be largely blocked by sustained treatment with rapamycin, (Fig.19). More surprisingly, we noticed that this “protection” of the young cell A/B landscape was maintained even after 2 weeks of rapamycin withdrawal. While these senescence associated changes in A/B compartmentalization could certainly occur with extended duration of rapamycin withdrawal, and are a subset of these senescent associated changes in A/B compartmentalization that do occur in rapamycin withdrawal, these results suggest that these changes in A/B compartmentalization are largely not necessary factors in the onset of either the proliferative or morphological and secretory phenotypes of replicative senescence.

Having identified the enhancer program of replicative senescence, as well as delineating a subset of this program as governing the SASP phenotype, we next sought to identify what transcription factors might govern these enhancer and transcriptional programs. We began by performing motif analysis of gained enhancer peaks. Given the broadness of H3K27ac and H3K4me² peaks, which increases the sequence length and thus background of peaks for motif finding, we defined different subsets of gained peaks, using H3k27ac and H3K4me² combined and independently, and setting multiple thresholds for fold-change of

peak intensity and minimum thresholds of peak intensity in senescent cells. We also generated multiple control sets of enhancers that were defined by similarly varying the aforementioned thresholds. Through these analyses and comparing to controls, we found that NF κ B motif was strongly enriched in gained senescence peaks but not in controls, and, when taking into account the extensive body of literature associating NF κ B and inflammation with aging and senescence, marked NF κ B as an ideal candidate for playing a role in the activation of enhancers in replicative senescence (Fig.20). We also identified the motif of the NFI family of factors, which includes *NFIA*, *NFIB*, *NFIC*, and *NFIX*, which have reported roles in differentiation, tumorigenesis, proliferation activation and repression, and altering chromatin accessibility (Denny et al., 2016; Fane et al., 2017; Harris et al., 2015) (Fig.21).

To determine the change in p65 binding during replicative senescence, we performed ChIP-seq against BJ fibroblasts at PD19 (young proliferating), PD30 (young proliferating), PD46 (early senescence), and PD57 (senescent). We observed both an increase in the number of peaks during increased population doubling, and an increase in binding of *p65* at our senescence induced enhancers. 284 of 2169 senescence induced enhancers show *p65* binding in PD57 senescent cells (Fig.22).

Additionally, we performed ChIP-seq against NFI transcription factors in early passage proliferating and senescent BJ fibroblasts using a pan-antibody targeting all four NFI family members. While we observed minimal difference in overall NFI binding between proliferating and senescent BJ fibroblasts, we observed a marked increase from 167 to 418 NFI peaks bound at the 2169 senescence induced enhancers (Fig.23).

To determine the function significance of *p65* and NFI family members upon the transcriptional program regulated by the senescence induced program of enhancers, we knocked down *p65*, *NFIA*, *NFIB*, *NFIC*, and *NFIX* in PD64 BJ fibroblasts and performed RNA-seq and ATAC-seq upon each after 2 weeks of knockdown. We observe a clear

reduction in the level of transcripts near senescence induced enhancers in knockdown of *p65* versus sh-GFP control, and we observed a clear difference with only two of the NFI family members (*NFIA* and *NFIC*) (Fig.24). Consistent with these findings, we observed that knockdown of *p65*, *NFIA*, and *NFIC* result in reduction of ATAC-seq signal at the enhancers induced during replicative senescence (Fig.25). Furthermore, knockdown of *p65* reduces the ATAC-seq signal at senesce-induced enhancers that are bound by p65. Similarly, knockdown of *NFIA* and *NFIC* show reduction of ATAC-seq signal at senescence-induced enhancers showing peaks in NFI ChIP-seq, while *NFIB* and *NFIX* show relatively minimal differences versus GFP control (Fig.26). These results suggest that *p65*, *NFIA*, and *NFIC* binding during replicative senescence contributes to the enhancer program changes during replicative senescence, as well as the resulting changes to the cell transcriptional profile.

To determine the role of NFI family members in the phenotype of senescence, we performed knockdown of *NFIA*, *NFIB*, *NFIC*, and *NFIX* in BJ fibroblasts at PD26 and observed whether each knockdown had an effect of the maximum population doublings of BJ fibroblasts. Consistent with expression and ATAC-seq data, we observed that *NFIA* and *NFIC* knock-down both dramatically increased maximum population doublings, while knockdown of *NFIB* and *NFIX* resulted in no effect or lowered population doublings, respectively (Fig.27).

Given that withdrawal of rapamycin from high passage BJ fibroblasts results in an induction of a SASP like phenotype without any alterations in proliferation, we reasoned that the genes induced in both rapamycin withdrawal and replicative senescence would be associated with the SASP phenotype, while those genes induced by replicative senescence but not induced during rapamycin withdrawal might be enriched for genes associated with proliferation. Indeed, chemical inhibition of the *p65* pathway seemed to have no effect upon the maximum population doublings of BJ (Fig.28), and knockdown of *p65* had a greater

impact versus control on the rapamycin induced subset of the 639 genes induced during replicative senescence (Fig.29). Accordingly, after ranking the 639 genes induced in replicative senescence based on their induction in rapamycin withdrawal, we observe that the top 300 least rapamycin-induced genes show enrichment for GO-terms associated with cell cycle arrest (Fig.30). Interestingly, we observe that *NFIA* and *NFIC* knockdown versus control in PD64 BJ fibroblasts have a significant reduction in the non-rapamycin induced senescence induced program (300 transcripts) of senescence (Fig.31,32). These results *NFIA* and *NFIC* may promote the proliferative, but not the SASP, aspects of replicative senescence.

To better isolate the role of *p65* in the SASP program of senescence, we decided to examine its dynamics in the context of rapamycin withdrawal. We performed ChIP-seq of *p65* in PD80 BJ fibroblasts either maintained in 500nM rapamycin or removed from 500nM rapamycin for 2 weeks. We observe a dramatic increase in *p65* binding (Fig.33). This result is confirmed by our observation that treatment of withdrawal cells with TPCA-1 IKK-2 inhibitor blocks this increase in binding. To confirm the functional importance of *p65* in the SASP transcriptional program of senescence, we compared knockdown of *p65* versus sh-GFP control in the rapamycin withdrawal condition, and observed a striking reduction in the levels of genes induced with rapamycin withdrawal (Fig.34). We also show that knockdown of *p65* has a clear effect in preventing the morphological changes that occur in rapamycin withdrawal, further confirming the importance of *p65* in the SASP phenotype (Fig.35). Importantly, we do not observe this striking inhibition of rapamycin-withdrawal transcript induction upon knockdown of NFI family members (Fig.34), further establishing the proliferative and SASP phenotypes of being dependent of distinct enhancer functions.

We then sought to use our novel isolation of the SASP program through rapamycin withdrawal to identify factors that may specifically regulate the SASP phenotype of senescence. Based on our RNA-seq and GRO-seq results, we showed that *INHBA* gene was

reduced by an average of 1.88 fold on rapamycin withdrawal. As the *INHBA* gene product can either homodimerize to form ActivinA, heterodimerize with *INHBB* to form Activin AB, or heterodimerize with *INHHA* to form Inhibin, we decided to treat cells with ActivinA, ActivinAB, or Inhibin in PD83 cells in rapamycin maintenance or withdrawal conditions. We observed that both ActivinA and ActivinAB strikingly reduced the genes being activated in rapamycin withdrawal, while Inhibin treatment had no discernible effect upon the rapamycin withdrawal program (Fig.36).

We then attempted to determine whether the altered enhancer program we identified in replicative senescence corresponds to global alterations in the levels of any histone modifications. We performed western blots in BJ fibroblasts at a range of population doublings over a large panel of histone modifications and came to identify H3K79me³ and H4R3me^(2as) as increasing in replicative senescence, while H4R3me^(2sym) exhibited a clear decrease (Fig.37). Previous studies from our lab had associated the symmetric to asymmetric switch at enhancers to increased pause release of associated genes (Liu et al., 2013), and other reports have widely associated the symmetric mark with transcriptional repression and the asymmetric mark with transcriptional activation (Baldwin et al., 2014; Kim et al., 2016; Xu et al., 2010). To determine the conservation of these changes across cell types, condition, and species, as well as the relevance of these modifications to aging, we examined these modifications using a ZMPSTE24 knockout mouse model of HGPS premature aging syndrome in isolated kidney tissue. Consistent with our results in human BJ fibroblasts, we observed that H3K79me³ and H4R3me^{2as} increased both in knockout of ZMPSTE24, as well as with increased age (Fig.38). Conversely, H4R3me^(2sym) showed a decrease in such conditions.

To further examine the role of H3K79me³ in replicative senescence, we performed ChIP-seq upon H3K79me³, its precursor H3K79me², and H2Bub, whose knockdown's

reduction of H3K79 methylation suggests a role upstream of H3K79me³ (Ma et al., 2011). While we observe a global increase in the levels of H3K79me³ (Fig.39,40), we observe that the increase in H3K79me³ is considerably more striking on genes up-regulated during replicative senescence (Fig.41). As we observe increase of both H3K79me³ and H3K79me² along gene bodies, with no change in the levels of the upstream H2Bub, we reasoned that the changes in H3K79 methylation were regulated through its writer, *DOT1L* (Fig.39,40). The role of *DOT1L* in the changes of H3K79me³ was confirmed, as treatment of BJ fibroblasts with EPZ-5676 resulted in a dramatic reduction of H3K79me³ signal in both ChIP-seq (data not shown) and western blot (Fig.42c). To determine the functional role of H3K79me³ and *DOT1L* on the onset of replicative senescence, we began treating early passage BJ fibroblasts with EPZ-5676, which resulted in an increase in the maximum population doubling (Fig.42a). Conversely, BJ fibroblast lines stably over-expressing *DOT1L* showed a decrease in maximum population doublings (Fig.42b). To determine the effect of H3K79me³ inhibition on the senescence transcription pattern, BJ fibroblasts continuously treated from early passage with either rapamycin or DMSO as a control were harvested for RNA-seq at early and late passage. The data showed that EPZ-5676 reduced expression of the genes induced during replicative senescence and increased expression of the genes decreased during senescence (Fig43a). Given that EPZ-5676 showed anti-senescence effects, we decided to explore whether EPZ-5676 might have additional effect on the senescence transcriptional program when combined with rapamycin treatment. Interestingly, we observe that that combined EPZ-5676 and rapamycin treatment resulted in decreased and increased expression for the senescence induced and repressed gene sets, respectively, when compared with either EPZ-5676 or rapamycin treatments alone (Fig43b). These results suggest that the H3K79me³ increase is a key epigenomic aspect of replicative senescence, and that inhibition of the mark's writer,

DOTIL, has potential clinical benefit in aging pathologies alone or in combination with rapamycin.

To determine the role of H4R3 methylation in replicative senescence, we stably overexpressed the following in young BJ fibroblasts: *PRMT1*, the writer of H4R3me^(2as); *PRMT5*, the writer of H4R3me^(2sym); and *JMJD6*, the eraser of H4R3me^(2sym). Consistent with the changes in H4R3 methylation observed in replicative senescence by western blot, we find that overexpression of both *PRMT1* and *JMJD6* result in reduced maximum population doubling, whereas *PRMT5* overexpression increased maximum population doubling (Fig.44). Conversely, stable knockdown of *JMJD6* results in increased maximum population doubling (Fig.45), which, combined with our overexpression and western blot results, suggest *JMJD6* may play a role in establishing replicative senescence. The role of *JMJD6* in establishing the senescent phenotype was further confirmed upon RNA-seq of *JMJD6* knockdown lines, as the senescence induced transcriptional program of 639 genes was significantly reduced in senescent cells with *JMJD6* knockdown versus non-silencing controls (Fig.46). We then performed ChIP-seq against 2 different stably tagged *JMJD6* BJ fibroblast cell lines at early and senescent population doublings. Both replicates show increased binding during in the senescent condition at the enhancers we identified as induced during replicative senescence (Fig.47). To confirm the effect of knockdown of *JMJD6* on genomic levels of H4R3me^(2sym), we performed native-ChIP-seq against H4R3me^(2sym) in sh-non-silencing control and *JMJD6* knockdown lines, at both early and late senescent passages. Consistent with our western blot results, we observe a dramatic decrease in total H4R3me^(2sym) peak number in senescent cells (Fig.48). Furthermore, we observe that regions showing loss of H4R3me^(2sym) levels in replicative senescence in control lines show increased level of H4R3me^(2sym) in *JMJD6* knockdown lines, further confirming the role of *JMJD6* in the global decrease of H4R3me^(2sym) observed during replicative senescence (Fig.49). We then sought to determine how the levels

of H4R3me^(2sym) change during senescence on the enhancers that we identified as induced during replicative senescence. Interestingly we observe a decrease during senescence of the repressive H4R3me^(2sym) mark on the enhancers gained during replicative senescence (Fig.50). Furthermore, this decrease in H4R3me^(2sym) is, in part, dependent upon *JMJD6*, as knockdown of JMJD6 reduced the depletion of H4R3me^(2sym) observed in senescence on the senescence gained enhancers (Fig.50). Taken together, these results suggest that H4R3 methylation dynamics play a functional role in replicative senescence, and that modulation of the writers and erasers of these marks have exciting potential for modification of the senescent phenotype.

Discussion

Our results show that not only do enhancers play a key role in driving the senescence phenotype, but that distinct factors regulate subsets of these enhancers to differentially control the proliferative and SASP programs of senescence. We show that NFIA and NFIC act on a subset of enhancers to regulate cell proliferation and its associated transcriptional program. The NFI family of transcription factors had largely been linked to differentiation and development (Harris et al., 2015) as well as oncogenesis (Fane et al., 2017), with NFIB being recently shown to promote metastasis through opening chromatin at enhancer like elements (Denny et al., 2016). The differential role in senescence of NFI family members in our study mirrors the varied cancer effect of NFI members in cancers, as different members have been linked to both oncogenic and tumor suppressor function depending on their system (Fane et al., 2017).

Conversely, NFkB functions on a different subset of the enhancers gained in replicative senescence to regulate the SASP program, independent of any effect upon senescence associated proliferative repression. While the role of NFkB in senescence has long been shown, our data clarifies the independence of NFkB program from the cell cycle arrest observed in replicative senescence (Chien et al., 2011; Salminen et al., 2012). This enhancer

based delineation of the proliferative and SASP features of senescence highlights their independence, which suggests the exciting potential for regulation of one phenotype independently of the other. Furthermore, abundance of transcription factors and complexity of signaling that occurs in senescence suggests we've just begun to scratch the surface of enhancer regulation, and that further exploration of the different enhancer programs that exist in replicative senescence may allow for further and more nuanced manipulation of the senescence phenotype.

We are further excited to explore the relationship of our changed enhancer program to super-enhancers. Super-enhancers are particularly key regulators of cell behavior (Hnisz et al., 2013b), and, consistent with recent observations (Tasdemir et al., 2016), we observe that many of our changed enhancers can be classified as super-enhancers. It remains to be seen how the super-enhancer code of senescence, and the factors affecting it, relate to the enhancer code we have observed.

A major goal of aging and senescence research is to develop treatments to increase lifespan and healthspan without negatively simultaneously promoting cancer. While overexpression of telomerase allows cells to proliferate indefinitely while bypassing cellular senescence (Stewart and Weinberg, 2002) and is sufficient to extend mouse lifespan (Bernardes de Jesus et al., 2012), the bypass of proliferative repression phenotype of senescence is a hallmark of cancers (Hanahan and Weinberg, 2011), with mice constitutively overexpressing telomerase showing concordant increases of oncogenesis (Artandi et al., 2002; Canela et al., 2004; González-Suárez et al., 2001; McKay et al., 2008). Studies showing extended life and healthspan upon clearance of senescent cells link the pathological influence of senescent cells to their SASP activity (Baker et al., 2016; Chang et al., 2016). The delineation of enhancer programs and factors uniquely regulating either proliferation or SASP in senescence provides a foundation for the discovery of SASP or proliferation selective

factors. ActivinA and ActivinAB, which were shown in our study to dramatically block SASP induction, is one such example of a factor whose treatment could have a major impact on aging pathologies. We are excited to explore what impact such treatments could have on in vivo models.

Complementing the identification of transcription factors that regulate distinct programs of senescence, our identification of the senescence switch of H4R3me2sym high to H4R3me2asym and H3K79me3 high during senescence has important clinical. Our panel of histone modifications shows the majority of histone modifications unchanged in BJ fibroblast replicative senescence, and many key senescence associated histone modifications show gene specific rather than global changes. However, the dramatic change in levels during senescence of our aforementioned modifications suggests they may function as a uniquely strong marker of aging tissue, which could have clinical value for the identification of aging pathologies. Furthermore, the impact of our manipulation of the histone writers and erasers of these modifications obviates the need for development of specific small molecule inhibitors of these enzymes. Our results also should encourage the exploration of the roles of additional under-studied histone modifications, as histone arginine methylation remains poorly explored in senescence and our results show this family of modifications and their modifiers can have striking effect on senescence.

Our results thus show a novel integration of the enhancer program, transcriptional dynamics, histone code, and activated transcription factors involved in senescence, and we use these findings to delineate and modify the clinically significant proliferation and SASP related features of senescence.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Suter, Thomas; Tazearslan, Cagdas; Merkurjev, Daria; Meluzzi, Dario; Suh, Yousin;

Rosenfeld, Michael. The dissertation author was the primary investigator and author of this paper.

CHAPTER 3 LIGAND-DEPENDENT ENHANCER ACTIVATION REGULATED BY TOPOISOMERASE-I ACTIVITY

Summary

The discovery that enhancers are regulated transcription units, encoding eRNAs, has raised new questions about the mechanisms of their activation. Here, we report an unexpected molecular mechanism that underlies ligand-dependent enhancer activation, based on DNA nicking to relieve torsional stress from eRNA synthesis. Using dihydrotestosterone (DHT)-induced binding of androgen receptor (AR) to prostate cancer cell enhancers as a model, we show rapid recruitment, within minutes, of DNA topoisomerase I (TOP1) to a large cohort of AR-regulated enhancers. Furthermore, we show that the DNA nicking activity of TOP1 is a prerequisite for robust eRNA synthesis and enhancer activation, and is kinetically accompanied by the recruitment of ATR and the MRN complex, followed by additional components of DNA damage repair machinery to the AR-regulated enhancers. Together, our studies reveal a linkage between eRNA synthesis and ligand-dependent TOP1-mediated nicking – a strategy exerting quantitative effects on eRNA expression in regulating AR-bound enhancer-dependent transcriptional programs.

Introduction

Research over the past few years, supported by data from GRO-seq analysis and the ENCODE project, has revealed that most developmental and regulatory transcriptional regulation programs are controlled by an extensive enhancer network (Kim et al., 2010; Shlyueva et al., 2014), with each cell type estimated to harbor 70,000-100,000 enhancers, located upstream and downstream of coding target gene promoters (Pennacchio et al., 2013). Enhancer signatures include mono-methylated H3K4 (H3K4me1) and H3K27-acetylated histones (Kim et al., 2010; Li et al., 2013a; Wang et al., 2011). These enhancers are usually characterized by a nucleosome-depleted core region where many of the cooperating

transcription factors bind (Andersson et al., 2014; Hah et al., 2013; Kaikkonen et al., 2013; Lai et al., 2013; Lam et al., 2013; Li et al., 2013a; Melgar et al., 2011; Melo et al., 2013; Mousavi et al., 2013). Most surprisingly, enhancers are also transcription units, wherein their effect on target coding genes correlates with the transcription of the lncRNAs, referred to as eRNAs (Andersson et al., 2014; De Santa et al., 2010; Hah et al., 2013; Kaikkonen et al., 2013; Kim et al., 2010; Lai et al., 2013; Lam et al., 2013; Li et al., 2013a; Melgar et al., 2011; Melo et al., 2013; Mousavi et al., 2013) adding a new layer of regulation to the fundamental mechanisms underlying enhancer action (Lam et al., 2014; Natoli and Andrau, 2012).

The current prevailing belief, based on chromosome capture assays, where looping constraints are inferred from interaction frequencies between a point of interest and distal loci of the genome is that the main mechanism by which enhancers affect their target gene expression is through chromatin looping. eRNAs transcripts seem to be functionally important by contributing to the stabilization of juxtaposed enhancer-target gene promoter loops to allow for optimal gene expression (Lai et al., 2013; Li et al., 2013a). However, both eRNA synthesis and nucleosome depletion are potential sources of topological strain on enhancers that can potentially hinder transcription. The movement and rotation of RNA polymerase complex (RNAP) along DNA template during the process of RNA synthesis (Liu and Wang, 1987) can generate positive supercoils in front of the advancing RNAP, and negative supercoils behind it (Darzacq et al., 2007; Kouzine et al., 2013; Kouzine and Levens, 2007; Liu and Wang, 1987). Because RNA polymerase is a powerful torsional motor, it can alter DNA topology by creating DNA supercoils, which can propagate and affect transcription elongation (Ma and Wang, 2014). While negative supercoiling can initially facilitate transcription initiation, either by helping RNAP to form an open complex or by helping to recruit transcription factors (Ma and Wang, 2014), it can subsequently lead to the generation of R-loops resulting from hybridization of nascent RNA to the DNA strand that is being transcribed, which in turn can impede

transcriptional elongation (El Hage et al., 2010). Positive or over-wound supercoiling can prevent transcription initiation and greatly diminish mRNA synthesis (Ma and Wang, 2014). Moreover, the very depletion of histones from the core region of enhancers releases unconstrained negative supercoils, which can impede transcription factor binding. One mechanism that resolves the undesirable effects of excessive supercoiling employs DNA topoisomerases, including topoisomerase I (TOP1). TOP1 can relax both negative and positive supercoils by transient single-strand breaks for the passage of individual DNA strands through one another, followed by the rejoining of the phosphodiester backbone of DNA (Pedersen et al., 2012; Pommier et al., 2006).

While TOP1 activity is well established in DNA replication, its potential functionality in enhancer activation and transcriptional initiation remains unclear. Most of the experiments hitherto examining the role of TOP1 in transcription have been limited to artificial promoter model systems which, if anything, have argued that TOP1 DNA nicking activity is not involved in transcriptional activation in such *in vitro* systems (Kretzschmar et al., 1993; Merino et al., 1993; Shykind et al., 1997).

However, the utilization of a nicking strategy for transcriptional initiation and enhancer regulated events would be in concert with the elegant explication of the molecular mechanisms underlying the expression of bacteriophage T4 late genes, with the participation of DNA-mounted activator of transcription, gp45 and RNAP-bound gp33. Here, a nick in the strands of the DNA and the actions of an exonuclease are required, with the DNA template single-strand nicks being essential for transcriptional activation and the nicked-DNA gp45-loading site located upstream or downstream of its target site (Herendeen et al., 1992). Also, in human cells, artificially-generated nicks (but not double-strand DNA breaks) have recently been found to be associated with transcription (Davis and Maizels, 2014). Together, these and

other experiments in prokaryotes and eukaryotes suggest an intriguing link between DNA nicking and transcription but the mechanism and the factors involved remain largely unknown.

Here, we describe a molecular mechanism that operates at functional androgen-regulated enhancers and identify DNA topoisomerase I as a critical DNA-nicking enzyme involved in the process of cell-specific, ligand-driven enhancer activation. Recruitment of TOP1 to these AR-bound enhancers is of functional consequence as knockdown of the enzyme in the prostate cancer cells results in inhibition of DHT-regulated eRNA and many coding gene transcriptional targets. Additionally, we provide evidence that recruitment of a significant repertoire of DNA damage response machinery occurs on these functional enhancers, potentially to prevent undesirable effects of persistent DNA damage.

Results

TOP1 Recruitment to AR-Regulated Enhancers Affects eRNA and Coding Gene Expression

To further investigate the mechanism of enhancer activation in ligand-regulated transcription, we employed an early prostate adenocarcinoma cell line, LNCaP, the growth of which is androgen-dependent (Horoszewicz et al., 1980). The cell line is exquisitely sensitive to androgen stimulation and arrests in the G1 phase of the cell cycle upon steroid depletion, despite the presence of peptide growth factors (**Fig. S1A**). Regulation of cyclin D expression and concomitant CDK4 activity represents one mechanism by which androgen impinges on the cell cycle to govern proliferation (Knudsen et al., 1998).

To investigate whether TOP1 played a role in ligand-regulated transcription, we undertook to examine the possible recruitment of TOP1 to enhancers; finding that it was recruited to several AR-enhancers early in response to androgen (5 α -dihydrotestosterone, DHT) treatment in the ligand-dependent LNCaP prostate cancer cell line (**Fig.51A, Figure S1B**). These data prompted us to study genome-wide localization of this protein by

performing chromatin immunoprecipitation (ChIP) coupled with next-generation sequencing (ChIP-seq). Because exhaustive efforts to identify a TOP1 antibody suitable for ChIP-seq proved unsuccessful, we generated a stable LNCaP cell line with inducible biotinylated TOP1 expression. We observed that TOP1 recruitment in response to DHT, generated enriched regions of a range of sizes (**Fig. 1B**), as opposed to point sources, as found for factors such as androgen receptor, or broad sources, such as observed for the H3K36me3 histone mark (Sims et al., 2014). Consistent with the observation that enhancers represent regulated transcription units, we noticed a hormone-dependent increase in RNA PolII (phospho-Ser5) occupancy predominately at these enhancers (**Fig. 51B**, **Figure S1C**). As expected, we also observed increased TOP1 occupancy over promoters and gene bodies of the representative DHT-induced genes (e.g., *KLK3* and *KLK2*), consistent with the possibility that TOP1 might be involved in both enhancer activation and transcriptional elongation events.

Preliminary analysis demonstrated that TOP1 binding overlapped in particular with that of liganded androgen receptor at enhancers (**Fig. 51B**, **Fig. S1C**). Genome-wide analysis revealed 6545 putative “AR-bound enhancer” sites based on the criterion of an AR-bound locus marked with H3K4me1, H3K27Ac, and more than 1kb away (in either direction), from the promoter of annotated genes, of which 96% bound TOP1, with 3921 (60%) exhibiting a DHT-stimulated increase in TOP1 binding (**Fig. S1D**).

To assess eRNAs induced by DHT, we took advantage of technological advances that permit mapping of the position, amount, and orientation of transcriptionally engaged RNA polymerase II on a genome-wide scale (Core et al., 2008). GRO-seq analysis (Core et al., 2008) of serum-starved LNCaP cells treated for 1h with DHT, identified 644 putative AR enhancers with significantly up-regulated eRNAs (**Fig. 1C**), which is the best mark of activated enhancers (Hsieh et al., 2014; Li et al., 2013a), amongst which 477 (~74%) were noted to have increased TOP1 occupancy in response to ligand at 30' (**Fig. 1D**); and virtually all

appear to exhibit DHT-increased TOP1 binding at 15' (**Fig. S5C**). Because TOP1 has been shown to affect the transcriptional activity of RNA PolII (Kretzschmar et al., 1993), we decided to investigate whether knockdown of TOP1 would alter eRNA synthesis from the androgen-regulated enhancers. Knockdown of endogenous TOP1 by small interfering RNA (siRNA) revealed that eRNA induction was reduced in at least 79% (507 of 644) of AR-regulated enhancers (**Fig.51C, E**), accompanied by a decrease in the induction of 368 coding target genes in the experiment shown (**Fig.51F**), with similar results in repeat experiments. 92% of DHT-induced eRNAs were up-regulated more than 2-fold, with FC average of 7.6x. Analysis of 100 randomly selected housekeeping genes not regulated by DHT in our GRO-seq experiments, confirmed that the specific siRNAs used for this study had no effect on their expression (**Table S3**).

To validate all major mechanistic points in this study, we chose four enhancers-gene pairs. Three of these enhancers (KLK3E, KLK2E and TMPRSS2E) are validated by previous studies (Andreu-Vieyra et al., 2011; Clinckemalie et al., 2013; Hsieh et al., 2014). The fourth one, NDRG1E, meets the criteria of others. It is an AR-bound element located not too far away from the *NDRG1* gene TSS (-29kb), it is H3K4me1+, H3K27Ac+ and following hormone stimulation the transcription unit produces DHT-dependent, bi-directional eRNA, making it a strong candidate. Using these enhancer sites, we found that recruitment of the nuclear receptor co-activators (p300 and SRC-1) at AR enhancers was diminished (**Figure S1E**). Thus, TOP1 knockdown attenuated the induction of eRNA (1h DHT treatment) and the production of mRNA of the corresponding target genes 5h after ligand addition (**Fig.51G, Figure S1F**). Importantly, the fold-induction (-/+ DHT) was similar between independent experiments in which eRNA levels were measured. Surprisingly, we noted that ATR (Ataxia telangiectasia and Rad3-related), a protein involved in DNA damage repair, was recruited to AR-regulated enhancers at ~15 min following addition of ligand (**Fig.51H, Figure S1G**).

Together, these data identify TOP1-bound genomic regions that bear enhancer marks and produce eRNA in a DHT-dependent manner. Knockdown of TOP1 reduces production of eRNA and coding gene RNA for most of these AR-regulated target genes.

NKX3.1 and TOP1 Co-occupy Enhancer Binding Sites and Regulate the AR Transcription Program

NKX3.1 is an androgen-regulated transcription factor (Bhatia-Gaur et al., 1999), which is a highly selective and specific marker of metastatic prostatic adenocarcinoma (Gurel et al., 2010). NKX3.1 has been found to interact with TOP1 to enhance formation of the TOP1-DNA complex and increase TOP1 nicking of DNA (Bowen et al., 2007). In fact, TOP1 activity in prostates of *Nkx3.1*^{+/+} and *Nkx3.1*^{-/-} mice is reduced compared with wild-type mice, but not in other organs that do not express *Nkx3.1* (Bowen et al., 2007). Overlap of the reported NKX3.1 ChIP-seq dataset (Tan et al., 2012) with that of AR and TOP1 revealed that NKX3.1 occupancy was highest at AR enhancers, with NKX3.1 binding sites located over regions with increased TOP1 binding (**Fig.52A, Figure S2A**). We observed that AR and TOP1 started to be recruited to AR-enhancers within a few minutes after DHT stimulation. Interestingly, siRNA-mediated knockdown of cellular *NKX3.1* inhibited recruitment of TOP1 at enhancers of DHT-regulated genes at 5 min following DHT stimulation (**Fig.52B**), in line with the previous data suggesting that NKX3.1 is needed for the formation of the TOP1-DNA cleavage complex (Bowen et al., 2007). We observed that following NKX3.1 knockdown in LNCaP cells, the DHT-dependent up-regulation of ~70% enhancer eRNAs were significantly reduced (**Fig.52C, D, Figure S2C**). We also noted significant reduction in the expression levels of 273 DHT up-regulated genes (**Fig.52E**), exemplified for two representative genes (**Fig.52F**). Additionally, knockdown of *TOP1* and *NKX3.1* reduced DHT-up-regulation of eRNA at the same 351 AR-enhancers in these experiments (**Fig.52G**), apparently without

affecting AR recruitment to the enhancer-binding sites (**Fig. S2E**). Together, these experiments demonstrate that NKX3.1 and TOP1 binding occurs at a subset of DHT-regulated enhancers and the knockdown of either diminishes transcription in response to ligand.

Catalytic Activity of TOP1 is Required for DNA Nicking and Enhancer Activation.

Based on its mechanism of action as a DNA nickase, by which TOP1 forms a covalent intermediate with DNA, and possesses intrinsic DNA ligase activity (Pommier et al., 1998; Champoux, 2001), it would be difficult to detect any such transient nick by available methods. Indeed, despite extensive attempts to detect such a nick in enhancers by primer extension approaches, only a few examples could be clearly visualized. Thus, using this approach to investigate whether AR-regulated enhancers might be the sites of DNA scission by the activated TOP1, we chose the *KLK3* enhancer as a model and examined a region overlapped by the AR and NKX3.1 peaks and flanked by two PRO-caps, which mark the transcription initiation sites at high resolution (Kwak et al., 2013), noting that PRO-cap sites could be located on AR-regulated enhancers following hormone stimulation from the AR binding sites. (**Fig.53A, Table S4**). Primer extension analysis of both DNA strands with [γ - 32 P]-ATP labeled oligonucleotides yielded several termination products consistent with a series of closely-spaced DNA nicks; the strongest band that became accentuated in response to DHT was seen on the lower strand, in support of the notion that it may be one of the major TOP1 binding/scission sites (**Figure S3B**). Moreover, detailed PRO-cap analysis to locate the precise start sites revealed that the RNA cap sites located, on average ~134 bp away from the center of AR peak binding site (**Fig. S3C**) are occupied by TOP1 (**Fig. S3D**), and as shown in GRO-seq experiments, continue to the end of eRNA-encoding sequence, however for the majority (75th percentile) of these transcripts the GRO-seq signal starts to fade away after 1000 bp from the TSS/cap site.

As another approach to infer the possibility of TOP1 DNA nickase actions in activation of AR-dependent enhancer we sought to mutate TOP1. TOP1 enzymatic activity depends on Tyr723 to relax superhelical DNA (Madden and Champoux, 1992). Specifically, Tyr723 of TOP1 initiates the nucleophilic attack on the backbone scissile phosphate resulting in nicked DNA and a phosphodiester link between the tyrosine and 3' phosphate (Champoux, 2001; Pommier et al., 2010). Subsequently, the covalent intermediate is re-ligated with concomitant release of Tyr723 from the DNA (Champoux, 2001; Stewart et al., 1998). We therefore tested whether the Y723F TOP1 mutant could rescue the defect caused by *TOP1* knockdown. For this purpose, endogenous *TOP1* was knocked-down with specific siRNA and either the wild-type or the Y723F TOP1 mutant was then expressed in LNCaP cells. Analysis of the enhancer RNA after 1h DHT treatment revealed that the wild-type TOP1 largely reinstated eRNA induction, whereas the catalytically-inactive mutant failed to do so (**Fig.53B**). The incomplete rescue with the wild-type construct most likely reflected the fact that not all cells could be efficiently electroporated with the DNA expression vectors, as LNCaPs are notoriously difficult to transfect with conventional cationic liposome reagents. Interestingly, wild-type TOP1 relaxes supercoiled DNA only in the presence of NKX3.1, whereas the active site mutant does not at all (Bowen et al., 2007), consistent with the presence of TOP1 on AR-bound enhancers. These findings are of particular interest based on previous *in vitro* transcription system analyses. TOP1 has been shown to be essential for transcriptional activation in a system containing RNA polymerase II and other cofactors (Kretzschmar et al., 1993; Merino et al., 1993; Shykind et al., 1997) but in these artificial *in vitro* transcription system, the Y723F mutant did not block the transcriptional activity of the complex at promoters. Therefore, in this context, TOP1 was proposed to modulate transcription by changing the conformation of DNA at the promoter or via interactions with TBP/TFIID (Kretzschmar et al., 1993; Merino et al., 1993; Shykind et al., 1997). In contrast, on AR-

regulated enhancers, the nicking activity of TOP1 appears to be required for its effects on eRNA transcription.

Incorporation of labeled nucleotide by terminal deoxynucleotidyl transferase (TdT) has been considered to label both DNA nicks and dsDNA breaks (Gavrieli et al., 1992); hence, we also employed this assay on specific enhancer sites to assess incorporation of Biotin 11-dUTP in response to DHT. Therefore, we fixed the cells with *Streck Cell Preservative* (Ju et al., 2006), a formulation shown not to cause DNA breaks during the fixation process. Biotin 11-dUTP incorporation with (TdT) was observed at 10 min following addition of DHT hormone treatment at the several enhancers tested, and this was strikingly reduced after TOP1 knockdown (**Fig.53C**) Together, these data suggested that TOP1 recruitment to enhancers co-occupied by AR and NKX3.1 occurred at regions proximal to transcription initiation sites and caused ssDNA nicks, although the possibility of a dsDNA break cannot be ruled out, especially as an unligated nick can be converted to a DSB for subsequent processing by the DSB repair pathway (Davis and Maizels, 2014).

Involvement of MRE11 in the Regulation of the AR-Program

The MRN complex, composed of the meiotic recombination 11 (MRE11), RAD50 and Nijmegen breakage syndrome 1 (NBS1) is central to the DNA damage response (DDR) pathway which is initiated upon recognition of the DNA breaks by sensor proteins (Stracker and Petrini, 2011). MRE11 regulates DNA repair by recruitment of DNA-repair proteins that load onto the chromatin at the site of the break (Price and D'Andrea, 2013).

Recent evidence shows that cleavage of the covalent 3' phosphotyrosyl bond that joins TOP1 to the DNA backbone by MRE11 generates a product carrying a 3' phosphate end, which MRE11-RAD50 can resect in an ATP-regulated reaction, producing a 3'-hydroxyl that can prime repair synthesis (Hamilton and Maizels, 2010; Sacho and Maizels, 2011). Interestingly,

the p300 transcriptional co-activator physically interacts with all three members of the MRN complex (Jung et al., 2005).

Based on these considerations and the results in **Fig.53**, we investigated whether MRE11 was present at AR-regulated enhancers. Kinetic ChIP experiments using a specific antibody (**Fig.54A, Figure S4A**) revealed that MRE11 recruitment at enhancer-binding sites peaked at 15 min of DHT treatment. On performing ChIP-seq, we identified 19,886 loci in the (–) hormone control and 30,636 loci in the cells treated with DHT for 15 min, observing that MRE11 sequencing tag density at enhancers increased with DHT treatment (**Fig.54B, C, Figure S4B**). We also observed similar recruitment of the RAD50 component of the MRN complex (**Figure S4C**). Genome-wide analysis showed indistinguishable alterations in the number of tags over promoters of these genes in response to DHT treatment, although a small increase in MRE11 occupancy at promoters of select DHT-regulated genes (e.g., *KLK3*, *KLK2*, *NDRG1*, and *TMPRSS2*) could be detected by ChIP-qPCR after DHT treatment (data not shown). GRO-seq analysis of nascent transcription revealed that induction of ~89% of detectible enhancer eRNAs induced by DHT were inhibited by MRE11 knockdown (**Fig.54D, E**). In addition, expression of 510 induced coding genes was reduced (**Fig.54F**). Knockdown of *RAD50* caused a similar effect on eRNA and mRNA expression levels (**Figure S4D**). Given the role of ATR in sensing single-strand DNA breaks, we also investigated the potential functional role of ATR following DHT. We found that ATR is rapidly recruited, by 15 min, to AR-bound enhancers after DHT (**Fig.51H**). This is of functional significance, as knockdown of either MRE11 or TOP1 caused dramatic decrease in ATR recruitment to enhancers (**Fig.54G**) and a reduction of DHT-induced enhancer and gene transcription (**Fig.54H**).

Recruitment of Components of DDR to AR-regulated Enhancers

Indeed, a mechanism that could be involved in the repair of single-strand nick would be the base excision repair pathway (BER), to process nicks that evaded TOP1 ligase activity.

Therefore, we investigated whether factors involved in this or other DNA damage repair pathways might also be recruited to AR-regulated enhancers. We performed kinetic ChIP experiments using antibodies against phospho-ATM (Ataxia telangiectasia mutated), Ku80 (part of the Ku heterodimer that binds to double-strand DNA break ends), Exonuclease 1 (EXO1), the Bloom syndrome DNA helicase (BLM), and DNA ligase IV (LIGIV). Additionally, we used antibodies to proteins involved in the base excision repair pathway, including XRCC1 (X-ray repair cross-complementing protein 1), DNA polymerases α and β , and Ligase I, observing an orderly and reproducible kinetics of recruitment after hormone treatment at enhancers including *KLK3*, *KLK2*, *TMPRSS2*, and *NDRG1* (**Fig.55, Figure S5A**), as well on other DHT up-regulated enhancers identified by the GRO-seq (**Figure S5B**). While TOP1 and ATR were essentially recruited simultaneously at enhancers at 15', XRCC1 was recruited between 15'-30', consistent with the recruitment of base excision repair pathway machinery that could process any unligated nicks. Interestingly, DNA ligase IV showed maximum occupancy after 30 min, while pATM (p-S1983), Ku80, EXO1, BLM and DNA ligase I were maximally recruited to enhancers ~60 min post DHT treatment (**Fig.55, Figure S5A**), indicating recruitment of multiple DNA repair factors that have been conventionally considered to function in DNA damage repair (Nimonkar et al., 2011). The sequence of events would be consistent with resolving any unligated DHT/TOP1-induced ssDNA nicks; the DDR machinery primarily recruited as a "safety net" against any DNA breaks that are not sealed by TOP1. From our data, the machineries of transcription and DNA damage repair seem to be intrinsically linked.

Discussion

Regulated gene expression has been a subject of intense investigation over the past few decades, yet the precise mechanisms by which enhancers orchestrate tissue-specific programs with such an astonishing precision remains unclear. In particular, the finding that

enhancers are also regulated transcription units, encoding eRNAs, has added to the mystery and raised new questions about how the subsequent topological strain on enhancers is handled. Both eRNA synthesis and nucleosome depletion at enhancers are potential sources of topological strain. Advancing RNA polymerase can generate both positive and negative supercoils. The amount of supercoiling is potentially enormous given that a positive and a negative supercoil is generated for every 10 bp transcribed and that the length of an eRNA transcript is typically 1-2 kbp in length. Indeed, it has been estimated that approximately seven supercoils may be generated by the transcribing polymerase per second, and that these supercoils can propagate >1 kbp from the transcription start site (Kouzine et al., 2013). At the same time, the depletion of histones from enhancers releases unconstrained negative supercoils, which, in principle, can parse to a change in DNA twist or unwinding to facilitate transcription and/or to a change in writhe that impedes transcription factor binding. To relieve torsional stress, it is tempting to predict that cells might employ actions of DNA topoisomerases, including topoisomerase I as an integral component of regulated enhancer transcription.

Here, we have elucidated the operation of just such a mechanism in prostate cell-specific enhancer activation by androgen receptor, using the LNCaP cancer cell line as a model. In a sense analogous to the role of TOP1 at origins of replication (Simmons et al., 1998; Tsao et al., 1993), we show here that this DNA nickase is rapidly recruited to a large cohort of AR/NKX3.1-occupied enhancers to putatively activate the enhancers and relieve torsional stress due to ongoing transcription (**Fig. 6**). Our results are consistent with observations that in yeast cells, Top1/Top2 play a role in the activation of genes characterized by high transcriptional plasticity (Pedersen et al., 2012). However, the beneficial effects of TOP1 have to be weighed against the negative effects of retention of TOP1 as an obstacle to further transcription and the deleterious effects of a single-strand nick if it is not quickly sealed by

TOP1 itself, or repaired by the base excision pathway. Unrepaired nicks could lead to the formation of DNA double-strand breaks (DSB) as, for example, when a replication fork runs into and collapses at a nick (Kuzminov, 2001; Wimberly et al., 2013). It has also been suggested that a co-directional collision between the replisome and backtracked RNA polymerase transcription elongation complexes leads to DNA double-strand breaks (Dutta et al., 2011). Thus one important role for the MRN complex and other components of the DDR machinery that we observe recruited to the TOP1-bound enhancers might be for the removal of any “stalled” TOP1 from the DNA substrate, as well as repair of any possible DNA breaks that might occur despite TOP1 or the BER actions (Hamilton and Maizels, 2010; Sacho and Maizels, 2011; Davis and Maizels, 2014).

TOP1 activity is likely to be modulated by factors other than NKX3.1, suggesting that the mechanism we describe here may not be restricted to prostate cells. In this regard, it has been shown that the catalytic activity of TOP1 is stimulated by large T antigen during unwinding of the SV40 origin (Simmons et al., 1998) and overexpression of the antigen rendered LNCaP cells androgen-independent for cell cycle progression (Knudsen et al., 1998). This raises the possibility that activation of TOP1 catalytic activity, may in part, trigger a switch to androgen independence. The Werner syndrome helicase, WRN, has also been found to enhance the ability of TOP1 to relax negatively supercoiled DNA and specifically stimulate the religation step of the relaxation reaction (Laine et al., 2003). It is therefore not unlikely that there exist other, yet undiscovered, activators of TOP1 catalytic activity to regulate eRNA synthesis and gene expression programs. Alternatively, there may be other DNA nickases that initiate enhancer activation in tissues other than prostate, in signal-dependent manner, and that the activities of those nickases are modulated by enhancer-bound factors.

While the finding that ligand-dependent enhancer activation strategy would involve a DNA nick may seem counter-intuitive in terms of cellular integrity, it is noteworthy that

cellular integrity is threatened daily by endogenous and extracellular agents that lead to the formation of single- and double-strand DNA breaks. For instance, the estimated number of single-strand breaks and spontaneous base losses in nuclear DNA together with other types of spontaneous damage may reach 10^5 lesions per cell per day (Hoeijmakers, 2009), yet the cells are programmed to survive. To maintain genomic integrity, cells constantly engage the DNA repair machinery. As such, the usage of a programmed DNA nicking/repair strategy in regulated transcription to relieve torsional stress and activate transcription in this case, while apparently surprising, is in keeping with growing evidence that components of DNA damage machinery do participate in transcriptional regulation. For instance, Reinberg and colleagues demonstrated that human RNA polymerase II complex contains components with roles in DNA repair, including Ku70, Ku80 and DNA Pol η (Maldonado et al., 1996) and Kung and colleagues (Mayeur et al., 2005) have identified heterotrimeric DNA-dependent protein kinase subunits: Ku70, Ku80 and DNA-PKcs, as well as poly(ADP-ribose) as proteins associated with the C-terminal domain of AR and demonstrated that in LNCaP cells, Ku70 and Ku80, recruited to the *KLK3* promoter and enhancer in a hormone-dependent manner. Interestingly, Ku70 and Ku80 can function outside of the Ku heterodimer that loads on double-strand DNA. Hasty and colleagues have shown that Ku80 deletion impairs the base excision pathway (BER) at the initial lesion recognition/strand scission step, arguing that free Ku70 and free Ku80, but not Ku heterodimers, associate with apurinic/apyrimidinic (AP) sites that BER corrects (Li, et al., 2013b; Choi, et al., 2014). Moreover, Mo and Dynan showed that in normally growing human cells, Ku80 associated with RNA polymerase II elongation sites. This association occurred independently of the DNA-dependent protein kinase catalytic subunit and was highly selective. In addition, there was no detectable association with the initiating isoform of RNAPII or with the general transcription initiation factors. The authors concluded that association of Ku80 with transcription sites is important for maintenance of global

transcription levels, as functional disruption of a discrete C-terminal domain in the Ku80 subunit inhibited transcription *in vitro* and *in vivo* (Mo and Dynan, 2002). Importantly, LigIV, like Ku80, is commonly associated with the NHEJ pathway, but its active site has been found to be highly permissive and capable of ligating atypical DNA substrates, including nicks with gaps (Gu et al., 2007). Interestingly, in the absence of RNase H2, the suppression of mutations arising from mis-insertion of ribonucleoside monophosphates (rNMP) during DNA replication, involves Top1-mediated cleavage at an rNMP, followed by unwinding of DNA by Srs2 and digestion by Exo1 (Potenski et al., 2014). Also, earlier studies showed that TOP1 enhanced TFIID-TFIIA complex assembly during activation of transcription; however, in these biochemical studies, the catalytic activity of TOP1 was not essential to activate transcription from promoters. It is also interesting to note that the AR itself has been shown to transcriptionally regulate a network of DNA repair genes, including those implicated in DNA damage sensing (MRE11, NBN and ATR), non-homologous end joining (XRCC4 and XRCC5), homologous recombination (RAD54B and RAD51C), mismatch repair (MSH2 and MSH6), base excision repair (PARP1 and LIG3) and the Fanconi pathway (FANC1, FANCC and USP1) (Polkinghorn et al., 2013). Moreover, p53 itself binds enhancers and regulates eRNA synthesis for transcription enhancement of neighboring genes (Melo et al., 2013). Together, the recruitment of DNA damage response machinery in specific transcriptional regulatory events is an emerging theme, from the regulation of pluripotency in embryonic stem cells by the trimeric XPC-nucleotide excision repair complex (Fong et al., 2011) to the regulation of human RAR α 2 gene via XPG induced DNA breaks at the promoter region (Le May et al., 2012). Moreover, experiments with yeast have revealed that the Rad1^{XPF}/Rad10^{ERCC1}, Mms4^{Emil} orthologs can catalyze the endonucleolytic cleavage of DNA immediately upstream from the Top1-DNA adduct (Pommier et al., 2010). Indeed, permissive chromatin architecture seems to be a crucial requirement for transcription initiation events

(Fong et al., 2013). While these events are quite distinct from the TOP1-dependent regulatory events described in the present manuscript, they do suggest a common usage of the DNA damage repair machinery to regulate gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture

LNCaP cells were purchased from ATCC and maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Omega Scientific), 2mM L-Glutamine and Penicillin/Streptomycin. For kinetic ChIP experiments, cells were starved in phenol-free DMEM (Lonza) supplemented with 5% charcoal:dextran stripped fetal bovine serum (Omega Scientific) for 72 hours. Cells were synchronized with 2.5 μ M α -amanitin (Sigma) for 2 hours, washed twice with PBS and released. 100 nM 5 α -dihydrotestosterone (DHT, Sigma) was added to the starvation media to stimulate the cells.

Small Interfering RNA

siRNA-mediated knockdown was achieved by transfecting cells with Lipofectamine 2000 and specific siRNAs. The following siRNAs were used for this study: AllStars Neg. Control siRNA (1027281) was from Qiagen. Human ON-TARGETplus SMARTpool siRNAs against TOP1 (L-005278-00-0020), MRE11 (L-009271-00-0020) and RAD50 (L-005232-00-0005) were purchased from Dharmacon. Single interfering RNAs targeting AR (SASI_Hs01_00224483, SASI_Hs01_00224484), TOP1 (SASI_Hs02_00335354, SASI_Hs01_00047440), ATR (SASI_Hs01_00176270, SASI_Hs01_00176271) and NKX3.1 (SASI_Hs02_00341026, SASI_Hs01_00018365) were obtained from Sigma. Multiple siRNAs were used during the course of the study to confirm data reproducibility. Additionally, siRNA sequences are listed in Supplementary Table 2. For transfection, LNCaP cells were seeded on dishes in RPMI-1640 supplemented with 10% FBS and allowed to attached overnight. The following day, the cells were washed twice with

PBS and fed with phenol-free DMEM supplemented with 5% charcoal:dextran FBS. One day later, the cells were transfected using Lipofectamine 2000 and 20 pmol mL⁻¹ siRNA diluted in Opti MEM reduced serum media without phenol red (Life Technologies). The transfection media was removed after 16 h incubation and the cells were washed twice with PBS. Fresh, phenol-free DMEM supplemented with 5% charcoal:dextran FBS and Penicillin/Streptomycin was added to the dishes. Cells were harvested 48-72 h post transfection. All siRNAs used in this study were validated by vendors or by us and used only if providing >70% knockdown efficiency. Relative quantities of gene expression level were normalized to the *GAPDH* gene. The relative quantities of ChIP samples were normalized by individual inputs, respectively.

ChIP-qPCR

Chromatin immunoprecipitation experiments were done as previously described (Garcia-Bassets et al., 2007). All ChIPs and qPCRs were repeated at least thrice and representative results were shown. *P*-values were calculated by using a two-tailed Student's *t*-test. Primers are listed in Supplementary Table 1.

GRO-seq and PRO-cap

Global run-on sequencing (GRO-seq) was performed as detailed (Wang et al., 2011) and precision nuclear run-on sequencing of transcription initiation sites (PRO-cap) was performed as described (Kwak et al., 2013).

Antibodies

AR (N-20), TOP1 (H-300), ATR (N-19), RAD50 (H-300), XRCC1 (H-300), BLM (H-300), DNA Ligase I (C-21), DNA Ligase IV (H-300), DNA POL α (C-21), DNA POL β (H-300), DNA POL γ (H-300), p300 (C-20), SRC-1 (M-341) were from Santa Cruz Biotechnology. MRE11 (Ab397) and p-S1983-ATM (Ab2888) were obtained from Abcam. Ku80 (A302-627A) and EXO1 (A302-639A) were purchased from Bethyl Laboratories.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for all sequencing data reported in this paper is GSE63202.

Supplemental Information

Supplemental Information includes Extended Experimental Procedures, five figures and four tables and can be found with this article online at <http://>

Author Contributions

J.P. and M.G.R. conceived the project. J.P., A.K.A and M.G.R. designed experiments. J.P. performed most of the experiments reported. P.K. performed bioinformatics analyses. W.L. performed GRO-seq experiments and Y.T. performed the modified PRO-cap experiments. T.S. carried out screening experiments, while Z.L. generated biotin-TOP1 stable cell line and carried out TOP1 ChIP-seq. J.Z. and K.A.O. assisted in deep-sequencing library preparations and sequencing. J.P., A.K.A and M.G.R. wrote the manuscript with input from P.K.

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CHAPTER 4 BARCODING OF CELL TYPE RESTRICTED ENHANCERS BY ESC TRANSCRIPTION FACTORS LICENSES THEIR ROBUST DEVELOPMENTAL ACTIVATION

Abstract

While cell-type-restricted enhancers are initially detected following cooperative binding of cell-type-restricted DNA binding transcription factors during determination/differentiation (Buecker and Wysocka, 2012; Heinz et al., 2015; Ong and Corces, 2011; Spitz and Furlong, 2012), it remains unknown whether there are preceding events in embryonic stem cells (ESCs) that are functionally important to activate cell-type-restricted enhancer networks. Here, using murine macrophages as a model, we report that, while largely devoid of characteristic enhancer marks (H3K4me¹, H3K4me², H3K27Ac, H3K27me³ and p300) in ESCs, macrophage enhancers are activated as transcription units mainly by the binding of a single, at most two, ESC-specific transcription factor. This provides “premarking” of these enhancers, as is also observed for other cell types. In contrast, ESC active enhancers are cooperatively bound by multiple ESC transcription factors. Interestingly, the strength of this signature in ESCs is functionally important for subsequent robust cell-restricted enhancer activation during differentiation events, as independently demonstrated by analysis of multiple *Esrrb* motif-deleted macrophage-restricted enhancers. The ENOS-determined location of hydroxymethylation of the enhancer in ESCs could serve as a potential molecular memory for subsequent robust binding of Pu.1 and enhancer activation in the mature macrophage. These findings suggest that the massive repertoire of cell-type-restricted enhancers are essentially hierarchically and obligatorily “barcoded” by binding of a single ESC transcription factors in ESCs, and strength of binding of this ESC transcription factor dictates enhancer activation in mature cells.

Enhancers function as critical regulatory elements that integrate genomic information

for cell fate transition and cell specific gene regulation programming, both in cell models and *in vivo* (Maston et al., 2012; Miguel-Escalada et al., 2015; Ong and Corces, 2011) often functioning as clustered/super-enhancers for many developmentally and pathologically important gene targets (Whyte et al., 2013)(Hnisz et al., 2013a). Their activation is key to understanding the mechanisms by which so many cell types emerge from stem cells harboring the identical genomes. Macrophage development provides a potent model for investigation of enhancer activation events because the sequential events leading to its differentiation and regulation by inflammatory signals are rather well understood (Baldridge et al., 2011; Epelman et al., 2014; Huang et al., 2013; Kaikkonen et al., 2013; Ostuni et al., 2013; Wu et al., 2014). Most of the ~22,000 macrophage enhancers present in terminally-differentiated macrophages compared to enhancers in ESC are ultimately activated based on the co-recruitment of the late lineage determining factors, Pu.1 and CEBP α , and are marked by H3K4me² and H3K27Ac marks (Creyghton et al., 2010; Glass and Natoli, 2016; Heintzman et al., 2007; Heinz et al., 2010a; van Oevelen et al., 2015). To begin to address the hypothesis that cell-type-restricted enhancers might be marked in ESCs, the full repertoire of macrophage-restricted enhancers in ESCs was examined. We found that the vast majority (18,405) exhibited an absence of H3K4me¹, H3K4me², H3K27Ac and H3K27me³ marks (referred to as “premarked in the Fig.1a), although ~4000 enhancers, active in both macrophages and ESCs and including “housekeeping genes”, did exhibit H3K4me² and H3K27Ac marks, generally within 200kb of coding genes. Finally, a small number of macrophage-restricted enhancers (214) had marks of “poised” enhancers, H3K27me³ (**Fig.1a,b**). To permit further understanding of enhancer features in ESCs, an ATAC-seq assay was performed both in mature macrophages and in ESCs. We found that the 18,405 “H3K4me¹, H3K4me², H3K27Ac, H3K27me³ and p300 unmarked” macrophage enhancers were in an “open” configuration compared to random regions (**Fig.1c**), consistent with

published DNase hypersensitivity analyses in ESCs (**Fig.1c**), but clearly these were not as robustly marked compared to active enhancer in ESCs (**Extended data Fig.1a**). To identify the transcription factor more specifically that might underlie the macrophage enhancer which has “open” chromatin configuration, we profiled the distribution of several of the most important ESC transcription factors -Esrrb, Nanog, Oct4 or Sox2 (ENOS)-, and interestingly, we observed that the binding of ENOS in 6,775 of macrophage enhancers with a -1kb/+1kb window- from Pu.1 bound enhancers (**Fig.57d**). Specificity of ENOS binding in macrophage enhancers in a -1kb/+1kb window was perceived by comparing ENOS binding in random regions (**Extended data Fig.57b**), and statistically significant binding of Esrrb, as one of example, in macrophage enhancer was observed by comparing Esrrb binding in random region and ESC active enhancer (**Extended data Fig.57c**). Cooperative binding of ESC factors is well studied; thus, we further explored their binding pattern in macrophage enhancers. To ensure that we were exclusively analyzing macrophage-restricted enhancers, ENOS binding in macrophage enhancer is examined in a -1kb/+1kb window, and interestingly, we found that ~80% of macrophage-restricted enhancers are bound by a single or mostly two ESC transcription factors, while active ESC enhancers exhibited binding of all four ENOS factors (**Fig.1e and Extended data Fig.57d**). Genome browser images show that macrophage enhancers are slightly open and bound by a single ESC transcription factor in the absence of enhancer histone marks in ESCs (**Extended data Fig.57e**). To determine whether cell-type-restricted enhancers in other cell types might also exhibit similar pre-marking in ESCs, we extracted cell-type-restricted enhancers from heart, kidney and N2A neuronal cells, finding that these enhancers in ESCs again exhibited binding of mainly a single ENOS factor and chromatin openness similar to that observed for the macrophage enhancers (**Extended data Fig.58a-d**).

To further study premarking events in macrophage enhancers in ESCs, we analyzed 12 of ESC transcription factors from the published literature in ESCs (Chen et al., 2008). We found that active ESC specific enhancers were characteristically bound by 4~8 of the 12 ESC transcription factors evaluated (Oct4, Sox2, Nanog, Esrrb, Smad1, E2f1, Tcfcp2l1, Zfx, Stat3, Klf4, c-myc and n-myc), consistent with their often cooperative binding (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008), while the great majority of the active macrophage-restricted enhancers exhibit binding of only one or two of these factors (**Fig.57f**). Taken together, these data indicate that cell type-restricted enhancers are in an “open” configuration, premarked by binding of generally a single ESC transcription factor. Therefore, it was important to investigate whether these open enhancer regions could interact with other genomic regions and mediate gene expression.

To begin to explore this question, we performed 4C-Seq (van de Werken et al., 2012). A macrophage-specific enhancer located 5' of *IL1a* transcription units on Chr.2 was selected and this Esrrb-bound “premarked” enhancer exhibited specific interactions with other genomic regions, including an upstream CTCF-bound site in ESCs (**Fig.57g**), but not with the cognate promoter it would ultimately regulate in macrophages (**Fig.57g**). In mature, KLA-treated macrophages, this enhancer exhibits robust interactions with the promoters of the cognate target coding gene (**Fig.57g**). An enhancer located in 5' of *Tnfaip3* was similarly found to exhibit specific interactions with other genomic regions, but not with the cognate promoter in ESCs (**Extended data Fig.59a**). In addition, we performed RNA-seq and examined proximal macrophage expressed genes of 6,775 macrophage enhancers in ESCs, and found 413 significantly expressed genes ($p < 0.01$) in macrophage, which has function under category of macrophage, but these genes are not expressed in ESCs (**Extended data Fig.59b,c**), with 4C-seq together suggesting that premarked macrophage enhancers are not functional even though there are ESC transcription factor binding with open chromatin configurations in ESCs.

Pu.1 is a transcription factor critically important in coding macrophage-specific enhancers (Heinz et al., 2010a). To further understand the potential impact of ENOS binding in ESCs with subsequent function on Pu.1-bound enhancers, we assessed the physical location of ENOS binding in premarked enhancers. When we plotted ENOS binding centered on Pu.1 binding sites in a -1kb/+1kb window in the macrophage enhancers, we observed that the ESC transcription factor binding varies in the 6,775 macrophage enhancers, corresponding to the location of cognate binding sites (**Fig.58a**).

As eRNA expression is a mark of enhancer activity (Li et al., 2013), the possibility that transcription units were present in the ENOS-bound premarked enhancers was examined. Because the GRO-seq data was insufficiently robust on these enhancer regions to draw clear conclusions, we performed PRO-cap in ESCs and macrophages. 2,336 of significant Cap sites were identified in -1kb/+1kb window in 6,775 premarked enhancers in ESCs and, interestingly, were found to be distinct from that observed in the active enhancers in mature macrophages (**Fig.58b**). By centering on the Pu.1 binding site, Cap sites were found to be located in close proximity to the Pu.1 site in the macrophage, while it is observed broadly, at various distances from the Pu.1 site, in ESCs (**Fig.58b**), with the sites correlating closely with the site of binding of the ENOS factors. The median distance from the Pu.1 binding site to the macrophage specific eRNA Cap was measured by selecting Cap sites within -500bp/+500bp from Pu.1 binding site to only consider significant transcriptional start sites, as expected, ~105 bp in the mature macrophage (Li et al., 2013), while the median distance from the ENOS binding site to the ESC specific eRNA Cap was found to be ~160 bp (**Fig.58c**), suggesting that ENOS factors serve in ESCs as transcription factors that activate the enhancer as a transcription unit in the stem cell state. This ESC-induced transcription unit for each enhancer is therefore distinct from the eRNA transcription units that will subsequently be nucleated by Pu.1 and CEBP α in the mature macrophage, the binding sites of which are located within a “core” enhancer region.

Indeed, ATAC-seq revealed chromatin openness in the region where ENOS factors bound to the enhancers (**Fig.58d**).

Given the well-established role of the Cohesin complex in chromatin architecture and gene regulation (Hadjur et al., 2009; Phillips-Cremins et al., 2013; Schmidt et al., 2010) (**Fig.57g**), we examined whether the Cohesin complex plays a role in premarked enhancers. We performed ChIP-seq for a Cohesin core subunit (Rad21) and used published data for Smc1 binding to examine their binding to premarked enhancers, finding that Cohesin colocalized with ENOS-bound regions (**Fig.58e**), and that high Rad21 binding was associated with high ENOS binding with a more open chromatin configuration (**Fig.58f**).

All observations were made from the experiments with ESCs cultured in serum, and ESCs in serum are not completely naïve (Ying et al., 2008). To escape any concern whether the serum culture condition impacted our results, we performed ChIP-seq with H3K4me² and H3K27Ac, and ATAC-seq under two different culture condition, 2i and serum. H3K4me² and H3K27Ac were not observed in macrophage enhancers in both 2i- and serum- cultured ESCs, and ATAC-seq signal was equivalently detected in both conditions (**Extended data Fig.60a,b**), suggesting that culturing ESCs in serum culture condition did not impact our observations.

To investigate the role of binding of stem cell transcription factors in ESCs to ultimate activity of the enhancers in mature macrophages, we first examined the possible correlation between recruitment of ENOS factors in ESCs to the ultimate activity of the enhancers in the mature macrophage. We first examined ~20% of ENOS-bound macrophage enhancers with the highest or lowest levels of ENOS transcription units among the 6,775 premarked enhancers, and then evaluated their ultimate activation in the mature macrophage. We found that the higher group of enhancers in ESC ultimately exhibited the higher “activation” as determined by binding of the macrophage transcription factors Pu.1, PRO-cap signal, eRNA

transcription, H3K4me², H3K27Ac and binding of Cohesin (**Fig.59a-d**). In addition, ~20% of the highest or least active macrophage enhancers were selected based on GRO-seq signal, reflecting the activity of the enhancers (Li et al., 2013), and ESC features were examined in these enhancer. ENOS binding and ATAC-seq signal in ESCs proved to be higher in the most active macrophage enhancers compared to less active macrophage enhancers (**Fig.59e,f**). Indeed, interestingly, we observed that putative functional macrophage enhancers defined by GRO-seq signal in macrophage are much more frequently found in 6,775 enhancers which have ENOS in a -1kb/+1kb window compared to the rest of 11,630 macrophage enhancers which do not have ENOS in a -1kb/+1kb window (**Fig.59g**).

To obtain a general idea whether the this positive tendency apply to the other tissues, we examined H3K4me¹ and H3K27Ac level at ~10% of the highest- or lowest- ENOS bound enhancers from four different tissues- spleen, lung, cortex and bone marrow- finding that high binding of ENOS in ESCs results in high levels of H3K4me¹ and H3K27Ac in differentiated tissues (**Extended Data Fig.61a-d**).

Because these data suggested that the actions of binding of stem cell transcription factors on macrophage-restricted enhancers in ESCs was actually predictive of the ultimate efficacy of the enhancer in the mature cell, it was important to test whether the binding of these factors in ESCs exerted a direct role on the ultimate activation of the cell-type-restricted enhancer in the mature macrophage. We therefore selected a series of macrophage-restricted enhancers that were bound by one of the ESC factors in ESCs, choosing enhancers linked to coding target genes that would not be predicted to influence macrophage development. We first selected the most prominent enhancer near the *Tlr1* locus and *Tnfaip3* locus, which was selectively bound by Esrrb, in proximity to the ultimate Pu.1 binding site, but that harbored no conventional histone enhancer marks at the ESC stage (**Fig.60a,e**). The experimental strategy was to use CRISPR-Cas9 technology to selectively delete the Esrrb binding site, and then to

initiate the programed differentiation protocol (Zhuang et al., 2012) to obtain “mature” macrophages in 20 days. The mature macrophages were selected based on their ability to adhere to the non-adherent culture plates, as confirmed by expression of Cd11b and F4/80 (**Extended data Fig. 6a**), permitting only a limited harvest of mature macrophages, thus precluding global genomic analyses. Significantly, three independently-derived individual clonal lines (#3, #10, #14) in Tlr1 enhancers were sequence-proven to harbor a deletion of the Esrrb site, with an 8 bp deletion (**Extended data Fig. 6b,c**). Consistent with the confirmed deletion, Esrrb binding in ESCs was inhibited in these mutant clones (#3, #10, #14) compared to wild type (wt) clones (**Fig. 60b**). The cells harboring the deleted Esrrb site were differentiated to macrophages with equivalent efficiency to those with the wild-type site; ChIP for Pu.1 and H3K4me² was performed, and eRNA was measured followed by Q-PCR of the enhancer in the ESC derived macrophages (ESDM). These analyses revealed that Pu.1 binding was inhibited, the KLA-induced transcription of the eRNA was lost, and there was a consistent decrease in the level of the H3K4me² mark with deletion of the Esrrb site (**Fig. 60c**). Mutant clonal cells in Tnfaip3 enhancer were constructed with either a 16 bp deletion (#26) or a 21 bp deletion (#45) encompassing the Esrrb (**Fig. 60e, Extended data Fig. 6d,e**). The wt and mutant (#26, #45) ESCs were differentiated to macrophage and enhancer activities were tested, finding again that the Pu.1 recruitment, eRNA transcription and H3K4me² level were all inhibited in mutant cells coinciding with inhibition of Esrrb binding in ESCs (**Fig. 60f,g**). As additional examples, two other loci, *Prdx5* and *Nod2*, were further studied (**Extended data Fig. 7a,b,d,e**). The wt and independently-derived Esrrb binding sites mutant clones were differentiated to macrophage, finding that eRNA transcription levels were inhibited in three different mutant clones (B11, F1, G11) at the *Prdx5* enhancer (**Extended data Fig. 7c**) and two different mutant clones (D2, D12) at the *Nod2* enhancer (**Extended data Fig. 7f**) in ESDM, consistent with Esrrb inhibition in ESCs (**Extended data Fig. 7c,f**). Because

modifying the genomic locus could potentially result in different genetic events, we performed RNA-seq in the Tlr1 enhancer clonal cells (wt, #10, #14) to test whether there is transcription change in these clonal cell lines and we found that normal ESCs and clonal ESCs (wt, #10 and #14) exhibited same pattern on transcription in the Tlr1 loci (**Extended data Fig. 6f**).

The enhancer harboring the Esrrb site deletion was evaluated by luciferase reporter assay for any potential impairment of enhancer function compared to the wt enhancers in mature macrophages due to genomic sequence disruption, finding that it was competent to increase reporter expression as wt enhancers in immortalized Raw 264.7 macrophage cells (**Fig.60d**).

Even though the number of enhancers we could target was constrained because of sequence requirements for CRISPR-Cas9 site-specific deletions, we were fortunately able to obtain mutated clones for four different regions, putative Tlr1 enhancer, Tnfaip3 enhancers, Prdx5 enhancers and Nod2 enhancers as described above, and this analysis corroborated a functional importance of ESC factor premarking in macrophage-restricted enhancers.

Poised enhancers, another class of well studied cell type specific enhancers, are often found near lineage-determining factors (Liber et al., 2010; Rada-Iglesias et al., 2011; Wang et al., 2015; Xu et al., 2007), which are usually regulated by super-enhancers (Whyte et al., 2013)(Hnisz et al., 2013a), and ~214 macrophage enhancers exhibit poised chromatin signatures in the ESCs (**Fig.57a**). Many are super-enhancers near genes encoding determining/pioneer transcription factors, exemplified by *Pu.1*. Interestingly, these poised enhancers are marked by binding of 2~4 ENOS factors in the core of their enhancers in ESCs, in contrast to the premarked cell-type-restricted enhancers generally bound by only one ESC factor. (**Fig.57f, Extended Data Fig. 8a,b**). For example, we noted that the *Pu.1* gene has four clustered enhancers with poised chromatin signatures in ESCs, in contrast to the two enhancers (enh2 and enh3) are active in mature macrophages, other two bound (enh1 and enh4)

by *Esrrb* and *Oct4* are not active in mature macrophage (**Extended Data Fig. 8b,c**). These two premarked enhancers in ESCs might participate in the early activation of *Pu.1*, which is known to exhibit a positive feedback loop on the two *Pu.1*-binding enhancers (Will et al., 2015).

A particularly intriguing question raised by the observations of enhancer “premarking” is how the marking of future cell type restricted enhancers might be “remembered” for their ultimate activation later in development. There are several non-exclusive possibilities that merit consideration. Based on the literature and Q-PCR analysis of the mRNA levels of the ENOS stem cell transcription factors, the temporal pattern of disappearance of these factors, such as *Oct4*, virtually coincides with the appearance of first lineage determining factors, *Tal1*, *Gata2* and *RunX1*, followed by the appearance of *Pu.1* and *C/EBP α* , which initiate the activation of the large macrophage enhancer activation program (Lichtinger et al., 2012) (**Extended data Fig. 8d**). Another explanation is that binding of one of the stem cell transcription factors on the future cell-type restricted enhancers might be accompanied by a specific DNA demethylation event that serves to ensure that an enhancer remains accessible to TFs, the binding of which may be impaired by DNA methylation. We therefore analyzed available data in ESCs regarding DNA methylation and, interestingly, we found that the 5-hmC mark in the 6,775 premarked macrophage enhancers (**Fig.61a**). Tet1 was also present in ENOS bound macrophage enhancers in ESCs (**Fig.61a**), reflecting the previous observation of presence of TET1 in a complex with *Esrrb* and *Oct4* (Gagliardi et al., 2013). Interestingly, we further found that knockdown of *Esrrb* inhibits 5-hmC levels in *Esrrb*- bound macrophage enhancers in ESCs (**Fig.61b**). To examine whether 5hmC is maintained during the differentiation process, so it could serve as a marker for molecular memory, we examined 5hmC during hematopoiesis using published data (Han et al., 2016), and finding that 5hmC is maintained during hematopoiesis (**Fig.61a,c,d**). The enhancer histone marks, H3K4me1 and

H3K27ac, are further studied during hematopoiesis (Lara-Astiaso et al., 2014) and as we observed, H3K4me¹ and H3K27Ac are not found in ESCs, and interestingly even not in mesoderm, and gradually gaining these marks early in hematopoiesis (**Fig.61c,e**).

Conclusions: Investigation of the premarking of cell type restricted enhancers in ESCs has provided a surprising insight into the process of genomic enhancer recognition underlying cell-type-specific transcriptional programs. Thus, it appears most, if not all, cell type restricted functional enhancers, perhaps $\sim 2-4 \times 10^5$ genome-wide, are marked in the stem cell stage by binding of a single, or occasionally two, canonical stem cell transcription factors, in a varied areas of the enhancers, while ESC enhancers are bound by multiple ESC transcription factors in the core of the enhancers.

ESC factor binding in ESCs appears to be of functional importance for its ultimate robust binding of the subsequently-expressed transcription factors required for activation in the differentiated cell. Indeed, the more robustly a “premarked” macrophage-restricted enhancer binds to the ESC transcription factor, along with Cohesin, and exhibits an “open” configuration and expression of a ncRNA transcript distinct from the ultimate eRNA, the more likely that enhancer is to be strongly active in the mature cell. It is tempting to speculate that the molecular memory is provided by the altered methylation state of the core region near the Pu.1 site consequent to the binding and activation in ESCs by binding of ESC transcription factors.

In this study, we used murine ESCs to propose premarking events, then, next consequent question was whether human ESCs also could have same premarking events because there are differences between mouse ESCs and human ESCs, such as poor expression of *Esrrb* in human ESCs. However, we think that since ESC factors are equally important

based on our observation from ENOS, other ESC factors assume this role in premarking cell type restricted enhancers in human ESCs.

The marking of the cell type restricted enhancers in ESCs thus serves as an obligatory code to license activation of these enhancers during development and differentiation, and apparently functions to not only to help preclude the premature interaction with the ultimate cognate coding gene promoter, but also to license the ultimate robust activation of the cell type restricted enhancer in the mature differentiated cell (**Fig.61f**).

Methods

Cell culture

46c murine ESC was kindly gifted by Austin Smith. It is grown in feeder free condition as previous described (Ying et al., 2003). ESCs were maintained in serum culture medium with DMEM-KO(Invitrogen 10829-018) supplemented with 15% ESC qualified-fetal bovine serum (Omega, FB-05), 2 mM nonessential amino acids (Invitrogen 11140-050), glutamax (Invitrogen 35050061), penicillin/streptomycin (Invitrogen 15140122), 2-mercaptoethanol (Sigma, M7522) and 1000 U/ml LIF (ESGRO, ESG1106). ESCs in 2i medium were grown in N2/B27 media with 50% Neurobasal (Gibco 21103-049) and 50% DMEM/F12 (Invitrogen 21331-020), 2 mM nonessential amino acids, glutamax, penicillin/streptomycin, 2-mercaptoethanol, N2 supplement (Invitrogen, 175020-01), B27 (Invitrogen, 17504-001), 1000 U/ml LIF, and 2i (ESGRO, ESG1121). Peritoneal macrophage is obtained from 6-8 weeks of female C57BL/6J mice obtained from Jackson laboratory. Mice are injected with thiogycollate 3-4 days before collecting day, and cultured in RPMI supplemented with 10% FBS and penicillin/streptomycin for overnight before collection. All animal care and experimental procedures were in accordance with the University of California, San Diego research guidelines for the care and use of laboratory animals.

Antibodies

Antibodies used for ChIP/ChIP-seq included: Rad21 (abcam, ab992), H3K4me2 (upstate, 07-030), Pu.1 (Santa cruz, sc-352) and H3K27Ac (active motif, 39133); for immunostaining, F4/80-FITC (ebioscience, 11-4801-81) and CD11b-PE-Cyanine5 (ebioscience, 15-0112-81); for MeDIIP, 5hmC (Active Motif, #39769).

Enhancer reporter assay

For construction of *Tlr1* enhancer reporter plasmids, 600bp of *Tlr1* locus spanning Esrrb binding site was PCR amplified and cloned into pGL4.23 at the KpnI/XhoI sites downstream of the luciferase reporter gene. Enhancer reporters were transfected into RAW264.7 macrophages using lipofectamin 3000 (invitrogen), using 200 ng of enhancer reporter and 5 ng of Renilla. Luciferase activity was measured 48 h after transfection using a Veritas microplate luminometer (Turner Biosystems) and normalized to Renilla activity to correct for differences in transfection efficiency. Experiment was performed five independent times. P-values were obtained using Welch's two t-test and data were represent with median $\pm$ s.d..

ESC differentiation to macrophage

ESC differentiation to macrophage was performed as described previously (Zhuang et al., 2012). Shortly, ESCs were trypsinized and transferred to bacteriological plates in macrophage differentiation medium A which has 15% L929 conditioned medium and 1ng/ml IL3 but lack of LIF for 6~8days to make embryoid bodies (EBs). EBs were transferred onto gelatin-coated tissue culture dishes for 3~4 days. After that, supernatants of adherents EBs containing floating macrophage progenitors were collected and plated onto bacteriological dishes for 7 days to obtain adherent macrophage in macrophage differentiation medium B which has 15% L929 conditioned medium). Medium was readed to the adherent EB plates and macrophage progenitors were obtained every 2 days.

CRSIPR/Cas9 assay

The online software (<http://crispr.mit.edu>) was employed to design optimal candidate sgRNAs to target proximal regions of Esrrb motif and these sgRNAs were cloned to vector PX459 (Addgene #48139), which co-expresses sgRNA and Cas9. This plasmid was transfected into ESCs with Lipofectamine 2000 and 1.0 ug/ml puromycin was added 2 days after transfection. Cells were cultured for another 3 days then diluted to pick up single cell-derived clonal lines. DNA was extracted from these cells and PCR was performed to amplify fragment containing sgRNA-targeted Esrrb motif. Sequencing was applied to identify Esrrb motif mutated/deleted clones. sgRNA sequences and primer sequences are listed in supplementary table 2.

RNA preparation and RT-qPCR

RNA was isolated using Trizol (Invitrogen) or RNeasy kit (Qiagen). RNA is reverse transcribed using SuperScript III Reverse Transcriptase kit (Invitrogen). Quantitative PCR was performed using SYBR Green Master Mix (Biorad). The experiments were repeated at least three times as a biological replicates and P values were obtained using Welch's two t-test. Primers are listed in supplementary table 2.

RNA-seq

RNA-seq sequencing libraries were made from 100 ng of DNase-treated total RNA samples using TrueSeq stranded mRNA Library Prep Kit (Illumina, RS-122-2101, RS-122-2102) and performed according to the manufacturer's instructions. RNA-seq libraries were quantified with Qubit, clustered and sequenced using HiSeq 3000/4000 SR cluster kit (Illumina GD-410-1001) and HiSeq 3000/4000 SBS kit (Illumina FC-410-1001). RNA-seq reads were counted by HOMER software considering only exonic regions for RefSeq genes. EdgeR software was the utilized to calculate the significant expressed genes in different samples ($p < 0.01$). Cluster 3.0 with K-Means Cluster generated clustering results in

differentially expressed genes and the output was viewed using Java Treeview. Gene ontology analysis and genetic association analysis was performed using Metascape (<http://metascape.org>).

Pro-cap

Pro-cap and library preparation for sequencing was described previously (Kwak et al., 2013). Nuclei were prepared from ~40 million cells for run-on assay. And then run-on reactions were stopped and RNA was extracted with Trizol LS reagent (Invitrogen). Following DNase treatment, the RNA was fragmented. Biotin incorporated fragmented RNA was immunoprecipitated with anti-streptavidin beads (Invitrogen). Then these RNA was treated with 3'-dephosphorylated with polynucleotide kinase (Enzymatics) and precipitated. The RNA was dephosphorylated with calf intestinal phosphatase (NEB) and 5'-de-capped with tobacco acid pyrophosphatase (Epicentre). The reaction was stopped and RNA was extracted with Trizol LS, and libraries were prepared by ligating Illumina TruSeq-compatible adapters to the RNA 3' and 5' ends with truncated mutant RNA ligase 2 (K227Q) and RNA ligase 1 (NEB), respectively, followed by reverse transcription, cDNA isolation and PCR amplification. Final libraries were size selected on TBE gels to 60–110 bp insert size. Pro-cap results were trimmed to remove A-stretches originating from the library preparation. Each sequence tag returned by the Illumina Pipeline was aligned to the mm9 assembly using Bowtie2 allowing up to 3 mismatches. Only tags that mapped uniquely to the genome were considered for further analysis. Each sequencing experiment was normalized to a total of 10^7 uniquely mapped tags by adjusting the number of tags at each position in the genome to the correct fractional amount given the total tags mapped.

ChIP-seq

Cells were fixed with 2mM disuccinimidyl glutarate (DSG) (proteochem) for 45 mins and 1% formaldehyde for 10 mins, and followed by glycine for 5 mins. Nucleus lysates are

prepared by using sonication buffer with 1% SDS, and immunoprecipitation is performed with several different antibodies. After overnight incubation with antibodies, beads are added for another 3hrs, and washing is performed. Reverse-crosslinking is done for overnight at 65°C and DNA is purified using QIAquick Spin column (Qiagen). For ChIP-seq, extracted DNA is ligated to adaptors and deep sequencing is performed with Illumina's HiSeq 2000, 2500, or 4000 system according to the manufacturer's instructions. The first 48bp for each sequence tag returned by the Illumina Pipeline is aligned to the mm9 assembly using BFAST or Bowtie2. Only uniquely mapped tags are selected for further analysis. The data is visualized by preparing custom tracks on the UCSC genome browser using HOMER. Genomic binding peaks for transcription factor were identified using the findPeaks.pl command from HOMER with 8-fold enrichment over the input sample, 4-fold enrichment over local background, a minimal tag number of 16, and normalization to 10^7 mapped reads per experiment. For histone marks, initial seed regions of 500bp were considered to calculate enriched reads. Regions of maximal density exceeding a given threshold with a $FDR < 0.001$ were called as peaks. The peaks within $\pm 1,000$ bp apart from the RefSeq gene TSS site were considered to be promoter, and to focus the analysis on enhancers, peaks within 3kb of a gene promoter were filtered out.

4C-Seq

Chromosome conformation capture was performed as described previously (Werken et al., 2012). Briefly, 10 million cells were cross-linked with 1% formaldehyde for 10 min. Soluble chromatin was incubated with 400 units of HindIII (NEB) for overnight, and then intramolecular ligations were performed using 1000 U of T4 DNA ligase (NEB) for 4hr at 16°C under dilution. Chromatin was decrosslinked at 65°C and purified using several phenol and phenol-chloroform extractions and ethanol precipitation. The second restriction digestion was also performed overnight, using 50units of DpnII (NEB). Intramolecular interactions were ligated overnight, and RNaseA was treated in chromatin. DNA was ligated overnight and

purified as before and ultimately using Qiagen columns and subjected to inverse PCR (expand long-range PCR system; Roche Diagnostics) using first primer designed on the viewpoint and second outer primer designed beside the DpnII site. Both primers contained Illumina sequencing adapters and barcodes for multiplexing. PCR samples were purified using a Roche kit and quantified using a Qubit. We analyzed data using a bioinformatics pipeline previously described (van de Werken et al., 2012).

MeDIP-Seq

Genomic DNA for hMeDIP was isolated from cells using Qiagen's DNeasy Blood and Tissue Kit. Isolated genomic DNA was then fragmented through sonication using Diagenode's Bioruptor platform to 100-300bp, and the size distribution was confirmed through gel electrophoresis. Barcoded adaptors for Illumina sequencing were added to 1µg of fragmented genomic DNA per experiment, using the NEBNext Ultra II DNA Library Prep Kit for Illumina, following manufacturer's instructions. This protocol was stopped after adapter ligation and cleanup (and before any amplification steps), and the adapter ligated fragmented DNA was then used for hydroxy-methylated DNA pulldown. Denaturing, immune-precipitation, washing, and purification of hydroxy-methylated DNA were performed as described previously (Thu et al., 2009), with the following modifications. 1µl of 5-hmC antibody containing serum was used per IP reaction. Washing was done five times, with each wash for 15 minutes at 4°C. Also, DNA was eluted from beads using 200µL digestion buffer, incubated overnight with Proteinase K at 50°C, and purified using Qiagen's QIAquick PCR Purification Kit. Purified hydroxy-methylated genomic DNA was then further processed with NEBNext Ultra II DNA Library Prep Kit for Illumina at the step of PCR Enrichment of Adaptor Ligated DNA, as per manufacturer's instructions, continuing with the entire protocol to prepare libraries for Illumina sequencing. Data was mapped to mm9 using Bowtie2 with standard settings and peaks were found by using MACS with default parameter.

ATAC-seq

ATAC-seq was performed as described previously (Buenrostro et al., 2013). Nuclei were prepared from 50,000 cells and transpose reaction was performed for 30mins at 37°C. DNA fragments were amplified by PCR, purified and deep sequencing was performed with Illumina's HiSeq 2000, 2500, or 4000 system according to the manufacturer's instructions. ATAC-seq data was mapped to mm9 using Bowtie2 with standard settings. Tag directories with reads mapped to the mitochondrial chromosome filtered out were created. ATAC-seq peaks were identified using findPeaks.pl in HOMER with the settings: -style histone -size 75 -minDist 75 -minTagThreshold 6 -L 8 -F 8. BED files were created from Tag directories using the HOMER package.

Deep-sequencing

For all ChIP-seq, RNA-seq, 4C-seq, ATAC-seq, MeDIP-seq and PRO-cap, the DNA libraries were sequenced for 50cycles on Illumina's HiSeq 200, 2500 or 4000 system according to the manufacturer's instructions. Sequencing experiments were visualized by preparing custom tracks for the UCSC Genome browser.

Bioinformatic characterization of enhancer

The criteria for identifying Pu.1-H3K4me2 co-bound enhancer regions is that the distance from the center of a Pu.1 peak to the H3K4me2 peak-occupied region is $\leq 1\text{kb}$. ESC factor bound macrophage enhancers are defined by calculating the distance $\leq 1\text{kb}$ between ESC factor peak spanning region and Pu.1 bound macrophage enhancers. The active ESC enhancers or active macrophage enhancers are defined using H3K27ac (over 100tags) to examine 12 different ESC factor binding. The functional macrophage enhancer was defined using GRO-seq (over 20 tags) in macrophage, and these enhancers were used to count number of functional enhancers in ENOS bound enhancer or non-bound enhancer in a $-1\text{kb}/+1\text{kb}$. Highly active or less active macrophage enhancers were created after excluding non-active

macrophage enhancers by using GRO-seq (less than 5 tags) in macrophage. The comparison of tag intensity of ChIP-seq, ATAC-seq, PRO-cap and GRO-seq or distances between different categories are presented as boxplots by using normal scales. P-values are calculated using Welch's two t-test. To profile the distribution of ESC factors surrounding Pu.1-H3K4me2 co-bound enhancer regions, ChIP-seq signals surrounding Pu.1 peak centers were separated by 40 bins, and then were sorted by the tag numbers based on the distance to Pu.1 peak center.

GEO Data

The GEO data bases used in this study are listed in Supplementary Table 1. All deep Sequencing DATA is deposited in GEO under accession number GSE81681.

Author Contributions

H.S.K. and M.G.R. conceived the project. H.S.K. performed most of the experiments reported, with particular contributions from W.M. (CRISPR-Cas9), Y.T.(Pro-cap) and E.D. (4C-Seq); D.M. and Y.T. performed most of the bioinformatics analyses; T.S. performed the DNA methylation experiment; K.O. prepared samples for deep sequencing assays; Q.M. analyzed 4C-Seq; additional experiments/methods were contributed by M.F.; H.S.K., and M.G.R. wrote the manuscript with extensive help from Y.T. All the authors declare no conflict of interests.

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The dissertation author contributed to this study through performance of experiments related to DNA methylation and hydroxymethylation, as well as intellectual input.

CHAPTER 5 CHROMOSOMAL ENHANCER SYNTAX: SPATIALLY-DISTRIBUTED SUPER ENHANCERS AND SUBNUCLEAR STRUCTURAL ASSOCIATIONS DICTATE ENHANCER ROBUSTNESS

Summary

Since their initial description, enhancers have been considered to be transferable elements that regulate proximal coding gene transcription based on their intrinsic properties. However, an important, but minimally explored, question is whether the strongest regulated enhancers, although separated by vast linear distances on a chromosome, might functionally cooperate to yield higher levels of transcriptional robustness. Here, we report that estradiol-17 β (E₂)- induces eRNA-dependent 3D physical proximity of a cohort of the most highly-induced MegaTrans- and ER α -bound enhancers, altering their relative positions in the remodeled chromosomal architecture. Genome editing approaches reveal that this gained physical proximity of “first tier” ER α enhancers imposes cooperative enhancement of activation on the interacting enhancers and their coding targets. Our data reveal a previously unappreciated “spatially-redistributed mega enhancers”, with robust enhancer transcription apparently requiring both Condensin-dependent E₂-induced enhancer proximity and association with an interchromosomal granule (ICG). These events, possibly augmenting liquid-liquid phase separation (LLPS) in the confined volume of this sub-nuclear architectural structure, would favor massively-increased concentrations of critical cofactor machinery to impart robustness to the first tier enhancers.

Introduction

Mammalian development requires the precise coordination of expression of tens of thousands of coding genes in space and time, giving rise to a myriad of cell types. Studies of transcriptional control have established the importance of many different types of cis-regulatory modules of gene expression located at greater distances from the proximal gene

promoter, including enhancer, insulators and silencers (Ayer and Benyajati, 1990; Bulger and Groudine, 2011; Gaszner and Felsenfeld, 2006; Giles et al., 2010; Levine, 2010; Petrykowska et al., 2008). Enhancers serve as critical regulatory elements for transcriptional programs directing development, homeostasis and disease states (Bulger and Groudine, 2011; Sakabe et al., 2012; Shen et al., 2012; Yue et al., 2014).

Global genomic approaches have illuminated the genetic, epigenetic and architectural features that define active enhancers: DNase I hypersensitivity (Gribnau et al., 2000), enrichment for unstable nucleosome variants H2AZ and H3.3 (Jin et al., 2009; Wang et al., 2008) specific post-translation modifications of adjacent nucleosomes including H3K4me¹, H3K4me² and H3K27ac (Calo and Wysocka, 2013; Creyghton et al., 2010; Heintzman et al., 2007; Heinz et al., 2015; Shen et al., 2012; Thurman et al., 2012; Zentner et al., 2011b) and finally by the active transcription of noncoding enhancer RNAs (eRNAs) (Kim et al., 2015; Lai and Shiekhatter, 2014; Lam et al., 2013; Li et al., 2013; Li et al., 2016). Many enhancers are also bound by nuclear proteins serving as key signal transducers for the various cell signaling pathways involved in development or homeostasis (Carroll et al., 2006; John et al., 2011). These studies estimated a total of 500,000 to 1,400,000 enhancers in the mammalian genome (Consortium, 2012; Hah et al., 2011;; Shen et al., 2012; Thurman et al., 2012).

Prior research had demonstrated the increased potency of multimerized DNA regulatory elements in proximity (Carey, 1998). This principle, when applied to whole enhancers suggests that groups of enhancers may have unique properties separate from their individual constituents (Hnisz et al., 2013; Hong et al., 2008; Parker et al., 2013; Perry et al., 2010; Whyte et al., 2013). The importance of collections of clustered enhancers in transcriptional regulation is now clear, a phenomenon alternatively referred to as “shadow enhancers (Hong et al., 2008) “stretch enhancers” (Parker et al., 2013) or “super enhancers” (Hnisz et al., 2013; Whyte et al., 2013). “Shadow enhancers” in *Drosophila* development

collectively regulate transcription of developmentally important genes (Perry et al., 2010). Super enhancers are largely composed of collections individual enhancers with less than 12.5 kb separation between them, producing synergistically high transcriptional output of their coding target genes (Whyte et al., 2013). However, ~15% of enhancers referred to as super-enhancers contain only a single enhancer element and are therefore best considered to be singularly strong individual enhancers (Pott and Lieb, 2015).

The genome is divided into thousands of non-random “topologically associated domains” (TADs) (Dixon et al., 2012; Nora et al., 2012). These megabase-sized regions separated by boundary elements place constraints on the interactions of enhancers and genes in different TADs (Benedetti et al., 2014; Dixon et al., 2012; Nora et al., 2012; Sexton et al., 2012). The Fractal-globule model for organization, which suggests that DNA is a fractal globule rather than an equilibrium globule (McNally and Mazza, 2010) at scales of up to 10 megabases, was supported by Hi-C results (Lieberman-Aiden et al., 2009; McNally and Mazza, 2010). More recent data have further suggested that loop domains, presumed to be formed by the loop-extrusion activity of extrusion complexes particularly cohesion (Fudenberg et al., 2016; Sanborn et al., 2015) isolate chromosomal segments into regions permitting local gene activation events. While emphasis has been placed upon the results of Hi-C and 5C data that indicate extensive local interactions (de Laat and Duboule, 2013; Gibcus and Dekker, 2013; Schwarzer and Spitz, 2014) what has remained incompletely explored is the possibility that even enhancers in distant TADs can interact or be brought into physical proximity due to three-dimensional chromatin folding potentially forming an additional transcriptionally-functional enhancer network. In addition to models generated by Hi-C (Dekker and Mirny, 2016; Gibcus and Dekker, 2013; Nagano et al., 2013) and FISH (Shopland et al., 2006; Yokota et al., 1995) approaches; recent multiplexed FISH experiments have also provided further evidence for cell to cell variability in 3D chromosomal architecture

(Wang et al., 2016). The introduction of microscopy/deep- sequencing genome architecture mapping (GAM) (Beagrie et al., 2017), provides evidence of longer-distance interactions in a crosslinking-independent fashion.

Transcriptional program regulated by 17 β -estradiol (E₂) in breast cancer cells provides an accessible model to investigate potentially overlooked mechanisms of enhancer activation and control of transcriptional programs. Estrogen receptor α (ER α) is a ligand-dependent sex steroid-regulated transcription factor that mediates most of the biological effects of estrogens, primarily at the level of gene transcription (Carroll et al., 2006; Lin et al., 2004). Others and we have investigated the E₂ mediated transcriptional program in MCF7 breast cancer cells (Hah et al., 2011; Hua et al., 2009; Li et al., 2013; Liu et al., 2014). ER α binds to 30,000 to 40,000 EREs genome-wide, a subset of which (7,000-8,000) harbor the identifying histone marks of enhancers (Li et al., 2013). Functionally important ER α bound enhancers recruit a variety of co-factors including P300, SRCs, Mediator, Cohesin, Condensin I and II. But the molecular signature of the most potent ER α enhancers has proved to be the ER α dependent assembly *in trans* of a large complex of DNA binding transcription factors including RAR α , FoxA1, AP2 γ and GATA3 (MegaTrans) at the enhancer (Bojcsuk et al., 2016; Li et al., 2015; Liu et al., 2014). The MegaTrans complex is required to activate eRNA transcription and target gene transcription. Only 30% of these MegaTrans-bound enhancers fit the commonly used definition of super-enhancers. The most robust ER α regulated enhancers in MCF7 cells also exhibited the highest levels of eRNA transcription (Li et al., 2013).

In this manuscript, we provide evidence suggesting that a subset of the most robust ER α enhancers on Chr.21, bound by the mega-dalton sized MegaTrans protein complex, are brought into non-simultaneous spatial proximity by E₂ to form a dynamic E₂-regulated network. These strong ER α -bound enhancers (referred to as “first tier” enhancers) exhibit rapid, dynamic and non-coincident alterations in topography. These E₂-dependent alterations

in enhancer proximity proved to be dependent upon the function of the Condensin complex, which is necessary for the E₂-induced proximity of the enhancers. Gene editing approaches have revealed that many enhancers in this network function in a cooperative fashion, with disruption of certain strong ER α enhancers resulting in decreased robustness for a network of first tier ER α enhancers. In response to E₂, enhancers located in two A compartments separated by a central, long B compartment, appear to be brought into proximity and to become fully transcriptionally active. Surprisingly, as determined by RNA FISH, they become most robustly transcribed when co-localized in the same phase-separated interchromatin granule structure (ICGs), perhaps further seeding the process of LLPS in these ICGs (Brangwynne et al., 2009; Feric et al., 2016; Lin et al., 2015; Molliex et al., 2015). Taken together, our data provides a largely overlooked structural regulatory strategy, involving 3D redistribution of MegaTrans enhancers, which associate with subnuclear RNP bodies. These events facilitate dynamic, ligand-induced networks of enhancers to cooperatively activate the ligand-regulated transcriptional program.

Results

To investigate any potential relationships between robust ER α -bound enhancers located at great linear genomic distances within a chromosome, we found it advantageous to initially focus on a set of ER α enhancers and coding target genes located on Chr.21. Chr.21, the smallest among human chromosomes, harbors some of the well-characterized ER α enhancers and coding genes that are located in a wide range of genomic distances (**Figure 1.A**). Chr.21 is an acrocentric chromosome that harbors a nucleolar organizing region (NOR) (Dekker and Misteli, 2015; Henderson et al., 1972; Zentner et al., 2011a) in its p-arm. On Chr.21 we identified 132 ER α bound, H3K27Ac, H3K4me¹ marked enhancers amongst the total of 479 enhancers defined by ATAC-seq peaks and eRNA transcription. 39 of these enhancers were highly active based on E₂ dependent recruitment of ER α and multiple key

transcription machinery and pioneer factors such as MED1, PolII and FoxA1 (**Figure S1.A-D**). We identify them as “first tier” ER α enhancers in this manuscript. The other 93 ER α bound enhancers had considerably less E₂ transcription machinery (**Figure S1.A-D**), and had a minimal MegaTrans complex recruitment (Data not shown). We also used 89 active non- ER α enhancers as a control group in these analyses. As expected enhancers in this group had strong basal, E₂ independent recruitment of MED1, PolII and FoxA1, but no ER α binding. An initial assessment of the effects of E₂ on enhancer function was provided by ATAC-seq (**Figure 1.D**). Using chromatin openness as a surrogate for enhancer robustness , meta analysis revealed that the first tier enhancers exhibited most robust responsiveness to E₂ in contrast to the 93 weak ER α -bound enhancers. E₂ had no effect on the basally robust non-ER α -bound enhancers. To further characterize the first tier ER α enhancers, we examined the distribution of factors, including several classical co-activators for estrogen receptor. In order to depict the data in a visually accessible manner by incorporating a combination of criteria, including relative chromosomal location and ER α abundance we present ‘triangle plots’ (Figure 1.E-I), in which the area of the triangle corresponds to the total tag density from ChIP-seq data at the indicated enhancer position. The line height is proportional to the level of ER α recruitment. Top ten of the 39 first tier enhancers are listed in order 5’ to 3’ along Chr.21 (*NRIP1e1-3*, *NCAM2e1*, *TIAM1e1*, *DOPEY2e1-2*, *SH3BGR1e1*, *DSCAM1e1-2*, *TMPRSS2e1*, *UMODL1e1*, *TFF1e1* and *COL18A1e1*).

First tier enhancers showed strong recruitment of classic factors associated with regulatory elements such as p300 and MED1 (**Figure 1.E, F**). p300, a well-established cofactor for the estrogen receptor, exhibited a decrease recruitment to weaker ER α enhancers, and non-ER α enhancers following E₂ treatment indicating re-distribution of p300 pool to most active enhancers. This is consistent with the haploinsufficiency phenotype of CBP/p300 in humans (Murata et al., 2001). First tier enhancers were also highly marked by components of

the MegaTrans complex (AP2 γ and GATA3, **Figure 1.G, H**). These factors were much less recruited to the remaining 93 ER-bound enhancers. This was also the case for FoxA1 (**Figure 1.I**), which is both a pioneer factor for ER α -binding to enhancers (Hurtado et al., 2011) and a component of the MegaTrans (Liu et al., 2014). To evaluate if the first tier enhancers might be premarked even prior to E₂ treatment, we evaluated their methylation and hydroxyl methylation status (Thu et al., 2009). First tier enhancers were the most demethylated enhancers in the basal and E₂ stimulated state, compared to the other two categories (**Figure 1.J**). The seemingly simultaneous binding of so many different transcription factors on ER α enhancers indicated that these loci are “hotspots”, as previously described in the literature (Junion et al., 2012; Rada-Iglesias et al., 2012; Siersbaek et al., 2014a; Siersbaek et al., 2014b).

To explore the basal and E₂-induced alterations in Chr. 21 chromatin architecture, we performed Hi-C, as modified by the *in situ* fixation method to increase efficacy (Rao et al., 2014). Hi-C libraries were sequenced to a depth of >200x10⁶ unique reads for each condition, analyzed by generating z-scores of contact counts. These analyses indicated the broad area of B compartment spanning roughly 14Mb in the middle of the Chr. 21 *q* arm (**Fig. 1B**) and treatment with E₂ did not substantially alter either the A/B compartment or the chromosomal boundaries, as determined by insulation scores (**Fig. 1B, 1C, respectively**). When analyzed at a resolution of 1Mb the Hi-C data also indicated the presence of extremely long distance interactions (**Fig. 1C**), although, not providing the resolution to permit precise detection. This suggestion of long-distance interactions is consistent with the findings in ES cells using GAM methodology (Beagrie et al., 2017).

E₂-Induced Long-distance Interactions of Chr.21.

Chromatin capture methods, including Hi-C and 5C, favor detection of relatively short-range interactions falling in the tail end of distribution of distances between two loci. But these technologies are inefficient in detecting the entire distribution in a population of

cells (Dekker, 2016; Giorgetti and Heard, 2016). Hence we elected to employ multi-region FISH analyses (**Figure S2.A**), to be more effective at detecting dynamic architectural changes and putative long-distance interactions across Chr.21.

E₂ treatment (50 min) of ER α expressing MCF7 cells induced an increased proximity of the first tier ER α enhancers located at a wide range of genomic distances (5.7Mb to 30Mb) (**Figure 2.A, B, Figure S2.B**). Spatial proximity is defined by number of interacting pairs coming below a cut off distance that is determined based on the median distance between these loci in a non- ER α cell type (see **Figure S2.E** and Methods). E₂ dependent reduction in the spatial distance between these loci is also evident from the cumulative distribution plot of the measured distances between them (**Figure 2.C, Figure S2.C**). 2-4 % of all loci pairs examined exhibited basal levels of interaction, which were increased by 2- to 4-fold following the addition of E₂ (**Figure 2D and Figure S2.B**), with 9-10% of loci pairs approaching each other at a distance closer than cut off distance. Even with large genomic separation between these loci, E₂ stimulation brings them in a spatial proximity that is comparable to a typical enhancer promoter distance by theoretical calculation. For instance, *NR1P1* and *DSCAM-AS1* enhancer loci were counted as interacting when they came within a radius of 590 nm (**Figure. 2D**). This radius is similar to the amount of space taken up by 100,000 base pairs of DNA (Dekker and Mirny, 2016), which is approximately the length of DNA separating the *NR1P1* promoter from the *NR1P1e1-3* super enhancer.

We examined the effect of E₂ stimulation in inducing proximity between several regions across Chr.21 (**Figure S2.D**). We found that most of the first tier enhancers showed decrease in median distance between them upon E₂ stimulation; in contrast there were no significance differences in the spatial distances between a weak ER α enhancer and first tier enhancer or a non- ER α target and a first tier enhancer (e.g. *UBE2G2/DSCAM-AS1* and

BCP26/*TFF1* respectively (**Figure S2.D**). This suggests that E₂-induced proximity happens preferentially between first tier enhancers.

These data point to a non-linear relationship between one-dimensional genomic distance and spatial distance. To better visualize the effects of E₂ on chromosome conformation, we plotted the relative 3D median spatial distances between the *NR1P1* and seven other first tier ER α enhancers in $-/+E_2$ conditions, using *NR1P1* as the “viewpoint” (**Figure 2E**). Under basal conditions, there is a high similarity between genomic distance and spatial distance (**Figure 2.E**, top vs. middle panel). However, after 50 min E₂ treatment, the spatial distances no longer correspond to the linear distances between the regulated genomic loci. Thus, in E₂ treated cells, *DSCR3* becomes spatially closer to *NR1P1* than to *DOPEY2*, and *TFF1* becomes closer than *DSCAM-ASI*, relative to their linear genomic distances. The *NR1P1* and *TFF1* enhancers have a median spatial distance of 1.86 μ m under basal conditions and 1.52 μ m following E₂ treatment. If geometrically modeled by placing one locus at the center of a sphere with a radius R equal to the median spatial distance of cells, after treatment of cells with E₂ ligand for 50 minutes, the volume of this sphere would decrease from 26.95 μ m³ to 14.71 μ m³. The rate of interaction between these loci should therefore increase by ~82%.

We next examined the ‘kinetics’ of long distance interaction by DNA FISH using *NR1P1* and *TFF1* probes (27.3Mb apart) at different time points after treating cells with E₂. We could observe the interactions between these genomic loci at the earliest time point examined (5 min) (**Figure.2 F**). Interestingly, we observed variation in the fraction of alleles coming below proximity cut off. An elevated induction in proximity was observed at 5-20 min followed by a decrease during the 20-30 min interval. Fraction of proximal alleles again increasing at 40-60 min before reaching the baseline at 120 min (**Figure.2 F**). These data are consistent with a similar reported periodicity of ER α and its co-factor binding (Liu et al., 2014;

Metivier et al., 2003) after E₂ treatment, further suggesting the role of ER α and its co-factors in mediating such long distance interactions.

Dynamic Interactions of First Tier Enhancers

Based on these observations, an important question was whether E₂-induce proximity of multiple first tier enhancers there by bringing them together in time and space, or whether these are dynamic, transient interactions that occur relatively independently, more in accord with a “random walk” model of chromatin interactions (Sachs et al., 1995; Yokota et al., 1995). We therefore performed three-color FISH studies using 40kb fosmid based FISH probes against several first tier loci of interest (*TFF1/NRIP1/TIAM1* and *TFF1/NRIP1/COL18A1*). These experiments revealed that E₂ dependent two loci interactions between any two pair of loci were observed at least 8.5 times more frequently than three loci interactions in the case of *TFF1/NRIP1/COL18A1* and no simultaneous 3 loci proximity were observed for *TFF1/NRIP1/TIAM1* (**Figure 2.G**). Therefore we concluded that the long distance interaction is likely to follow a random walk model.

Next we explored whether any of these long distance interactions could be observed by complementary approaches utilizing proximity mediated ligation based strategies. We performed a 4C experiment using *TFF1e1* as the viewpoint (Stadhouders et al., 2013). This analysis revealed that there was a clear E₂-induced interaction between the *TFF1e1* and the *DSCAM-AS1e1* region that is located 2 Mb apart in two separate TADs (**Figure 2.H**). FISH analysis also confirmed a statistically significant E₂ induced proximity between *DSCAM-AS1/TFF1e1* interactions (**Figure 2.I**). It should be noted that according to FISH data 10-15% of *TFF1e* and *DSCAM-AS1e* loci came closer than 330 nm in spatial distance (**Figure 2.I**) that allowed this interaction to be effectively captured by 4C. This demonstrated that the long distance interactions can be captured by proximity- mediated ligation approaches if the loci come in close spatial proximity (~300nm) in a sufficient population of nuclei. However,

proximity of this extent in such a large fraction of nuclei was rarely noted in other much longer interactions we observed.

Effects of Induced Proximity on Gene Transcription

In order to test the functional relevance of E₂ induced architectural changes in chromosome, we asked if the induced proximity influence the transcriptional outcome of the proximal loci. We resorted to RNA FISH to visualize and localize the transcription. eRNAs are generally shorter species of non-coding RNA that make their visualization challenging. Therefore, we designed intronic probes against coding genes regulated by two strong enhancers that undergo E₂ induced proximity, namely *NR1P1* and *TFF1e*. Intronic probes allowed us to visualize the nascent RNA at the site of transcription. We used the intensity of the signal as readout for transcriptional robustness. Indeed, we observed a negative correlation between the median spatial distance between actively transcribing *NR1P1/TFF1* loci and robustness of transcription, when these loci were simultaneously expressed in the same nucleus (**Figure 2J and K**). Based on these data, we concluded that E₂ induced proximity enhances the robustness of the participating transcribing units in a cooperative manner. Interestingly, we also noted that active transcription of any of these loci occur only in ~ 30% of nuclei (**Figure S2.F**) and simultaneous expression of at least one allele each of *NR1P1* and *TFF1* in same nucleus is observed ~10% of cells. This in agreement with the fraction of *NR1P1* and *TFF1* loci that showed induced proximity in DNA FISH (**Figure 2.D**).

Functional Significance of The Induced Enhancer Interactome

The induced interactions between first tier enhancers separated by multiple TADs contrasts with paucity of similar observations from extensively available Hi-C datasets. Therefore, it became critical to determine the functional importance, if any, of E₂-induced interactions we observed using microscopy. We employed CRISPR-Cas9 based gene editing approaches (Cong et al., 2013; Jinek et al., 2013; Ran et al., 2013) to delete *NR1P1e3* and

TFF1e1 enhancer regions (**Fig. S3A,B**). To minimize potential off-target effects of gRNA used, we generated at least 4 different lines for each deletion using independent sets of gRNAs pairs. Homozygous genotype of the clones used was confirmed with PCR (**Figure S3.A, B**) and Sanger sequencing of the genomic DNA (Figure S3.C-S3.L). All the clones used in functional assays expressed comparable level of ER α protein and responded robustly to E₂ (**Figure S3.M**)

First, we used QPCR to test the transcriptional effect of first tier enhancer deletion on other first tier enhancers in Chr.21. To reduce clonal variation in gene expression levels, experiments were repeated using four independent clones. The largest effect of the enhancer deletion was observed on the cognate target genes. *TFF1e1* knockout (KO) resulted in 98% reduction in *TFF1* mRNA (**Figure 3.B**) and *NR1P1e3* KO reduced the *NR1P1* mRNA level by 50% (**Figure 3.A**). A relatively weaker impact of *NR1P1e3* deletion could be due to the fact we deleted only 1 out of 3 enhancers in the *NR1P1e3* super enhancer cluster. Interestingly, *TFF1e1* KO reduced the *TMPRSS2* transcript, a first tier ER α enhancer located 1 Mb away by 55% and *NR1P1e3*, that is located 27.3Mb away by 30% both under E₂ treated condition (**Figure 3.B**). Deletion of the *NR1P1e3* also demonstrated long distance effect; *TFF1* transcript was reduced by 35% and *TMPRSS2* reduced by 50% (**Figure 3.A**). Intriguingly, *NR1P1e3* enhancer deletion had a more striking effect on *TFF1e1* eRNAs, where both sense and antisense strand reduced by ~ 55% (**Figure 3.C**).

To quantitate the effects of the *TFF1e1* and *NR1P1e3* deletion genome-wide, we performed GRO-seq on the KO lines and compared them to wild type MCF7 cells that had undergone the same transfection and selection regimens as KO cells. A meta-analysis of the GRO-seq data found that both enhancer deletions had a significant effect on the transcription of E₂ induced eRNAs from the first tier ER α enhancers on Chr.21; in contrast, the less robust ER α enhancers and non-ER α bound enhancers were not affected (**Fig. 3D**). To better visualize

the effects of the *TFF1e1* and *NRIP1e3* deletions on the transcription of individual enhancers on Chr.21, we use “circle plots” where area of the circles correspond to the RPKM values of indicated first tier eRNA and length of the lines correspond the ER α ChIP-seq tag counts (**Fig.3E-J**). The raw data used to generate this plot is presented in Table 4. *TFF1e1* deletion affected the robustness of the E₂ induced eRNA transcription on many distal first tier ER α enhancers on Chr.21, most notably on *NRIP1*, *NCAM2*, *DSCAM1*, *UMODL1* and *COL18A1* enhancers (**Figure 3.G,H**) Similarly *NRIP1e3* deletion reduced transcription to varying degree of multiple tier1 enhancers, such as *TFF1e*, *DSCAM2e1*, *SH3BGR*, *DOPEY2e1-2*, *UMODL1e*, *TMPRSS2e1*, and *COL18A1e1*(**Figure 3.I, J**). Both deletions also affected coding transcription units controlled by the affected enhancers. The effects of the *TFF1e1* and *NRIP1e3* deletions on the *TFF1e1* eRNA and mRNA is illustrated by a browser image given in **Fig.3K**, that clearly shows nearly complete absence of transcription from *TFF1* coding genes in *TFF1e1* KO and a visible reduction in both coding transcript and eRNA in *NRIP1e3* KO. **Figure 3.L** shows the actual RPKM values of plus and minus *TFF1e* strands in indicated genotypes. Qualitatively similar data was observed in an independent GRO-seq experiment, with meta analysis of 39 first tier enhancers showing a ~80-90% Pearson’s correlation coefficient in each condition. Further, to exclude any indirect effects from reduction in *TFF1* gene product, we deleted the *TFF1e1* promoter, which abolished transcription of the *TFF1* coding gene. GRO-seq showed that this had no effects on transcription units outside of the specific TAD in which *TFF1* was located (data not shown) indicating that the effects of *TFF1e1* deletion reflected actions of the enhancer rather than any indirect effects of loss of the TFF1 protein. To test if the transcriptional effect seen extended beyond the first tier enhancers; we examined the eRNA expression from 82 non- ER α enhancers present in Chr.21 (**Figure S3.P**). This analysis revealed no change in eRNA transcription from these enhancers, further proving the specificity of effect on first tier ER α enhancers. Taken together we observed a

striking impact of deletion of first tier enhancers on other first tier enhancers that exhibit an induced proximity in E₂ treatment. These data validated the functional importance of individual first tier enhancers in contributing to the robustness of the regulated transcriptional program.

Effects of Enhancer Deletions on Long Distance Interactions.

In order to examine the contribution of first tier enhancer elements in E₂ induced proximity of first tier enhancers we performed DNA FISH analysis using these cells. These series of experiments demonstrated that both *TFF1e1* and *NR1e3* enhancer deletion significantly attenuated the E₂ induced proximity of *NR1e1* and *TFF1* (**Figure 4.A-C**). We also found that a minimal, 51-nucleotide deletion encompassing a FoxA1 motif at *TFF1e1* core (*TFF1e1*FM KO) 1.6 kb deletion thus alleviating the concern of non-specific effects resulting from larger genomic deletion (**Figure 4.A**). Similarly, E₂-induced proximity between *NR1e1* and *DSCAM-AS* was virtually abolished on deletion of the *NR1e3* enhancer (**Figure S4.A**), as was the closer proximity between *DSCAM-AS1* and *TFF1* upon *TFF1e1* KO (**Figure S4.B**).

We next wanted know if a first tier enhancer contributed to the induced proximity between two other first tier enhancers. Deletion of the *TFF1e1* resulted in a modest, but statistically significant, decrease in induced proximity between *NR1e1* and *DSCR3* (**Figure 4.D**). Similarly, in *NR1e3* KO cells E₂ induced proximity between *TFF1* and *DOPEY2*, another robust first tier ER α enhancer, was compromised (**Figure 4.E**). Based on these results we concluded that E₂ induced proximity between long distant first tier enhancers are driven by enhancer activity and that each enhancer contribute to the robust architectural changes of other component enhancers.

Potential Roles of eRNA in the Formation of the Connectome

Prior studies have shown that eRNAs are functionally required for the enhancer

activity and enhancer promoter looping (Blinka et al., 2016; Hsieh et al., 2014; Li et al., 2013). In order to examine the contribution of eRNA generated from first tier enhancers in transcription and induced proximity, we used ASOs that function to knockdown eRNA transcripts in a RNase H dependent fashion. FISH analysis revealed that *TFF1e1* knockdown caused a clear decrease in the interactions between *NR1P1* and *TFF1* regulatory regions (**Figure 4.F**).

We next addressed the contribution of first tier eRNAs in transcriptional output from other enhancers. We performed GRO-seq experiment after knockdown of *TFF1e1* and *NR1P1e3*. These data revealed that, analogous to effects of enhancer deletions, knockdown of the *TFF1e1* or *NR1P1e3* eRNAs caused impairment in the activation of the other 39 first tier enhancers, while having no effect on the other ER α -bound enhancers or the non-ER-bound enhancers (**Figure 4.G, Figure S4.C**). When plotted to visually depict the quantitative effects on ten of the most robust E₂-regulated enhancers, we observed that the pattern highly resembled those observed following genetic deletion of the *TFF1e1* and *NR1P1* enhancers (**Figure S4E-J compare to Figure 3.E-J**). These data suggest that eRNAs or/and enhancer transcription play critical roles in these extreme long-distance enhancer interactions, as well as in short range enhancer: promoter interactions (Blinka et al., 2016; Hsieh et al., 2014; Li et al., 2013). eRNA knockdown experiments provided independent validation that both transcriptional and chromosomal architectural phenotypes observed in CRISPR enhancer KO cells are genuinely contributed by enhancer mutations, not due to clonal variation. All these data support the hypothesis that transcriptional robustness of first tier ER α -bound enhancers is imparted by the long distance interactions between component enhancers.

The B Compartment of Chromosome 21

Because of its large size and strategic location close to center of q-arm of Chr.21, we surmised that the extended B-compartment might contribute to E₂ induced architectural features of Chr.21. Hi-C analysis revealed the presence of a B-compartment in Chr.21 spanning

mostly the region between 18-32 Mb (**Figure 1.B**). ChIP-seq analysis revealed that the extended B-compartment is decorated with H3K27Me³ and H3K9Me³ (**Figure S5.E**) thus fitting the epigenetic features of B compartments (Lieberman-Aiden et al., 2009; Rao et al., 2014). This 14Mb stretch of B-compartment is interrupted at three regions by small patches of A-compartment that showed depletion of heterochromatic marks, but recruitment H4K20me³ (**Figure S5.E**). A number of first tier ER α -bound enhancers (*NCAM2e1*, *TIAM1e1* and *NRIP1e3*), fall in the A-compartment near the extended B-compartment (**Figure 1.B**). While *NCAM2e1* falls in the A-compartment patch flanked by B-compartment, *NRIP1* is ~ 2.5Mb 5' and *TIAM1* is ~1Mb 3' to the extended B-compartment. We examined if the close positioning to B-compartment could alter the interaction pattern of these enhancers. DNA FISH studies revealed that these first tier enhancers had high basal interaction (10% between *NRIP1/NCAM2* and 13% between *NRIP1/TIAM1*). These regions do not show an E₂ induce movement, possibly because they are already in spatial proximity (**Figure 5.A-B**). Consistent with previous report (Lieberman-Aiden et al., 2009) we also noted that A and B compartment associate with each other at a much less frequency than two A-compartment regardless of the genomic distance. FISH experiment using a probe against *TFF1* (A compartment) and a B compartment (BCP26) revealed that 1.2 % alleles pairs came below cut off distance (+E₂ condition), while under same condition 9% of *TFF1-NRIP1* (both A compartment) that are located 27.3 Mb apart were below the cut off distance (7.5 fold increase over the former) (**Figure 5.C**). The difference was also evident at the median spatial distance between these loci (1.53 μ m for *NRIP1/TFF1* vs. 2.25 μ m for *TFF1-BCP* under + E₂) (**Figure S2.D**). Same trend was seen we did the experiment using probe against *NRIP1* (A-compartment) and B compartment (**Figure S5.A, B**).

We observed that B-compartment in Chr.21 significantly overlap with published Lamin Associated Domain data (**Figure S5.F**), while the first tier enhancers that are proximal

to this region is associated with A-compartments and avoid LADs (**Figure S5.F**). Since NOR in Chr.21 can associate with nucleolar compartment we examined the relative position of extended B-compartment in the nucleus. Immuno DNA FISH using B-compartment probe (BCP), Lamin B1 and Fibrillarin (a nucleolar protein) revealed that 55% of B-compartment is in close proximity with Lamin B1 or nucleolar surface (**Figure S5.G, H**). Using pairs of FISH probes inside B-compartment region we found that E₂ stimulation can result in a modest expansion of the B-compartment (**Figure 5. D, E, S5.D**). We hypothesize that tethering of extended B-compartment to two prominent nuclear structures provide a unique 3-D architecture to Chr.21. The relatively fixed association of B-compartment with these structures and the E₂ depended expansion might result in altered flexibility of the B compartment and thereby bring the flanking A compartments in close proximity (**Figure S5.C**).

Estrogen Reconfigures Chr.21 Topography of First Tier Enhancer Networks

Chr.21 is one of 5 acrocentric chromosomes (Chr.13,14,15,21,22) in the human genome that carry rDNA repeat sequences for ribosome biogenesis and those rDNA-containing regions tend to be important in forming a subnuclear structure, the nucleolus, for rDNA transcription, rRNA processing, and ribosome assembly (Henderson et al., 1972; Zentner et al., 2011a). ImmunoFISH experiments using *NRIP1*/*TFF1* probes and anti-Fibrillarin antibody indicated that *NRIP1* is located immediately adjacent to the outer surface of the nucleolus in the untreated cells. It relocates further into the peri-nucleolar region in E₂-treated cells when it interacted with *TFF1*. In turn, a sub-population of *TFF1* appears to relocate to the nucleolar periphery, where it engaged with *NRIP1* (**Fig.6A,B**).

We wished to determine whether structural proteins might be important determinants of the E₂-induced proximity of first tier enhancers. Because the binding of Cohesin to ER α -bound cognate enhancers was minimally altered by E₂, while binding of Condensins I and II were highly E₂-dependent (Li et al., 2015), it was important to test whether these first tier

long-distance interactions might require the actions of Condensins. By ChIP-seq analysis, we determined that the 39 first tier enhancers on Chr.21 exhibited striking E₂-dependent recruitment of both NCAPG and NACAPH2 (**Figure 6.J; Figure S.6D**), in contrast to minimal induction on the other 93 ER α -bound enhancers (**Figure 6.J; Figure S.6D**). Following knockdown of *RAD21* or *NCAPG* using specific siRNAs (**Figure S.6E**) and performing FISH analyses we found that knockdown of Condensin subunits caused a dramatic loss of E₂-induced proximity of first tier enhancers, while *RAD21* (Cohesin) knockdown caused only minimal effects (**Figure 6.K**). These data stand in contrast to the strong effects of Cohesin knockdown on short-range enhancer: promoter interactions (Li et al., 2013) indicating a specific, strong role of Condensin in these dynamic “long distance” induced proximity. This is consistent with recent observations in yeast, where Condensins are much more important than Cohesin for long range interactions (Kim et al., 2016). Given the initial data indicating that Condensins may be associated with matrix-like structures, these findings suggest that interactions with a subnuclear architectural structure may be a functional aspect of these first tier enhancer networks.

Interactions with Subnuclear Architectural Structures

Indeed, we had previously noted that a POU domain factor activating a broad enhancer program in developing pituitary cell lineages required interactions with specific subnuclear architectural structures Matrin3 network to permit enhancer activation and function (Skowronska-Krawczyk et al., 2014). These data indicated that at least a subset of enhancers require association with specific subnuclear architectural structures to be functionally activated, and it provoked the possibility that enhancer interactions occurring in the context of a subnuclear architectural structure that permits concentrating coactivator to contribute to the robustness of enhancer transcription. Based on these data, we initiated exploration of the potential E₂ dependent interactions of first tier enhancers in SC35-

containing interchromatin granules (ICGs), an RNA/RNP-generated representative of a Liquid liquid Phase Separated (LLPS) structure (Han et al., 2012; Kato et al., 2012; Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015).

By DNA FISH, we observed that *TFF1* loci associate with SC35 at a higher frequency (~22%) compared to *NRIP1* loci (**Figure 6.C,D**). We also observed a position- depended association of genomic loci with ICGs. Loci at 5' of Chr.21 (E.g. *NRIP1*) showed low association (5%) with speckle domain while those loci in 3' region associate a significantly higher rate (e.g. 50% association for *COL18A1*) (**Figure S.6A**). We next explored several first tier interacting enhancers (*COL18A1/DSCR3* and *TFF1/DSCR3*), finding that when these regions were in close proximity by FISH, they were located in the same SC35 granule in 50-55% of nuclei, as opposed to 8 -20% associations of more distant allele pairs with same structure when they did not exhibit proximity by FISH (**Figure 6.E, F**). The more pertinent question, whether the localization in SC35 granules was linked to transcriptional activity, was investigated using immunoRNAFISH (mNRIP1/ mTFF1/SC35). The data revealed that actively-transcribing *TFF1* was markedly associated within ICGs with 78% of the robust *TFF1* RNA loci colocalized with SC35 granules (**Figure S6.B, C**). The RNA FISH signal for NRIP1 was much less robust, but it was found that the signal was most robust when the *NRIP1* and *TFF1* loci were colocalized to the same SC35 granule, compared NRIP1 alone associating with ICG or when they are not in proximity to ICG (**Figure 6.G, H**). Similar observation was made for *TFF1* transcript too (**Figure 6.I**). This data, along with the observation that transcription of *NRIP1* and *TFF1* was enhanced by E₂ induced physical proximity (**Figure 2.J, K**), strongly support the idea that first tier enhancer cooperatively increase the transcriptional activity of each other in the presence ICG.

Many subnuclear architectural structures are themselves RNA:protein complexes that have undergone phase separation (Feric et al., 2016). This raised the possibility that

interaction of active enhancers on nuclear structures would raise the local concentration of various ER α co-activations, many which contain low complexity RNA binding domains. Based on the observation of induced proximity of associating loci in ICG enhancing transcriptional output we decided to test the hypothesis that LLPS might be an underlying mechanism that bring these first tier enhancers separated by vast genomic space to close physical proximity. To test this hypothesis we first disrupted the ICG using siRNA against SRSF1 (Pandit et al., 2013) finding that even with ~65% knockdown efficiency, the eRNA transcription of first tier enhancer was decreased >60 % (**Figure 6.L,M; Figure S6.G,H**). Finally, to test the effects of a aliphatic alcohol that melt the low complexity domain structures, we treated MCF7 cells with 1,6-Hexanediol (1,6-HD) (Lin et al., 2016). An exposure to 1,6-HD as short as 5 min virtually abolished robust transcription of the *NR1P1e3* and *TFF1e1* and a number of other first tier eRNA in a time frame of 50 min post exposure (**Figure 6.L,M; Figure S6.G,H**). These data strongly suggest the involvement of proteins harboring low complexity domains and LLPS in E₂ induced eRNA transcription and enhancer robustness.

Discussion

In this paper, we have uncovered a currently unappreciated functional consequence of the ligand-induced interactions between robust first tier enhancers separated by vast linear genomic distances, dictating the robustness of chromosome wide enhancer driven transcriptional regulation programs. While enhancers are well established to have a transferable ability to activate transcription of reporter genes (Banerji et al., 1981; Moreau et al., 1981), the observations described here reveal that the physiological robustness of the regulated first tier enhancers, at least in part, derives from their interactions with other strong, regulated enhancers located on the same chromosome. Using the actions of liganded estrogen receptor (ER α) in breast cancer cells as a model, our data indicates that ligand induces dynamic, asynchronous, increased proximity of a cohort of the most robust ER α bound

enhancers located in two A compartments on Chr.21 that are separated by a centrally located 14Mb long B compartment, which itself preferentially associated with the nucleolar surface and LaminA-rich nuclear membrane. Surprisingly the E₂-induced increased proximity of first tier enhancers caused an increased robustness of activation for the entire set of interacting enhancers.

Specifically, we find that proximity and interactions between *NR1H3* and *TFF1* enhancers, and several other first tier enhancers, are increased in response to E₂ in MCF7 cells and this appears to occur in a non-synchronous fashion after ligand treatment. We have found that, in the context of the intact chromosome, robustness of the E₂-induced strong enhancers, marked by the MegaTrans complex, substantially depends on cooperative interactions with other enhancers, in effect functioning as a dynamic chromosome-wide, ligand-induced, Megaenhancer. Specificity of these interactions is indicated by the fact that non-E₂ regulated enhancers and weak ER α bound enhancers are essentially unaffected by these interaction-dependent events.

Roles of eRNAs in Dynamic Induced Enhancer Interactions

Our study additionally revealed that the eRNAs of the strongest and most actively transcribed enhancers are involved with facilitating the actions of proteins/RNAs involved in long-distance looping interactions between enhancers and other regulatory elements. Our data offers further proof in support of prior examples of functional long-distance interactions between regulatory regions in diverse organisms and tissues that might be related to transcriptional efficacy, including examples of interchromosomal interactions (Beagrie et al., 2017; Fanucchi et al., 2013; Markenscoff-Papadimitriou et al., 2014; Proudhon et al., 2016). These are exemplified by experiments that inserted an extra β -globin locus control region (LCR) into the mouse genome (Noordermeer et al., 2011), with the ectopic LCR interacting with the endogenous *β h1* gene, interchromosomally, in 5-10% of cells. Indeed, the interaction

frequencies for distal ER α enhancers on Chr.21 are also in the range of 5-10% following E₂ induction. It has been suggested that the aggregation of olfactory receptor (OR) enhancers around an OR promoter results in the formation of a stable nucleoprotein complex, which is sufficient to cause a feedback-eliciting level of OR gene transcription resulting in a permanent OR choice in that specific sensory neuron (Markenscoff-Papadimitriou et al., 2014) however, whether this interaction occurs in a preexisting subnuclear structure or generates a novel one is not known.

Because of the observation that the induced interactions between first tier enhancers are dynamic and not simultaneous, our data is most consistent with a random walk, and implies a molecular memory of initially transient interactions. We suggest that these enhancer-driven events might therefore have some mechanistic similarities to V-D-J recombination events in B cell development, which involves a condensation of the genomic region, followed by a random walk until interaction of the DJ region with the V region results in cleavage and translocation (Lucas et al., 2014).

Implications of Functional Enhancer Network for Future Mechanistic Studies

The unexpected contributions of temporally transient enhancer interactions to modulating robustness of other first tier enhancers in the network raise the intriguing question: What are the underlying molecular mechanisms that impart enhancer robustness? Given the extensive linear distances between the first tier enhancers, it would seem unlikely that this process could be controlled by loop-extrusion (Bouwman and de Laat, 2015; Fudenberg et al., 2016; Sanborn et al., 2015) because that process is naturally limited by the presence of boundary elements (Fudenberg et al., 2016). Therefore, the challenge now is to unravel the mechanistic basis of the ability of these long-distance interactions to regulate the robustness of first tier enhancers brought into further proximity in response to ligand. In this regard, ideas about liquid-liquid phase separation (LLPS), as an underlying mechanism for enhancer

robustness becomes a tempting hypothesis, a problem that will require years of further investigation by the scientific community, using new technologies, to be fully resolved.

However, the findings in this manuscript do provide some initial clues regarding these questions, including several non-exclusive possibilities. First, even transient ligand-induced interactions between these enhancers could allow for rapid exchange/interaction of transcription factors/cofactors/chromatin remodeling components at higher local concentrations in the "first tier" enhancers (Tropberger et al., 2013). A highly attractive, complementary possibility of potential eRNA-dependent seeding of LLPS events centered at first tier enhancers which can ultimately lead to phase transitions in ICG. This might prove to be analogous to the formation of the nucleolus, resulting from the process of LLPS induced by transcription of the rRNA loci found in the nucleolar organizing regions (NORs) (Shav-Tal et al., 2005).

As RNP bodies tend to exhibit liquid-like physical properties after the process of LLPS, these suspended liquid droplets can fuse and split apart at rates determined by a variety of factors including viscosity (Brangwynne et al., 2011) as described for P bodies. The nucleolus, P bodies and probably other ribonucleoprotein (RNP) nuclear bodies, are membrane-less structures assembled through phase separation of their molecular components into internal subcompartments that represent distinct, coexisting liquid phases. We are tempted to speculate that the enhancers colocalizing at ICGs may participate in the information exchange underlying enhancer robustness (Feric et al., 2016; Guo and Shorter, 2015; Shukla and Parker, 2016; Weber and Brangwynne, 2015). An additional example is provided by enrichment of germplasm proteins and effector RNAs that creates a steep transition between aggregated germ plasm components at the posterior pole and a low concentration of these components throughout the rest of the embryo (REF). Such a transition may involve a phase shift from a fluid to a more gel-like state, as suggested for other RNP particles (Lehmann,

2016). We suggest that the eRNA-dependent formation of architectural structures at the enhancer can alternatively nucleate, or become embedded in, structures including the SC35/interchromatin granule.

Given the data suggesting that RNP-dependent LLPS could massively increase local protein concentrations (Zhang et al., 2015), we propose that dynamic, increase in coactivator complexes associated with the regulated MegaTrans enhancers (Liu et al., 2014) is a key principle driving the increased robustness of first tier enhancers in the network. It is evident that RNAs are critical to define the localization and function of granules (Zhang et al., 2015). Many proteins in the coactivator complex harbor the prion-like LC domains linked to LLPS. For instance, two of the most robustly recruited cofactors to first tier ER α enhancers, P300 and BRD4, have low complexity RNA binding motifs, and are known to form fibers *in vivo* (Olzscha et al., 2017). Therefore, it becomes tempting to speculate that eRNAs of interacting enhancers, with other lncRNAs, such as *DSCAM-ASI*, a first tier enhancer locus on Chr.21, can stabilize interactions of proteins (Li et al., 2013; Sigova et al., 2015) with enhancers, assist in nucleation of novel multi-mega dalton sized phase separated RNP bodies that become essential components in formation of the interacting enhancers, This process could increase the robustness of enhancers by concentrating transcription factors and cofactors via LLPS demixing. With fusion to the ICGs, also an LLPS structure, a significantly higher concentration of coactivators could be achieved within the ICGs relative to the solution phase (Banani et al., 2017; Li et al., 2012). We posit that this increased concentration of coactivators would critically augment the robustness of the first tier enhancers in the subnuclear structure.

An additional feature of regulated chromosomal architecture in our study is that first tier loci tend to colocalize in the basal state with certain favored regions, but not with disfavored regions, even when they are far closer by 1D genomic distance. E₂ further reinforces these existing biases, such that it becomes possible for two loci located on opposite

ends of a chromosome arm like *NR1P1* and *TFF1* to interact with a much higher frequency than many loci with far less 1D genomic distance. Given past findings that the structure of DNA in the nucleus resembles that of a “fractal globule”(Lieberman-Aiden et al., 2009; Mirny, 2011), it should not come as a surprise that 1D genomic distance and 3D spatial distance are not well correlated in the nucleus. Our study reveals the functional significance of low frequency mega base scale long distance interactions in the vicinity of membraneless organelles. These findings also pave way to future explorations into the role of such *de novo* assembled structures as organizers linking large-scale chromatin architecture and regulated transcriptional program in 3-D nuclear space.

Author Contributions

M.G.R., S.J.N., D.N., and L.Y conceived the project. L.Y and S.J.N performed the CRISPR-Cas9 enhancer deletions. S.J.N and J.Z performed FISH experiments. D.M generated codes and performed informatic analyses on FISH images. S.O, W.L and S.J.N did GRO-seq assays with help from L.Y and R.J. L.Y. and S.J.N. performed Q-PCR assays. D.M and Q.M performed the bioinformatic analyses. A.G. performed the *in situ* Hi-C. S.J.N. and L.Y performed ChIP-seq experiments. S.O. performed 4C assay. S.J.N and F.Y. performed the knockdown experiments. R.J and H.S.K performed ATACseq, T.S performed Methylation Seq. K.O prepared most samples for deep sequencing. M.G.R., S.J.N, L.Y and D.N. wrote the manuscript.

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Materials and Methods

Antibodies: The antibodies in this study were: anti-ER α (HC-20, Santa Cruz); anti- α -tubulin (T5168, Sigma), anti-H3K27Me3 (C15410195, Diagenode), anti-H4K20me3 (ab9053, Abcam), anti-H3K9me3 (ab8898, Abcam), anti-SC-35 (ab11826, Abcam), anti-SON (ab121759, Abcam), anti-Lamin-B1 (ab16048, Abcam), anti-Fibrillarin (sc-25397, Santa Cruz Biotechnology), anti-NCAPG (Bethyl Laboratories, A300-602A).

Cell Culture: MCF7 obtained from ATCC were cultured in DMEM media with 10% FBS in a humidified incubator with 5% CO₂ at 37°C. To prepare cells for estrogen stimulation, they were cultured in phenol-red free DMEM with 5% charcoal stripped FBS for 3-5 days. To induce estrogen signaling, these cells were treated with 100nM 17 β -estradiol (E₂) for indicated time points. Control samples were treated with ICI 182,780, a high affinity ER α antagonist to reduce basal ER α protein level, for a period of 3 hrs.

CRISPR MCF7 Enhancer Deletion Line Generation: Guide RNA sequences were designed to target the 5' and 3' of enhancer sequences by entering these sequences into the website crispr.mit.edu. Top results from the website were cloned into the pX459-puro vector. Pairs of pX459 gRNA targeting plasmids were transfected into wild type MCF7 cells using lipofectamine 3000 reagent. 72 hours after transfection, MCF7 cells were treated with TrypLE Select reagent and trypsinized for 30 minutes and then pipetted up and down 50 times through a filtered P100 tip to break up any large clusters of adherent cells. The single cell suspension was then plated onto many 10cm plates at a density of roughly 50,000 to 100,000 cells per plate. 12 hours after plating, puromycin selection reagent was added to a final concentration of 400-600ng/mL in multiple 10cm plates in increments of 50ng/mL. The puromycin selection continued for 72 hours, after which the puromycin media was removed, and non-selective media with pen/strep and Normocin antibiotics was added. The remaining cells are regrown

for between 14-28 days, until colonies made up of 50-100 cells could be observed with a microscope. At this point, individual colonies of MCF7 cells were lifted from the plate with a P100 pipette under the observation of a microscope inside of a sterile tissue culture hood. Colonies were then moved to individual wells of a 96-well or 48-well plate. After reaching confluence within these wells, the cells were again trypsinized and 50% of the cells were taken for genotyping while the remaining 50% could regrown. After genotyping, any wild type, heterozygous and homozygous cell lines deemed fit for further study were systemically expanded until enough cells were available for freezing stocks at -80°C or in liquid nitrogen.

RT-QPCR: RNA was isolated using Trizol (Invitrogen) or RNeasy column (Qiagen), and total RNA was reverse-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen). Quantitative PCRs were performed with MX3000P (Stratagene) using VeriQuest Fast SYBR Green qPCR master mix (Affymetrix, Cat# 75690). The relative gene expression was normalized to GAPDH or beta actin. Experiments were performed with at least three independent biological replicates and three technical replicates for each experiment. Statistical analysis was performed using a paired two-tailed Student's t-test.

ChIP-seq: Briefly, approximately 10^7 cells were cross-linked with 1% formaldehyde at room temperature for 10 min and neutralized with 0.125M glycine. After sonication, $\sim 75\mu\text{g}$ soluble chromatin was incubated with 1-5 μg of antibody at 4°C overnight.

Immunoprecipitated complexes were collected using Dynabeads A/G (Invitrogen). Subsequently, immuno-complexes were washed, DNA extracted and purified by QIAquick Spin columns (Qiagen). For ChIP-seq, the extracted DNA was ligated to specific adaptors followed by deep sequencing with the Illumina's HiSeq 2000 system according to the manufacturer's instructions. Usually, the first 48bp for each sequencing read was aligned to the hg18 assembly using BFAST or Bowtie2. Only uniquely mapped tags were selected for further analysis. The data was visualized by preparing custom tracks on the University of

California, Santa Cruz, (UCSC) genome browser using HOMER (<http://biowhat.ucsd.edu/homer>). The total number of mapable reads were normalized to 10^7 for each experiment presented in this study.

Identification of ChIP-seq Peaks: The ChIP-seq peaks were identified by HOMER. Given different binding patterns of transcription factors and histones, parameters were optimized for the narrow tag distribution characteristic of transcription factors by searching for high read enrichment regions within a 200bp sliding window. Regions of maximal density exceeding a given threshold were called as peaks, and adjacent peaks were set to be >500bp away to avoid redundant detection. The common artifacts from clonal amplification were circumvented by considering only one tag from each unique genomic position. The threshold was set at a false discovery rate (FDR) of 0.001 determined by peak finding using randomized tag positions in a genome with an effective size of 2×10^9 bp. For ChIP-seq of histone marks, seed regions were initially found using a peak size of 500bp (FDR<0.001) to identify enriched loci. Enriched regions separated by <1kb were merged and considered as blocks of variable lengths. All called peaks were then associated with genes by cross-referencing with the RefSeq TSS database. Peaks from individual experiments were considered overlapping if their peak centers were located within 200bp (for some analysis may extend to 1kb). The peaks within ± 1 kb apart from RefSeq gene TSS site were considered as promoter-bound

GRO-seq: GRO-seq experiments were performed as previously reported⁽¹⁾. Briefly, MCF7 cells were swollen in swelling buffer (10mM Tris-Cl pH7.5, 2mM MgCl₂, 3mM CaCl₂) for 5 min on ice and then lysed in lysis buffer (swelling buffer with 0.5% IGEPAL and 10% glycerol), before being re-suspended in 100μl of freezing buffer (50mM Tris-Cl pH8.3, 40% glycerol, 5mM MgCl₂, 0.1mM EDTA). For the run-on assay, re-suspended nuclei were mixed with an equal volume of reaction buffer (10mM Tris-Cl pH 8.0, 5mM MgCl₂, 1mM DTT, 300mM KCl, 20 units of Superase.In, 1% sarkosyl, 500μM ATP, GTP, and Br-UTP, 2μM

CTP) and incubated for 5 min at 30°C. The nuclear-run-on RNA (NRO-RNA) was then extracted with TRIzol LS reagent (Invitrogen) following manufacturer's instructions. After base hydrolysis on ice for 40min and followed by treatment with DNase I and antarctic phosphatase, the Br-UTP labeled NRO-RNA was purified by an anti-BrdU agarose beads (Santa Cruz Biotech) in binding buffer (0.5XSSPE, 1mM EDTA, 0.05% tween) for 3hr at 4°C while rotating. Then T4 PNK (NEB) was used to repair the end of NRO-RNA. Subsequently, cDNA synthesis was performed as reported^(9,33) with few modifications. The RNA fragments were subjected to poly-A tailing reaction by poly-A polymerase (NEB) for 30 min at 37°C. Reverse transcription was then performed using superscript III (Invitrogen) with oNTI223 primer. The cDNA products were separated on a 10% polyacrylamide TBE-urea gel with right product (~100-500bp) being excised and recovered by gel extraction. After that, the first-strand cDNA was circularized by CircLigase (Epicentre) and re-linearized by Ape1 (NEB). Re-linearized single strand cDNA were separated by TBE gel and the products of desired size was excised (~120-320bp) for gel extraction. Finally, cDNA template was amplified by PCR using the Phusion High-Fidelity enzyme (NEB) with primers oNTI200 and oNTI201 for deep sequencing.

Computational analysis of GRO-seq: The sequencing reads were aligned to hg18 using Bowtie2. For analyzing estrogen effects and enhancer deletion effects on gene transcription, we counted the reads from the first 30kb (assuming a RNA polymerase speed of ~0.5 kb/min during 1hr E₂ treatment) of entire gene body, excluding the promoter-proximal region on the sense strand with respect to the gene orientation by using BED Tools or HOMER. EdgeR (<http://www.bioconductor.org/>) was used to compute the significance of the differential gene expression ($FC \geq 1.5$, $FDR \leq 0.01$). Additionally, a read density threshold (*i.e.* GRO-seq normalized read counts/kb) was used in order to exclude lowly expressed genes.

De novo identification of GRO-seq transcripts: GRO-seq read densities were

analyzed in a similar manner to ChIP-seq. Provided GRO-seq generates strand-specific data, separate tracks were uploaded onto the UCSC genome browser; tag-enriched sites were identified using a sliding window of 250bp. Transcript initiation sites were identified as regions where the GRO-seq read density increased threefold relative to the preceding 1kb region. Transcript termination sites were defined by either a reduction in reads below 10% as compare to that of TSS or when another transcript's start was identified on the same strand. Individual high-density peaks spanning a region less than 250bp were considered artifacts and removed from the analysis. Transcripts were defined as putative eRNAs if their *de novo* called start sites was located distal to RefSeq TSS ($\geq 3\text{kb}$) and were associated with ER α and H3K27ac co-bound regions.

Bioinformatics characterization of ER α enhancers: The ER α -H3K27ac co-bound regions are defined as that the distance from the center of an ER α peak to the H3K27ac peak-occupied region is $\leq 1\text{kb}$. Overall, two methods were used to assign the ER α bound enhancers to E₂ upregulated genes: 1) identifying the E₂-upregulated coding genes from GRO-seq and coupling each of them to their closest ER α -H3K27ac co-bound enhancer within first distance (200kb) (a “gene-centric” view); 2) characterizing the ER α -H3K27ac co-bound enhancers first and then coupling each of them to their closest TSS that belongs to 1,309 E₂ upregulated coding genes (an “enhancer-centric” view).

DNA FISH and RNA FISH: MCF7 cells grown on acid-washed poly-lysine coated coverslips were fixed with freshly made 4% paraformaldehyde in PBS for 8 min. Excess formaldehyde was quenched with 0.1M Tris-HCl (pH 7.4) for 5 min. Coverslips were washed with PBS and stored at 4°C until used. Prior to hybridization coverslips were incubated in 0.1N HCl for 5min at room temperature. Washed twice with PBS. Coverslips were incubated in PBS containing 100 $\mu\text{g/ml}$ RNase A for 1 hr at 37°C-followed by equilibration in 50% formamide/2XSSC for 1hr. 125ng of probe in equal volume mixture of formamide and 2X

hybridization buffer mix (4XSSC/40%Dextran Sulphate) was used per coverslip. Coverslips on glass slides were heated for 6 min on a hotplate with temperature set at 80°C followed by overnight hybridization at 37°C in a humidified dark chamber. The coverslips were then washed twice with pre-warmed buffer containing 50% formamide/2XSSC and twice with 2XSSC before being finally mounted with Vectashield antifade mounting medium with DAPI (Vector Laboratories). For ImmunoFISH (DNA), cells were incubated first with PBS containing 0.5% Triton-X-100 and 5%BSA for 15 min at room temperature. Primary antibodies were used at a dilution of 1:50 in blocking buffer (0.1% Triton-100/5% BSA in PBS) for 1 hr. at 37°C. Washed 3 times in PBST (PBS containing 0.1% Triton X-100). Incubated with appropriated fluorescent conjugated secondary antibody (1:1000) dilution for 30 min at room temperature. Cells were fixed for a second time with freshly prepared 2% paraformaldehyde for 10 min at room temperature followed by treatment with 0.1M Tris-HCl (pH 7.4) for 5 minutes. Washed twice in PBS and DNA FISH protocol described above was resumed.

For RNA FISH, cells containing coverslips were fixed in 4% freshly prepared paraformaldehyde. Washed twice with PBS with freshly added 2mM Ribonucleoside vanadyl complexes (Sigma-Aldrich, St. Louis, MO, USA). Cells were permeabilized and stored in 70% ethanol at 4°C. Prior to probe hybridization coverslips were incubated with wash buffer (10% formamide/2XSSC) for 30 minutes at room temperature. RNA FISH probes were resuspended in hybridization buffer (10% formamide and 10% Dextran sulphate in 2XSSC). Coverslips were incubated with probes overnight at 37°C in a humidification chamber. Post incubation washes were done using pre-warmed wash buffer twice at 37°C. Immuno RNA FISH were performed using the protocol described above with addition of primary antibody mixed along with the RNA FISH probes and incubated overnight. Probes and primary antibody was washed off using wash buffer at 37°C followed by fluorescent conjugated secondary antibody

incubation. Nuclei were counterstained by incubating in wash buffer containing Hoechst 33342 at a concentration of 1 µg/ml for 15 min.

DNA and RNA FISH probes: All the BAC based probes for DNA FISH were purchased in the fluorescent labeled from Empire Genomics (Buffalo, NY, USA). Fosmids were obtained from CHORI (Oakland, CA, USA). Fosmid based hybridization probes for DNA FISH were generated from 1 µg fosmid using Nick Translation kit (Abbot Molecular), Green 496, Oragne 552 or Red 650 conjugate dUTP following manufacture recommended protocol. 125ng of each labeled probes, 4 µg human Cot1 DNA (Thermo Fisher Scientific) and 10 µg salmon testis DNA (Sigma-Aldrich) were used per coverslip. They were co-precipitated in ethanol and were resuspended in equal volume mixture of formamide and 2X hybridization buffer mix (4XSSC/40%Dextran Sulphate) prior to hybridization reaction. BAC and Fosmid clone ID used in this study is given as a separate table ().

RNA FISH probes were designed using Stellaris Probe Designer tool (Biosearch Technologies). Repeat masked intronic sequences of TFF1(intron 1), NRIP1 (Intron2), DSCAM-AS1(Intron 1) were used as template for probe design. Probes were labeled with FAM, Quasar 570 or Quasar 670 dyes.

Microscopy:

Images were acquired using a Perkin Elmer Spinning Disk Confocal Microscope (100x Nikon Plane Achromatic oil immersion objective, numerical aperture: 1.40). The microscope was equipped with a Piezo-Z drive and EMCCD Hamamatsu 14-bit 1Kx1K camera. Z-stack data was acquired at a step size of 150 nm. The 3D images were reviewed and processed using Volocity software (Perkin Elmer, v6.0.1). Background-subtracted image stacks were used for downstream analysis using Volocity software and custom software.

Image analysis:

3D image stacks were initially analyzed using Volocity software. The functions “Find

Object” and “Exclude Objects by Size” were combined for automatic detection of the FISH probe signals. For accurate and automated calculation of spatial distances between the probed loci, the 3D coordinates of FISH signals were exported to CSV files using the Volocity software and were analyzed using custom software implemented with Python, NumPy, and SciPy. To estimate the 3D distance distribution between any two genomic loci, the centroids of the FISH signals from those loci were used to calculate a number (see below) of shortest distances for each nucleus, and those distances were then pooled from all examined nuclei. This procedure assumed that each of the shortest distances obtained from each nucleus corresponded to loci located on the same chromosome. For experiments probing one diploid and one aneuploid locus, up to two shortest distances were obtained per nucleus. For experiments probing two aneuploid loci, the maximum number of shortest distances obtained per nucleus was equal to the smallest known copy number among the two loci in MCF-7 cells. The median distances between control and test conditions were compared using the Mann-Whitney U test. The empirical cumulative distributions of distances were compared using the Kolmogorov-Smirnov test. Custom software used for this study is available upon request.

3D Proximity Calculations:

To estimate the E2-induced changes in 3D proximity between different loci on Chr.21, we calculated the fraction of pairs of loci whose spatial distance is less than a well-defined cut-off distance. Because the loci considered in this study were separated by a wide range of genomic distances (from 1.9 Mb to 33 Mb), the corresponding median spatial distances were also expected to vary greatly. Therefore, using a single cut-off distance as a measure of proximity was likely to yield excessively large or small fractions of proximal locus pairs at small or large genomic separations, respectively. To address this problem, each pair of loci was assessed using a cut-off distance that depends on the genomic separation between the loci. Specifically, the cut-off distance was taken as 40% of the predicted median spatial distance

between those loci under basal conditions. In turn, the predicted median spatial distance was obtained from a fit of the power-law function $y = a x^S$ to experimental data, where y is the median spatial distance, x is the genomic distance, and a and S are fit parameters. To determine the fit parameters, two experimental data sets were considered. The first data set consisted of the 3D-FISH distance measurements obtained in this study for various locus pairs on Chr.21 of E2-depleted MCF-7 cells. The second data set was taken from (Wang et al., 2016) and consisted of the 3D coordinates of 34 TADs on Chr.21 of IMR90 cells, which do not respond to estrogen. Such coordinates were obtained by a novel multiplexed-FISH method capable of capturing the architecture of entire chromosomes in single cells. Power-law fits obtained from the two data sets produced qualitatively similar results.

For a given pair of loci, the fraction of observed spatial distances less than the appropriate cut-off distance was used to generate barplots of fold changes in 3D proximity between those loci. The theoretical sample standard deviation s for each fold change $c = a/b$ of fraction a relative to fraction b was estimated using the error propagation formula $(s/c)^2 = (v_a/a^2 + v_b/b^2)$, where $v_a = a(1-a)/N_a$ is the theoretical sample variance of fraction a , N_a is the number of observations used to calculate a , and similar definitions apply to v_b . The p-values for the changes in fractions of distances less than the cut-off were calculated using a previously described bootstrapping method in which the observed spatial distances for the two conditions being compared are combined and sampled with replacement to calculate the null distribution of changes in fractions.

In addition to the cut-off distance approach described above for measuring the 3D proximity of genomic loci, the “Intersect” function of the Volocity software was also used as an alternative approach. This function employs the “voxel overlap” between two objects as an indication of spatial interaction. To improve the reliability of the detected interactions, a minimum overlap volume of $0.003 \mu\text{m}^3$ was used. The results from this voxel overlap

approach were found to agree qualitatively with those from the cut-off distance approach, in terms of the induced proximity changes reported in this study.

In-situ Hi-C: In situ Hi-C was essentially performed as described (Rao et al., 2014). Briefly, for each experiment, 2×10^6 cells, fixed for 10 minutes with 1 % formaldehyde/PBS and washed twice with PBS, permeabilized for 7 minutes at 62°C in a PCR cycler with 200 µl lysis buffer (0.5% SDS, 50 mM Tris-HCl pH=7.5, 10 mM NaCl, 1mM EDTA, and 1X protease inhibitors solution (Roche)) and pelleted at 2500x g for 5 minutes. Supernatant was removed and nuclei were resuspended in 25 µl 10% Triton X-100, 25 µl NEB 2 buffer, 195 µl water, and rotated for 15' at 37°C. Chromatin was digested overnight at 37°C after adding 0.5 µl 1 M DTT and 4 µl 25 U/µl Mbo I and rotated at 8 RPM. MboI was inactivated by incubation at 62°C for 20 minutes. Nuclei were centrifuged for 5 minutes at 500 x g and 200 µl supernatant was discarded. Overhangs were filled in by adding 32 µL water, 5 µl of 10X NEBuffer2, 0.35 µl of 10 mM dATP, 0.35 µl of 10 mM dTTP, 0.35 µl of 10 mM dGTP, 7.5 µl 0.4 mM Biotin-14-dCTP (Invitrogen), 4 µl 10% Triton X-100, and 5 µl of 5 U/µl Klenow enzyme (Enzymatics) and rotating for 40 minutes at room temperature. The reaction was stopped by adding 2.5 µl 0.5 M EDTA. DNA was ligated under rotation overnight at 16°C in a total volume of 400 µl ligase mix containing 40ul 10x T4 DNA ligase buffer (Enzymatics), 36 µl 10 % Triton X-100, 5 µl 100x BSA (10 mg/ml), 1 µl (1200 U) T4 DNA ligase (Enzymatics). The reaction was terminated by adding 20 µl 0.5 M EDTA. Samples were digested for 15 minutes at 42°C with 1 µl 10 µg/µl RNase A. 33 µl of 5 M sodium chloride and 55 µl of 10% SDS were added and reverse crosslinked for 4 h at 65°C. Protein was digested with 10 µl of 20 mg/ml proteinase K (Life Technologies), incubated at 55°C for 120 minutes, shaking at 800 RPM, then 65°C for 90 minutes. DNA was extracted once with 600 µl phenol/chloroform/isoamylalcohol (25:24:1) Tris-buffered to pH 8.0 and once with 300 µl CHCl₃, and precipitated overnight at -20°C with 1.5 µl 20 mg/ml glycogen and 1412 µl 100%

ethanol overnight. DNA was pelleted for 20 minutes at 16000x g, 4°C and washed once with 1 ml 80% ethanol for 5 minutes, 8000x g, 4°C. Pellets were dissolved in 131 µl TT (0.05% Tween 20/10 mM Tris pH=8) each. DNA was sheared with 300 bp Covaris protocol in snap cap tube in a Covaris E220 at 10 % duty cycle, intensity 140 W, 200 cycles/burst for 80" total time. Large DNA fragments (>400 bp) were depleted with 5 µl speedbeads and 6.45% PEG8000/2.5 M NaCl. Supernatant was transferred to fresh tubes and small DNA fragments were collected with 9.5% PEG8000 by adding an additional 60 µl PEG8000/2.5 M NaCl and 3 µl Speedbeads. DNA was eluted in 50ul TT for 5 minutes. DNA was captured with 50 µl 2x B&W buffer containing 0.2 % Tween 20 and 15 µl T1 Dynabeads (Invitrogen, washed twice with 1x B&W buffer (10 mM Tris-HCl pH 7.5, 01 mM EDTA, 2 M NaCl, then suspended in 51 µl 2x B&W buffer containing 0.2% Tween 20), rotating for 30 minutes at room temperature. Beads were washed once with 500 µl each of 1x B&W/0.1 % Triton-X100, once with TET (0.05% Tween 20/TE). Beads were resuspend in 100 µl end repair mix (KAPA Library Preparation for Illumina) Incubated for 30 minutes at 20°C. Reaction was stopped by adding 2.5 µl 0.5 M EDTA. Beads were collected and washed twice with 150 µl 1x B&W/0.1% Triton-X100, once with 180 µl TET. Beads were resuspend in 50 ul A-tailing reaction mix (KAPA Library Preparation for Illumina), incubated 30 minutes at 30°C. Reaction was stopped by adding 1.5 µl 0.5 M EDTA. Beads were collected and washed twice with 150 µl 1x B&W/0.1% Triton-X100, once with 180 µl TET. Sequencing adapters were ligated to the bead-bound DNA in 100 µl 1x rapid ligation buffer (Enzymatics) containing 0.1% Tween 20, 2ul 1:20 Truseq adapters (Illumina), 1 µl (3000 U) T4 DNA ligase (Enzymatics) for 20 minutes at room temperature. The reaction was stopped with 5 µl 0.5 M EDTA, beads washed twice with 1x B&W, twice with 0.1% Tween 20/TE, then resuspended in 30 µl 0.033% Tween20/LoTE, (TE diluted 1:4 with water). Libraries were PCR-amplified using the 10 µl of the bead suspension as template for 10 cycles using KAPA HiFi, size-selected to 225-425 bp

insert size using speedbeads PEG8000/2.5 M NaCl solutions, and paired-end sequenced on an Illumina HiSeq 2500.

4-C: The protocol of 4C-seq largely followed a published protocol (Stadhouders et al., 2013) with following modifications. Briefly, 10 million cells were cross-linked with 1% formaldehyde for 10 min and nuclei were extracted. Nuclei were resuspended in restriction enzyme buffer and incubated with 0.3 % SDS for 1h at 37°C and further incubated with 2% Triton X-100 for 1h. 400U of DpnII restriction enzyme was added and incubated overnight. Restriction enzyme was heat inactivated at 65°C for 20 min. Ligation of DNA regions in close physical proximity was performed using 1000U of T4 DNA ligase (NEB) for overnight. After de-crosslinking, the second digestion and ligation was performed using restriction enzyme NlaIII and T4 DNA ligase. 4C-seq libraries were amplified using PCR with the first primer designed on each viewpoint and the second primer designed beside the NlaIII site. Both primers contained illumina sequencing adaptors and barcode. 4C libraries were sequenced on the Illumina Hi-Seq 2500 using single-read 100-cycle runs.

ATAC-seq: ATAC-seq was performed as previously described (Buenrostro et al., 2015). Briefly, wild-type or CRISPR deletion clones of MCF7 cells were harvested after indicated time of ICI/estradiol treatment, washed once with PBS and 50,000 cells were used for each ATAC-seq sample preparation. Transposition reaction was performed at 37°C for 30 min using Nextera DNA Library Preparation Kit (Illumina Cat No. 15028212) in 50µl volume (2.5 µl Transposase enzyme, 25 µl 2X TD buffer and 22.5 µl nuclease-free water). The reaction mixture was immediately purified using MinElute PCR purification Kit (Qiagen, Cat No. 28006) and eluted in 10 µl nuclease-free water. The tagmented DNA was then PCR amplified using KAPA Real-Time Library Amplification Kit (Kapa Biosystems, Cat No. KK2701) in a 50 µl reaction (10 µl tagmented DNA, 2.5 µl 25 µM PCR primer 1, 2.5 µl 25 µM barcoded PCR primer 2, 25 µl KAPA HiFi HotStart ReadyMix, 10 µl nuclease-free water), using the

following program (One cycle at 72oC: 5 min, 98oC: 30sec; 8-12 cycles at 98oC: 10 sec, 63oC: 30 sec and 72oC: 1min). PCR standards, supplied in the kit, were included in separate wells and the reaction was stopped between standard 2 and 3. The reaction mixture was subjected to size selection (to select fragments ranging 150-800bp) using 1.6 volume of Agencourt AMPure XP magnetic beads (Beckman Coulter, Cat No. A63880), washed once with 80% ethanol, air-dried and eluted in 15 µl of nuclease-free water. The resulting ATAC-seq library was quantified using Qubit Fluorometer (Thermo Fisher) and the quality was analyzed by resolving 1 µl on Tapestation (Agilent). Ten nmol of the library was sequenced on HiSeq 4000 sequencer (Illumina). DATA was mapped to hg18 using Bowtie2 with standard settings. Tag directories were created after removing the reads mapped to the mitochondrial chromosome. ATAC-seq peaks were identified using HOMER.

Methylated DNA Immunoprecipitation: Genomic DNA from cells was isolated using DNeasy Blood and Tissue Kit (Qiagen, Cat#69504). DNA was sonicated to fragment size of 100-300bp using Bioruptor (Diagenode). NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, E7645) was used to ligate barcoded adaptors for Illumina sequencing to 1ug of sonicated genomic DNA for each pull down experiment. After size selection and clean-up, immunoprecipitation of methylated and hydroxymethylated DNA was performed as previously described (Thu et al., 2009) with the following modifications. 1ug of 5-mC antibody was used per MeDIP reaction (Active Motif # 39649), and 1uL of 5-hmC antibody containing serum was used per hMeDIP reaction. DNA was then eluted from beads with 200uL Proteinase K digestion buffer overnight at 50°C. Samples were then purified using QIAquick PCR Purification Kit (Qiagen, Cat#28104). These MeDIP and hMeDIP libraries then resumed processing with the NEBNext Ultra II DNA Library Prep Kit for Illumina at the step of PCR Enrichment of adaptor ligated DNA, and the manufacturer protocol for Illumina library preparation was followed to completion.

Chapter 5 is an adaptation of a manuscript that is being submitted for publication.

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CHAPTER 6 DISCUSSION

Global versus gene specific epigenomic changes in senescence

While both literature and the study presented in this dissertation have shown a wide array of epigenomic changes to occur in the process of senescence, key questions remain unanswered as to the degree to which each of these factors are causative of the senescence phenotype. A variety of striking and global epigenomic changes have been observed to occur during senescence that have led to proposals that senescence may be driven by a tendency of the senescent chromosome to be more open and dysregulated. However, I argue that our studies and the body of literature regarding senescence epigenomic suggest that more targeted and gene specific changes are playing at least as significant a role in driving the senescence phenotype.

A host of global patterns are observed during senescence. Hypomethylation is observed generally throughout non-CpG promoter regions (Cruickshanks et al., 2013). Global loss of total histone levels are observed (Roderick J. O'Sullivan et al., 2010). Large scale deterioration of local intra-chromosomal interactions are observed (Chandra et al., 2015; McCord et al., 2013). Large scale deterioration in lamin associated domains are observed (Shah et al., 2013). While there is inconsistency about the global change of H4K16ac changes during senescence that seem to depend upon the system being used (Contrepois et al., 2012; Miller et al., 2010), genome wide analyses showed a depletion of H4K16ac at late replicating regions and an increase at the promoters of senescent genes regardless of their change in expression during senescence (Rai et al., 2014). H3K56ac was shown to globally decreasing during senescence due to the activity of HDAC1 and HDAC2 in response to DNA damage (Miller et al., 2010). These changes in higher level structure are also suggested to be in part causative of the senescence phenotype, as knockdown of lamin B1 is sufficient to induce senescence, and overexpression of histones has been sufficient to extend lifespan of yeast

(Feser et al., 2010b). While the data indicating functional impact of these global changes upon the senescence state is, however, rather limited, it is difficult to determine whether that is due many of these features are downstream of other events, or because of the technical difficulty of cleanly manipulating these feature. For example, as the three dimensional architecture of chromosomes is the result of many interworking components, it is technically difficult to manipulate the chromatin architecture at this level and determine if such manipulations have any effect upon the senescent phenotype. Regardless, the correlation of these global features with senescence is clear, and certain features, such as the sufficiency of LMNB1 knockdown in inducing senescence, suggest that some of these global features of senescence play a major role in the senescent phenotype.

Conversely, a host of gene and region specific epigenomic changes are also observed during replicative senescence. Hypermethylation of specific promoters associated with proliferation was observed, including CCNA2, CENPA, and TOP2A (Cruickshanks et al., 2013). H3K4me3, a histone modification classically associated with active promoters, has been shown to increase at genes associated with SASP during senescence (Shah et al., 2013), and its writer MLL1 has been shown to be essential for associated SASP genes (Capell et al., 2016, p. 1). Enhancer gain as marked by H3K27ac was shown to correspond with genes induced during senescence, with a substantial gain in clusters of high H3K27ac marks known as super-enhancers (Hnisz et al., 2013b; Tasdemir et al., 2016). Furthermore, inactivation of these super-enhancers through knockdown or small molecule inhibition of BRD4, a transcriptional activator shown to be necessary for super-enhancer function, showed inhibition of SASP. Given that super-enhancers are small in number and have been shown to regulate key cell state defining genes, these results confirm the relevance of gene specific features on the senescent phenotype. GATA4 has also been shown to play a significant impact upon the senescence phenotype in a gene specific man

Further emphasizing the relevance of gene specific factors as causative of the senescent phenotype is the interrelation between global and gene specific epigenetic factors. HIST1H2AE and HIST1H2BM are among genes shown to gain promoter methylation (Cruickshanks et al., 2013), which corresponds clearly with core histone reduction observed in replicative senescence (Roderick J. O'Sullivan et al., 2010). Gene and region specific changes in H3K27me3 and H3K4me3 are observed in senescence and correlate with senescence associated genes that include those of the SASP program. However, these regional and gene specific changes are observed to correlate with and be instigated by senescence associated depletion of LADs (Shah et al., 2013), which is in turn influenced by reduced levels gene expression of LMNB1, or can be activated by an autophagy response to oncogenic stress (Dou et al., 2015).

Our own data also shows support for the importance of gene specific changes instigating the senescent phenotype, rather than the state being merely the result of global opening of the genome. When we performed our screen of histone modifications that might be altered in replicative senescence, we did indeed observe a global decrease in histone content of senescent samples versus young samples when normalizing all samples to total levels of protein. When we used ponceau staining and western blotting of total H3 and H4 levels, we found that these core histones were at levels around 2.5 fold higher in young versus senescent cells, broadly consistent with previous findings (Roderick J. O'Sullivan et al., 2010). However, we found that, when performing ChIP-seq experiments on a similar number of senescent and young cells and normalizing DNA content between samples, we found a similar fold change in genomic content versus total protein levels in young versus senescent cells. This is consistent with the observation of multinucleation in senescent cells (Akakura et al., 2010; Salama et al., 2014; Vergel et al., 2010). When looking at the normalization used in the O'Sullivan paper, we observe that samples are normalized to levels of γ -tubulin. While γ -

tubulin is a classic standard for histone normalization and is closely associated with the centrosome (Manning and Kumar, 2010), it is possible that this normalization approach may overstate the significance of histone level change observed during senescence.

Additionally, altered levels of total histone levels without concomitant changes in the writers and erasers of each modification might predict that there would be major differences in the levels of different modifications throughout senescence. However, our panel of histone modifications, once normalized to total histone levels, shows remarkable consistency between the majorities of modifications throughout senescence. This would seem unlikely under the proposed model, as different writers and erasers would likely have dramatically different kinetics, and alterations in substrate would thus suggest relative differences in modifications would become apparent during senescence. While our findings do not directly or necessarily conflict with the model of global histone loss during replicative senescence in BJ fibroblasts, they raise questions as to the appearance and impact of these changes in senescence. Furthermore, while it is likely that some change in total histone levels occurs in mammalian senescence, these changes in total levels are indirect evidence of their occupancy upon chromatin.

The uncertainty regarding changes in total histone levels during senescence could be better resolved through appropriately normalized genome wide methods. Spike in ChIP-seq techniques account for different levels of material being pulled down even in the absence of different distribution of the factor being pulled down (Egan et al., 2016). Use of spike in ChIP-seq with core histone marks could provide evidence of their altered occupancy or distribution on the senescent genome. Spike-in MNase-seq has been used in yeast and showed altered histone occupancy patterns with senescence that were reduced upon histone overexpression (Hu et al., 2014), which do further support the view that histone levels change with senescence. However, general differences in the epigenomics of yeast versus

mammalian senescence that have been discussed above would emphasize the importance of these studies being performed in mammalian cells.

Our study further emphasizes the role of gene specific changes in senescence through our findings regarding enhancers. Consistent with western blots of H3K27ac and H3K4me3 on young and senescent samples, we find that the majority of histone modifications show no change, and that a similar number of peaks are gained as are lost. Furthermore, we don't notice any appreciable difference in the shapes of these peaks, which might be expected with altered nucleosome density. Our study also showed the impact of specific transcription factors on both the proliferative and SASP features of senescence.

Additionally, our HiC experiments in rapamycin withdrawal conditions seem to show the maintenance of young genomic organization even in high passage cells after 2 weeks of withdrawal from rapamycin, even though these cells are now showing senescence hallmarks of cell cycle arrest, SASP gene expression, altered morphology, and beta gal staining. The presence of all the phenotypes of senescence in these cells without any concomitant alterations in global chromosomal A/B compartmentalization would seem to suggest that the deterioration of interactions observed in cellular senescence is not a necessary causative factor in senescence.

Further hints at the role of various epigenomic processes associated with senescence can be extrapolated from experiments done more broadly upon organismal aging using various model systems. While a general correspondence exists between aging and senescence, there are key difference in the epigenetic trends and causality observed between the aging of different model organisms and cellular senescence. Aspects of the "open-dysregulated chromosome" model of senescence are apparent in the global epigenomic changes observed in yeast and worms. This is shown in the manipulation of H3K4me3 levels in worms, as increasing levels of H3K4me3 results in decrease lifespan, and extension in lifespan is

observed upon H3K4me3 reduction (Han and Brunet, 2012). Additionally, knockdown of the H3K4 demethylase Lid in flies further extended lifespan, showing support for this model in flies (Li et al., 2010). Consistently, the opposite phenomena holds true in manipulations of H3K27me3, as increases of this repressive mark tend to extend lifespan, while decreases reduce it (Jin et al., 2011). Similarly, the histone lysine deacetylase activity of the Sir family of enzymes has been long linked to extension of lifespan in organisms ranging from yeast to mouse (Herranz and Serrano, 2010b; Rogina and Helfand, 2004b; Heidi A. Tissenbaum and Guarente, 2001), with knockout and overexpression of family members in yeast reducing or extending lifespan, respectively (Howitz et al., 2003; M. Kaeberlein et al., 1999); and deletions in the H4K16 acetyltransferase SAS2 have additionally been linked to lifespan extension in yeast (Kozak et al., 2010).

However, the model of global genome opening during senescence also paints in broad strokes that undervalue the gene specific nature of the changes in model organisms. While a global H3K27me3 change is indeed observed in worms, it is suggested that changes in aging occurred largely through alteration in DAF-16/FOXO (Jin et al., 2011), suggesting that the causative factor in lifespan extension in this instance may more lie with the insulin signaling pathway, a hallmark regulator of lifespan extension in worms (Cynthia J. Kenyon, 2010), rather than more general chromatin openness. Furthermore, the observation that reduction in the levels of H3K27me3 in flies extends lifespan, suggests the incompleteness of the model. Histone acetylation also fails to follow a consistent trend. While Sir family members have had a consistent positive impact on lifespan extension throughout multiple organisms, the evidence of that this impact occurs through its deacetylase activity upon histones specifically is limited largely to yeast models. While yeast studies have linked Sirtuin histone deacetylase activity in part to H4K16ac and telomeric shortening induced aging, *C. elegans* aging has been shown to be independent of telomere dynamics (Raices et al., 2005) and the impact of Sirtuins could

easily explained through their targeting of the FOXO family of transcription factors (Daitoku et al., 2004). In fact, mammalian studies have typically linked Sirtuin function to its activity on FOXO family members, rather than its activity upon histone (Brunet et al., 2004; Frescas et al., 2005). Furthermore, overexpression of SIRT6 in mice resulted in lifespan extension in males but not females, which is consistent with it impacting specific pathways rather than globally stabilizing the genome of senescent cells through deacetylation (Kanfi et al., 2012b).

While the importance of sequence specific transcription factors, and therefore specific enhancers and genes, in senescence does emphasize the importance of gene specific features in senescence, they in no way preclude more global phenomena from playing a role in the senescent genome. However, while certain causative global patterns in senescence do exist, the data in both our study and literature seems to relatively emphasize gene specific changes in the epigenome driving the senescence cell state.

Cellular Rapamycin Addiction

While our experiments with rapamycin withdrawal showed activation of the SASP program that is also seen in our replicative senescence experiments, we noticed that rapamycin withdrawal samples both showed GO terms more strongly associated with SASP, induction of IL1A, IL1B, and IL6 hallmark SASP genes not found in our replicative senescence experiments (Laberge et al., 2015), as well as higher binding of NFkB than was ever observed in replicative senescence experiments. This suggests that, while treatment of rapamycin can result in extended lifespan, cessation of rapamycin treatment could result in a more severe aging and SASP phenotype than would have been observed if rapamycin had never been given. Possible explanations for such a phenomena could include the fact that rapamycin treatment allows for increased doublings of treated cells, allowing for potential further shortening of telomere, which could resulting in higher chromosomal instability, activation of DNA damage machinery, and activation of the senescent phenotype. Alternatively, telomeric shortening

during replicative senescence activates DNA damage machinery in a slow and progressive manner that is kinetically limited by the rate of cell division. As rapamycin treatment may block the onset of the SASP phenotype through inhibition of DNA damage machinery (Krześniak et al., 2014; Rai et al., 2013), rapamycin withdrawal may result in immediate activation of damage machinery in response to fully shortened telomeres, in contrast to the gradual activation of DNA damage machinery observed in replicative senescence. While these hypotheses need to be tested experimentally, the clinical use of rapamycin and other mTor inhibitors in organ transplants, cancer treatments, and other conditions highlights the importance of understanding these potentially medically significant consequences of withdrawal.

Methylation dynamics on enhancers in activation and damage

In the course of examining the role of DNA damage repair machinery at androgen receptor regulated enhancers, we found some limited role in nuclear receptor activated transcription with factors that are also implicated in active DNA demethylation and conversion of 5mC into 5hmC, including GADD45, TET enzymes, and several base excision repair glycosylases (Cortellino et al., 2011; Kangaspeka et al., 2008). In the studies on ESC pre-marked enhancers and on enhancers as organizing centers from intra-chromosomal chromosomal interactions, we observed patterns in methylation and hydroxy-methylation at enhancers that correspond broadly with each's reported role in repression and activation, respectively (Ehrlich and Ehrlich, 2014). However, interestingly, while we noticed enhancers that were generally active were positive for 5hmC and depleted for 5mC, we noticed in both studies that the most highly activated enhancers were also depleted in both 5hmC and 5mC. While we were initially puzzled by this breakdown in the expected correlation of higher activation leading to higher levels of 5hmC, this observation has led to our formulation of a novel working model for the dynamics of DNA methylation marks over the process of

enhancer activation. While 5hmC is associated with activated enhancers, 5hmC cannot be generated de novo and must instead be formed through enzymatic activity of enzymes such as the TET family members upon 5mC (Guibert and Weber, 2013). It thus follows that sufficiently low levels of 5mC would result in depletion of the 5hmC mark. As the most active enhancers are also particularly depleted in 5mC, it stands to reason that they would also show 5hmC depletion. Thus, a proposed model for methylation dynamics on pre-marked enhancers would have them being initially high in 5mC, transitioning to a state of high 5hmC and low 5mC as they become active, followed by the final transition to a state of low 5hmC and low 5mC as they become highly active enhancers. We propose that 5hmC may serve as a molecular memory for pre-marked enhancers as they transition from inactive to active, as may serve as a bridge between these two states. While we are performing further analysis to confirm this finding, our early kinetic analyses of enhancer activation over progressive developmental stages seems to be broadly consistent with our model.

APPENDIX

Table 1. Histone modifying factor changes during senescence.

Factor	Activity	Change During Senescence	Affect upon senescence
DNMT1	maintenance DNA methyltransferase	Down (Casillas et al., 2003)	
DNMT3a	de novo DNA methyltransferase	Down (Casillas et al., 2003)	
DNMT3b	de novo DNA methyltransferase	Up (Casillas et al., 2003)	
MECP2	Methylated DNA binding; adapter		Mutation accelerates senescence in humans (Squillaro et al., 2010)
EZH2 (PRC2)	H3K27 and H1K26 methyltransferase	Down (Adrian P Bracken et al., 2007)	
JMJD3	H3K27me3 lysine demethylase	Up (Agger et al., 2009)	
BMI1	PRC1 component		Deficient mice show accelerated senescence (Jacobs et al., 1999)
CBX7	PRC1 component		Deficient human line show accelerated senescence (Gil et al., 2004)
			Deficient mouse fibroblasts showed delayed senescence (Forzati et al., 2012)
CBX8	PRC1 component		Deficient mouse fibroblasts show accelerated senescence (Dietrich et al., 2007)
p300/CBP	Lysine acetyltransferase (multiple substrates)	Down (Bandyopadhyay et al., 2002)	p300 inhibition accelerated senescence in human melanocytes (Bandyopadhyay et al., 2002)
SIRT1	Lysine Deacetylase (multiple substrates)	Down (Han et al., 2010)	Overexpression reduces senescence associated phenotypes in mice (Herranz and Serrano, 2010a; Kim et al., 2007; R.-H. Wang et al., 2008)
			Deficient mice show signs of premature aging (Cheng et al., 2003)
SIRT6	Lysine Deacetylase (multiple substrates)		Overexpression extends male mouse lifespan (81)
			Deficient mice show reduced lifespan (80)
HDAC1	Lysine Deacetylase (multiple substrates)	Up (Bandyopadhyay et al., 2002)	Drug inhibition accelerates in human cells (108, 109)
			Oxerexpression accelerates senescence in human cells (63)

Table 1: Continued

G9a/GLP	H3K9 methyltransferases	Down (Takahashi et al., 2012)	
HIRA (complex)	H3.3 deposition	Up (38, 39)	
ATRX/DAXX	H3.3 deposition		
CAF-1	Replication dependent histone assembly	Down (Roderick J O'Sullivan et al., 2010)	
ASF1A/B	H3/H4 deposition	Down (Roderick J O'Sullivan et al., 2010)	
SLBP	Stabilizes canonical histone transcripts	Down (Kaygun and Marzluff, 2005)	
KMT5B/C	H4K20 methyltransferase		Deficient mouse fibroblasts show impaired proliferation (Schotta et al., 2008)
RBBP4	NuRD subunit		Deficient human fibroblasts recapitulates HGPS phenotype (Pegoraro et al., 2009)
RBBP7	NuRD subunit		Deficient human fibroblasts recapitulates HGPS phenotype (Pegoraro et al., 2009)
SUV39h1	H3K9 methyltransferase		Deficient mouse cells avoid oncogene induced senescence (Braig et al., 2005; Reimann et al., 2010)
			Drug inhibition induces cell cycle arrest in human cells (Cherrier et al., 2009)

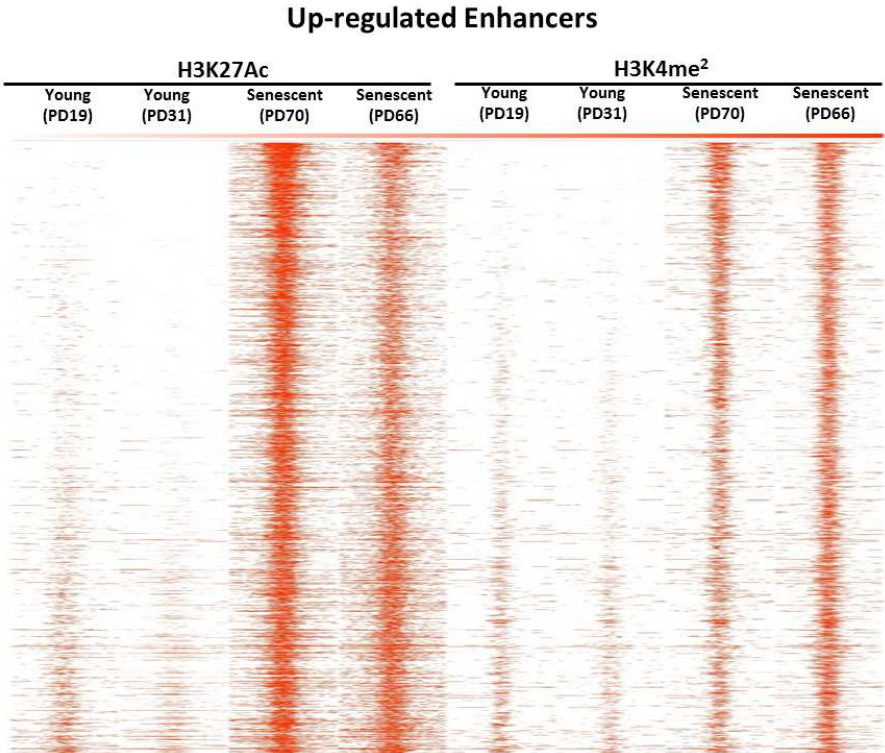


Figure 1. Heatmap of H3K27ac and H3K4me3 ChIP-seq enrichment in young and senescent cells on 2169 enhancers gained in replicative senescence. Each column shows $\pm$ 3kb window from mid-point of enhancer. Sorted based on averaged fold change of HOMER determined peakscores (Heinz et al., 2010b) of enhancers in replicative senescence.

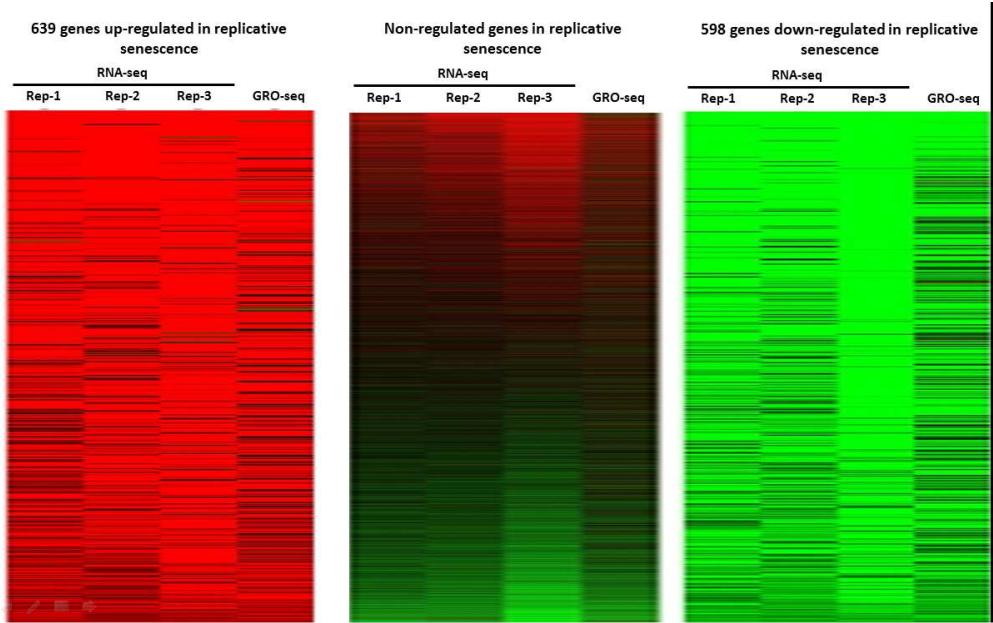


Figure 2. Heatmap of transcriptional changes observed in replicative senescence over up, down, and non-regulated gene sets. Increases (red) or decreases (green) in normalized tags over gene exon (RNA-seq) or body between from young to senescent are shown.

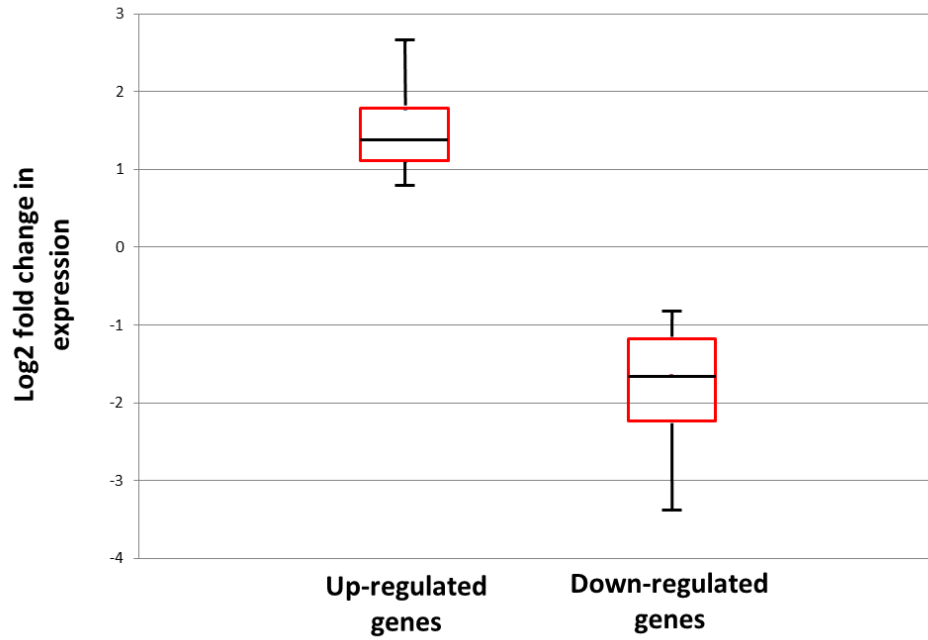


Figure 3. Box plot of genes in defined as up or down-regulated in replicative senescence. Whiskers show 5th and 95th percentiles, boundaries of boxes show 25 and 75th percentiles, and line within box shows 50th percentile of population. Values correspond to averaging of fold changes from young to senescent of RNA-seq triplicates and single GRO-seq replicate.

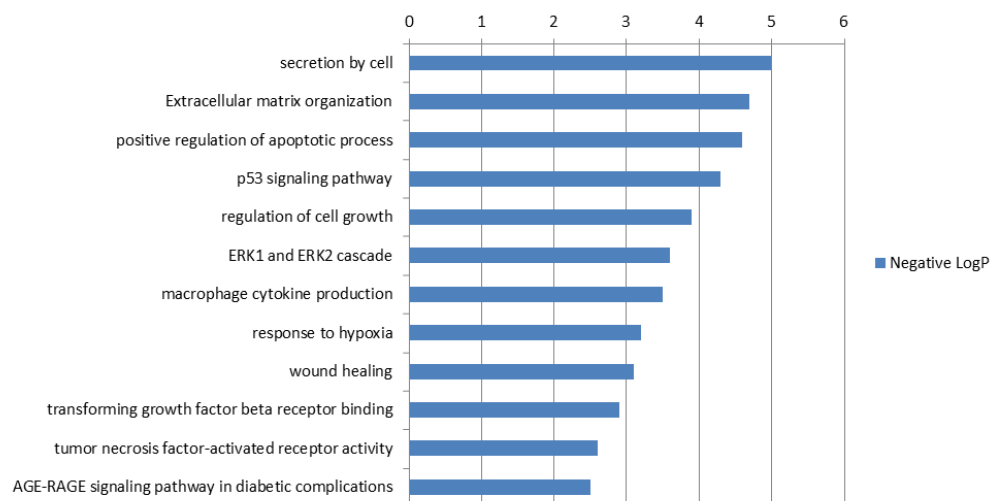


Figure 4. Selected GO-terms from Meta-scape analysis of 639 genes up-regulated during replicative senescence. Values listed are negative logP values of the term.

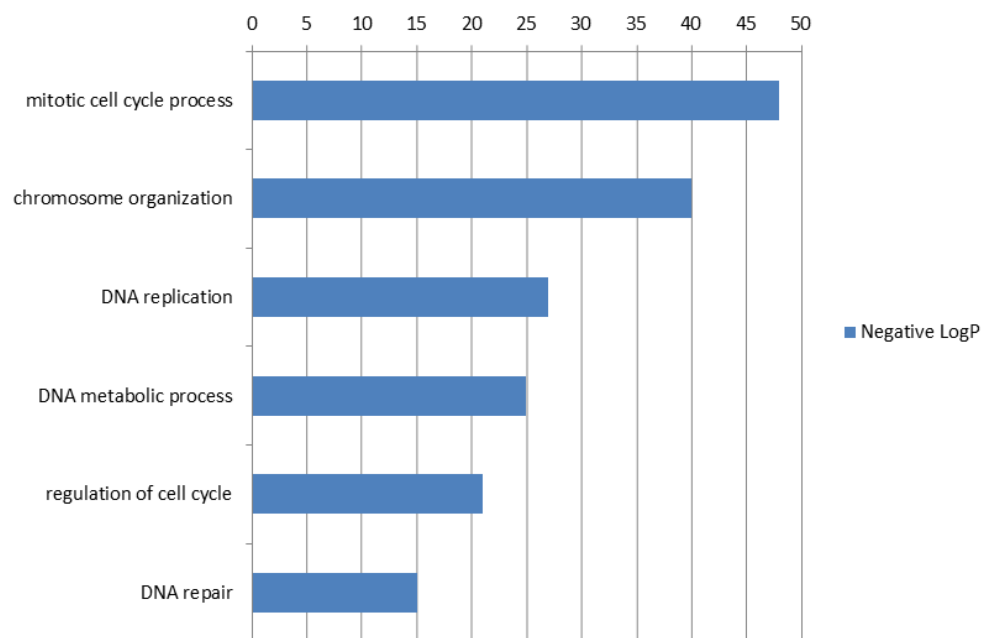


Figure 5. Selected GO-terms from Meta-scape analysis of 598 genes down-regulated during replicative senescence. Values listed are negative logP value of term.

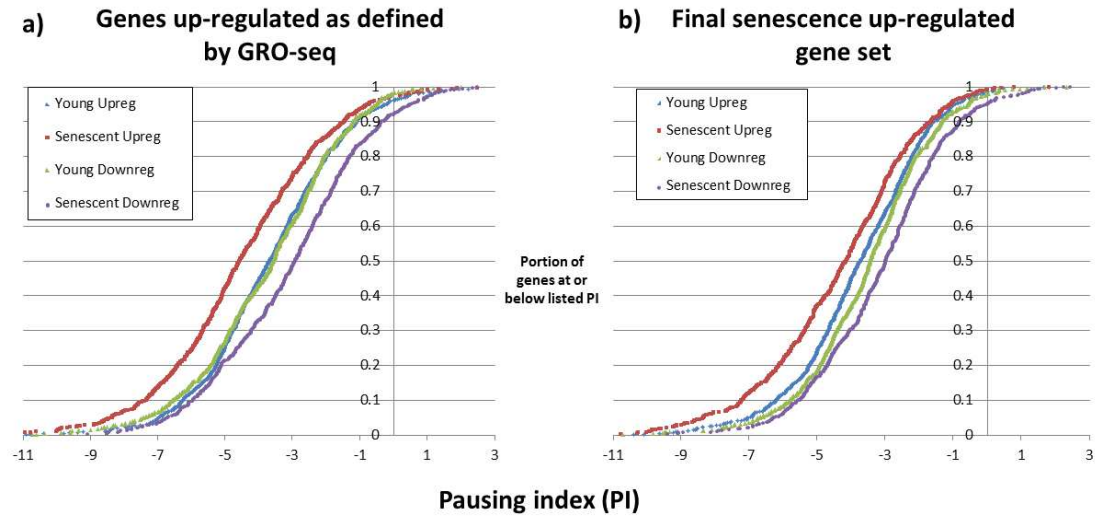


Figure 6. Pausing index curves determined by GRO-seq analysis in young and senescent cells **a)** over gene sets of up and down-regulated genes as defined by GRO-seq analysis along, or **b)** over 639 up and 598 down-regulated genes defined by RNA-seq and GRO-seq analysis combined.

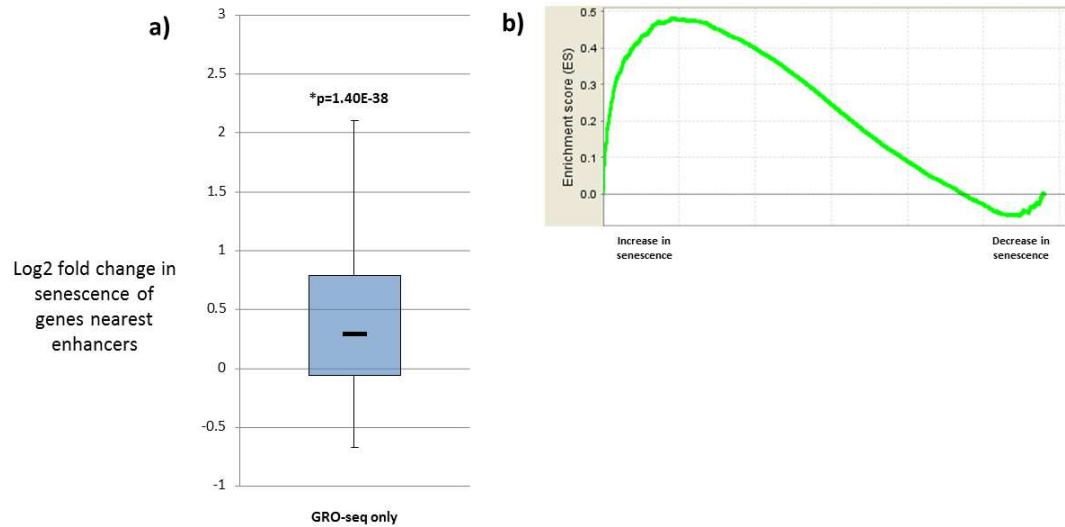


Figure 7. Analysis of transcriptional profile of genes above expression threshold nearest to 2169 senescence gained enhancers. **a)** box plot showing fold change of gene set during senescence **b)** Gene Set Enrichment Analysis (GSEA) enrichment score plot (Mootha et al., 2003; Subramanian et al., 2005) of gene set over expression dataset of 14,493 genes in BJ fibroblast over minimum expression threshold, ranked by fold change in replicative senescence based on averaging of fold changes of 3 RNA-seq and 1 GRO-seq replicate

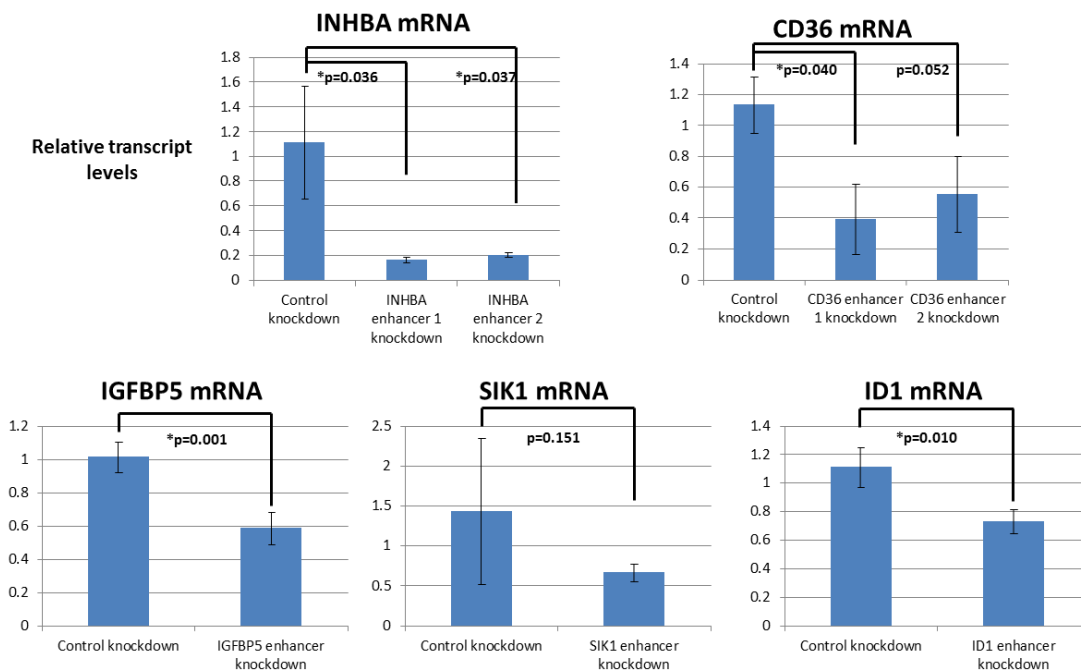


Figure 8. Fold change of senescence induced transcripts upon shRNA-mediated knockdown of senescence gained enhancers. Enhancers were selected based on proximity to genes induced during replicative senescence, significant gain of H3K27ac binding, and induction of GRO-seq signal in senescence gained H3K27ac peak. shRNAs were targeted to senescence induced GRO-seq signal, RNA was harvested after 2 weeks of knockdown and selection, and qPCR was performed on senescence induced gene. Relative levels of transcript provided in arbitrary units.

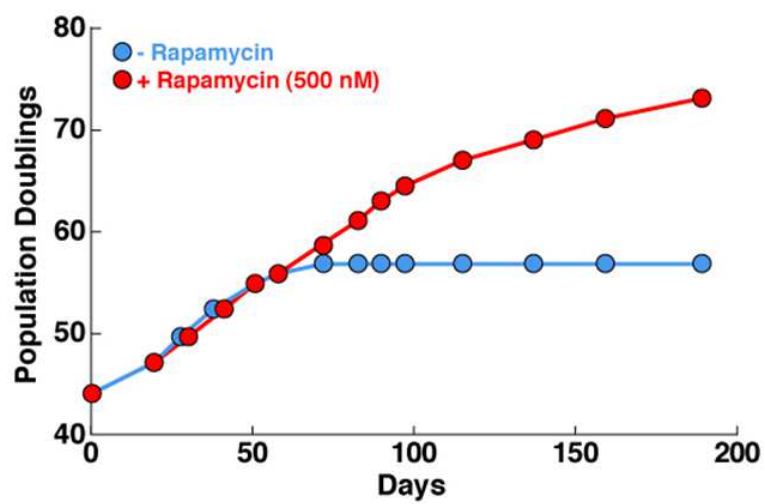


Figure 9. Growth curves in BJ fibroblasts treated with 500nM rapamycin or control.

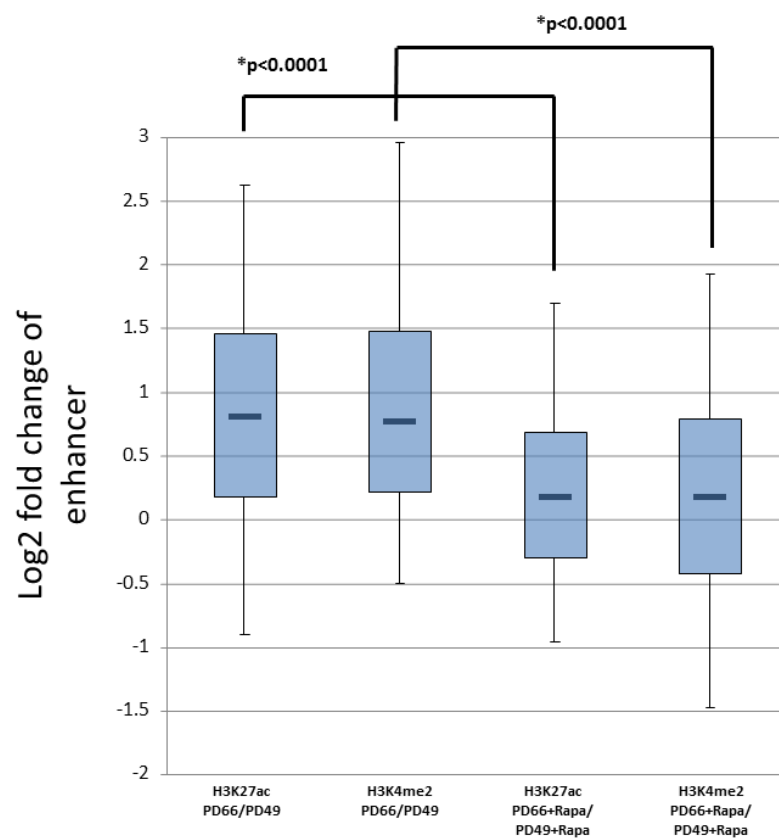


Figure 10. Box plots of fold change in enhancers between conditions listed on x-axis. Fold change based upon log2 ratio of peakscores of listed conditions.

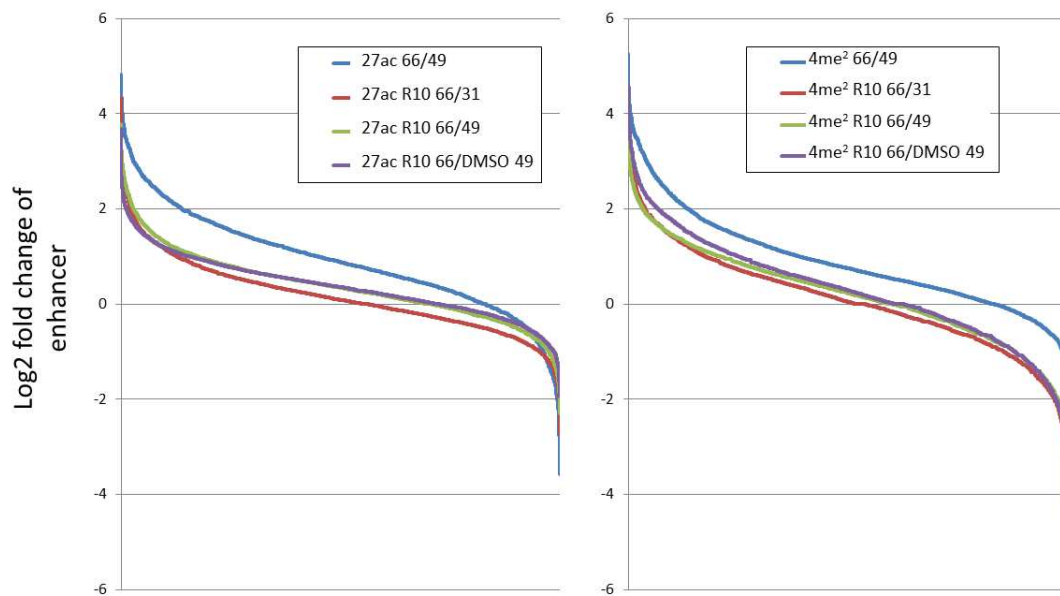


Figure 11. Ranked fold change of peakscores from conditions listed over enhancers gained in senescence. Set of enhancers gained in senescence was determined from independent replicate of H3K27ac and H3K4me2 ChIP-seqs from those used in conditions plotted in figure.

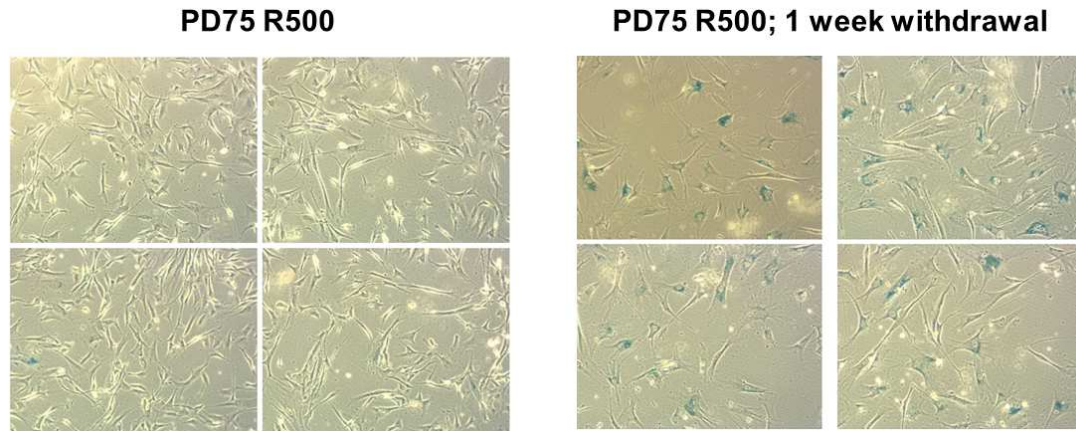


Figure 12. Beta-galactosidase staining of population doubling 75 BJ fibroblasts maintained in 500nM rapamycin, or withdrawn from rapamycin maintenance for 1 week.

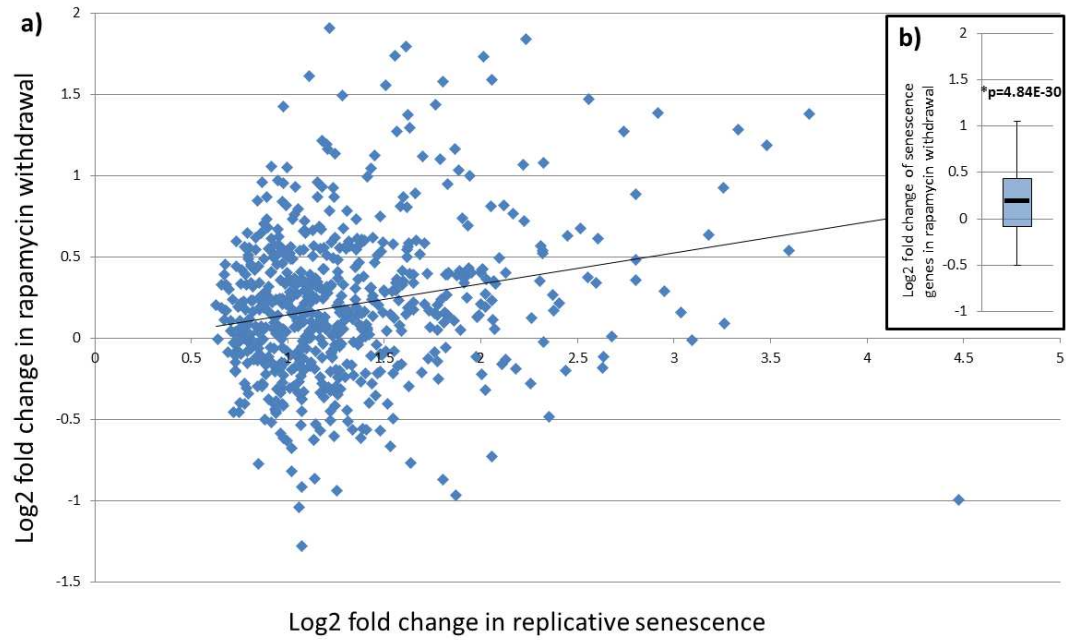


Figure 13. Fold change in rapamycin withdrawal of genes upregulated in replicative senescence. **a)** Scatterplot of fold changes between conditions **b)** box plot of fold changes in rapamycin withdrawal of 639 genes induced in replicative senescence

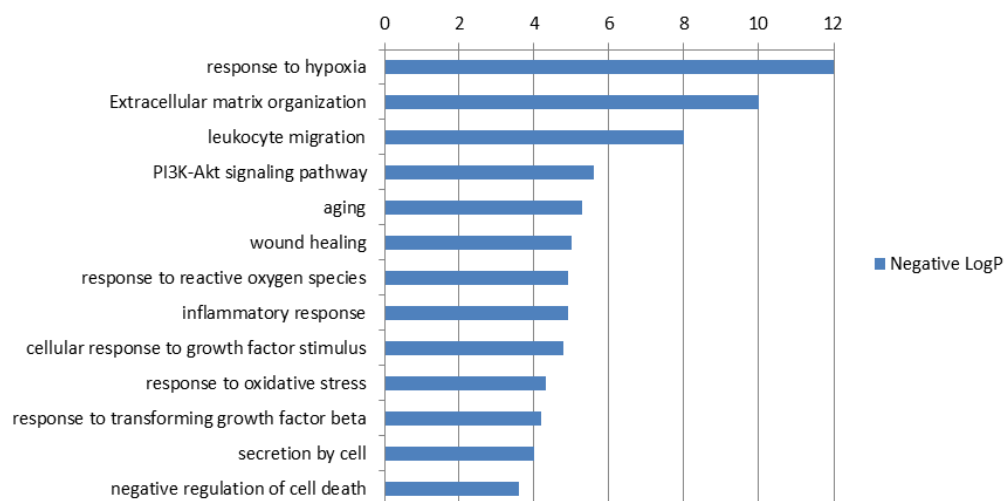


Figure 14. Selected GO-terms from Meta-scape analysis of 252 genes up-regulated during rapamycin withdrawal. Values listed are negative logP value of term.

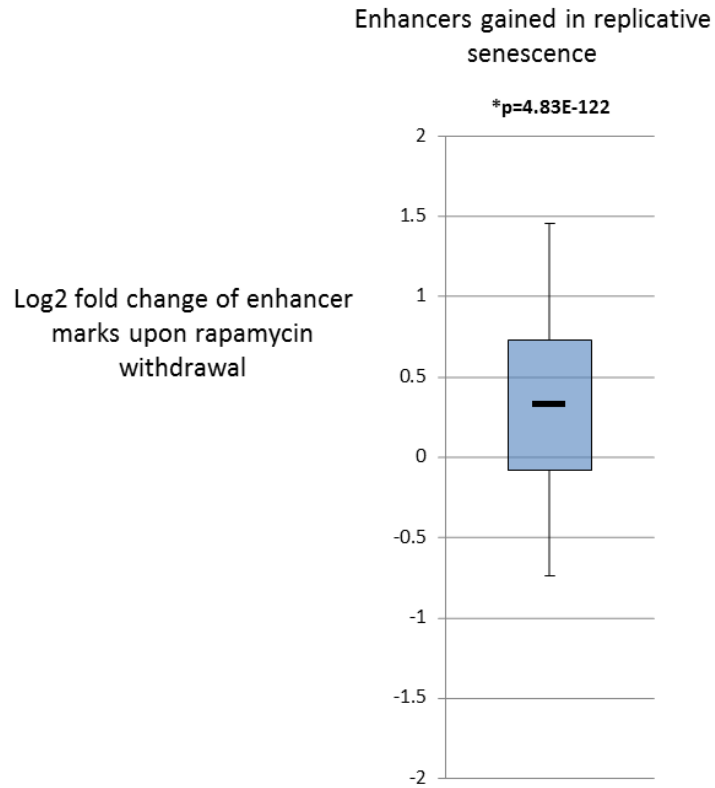


Figure 15. Box plots of fold change of enhancers marks upon rapamycin withdrawal over 2169 enhancers gained during replicative senescence. Fold change listed is an average of the fold changes in rapamycin withdrawal of the H3K27ac and H3K4me2 peakscores of these enhancers.

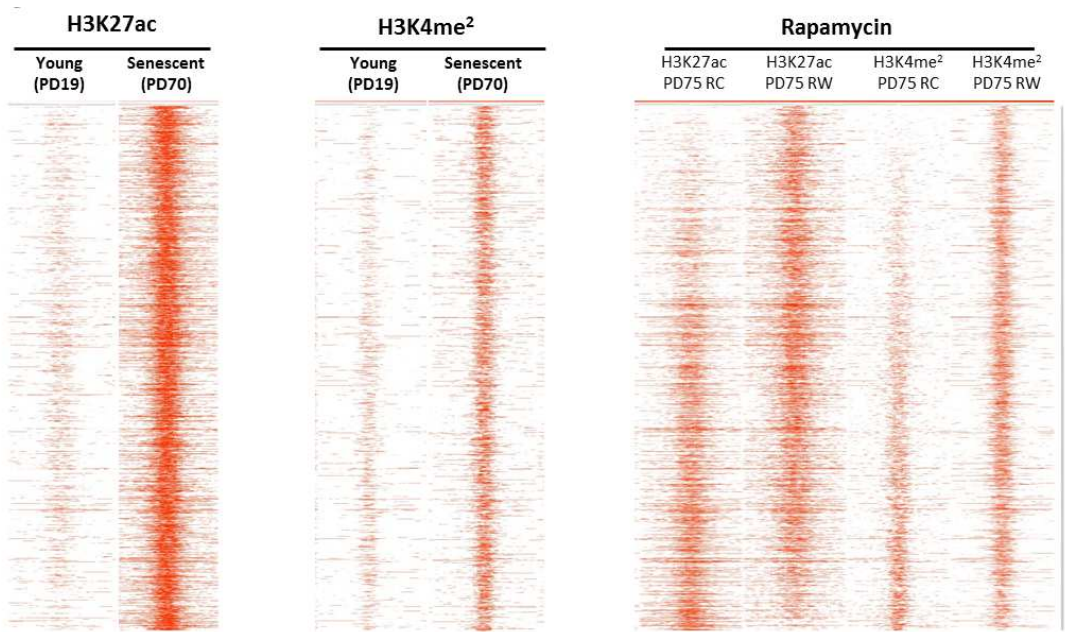


Figure 16. Heatmap of H3K27ac and H3K4me3 of 2169 enhancers gained in replicative senescence in young, senescent, rapamycin maintained, and rapamycin withdrawal conditions. Sorted based on fold change of enhancers in rapamycin withdrawal. Each column shows +/- 3kb window from mid-point of enhancer.

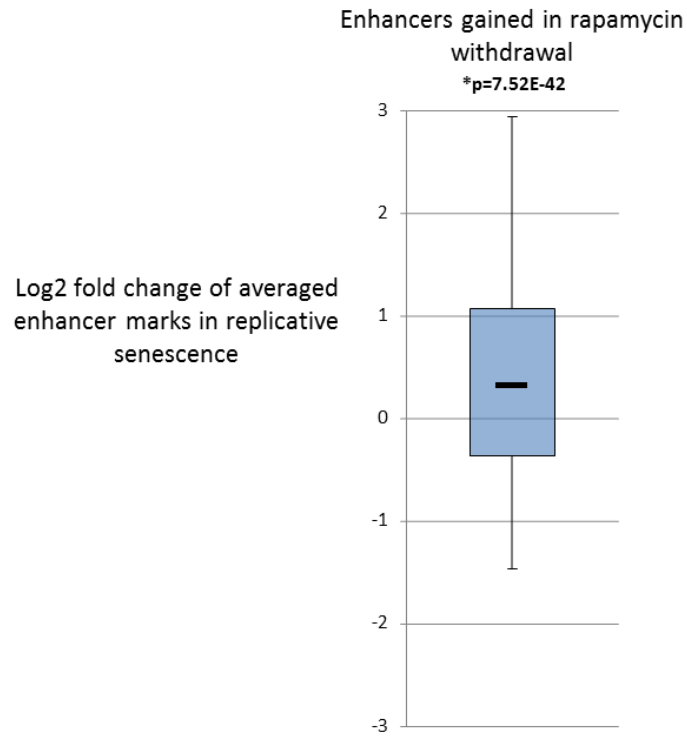


Figure 17. Box plots of fold change of enhancer peaks during replicative senescence over 1512 enhancers gained during rapamycin withdrawal. Fold change listed is an average of the fold changes in replicative senescence of the H3K27ac and H3K4me2 peakscores of these enhancers.

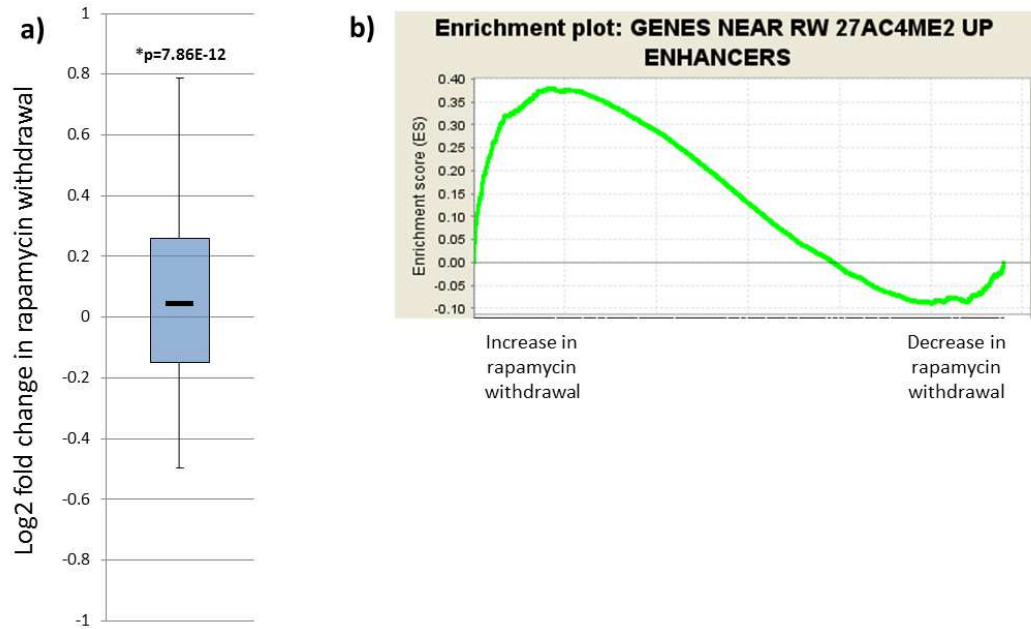


Figure 18. Analysis of transcriptional profile of genes above expression threshold nearest to 1512 rapamycin withdrawal gained enhancers. **a)** box plot showing fold change of gene set during rapamycin withdrawal **b)** GSEA enrichment score plot of gene set over expression dataset of 14,493 genes in BJ fibroblast over minimum expression threshold, ranked by fold change in rapamycin withdrawal

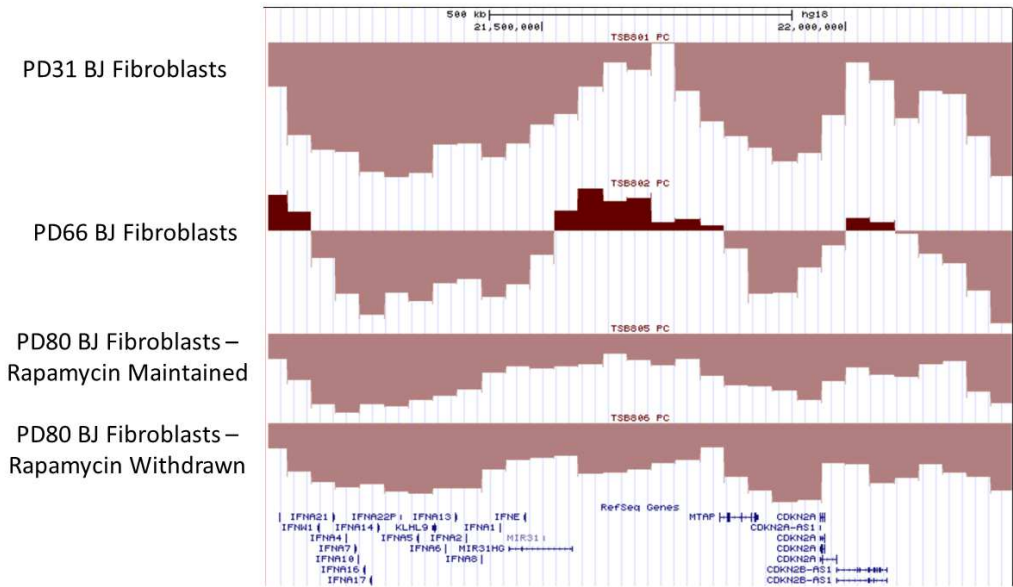


Figure 19. A/B compartments from PCA analysis of *in situ* HiC.

Rank	Motif	P-value	Best Match Details
1		1e-198	HIF1b(HLH)O785-HIF1b-ChIP-Seq(GSE34871)/Homer More Information Similar Motifs Found
2		1e-69	TEAD(TEA)Fibroblast-PU.1-ChIP-Seq Homer More Information Similar Motifs Found
3		1e-26	NFkB-p65-Rel(RHD)LPS-exp Homer More Information Similar Motifs Found
4		1e-26	MA0124.1_NKX3-1/Jaspar More Information Similar Motifs Found
5		1e-25	Smad2(MAD)ES-SMAD2-ChIP-Seq(GSE29422)/Homer More Information Similar Motifs Found
6		1e-21	EKLF(Zf)Erythrocyte-Klf1-ChIP-Seq(GSE20478)/Homer More Information Similar Motifs Found
7		1e-19	MA0052.1_MEF2A/Jaspar More Information Similar Motifs Found

Figure 20. Motif analysis performed upon H3K27ac peaks showing 3-fold increase in peakscore during replicative senescence.

Rank	Motif	P-value	Best Match Details
1		1e-28	HIF1b(HLH)O785-HIF1b-ChIP-Seq(GSE34871)Homer More Information Similar Motifs Found
2		1e-27	TEAD(TEA)Fibroblast-PU.1-ChIP-Seq Homer More Information Similar Motifs Found
3		1e-14	PB0145.1_Mafk_2/Jaspar More Information Similar Motifs Found
4		1e-13	PB0082.1_Tcf3_1/Jaspar More Information Similar Motifs Found
5		1e-12	MA0161.1_NFIC/Jaspar More Information Similar Motifs Found
6		1e-12	MA0041.1_Foxd3/Jaspar More Information Similar Motifs Found
7		1e-11	MA0075.1_Prrx2/Jaspar More Information Similar Motifs Found

Figure 21. Motif analysis performed upon H3K27ac peaks showing 4-fold increase in peak score during replicative senescence.

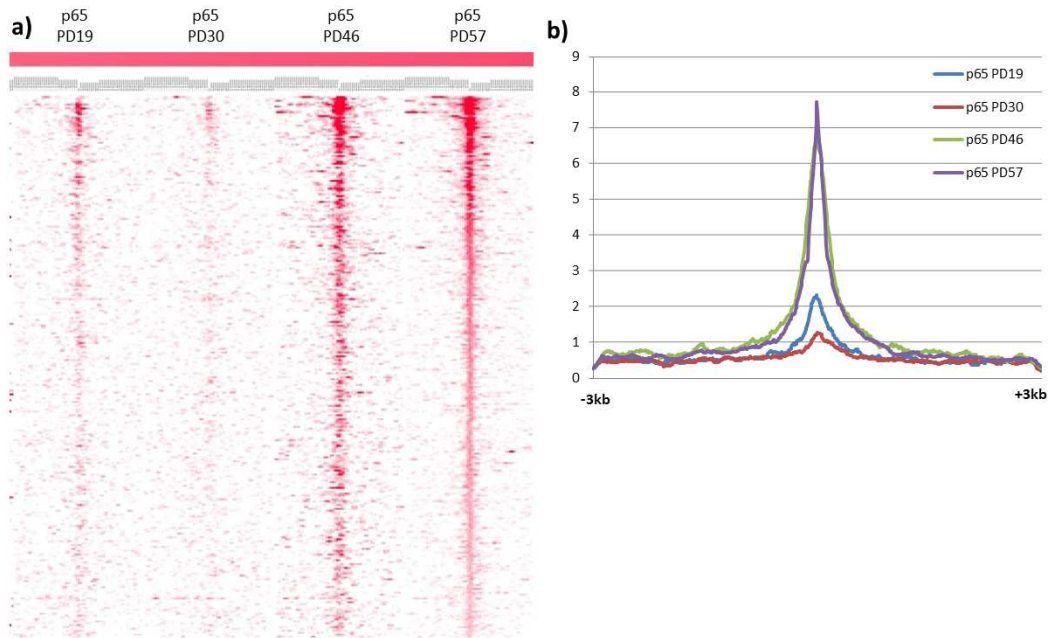


Figure 22. p65 ChIP-seq binding profile at p65 bound subset of 2169 gained senescence enhancers **a)** Heatmap of p65 ChIP-seq enrichment at listed population doublings. Each column shows ± 3 kb window from mid-point of p65 peak. Sorted based on p65 peakscore at population doubling 57 **b)** meta-analysis of p65 tag density from p65-ChIP at listed population doubling over p65 bound enhancers

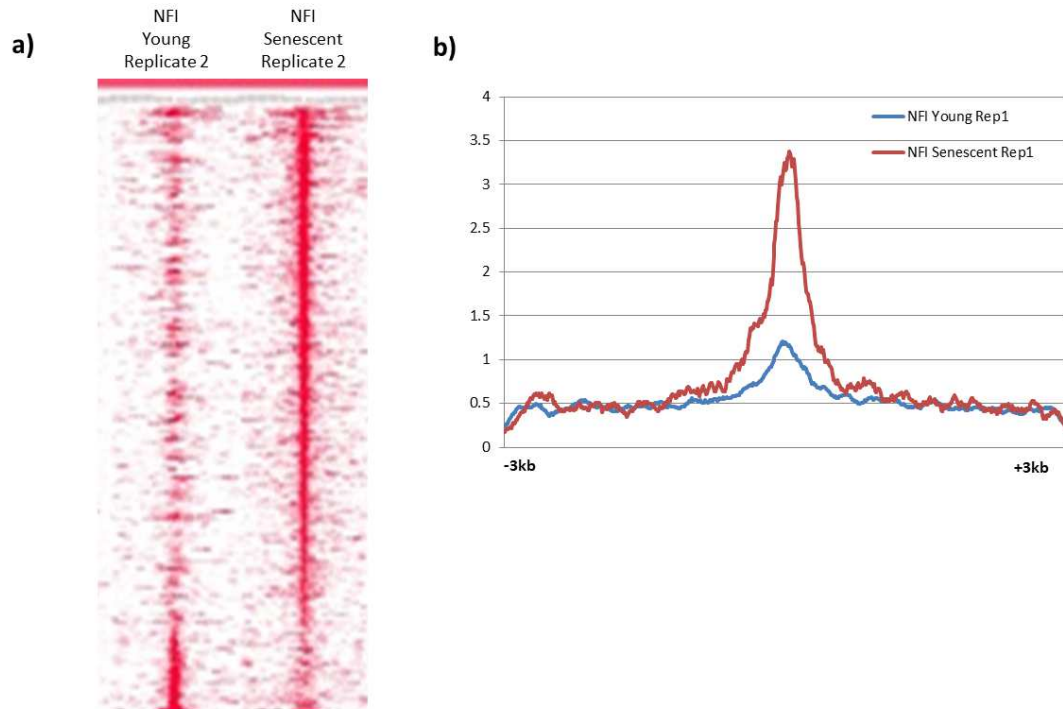


Figure 23. NFI ChIP-seq binding profile at NFI bound subset of 2169 gained senescence enhancers **a)** Heatmap of replicate 2 of NFI ChIP-seq enrichment from young or senescent cells. Each column shows $\pm$ 3kb window from mid-point of NFI peak. Sorted based on NFI peakscore **b)** meta-analysis of NFI tag density from replicate 1 of NFI ChIP-seq at listed population doubling over NFI bound enhancers

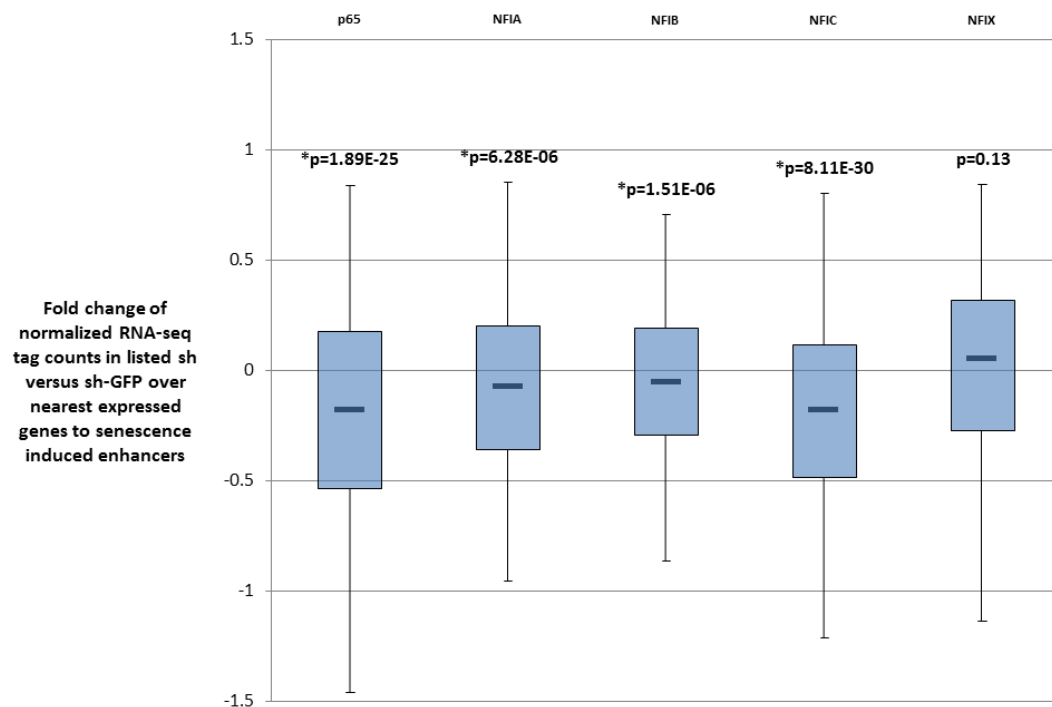


Figure 24. Box plots showing change in RNA-seq tag density over nearest expressed genes to 2169 senescence gained enhancers upon 2 week knockdown of listed factor in PD64 BJ fibroblasts. Fold change represents ratio of normalized tag count in knockdown listed factor versus sh-GFP control.

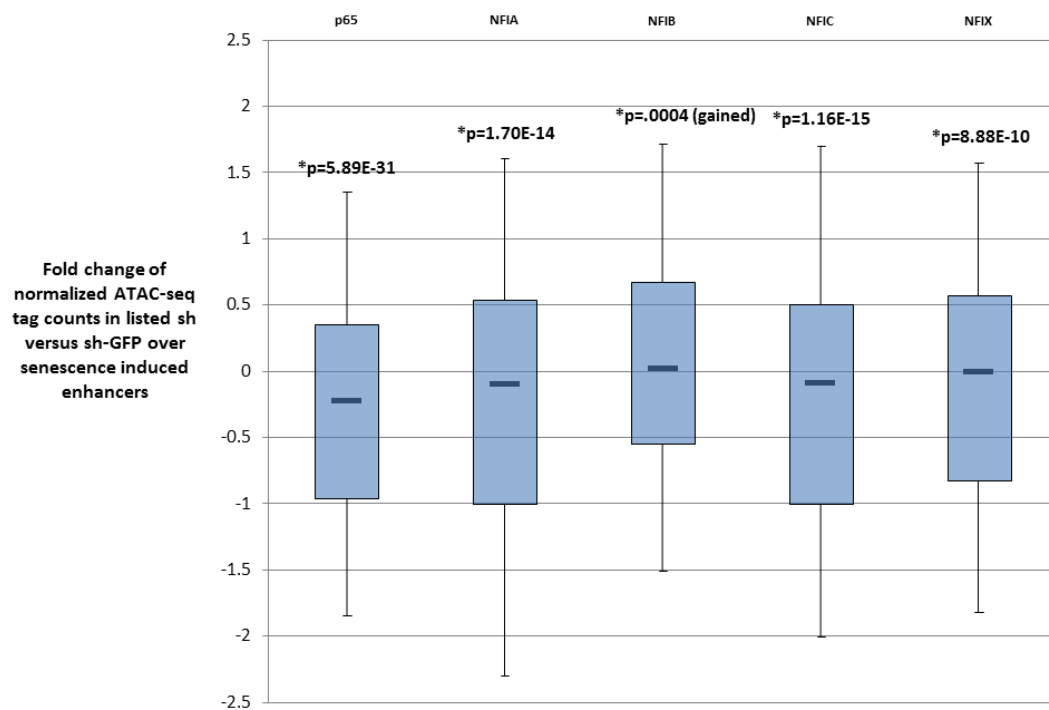


Figure 25. Box plots showing change in ATAC-seq tag density over 2169 senescence gained enhancers upon 2 week knockdown of listed factor in PD64 BJ fibroblasts. Fold change represents ratio of normalized tag count in knockdown listed factor versus sh-GFP control.

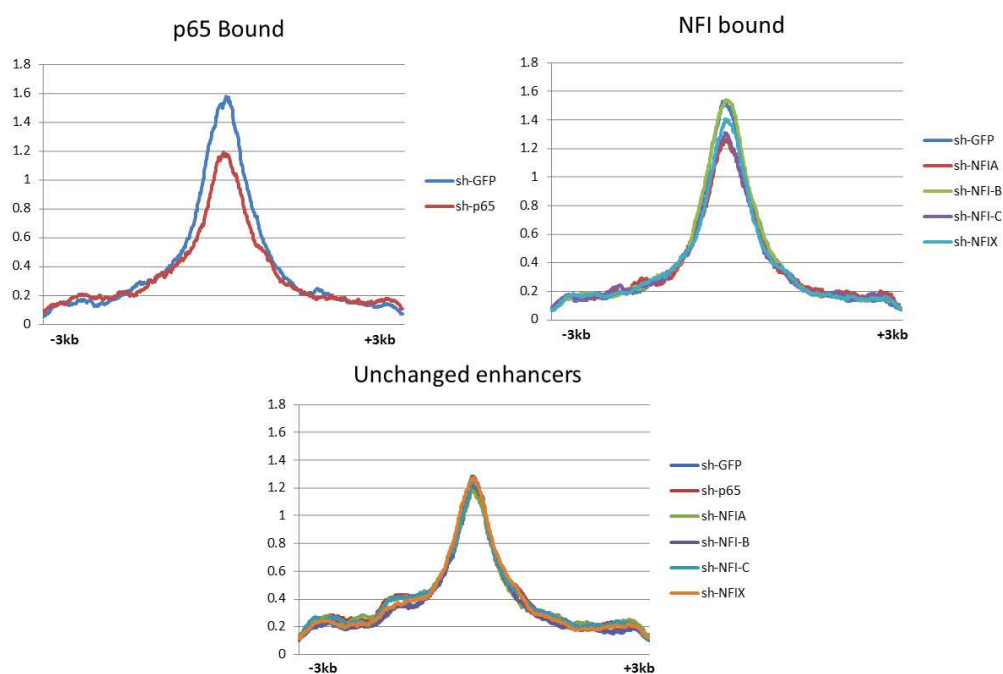


Figure 26. Meta-analysis distribution of normalized ATAC-seq tag density over listed transcription factor bound subset of peaks showing H3K27ac gain during replicative senescence, or control enhancers showing no change in H3K27ac.

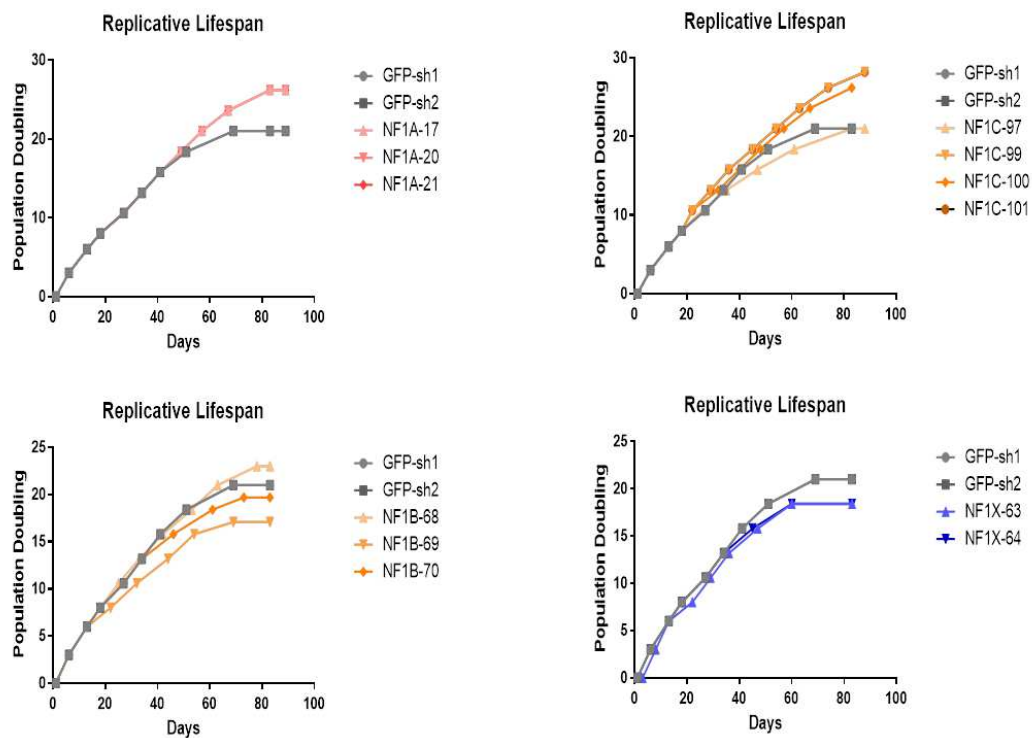


Figure 27. Population doublings over time of BJ fibroblasts after shRNA mediated stable knockdown of NFI family member or sh-GFP control. Different replicates of NFI family members represent distinct shRNA constructs.

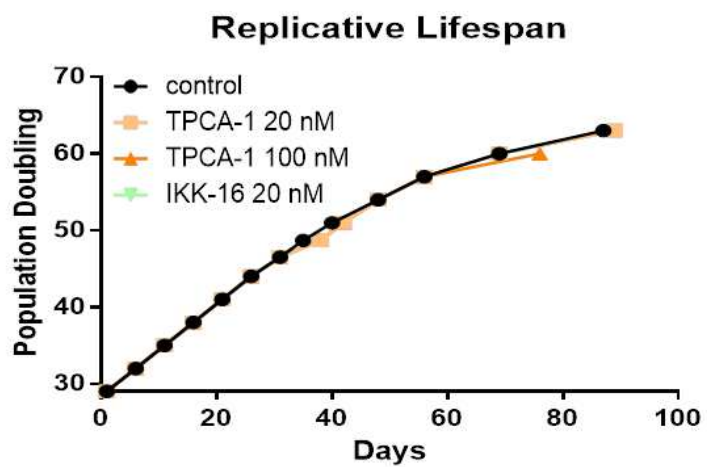


Figure 28. Population doublings over time of BJ fibroblasts after continued treatment with listed NFkB pathway inhibitor or vehicle control.

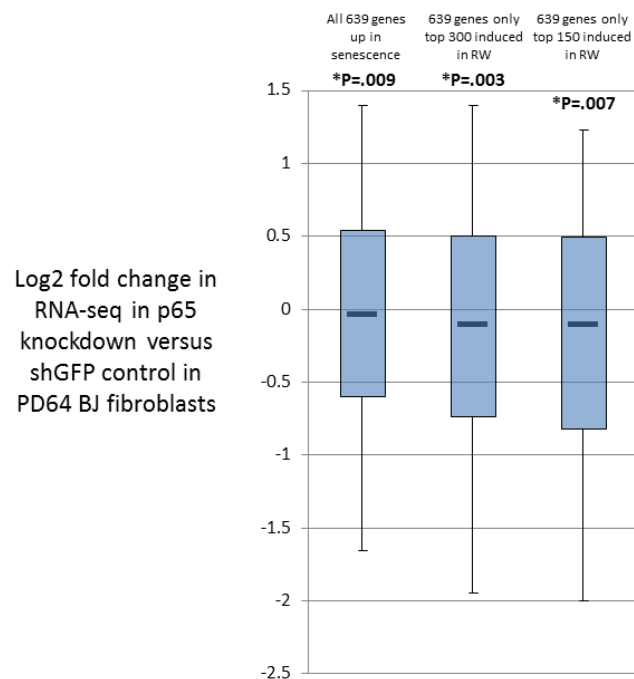


Figure 29. Box plots showing change in RNA-seq tag density over listed sets and subsets of 639 genes induced in replicated senescence in listed p65 stable knockdowns versus sh-GFP control in PD64 BJ fibroblasts. Fold change represents ratio of normalized tag count in knockdown of p65 versus sh-GFP control.

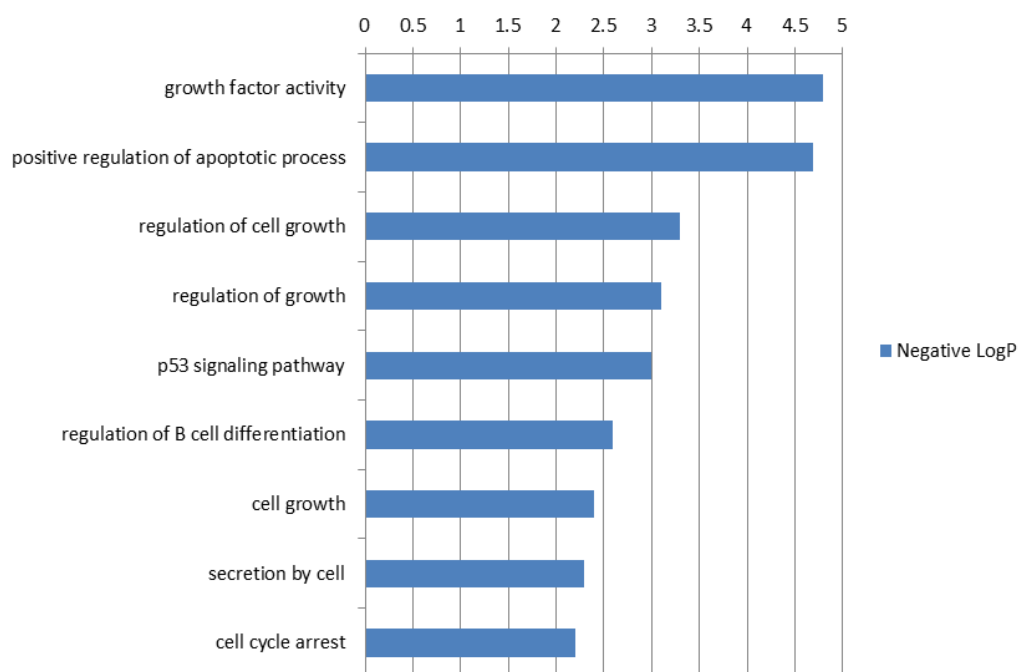


Figure 30. Selected GO-terms from Meta-scape analysis of the 300 genes up-regulated in senescence that are least induced by rapamycin withdrawal. Values listed are negative logP values of the term.

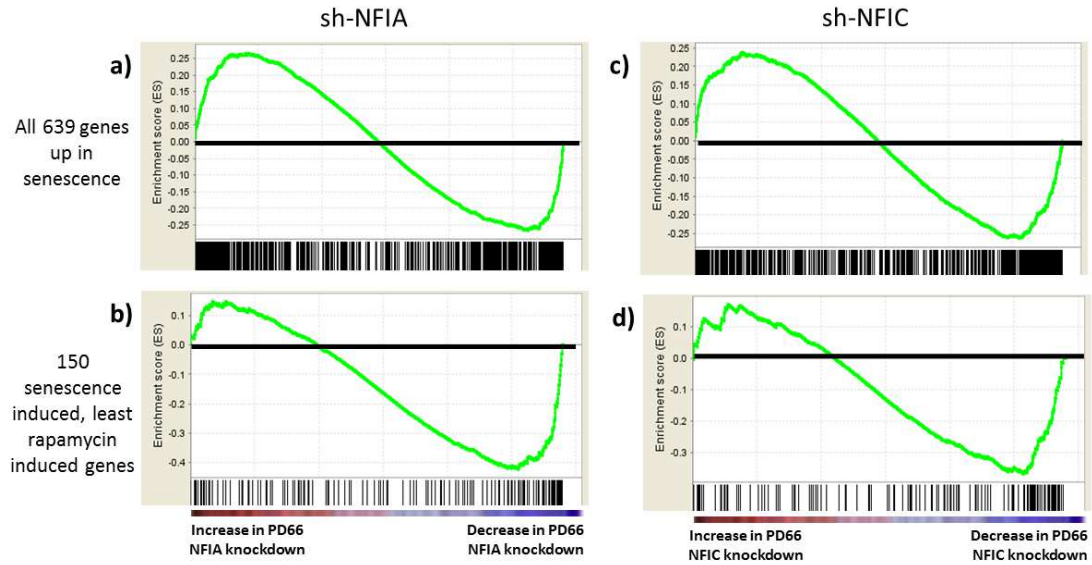


Figure 31. GSEA enrichment score plot of gene sets of either **a and c)** all 639 genes up-regulated in replicative senescence or **b and d)** the 150 genes up-regulated in senescence that are least induced by rapamycin withdrawal over expression datasets of 14,493 genes in BJ fibroblast over minimum expression threshold, ranked either by fold change in knockdown of either **a and b)** NFIA or **c and d)** NFIC versus sh-GFP control in PD64 BJ fibroblasts

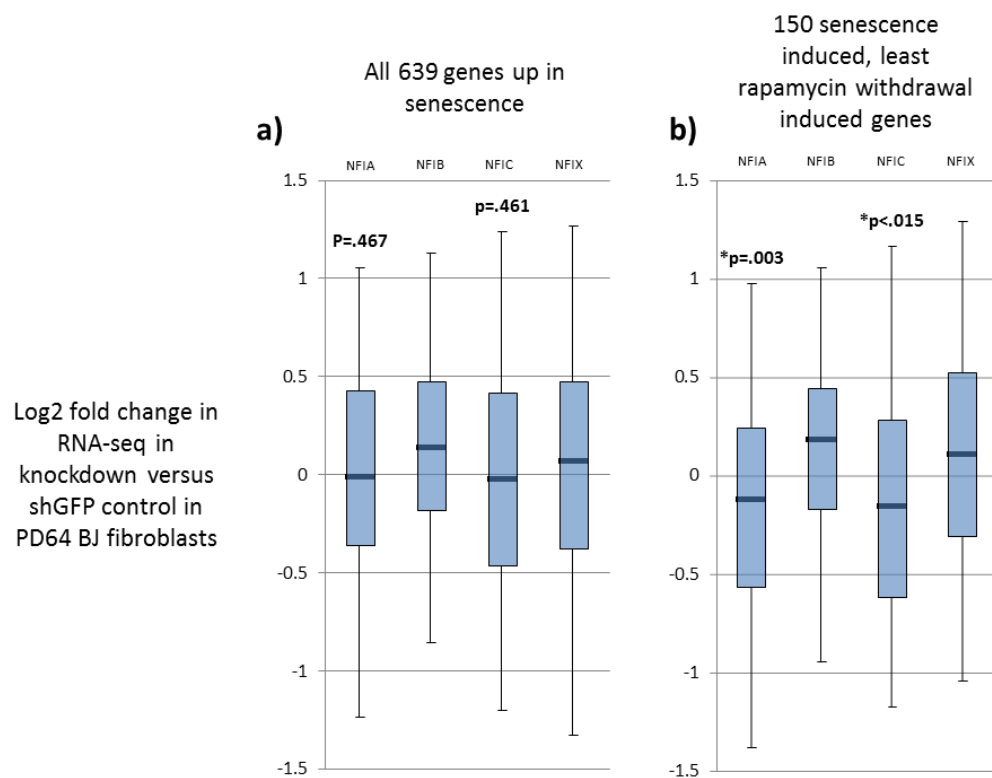


Figure 32. Box plots showing change in RNA-seq tag density over **a)** all 639 genes up-regulated in replicative senescence or **b)** the 150 genes up-regulated in senescence that are least induced by rapamycin withdrawal in listed NFI family member stable knockdowns versus sh-GFP control in PD64 BJ fibroblasts. Fold change represents ratio of normalized tag count in knockdown of listed NFI family member versus sh-GFP control.

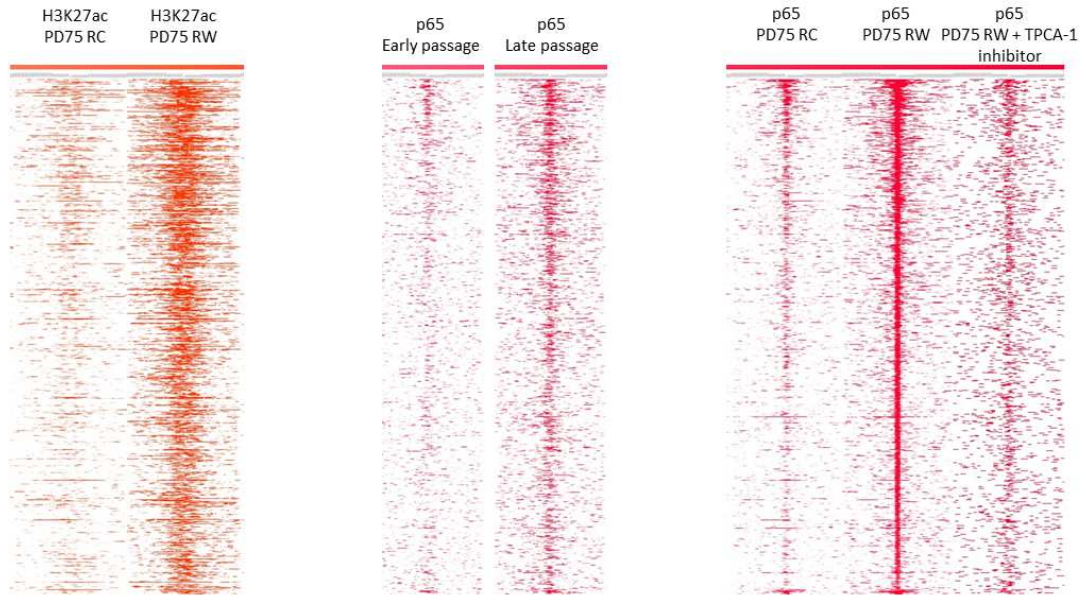


Figure 33. Heatmap of p65 or H3K27ac ChIP-seq enrichment on p65 bound subset of 1512 rapamycin withdrawal gained enhancers at listed conditions. Each column shows ± 3 kb window from mid-point of p65 peak. Sorted based on p65 peakscore in rapamycin withdrawal.

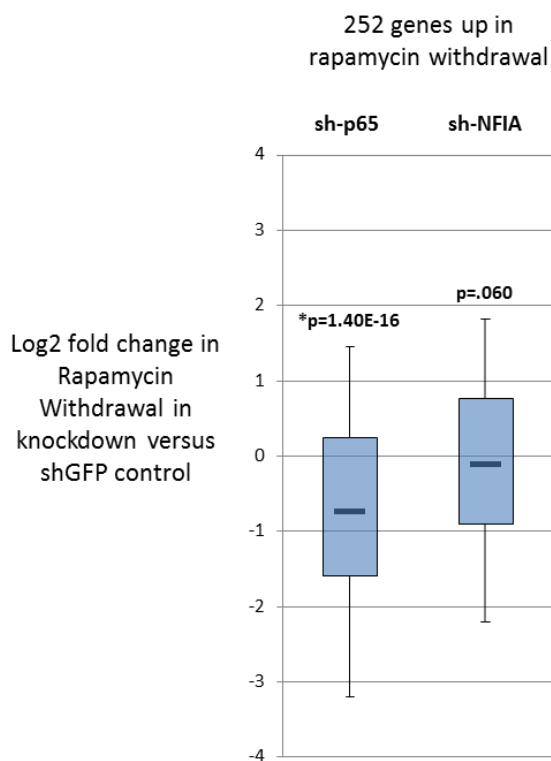
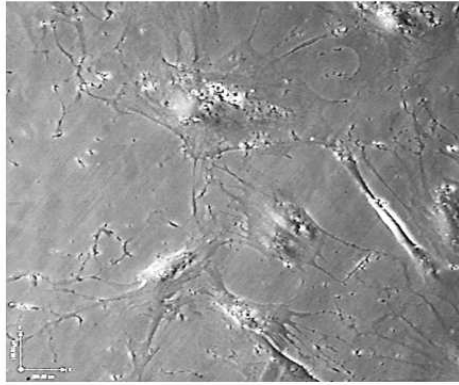


Figure 34. Box plots showing change in RNA-seq tag density in listed p65 or NFIA stable knockdowns after 2 weeks of rapamycin withdrawal versus sh-GFP control after 2 weeks of rapamycin withdrawal, both in PD75 BJ fibroblasts, over 252 genes up-regulated in rapamycin withdrawal. Fold change represents ratio of normalized tag count in knockdown of p65 or NFIA versus sh-GFP control.



**Senescent shGFP Control
after rapamycin withdrawal**



**Senescent sh-p65 after
rapamycin withdrawal**

Figure 35. Phase contrast images of PD75 BJ fibroblasts under stable knockdown of either p65 or shGFP control after 2 weeks of rapamycin withdrawal.

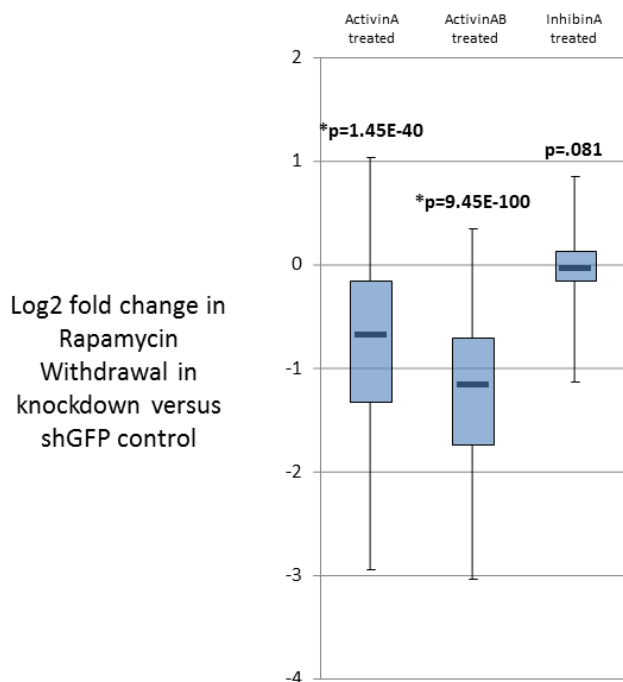


Figure 36. Box plots showing change in RNA-seq tag density in listed treatment after 2 weeks of rapamycin withdrawal versus control DMSO treatment after 2 weeks of rapamycin withdrawal, both in PD75 BJ fibroblasts, over 252 genes up-regulated in rapamycin withdrawal. Fold change represents ratio of normalized tag count in treatment versus DMSO control.

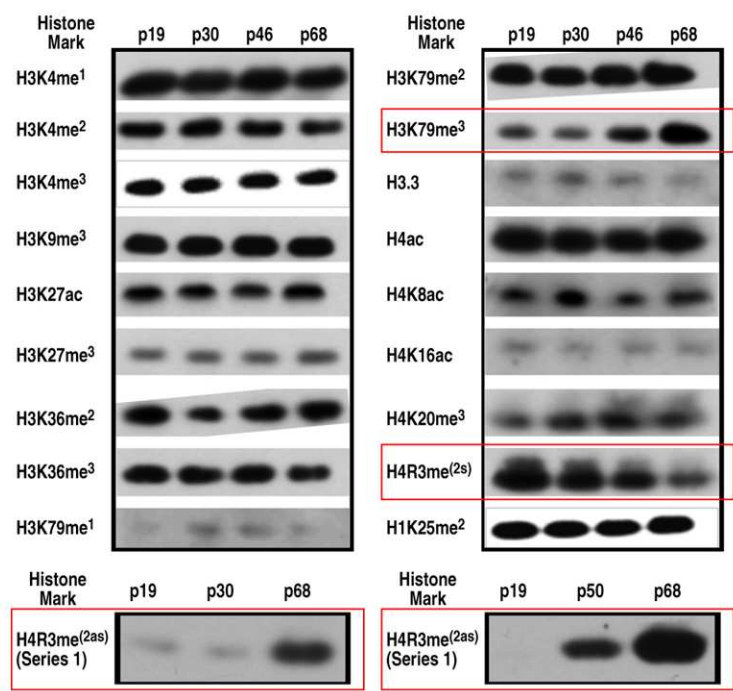


Figure 37. Western blots of listed histone modifications at listed population doubling. Samples were normalized by total histone levels, using Ponceau S staining and blotting of histone H3.

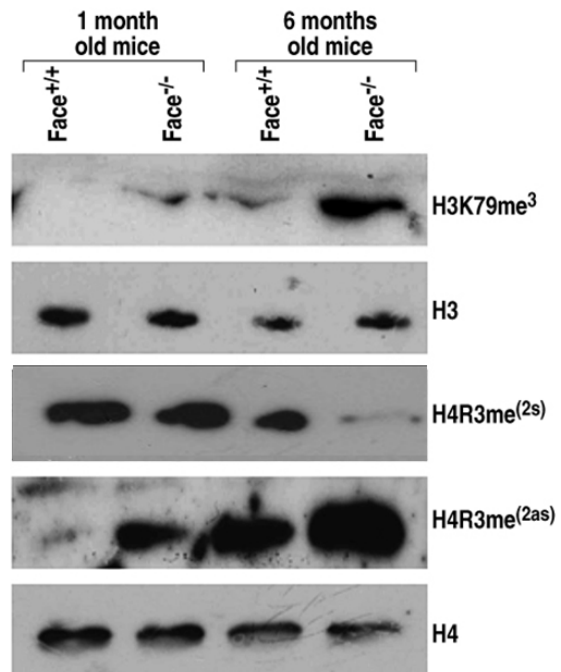


Figure 38. Western blots of lysates harvested from WT or ZMPSTE24 (Face^{-/-}) mice at either 1 or 6 months of age. Lysates blotted for H3K79me³ were normalized to total H3 levels, while lysates blotted for H4R3 methyl marks were normalized to total H4 levels.

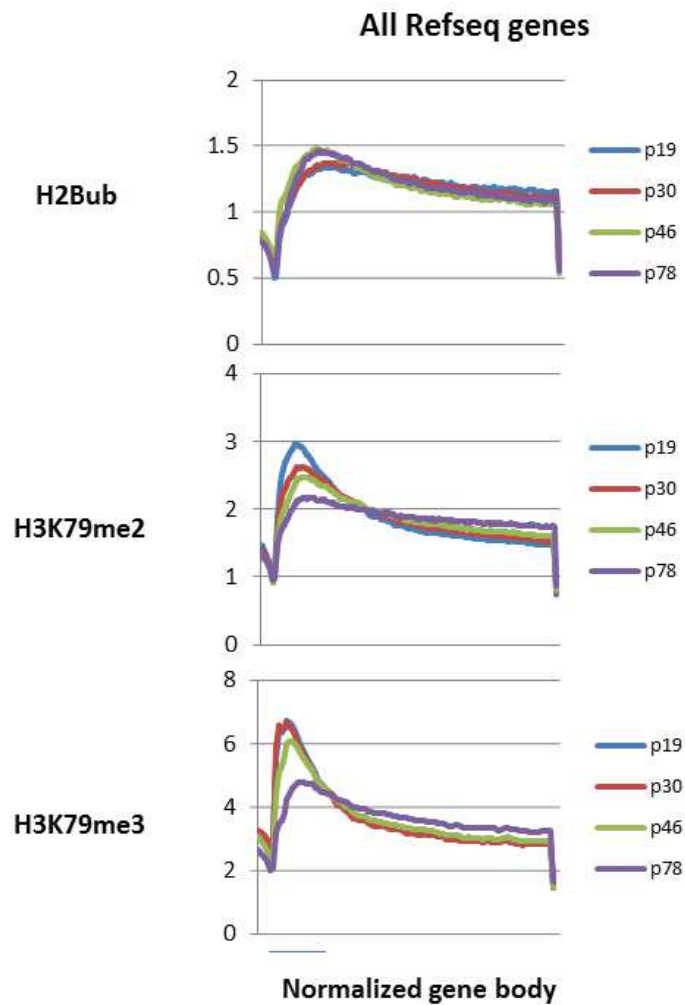


Figure 39. Meta-analysis of H2Bub, H3K79me2, or H3K79me3 levels across normalized gene bodies of all Refseq genes a listed population doublings. Underlined region corresponds to 2kb region downstream of TSS.

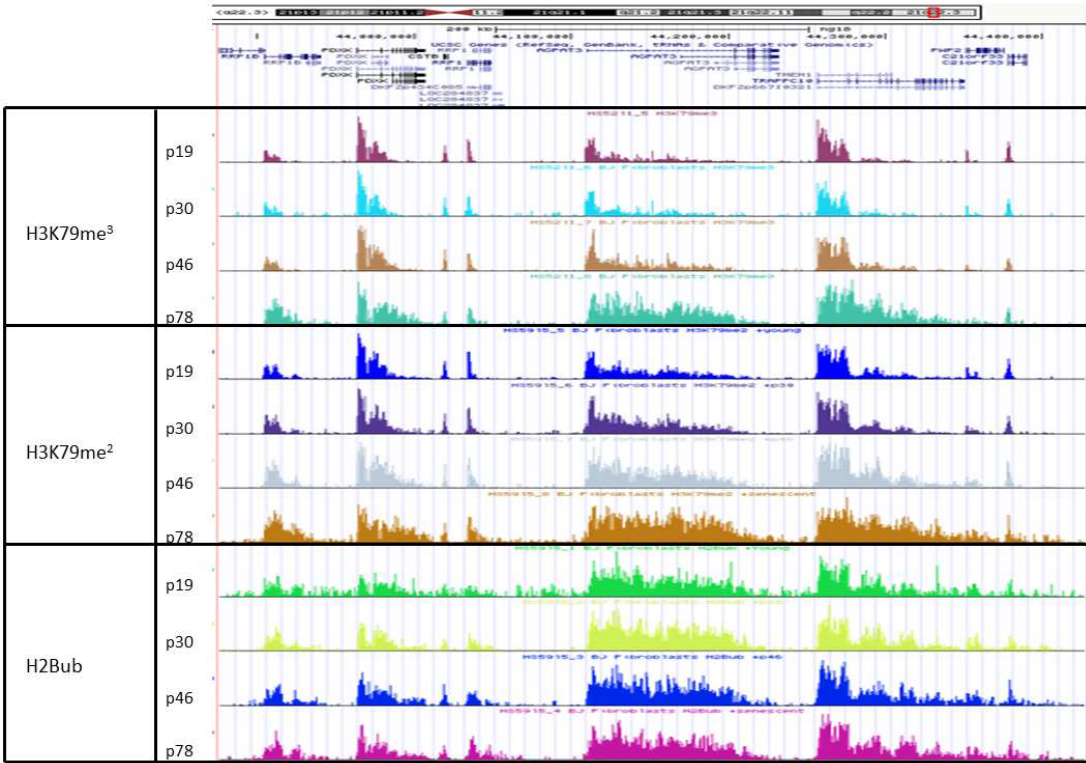


Figure 40. UCSC genome browser bedgraphs of H2Bub, H3K79me2, or H3K79me3 levels at representative genomic regions.

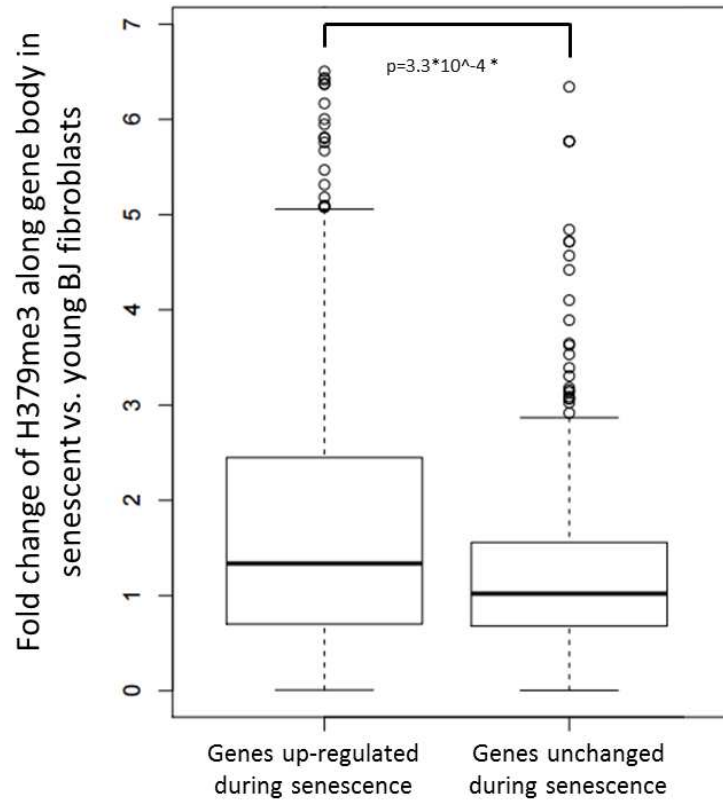


Figure 41. Box plot of the fold change during senescence of the total normalized tags of H3K79me3 across the gene bodies of genes up-regulated during senescence versus unchanged genes.

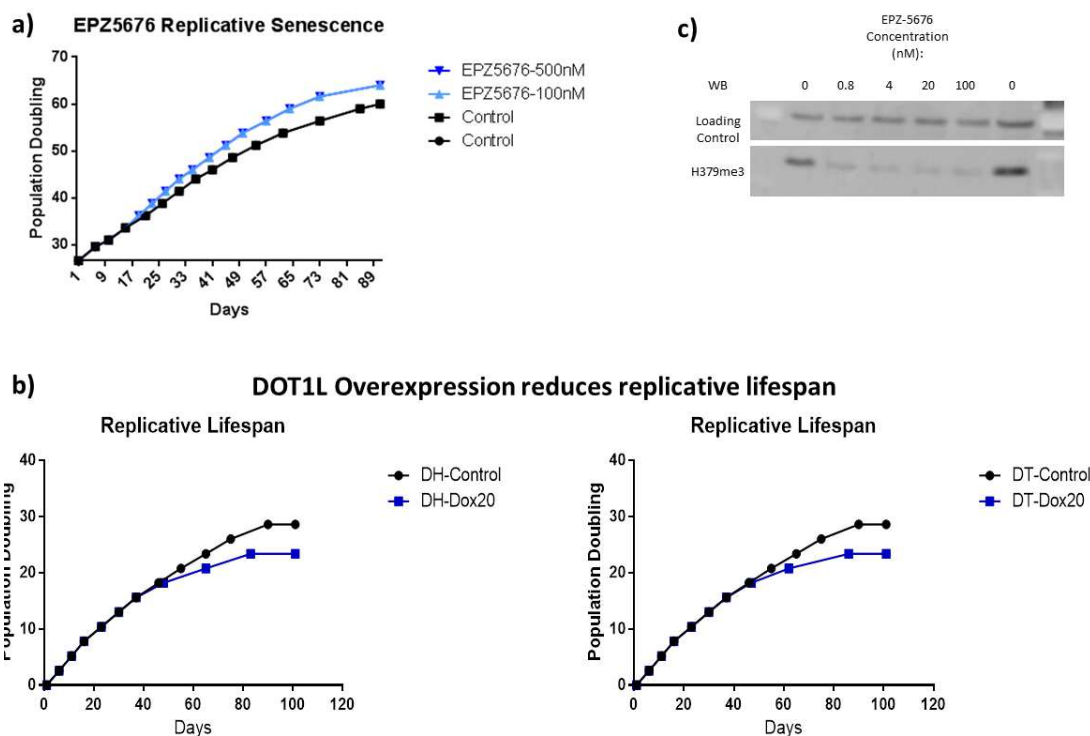


Figure 42. Role of H3K79me3 in replicative senescence. **a)** Population doublings over time of BJ fibroblasts with continued treatment with EPZ-5676 DOT1L inhibitor versus DMSO control. **b)** Population doublings over time of BJ fibroblasts expressing doxycycline inducible HA (DH) or TY1 (DT) tagged DOT1L or empty vector control in the continuous presence of 20nM doxycycline. **c)** Western blot of effect of EPZ-5676 on H3K79me3 levels in BJ fibroblasts at listed doses.

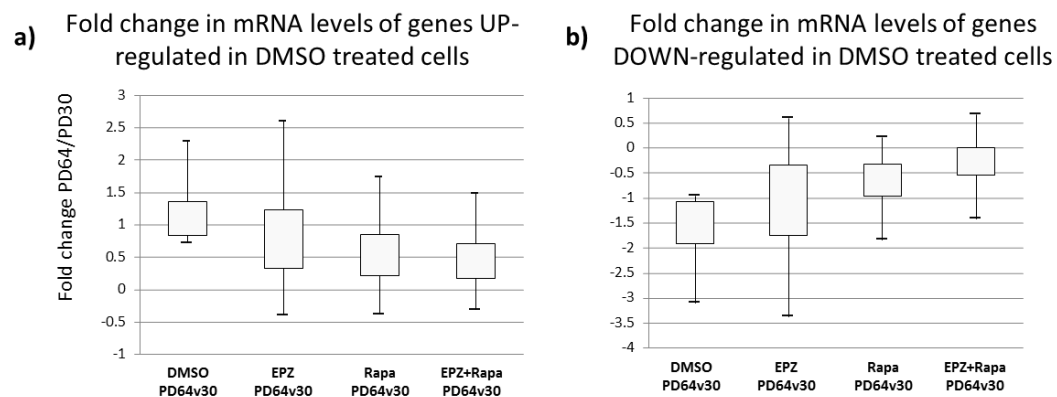


Figure 43. Box plots showing change in RNA-seq tag density in PD64 versus PD30 BJ fibroblasts in listed treatments across genes either **a)** up or **b)** down-regulated during replicative senescence. Fold change represents ratio of normalized tag count in treatment in genes at PD64 versus PD30.

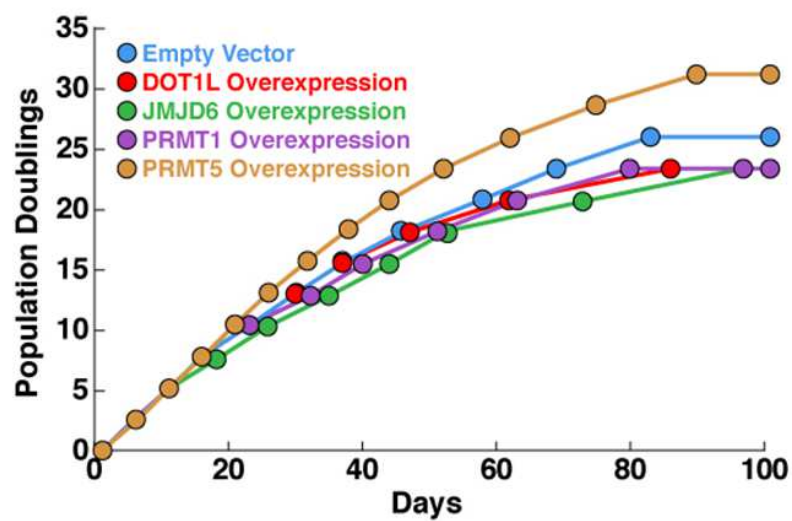


Figure 44. Population doublings over time of BJ fibroblasts expressing doxycycline inducible HA DOT1L, JMJD6, PRMT1, PRMT5, or empty vector control in the continuous presence of 20nM doxycycline.

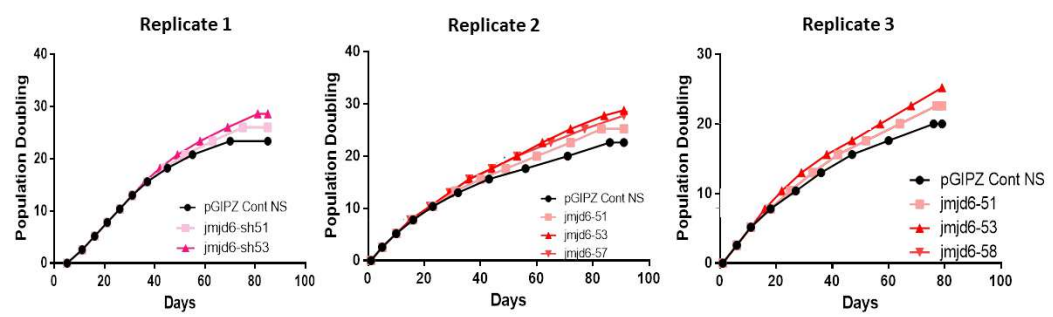


Figure 45. Population doublings over time of BJ fibroblasts after shRNA mediated stable knockdown of JMJD6 or sh-non silencing (NS) control. jmjd6-51, 53, 57, and 58 each represent distinct shRNA constructs.

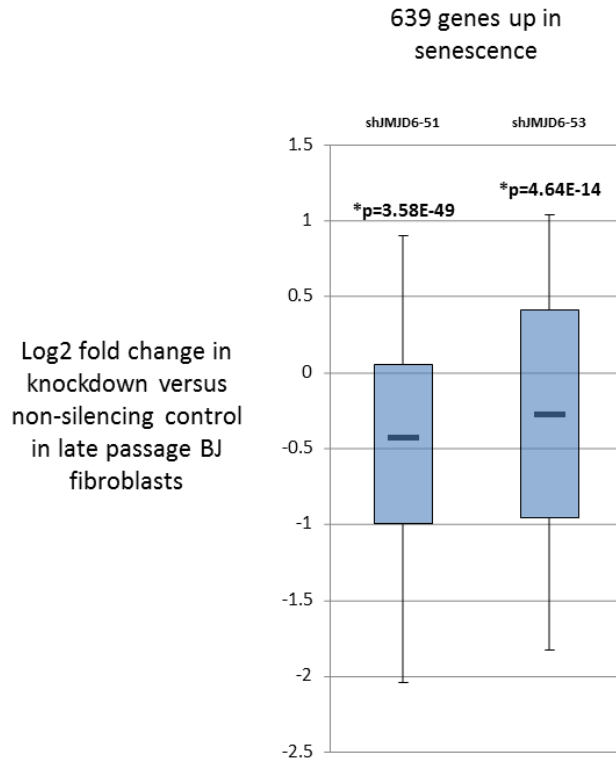


Figure 46. Box plots showing change in RNA-seq tag density over 639 genes induced in replicative senescence in listed JMJD6 stable knockdowns versus sh-non-silencing control in senescent BJ fibroblasts. Fold change represents ratio of normalized tag count of genes in JMJD6 stable knockdowns versus sh-non-silencing control. shJMJD6-51 and shJMJD6-53, each represent distinct shRNA constructs.

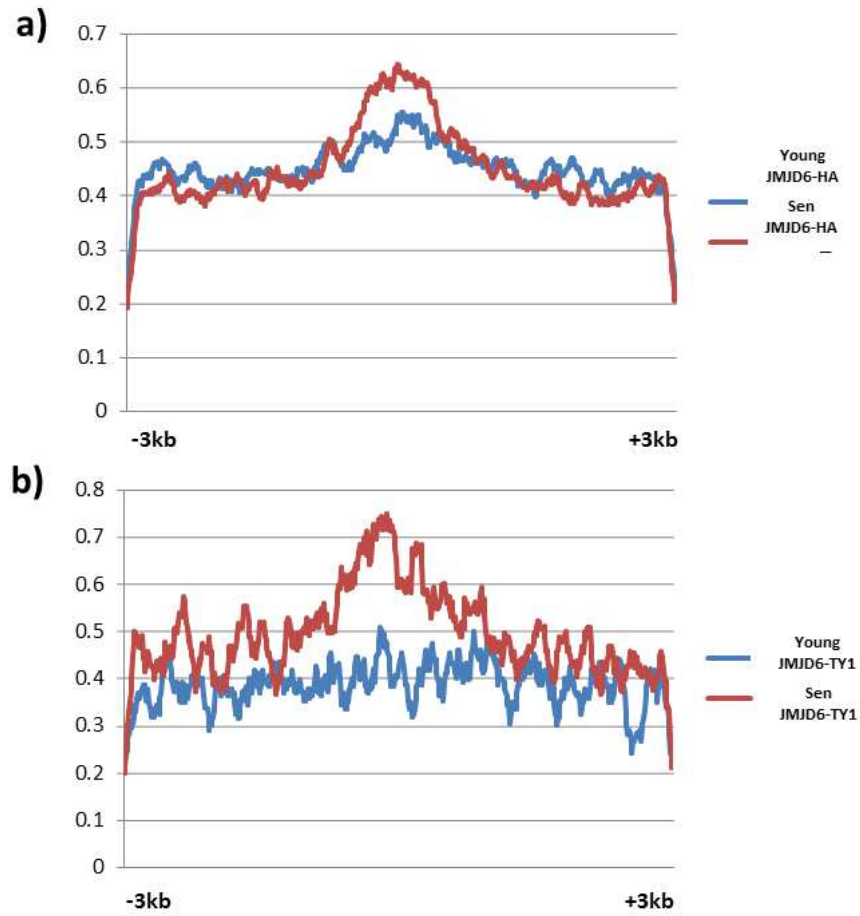


Figure 47. Meta-analysis of the normalized tag density of JMJD6 based on ChIP-seq using **a)** HA or **b)** TY1 epitope tags to determine JMJD6 binding in young or senescent BJ fibroblasts.

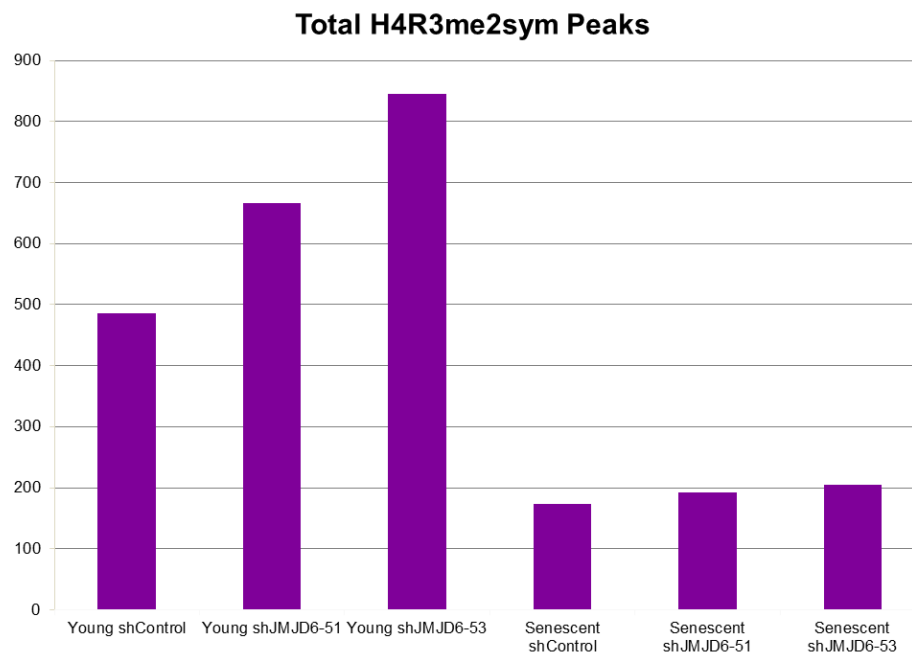


Figure 48. Total peak numbers of native ChIP seq of H4R3me2sym in young and senescent BJ fibroblasts after shRNA mediated stable knockdown of JMJD6 or expression of sh-non silencing (NS) control. shJMJD6-51 and shJMJD6-53 each represent distinct shRNA constructs.

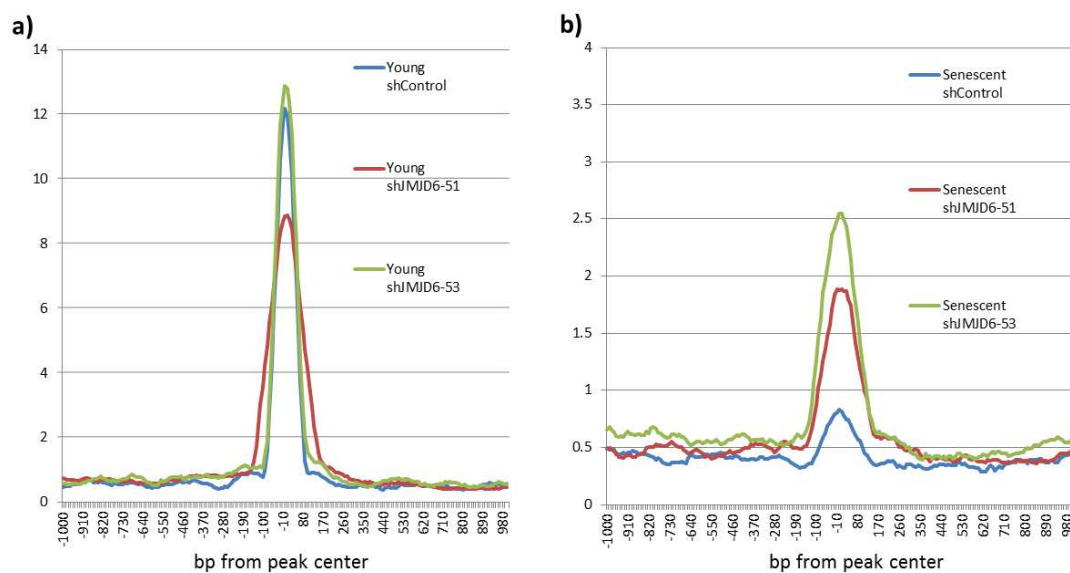


Figure 49. Meta-analysis of the normalized tag density of H4R3me2sym at peaks lost in the sh-non-silencing control samples during replicative senescence. Tag density plots correspond to **a)** young or **b)** senescent BJ fibroblasts after shRNA mediated stable knockdown of JMJD6 or expression of sh-non silencing (NS) control. shJMJD6-51 and shJMJD6-53 each represent distinct shRNA constructs.

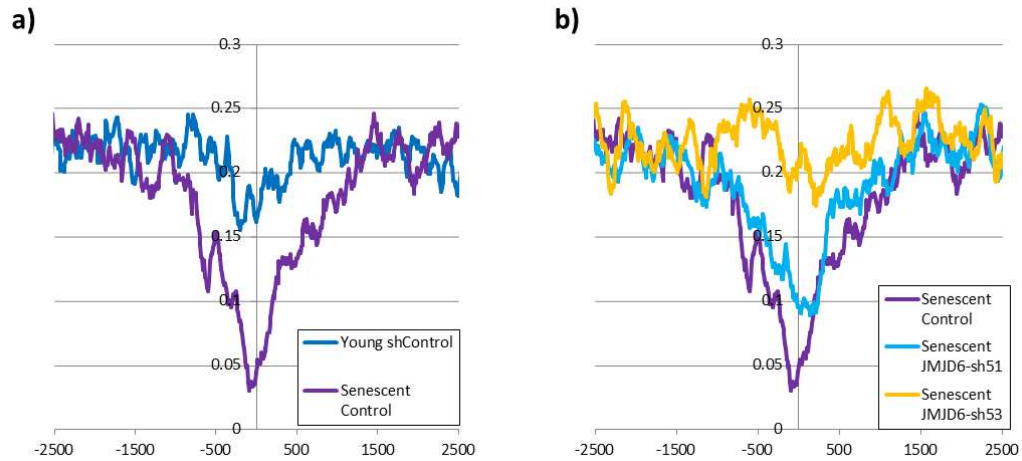


Figure 50. Meta-analysis of the normalized tag density of H4R3me2sym at the 2169 enhancers gained during replicative senescence. Tag density plots correspond to **a)** sh-non-silencing control expressing young or senescent BJ fibroblasts or **b)** senescent BJ fibroblasts after shRNA mediated stable knockdown of JMJD6 or expression of sh-non silencing (NS) control. shJMJD6-51 and shJMJD6-53 each represent distinct shRNA constructs.

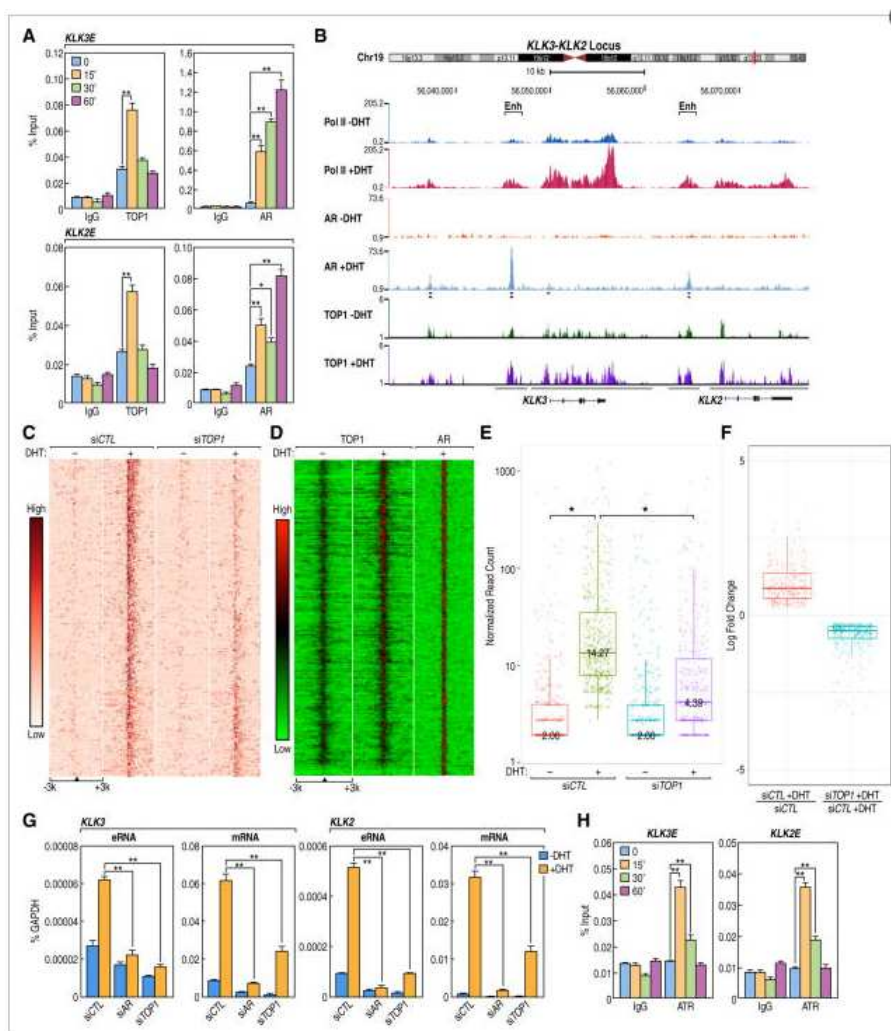


Figure 51. TOP1 Occupies AR-enhancers and Affects the Transcriptional Program of the Prostate Cancer Cell Line LNCaP. (A) Recruitment of AR and TOP1 to the KLK3 and KLK2 enhancers. The highest TOP1 binding is detected at 15 min DHT treatment. Data points show mean $\pm$ s.d.; (n=3), *P<0.05, **P<0.01. (B) The UCSC genome browser screenshot of the KLK3-KLK2 locus showing the occupancy of p-S5-RNA PolII (Pol II), AR and TOP1 (all tested with and without DHT treatment). (C) GRO-seq analysis of the effect of TOP1 knockdown on nascent RNA levels shown as a heatmap for 579 (out of 644, which were upregulated by DHT treatment) with the most affected AR-enhancers at the top. (D) Heatmap showing DHT-induced TOP1 sequencing tags density increase around 644 AR-enhancer binding sites (centered on AR). (E) Boxplot: siTOP1 reduced transcription at ~ 80% of DHT-up-regulated AR-enhancers. *P $\leq$ 2.2e-16 (Wilcoxon test). (F) Boxplot: The response to DHT of 368 DHT-up-regulated genes was reduced after TOP1 knockdown by siRNA. (G) Knockdown of TOP1 affects the induction of both eRNA and mRNA. LNCaP cells, hormone-starved for 1 day and transfected with the indicated siRNA, were stimulated with 100 nM DHT for 1h (eRNA) or 5 h (mRNA) 48 h post transfection. Quantitative RT-PCR was performed with SYBR Green using reverse-transcribed RNA. Data represent mean $\pm$ s.d.; (n=3), **P<0.01. (H) Recruitment of ATR to the KLK3 and KLK2 enhancers following DHT stimulation of starved cells. Data represent mean $\pm$ s.d.; (n=3). **P<0.01. See also Figure S1 and Table S3.

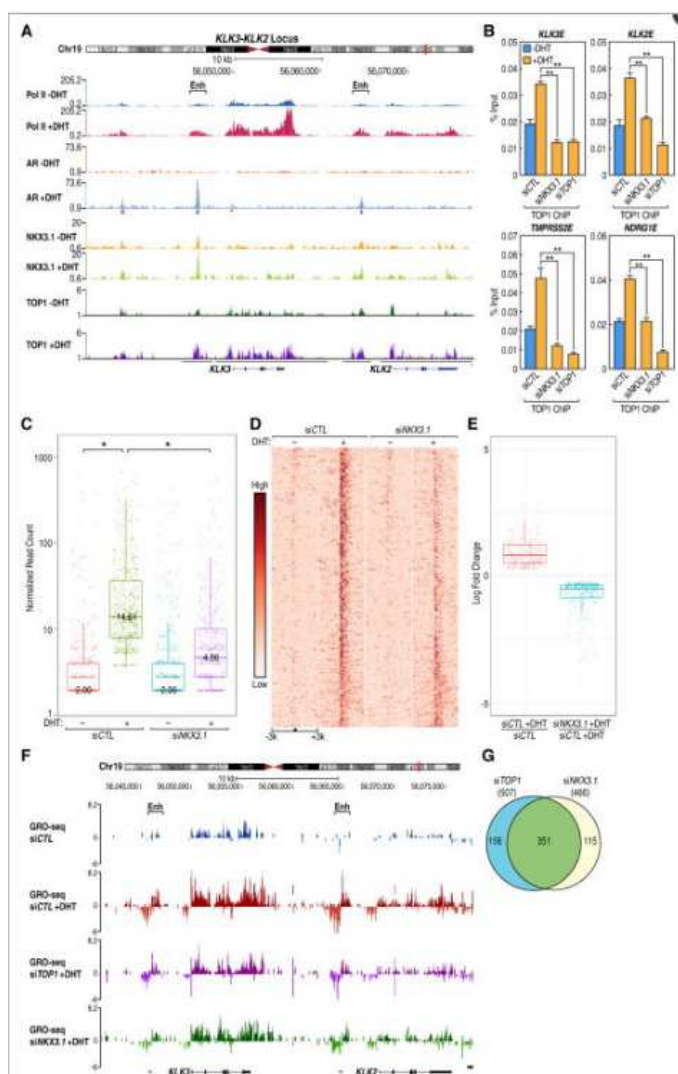


Figure 52. NKX3.1 and TOP1 Co-occupy a Subset of AR Enhancers and Co-regulate the Enhancer Program. (A) The UCSC genome browser screenshot displaying a direct overlap between AR, NKX3.1 and TOP1 binding at enhancers of KLK3 and KLK2 genes. Regions with increased (after DHT) TOP1-binding (except regions present in the background control) are underlined. (B) Knockdown of NKX3.1 prevents TOP1 from binding at AR-regulated enhancers; siCTL-, siNKX3.1- and siTOP1-treated cells were stimulated with DHT for 5 min. Chromatin immunoprecipitation was performed with an antibody against TOP1. Data represent mean ± s.d. (n=3). **P<0.01. (C) Knockdown of NKX3.1 by siRNA affects the induced transcription of ~69% of the regulated eRNAs. *P ≤ 2.2e-16 (Wilcoxon test). (D). Heatmap of AR-enhancers sorted from most-to-least affected by siNKX3.1. (E) siNKX3.1 reduces induced transcription of 273 genes in this experiment determined by GRO-seq. (F) The UCSC genome browser screenshot showing the KLK3-KLK2 locus. Knockdown of TOP1 or NKX3.1 by siRNA reduces eRNA and genic RNA induction. (G) Knockdown of either TOP1 or NKX3.1 affects induction of the same 351 eRNAs in the same experiment, as measured by GRO-seq. See also Figure S2 and Table S3.

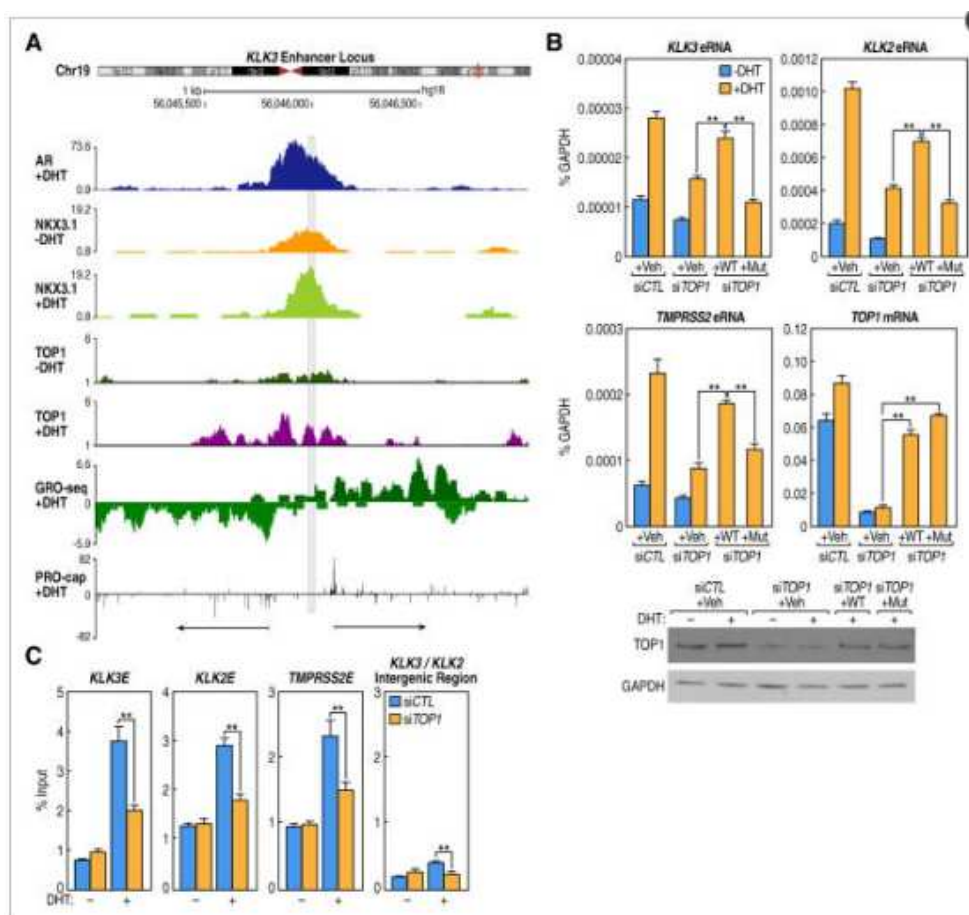


Figure 53. TOP1 Recruits to AR-Regulated Enhancers and Nicks the DNA

(A) UCSC browser screenshot displaying the KLK3 enhancer. Arrows indicate the PRO-caps representing (putative) eRNA TSS flank the NKX3.1 peak. (B) eRNA readout assay showing that Tyr723 of TOP1 is required for eRNA induction. LNCaP cells were hormone-starved for 24 h, transfected with siTOP1 to deplete the endogenous protein and then electroporated with either empty expression vector (Veh), wild-type TOP1 (WT), or the Y723F-TOP1 mutant (Mut) before treatment with either ethanol or DHT for 1h. eRNA for KLK2, KLK3 and TMPRSS2 gene enhancers was quantified by RT-PCR. TOP1 mRNA and protein levels are also shown. qPCR data show mean $\pm$ s.d.; (n=3). **P<0.01. (C) Knockdown of endogenous TOP1 affects nick/break formation as measured by incorporation of Biotin 11-dUTP at selected AR-enhancers after 10 min DHT treatment. Data represent mean $\pm$ s.d.; (n=3). **P<0.01.

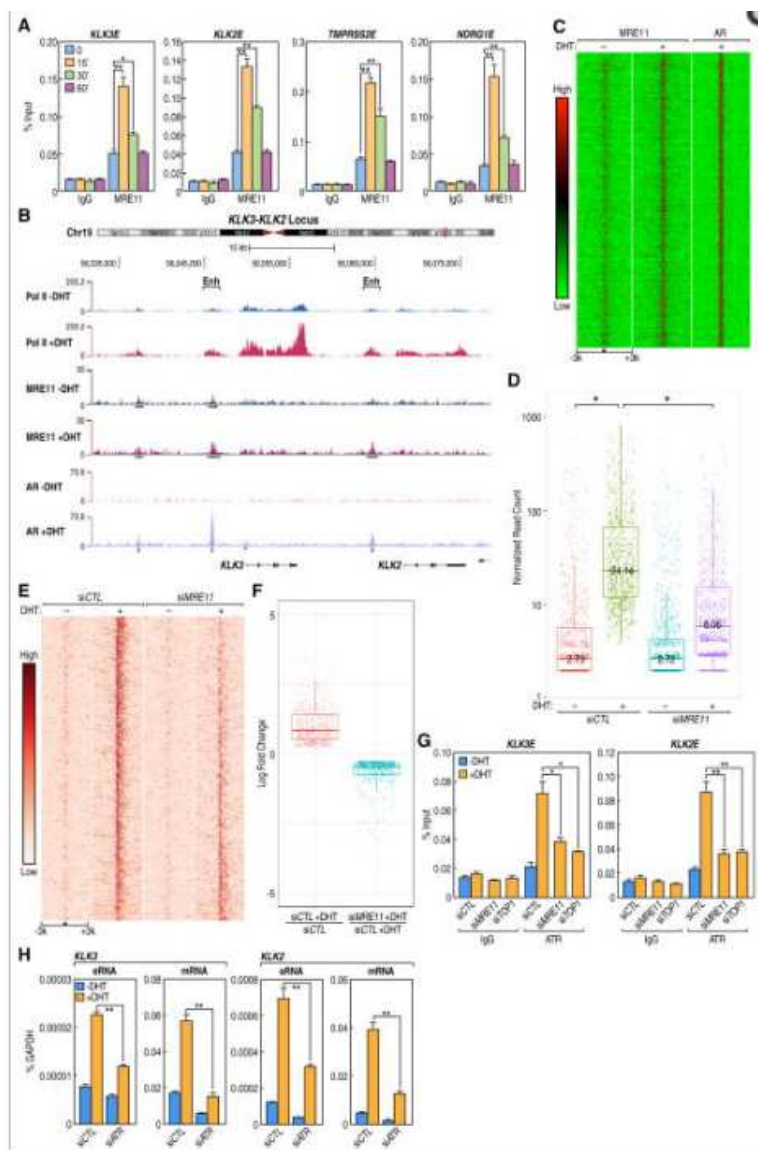


Figure 54. MRE11 Regulates the AR Transcription Program. (A) Recruitment of MRE11 to the selected DHT-regulated AR-enhancers. Data points show mean $\pm$ s.d. (n=3). * $P < 0.05$, ** $P < 0.01$. (B) MRE11 binding (sequencing tags density) increases over AR-enhancers in a DHT-dependent manner (KLK3 and KLK2 genes shown). (C) Distribution of MRE11 and AR binding (sequencing tag density) centered over AR-enhancer binding sites with DHT-induced eRNA. (D) MRE11 knockdown reduces eRNA expression levels of 89% of DHT up-regulated eRNAs. * $P \leq 2.2e-16$ (Wilcoxon test). (E) Heatmap for AR-enhancers sorted from the most downregulated by siMRE11 at the top to the least, at the bottom. (F) Boxplot showing 510 genes, where DHT-induced up-regulation of transcription (determined by GRO-seq) was reduced by MRE11. (G) Knockdown of either MRE11 or TOP1 affects recruitment of ATR at enhancers following hormone stimulation of the starved cells, measured after 15 min DHT stimulation. Data show mean $\pm$ s.d. (n=3). * $P < 0.05$, ** $P < 0.01$. (H) siATR affects induction of eRNA (1h DHT) and mRNA (5h DHT treatment) of the corresponding gene. Data are the mean $\pm$ s.d. (n=3). * $P < 0.05$, ** $P < 0.01$. See also Figure S4 and Table S3.

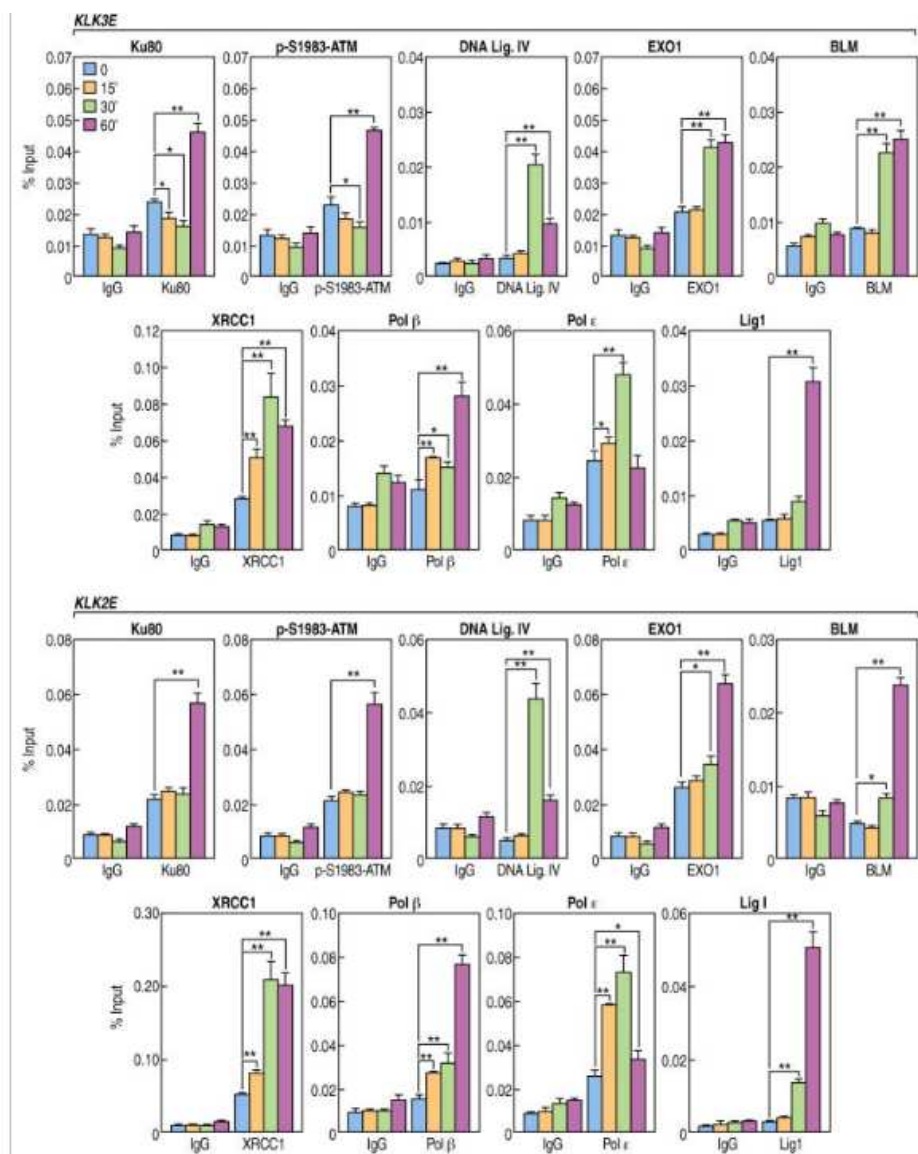


Figure 55. Canonical DNA Damage/Repair Machinery Components Recruit to AR-Regulated Enhancers. Kinetic recruitment of factors implicated in the DNA damage response (DDR) to AR- enhancers. All kinetic ChIP experiments were performed at least twice with cells of similar passage number to ensure data reproducibility. Data shown as mean $\pm$ s.d. (n=3). *P<0.05, **P<0.01. See also Figure S5.

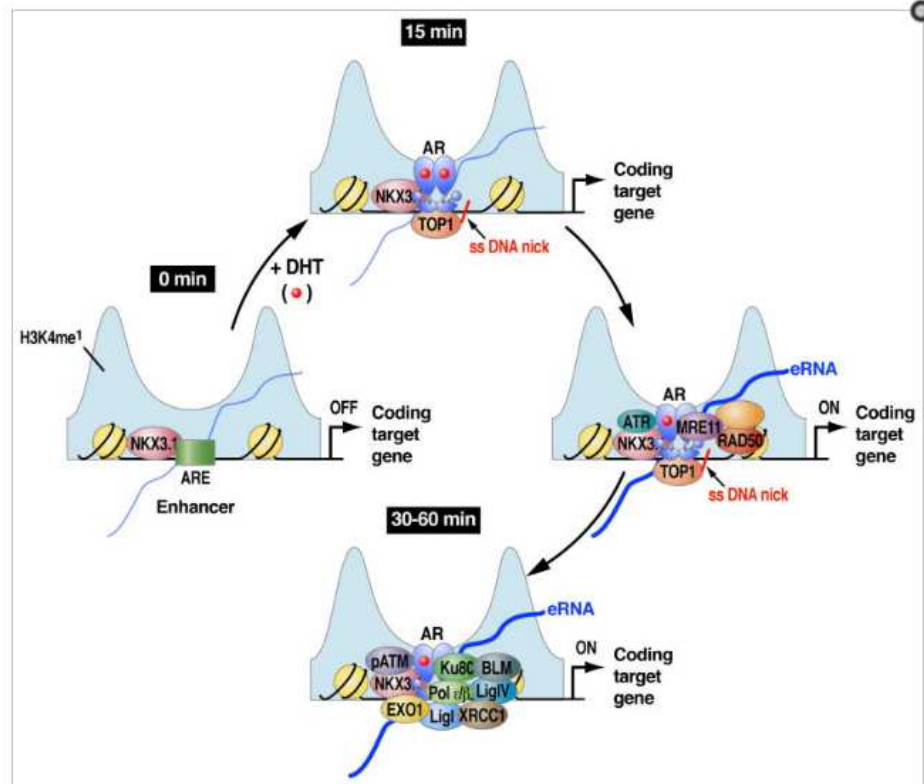


Figure 56. A Model for TOP1-mediated Activation of the AR-Enhancer. Following androgen stimulation, AR and DNA topoisomerase I recruit to the enhancer region, premarked by the NKX3.1 pioneer transcription factor. NKX3.1 to TOP1 stimulates enzymatic activity of the topoisomerase, resulting in nicking of DNA on a single strand, followed by recruitment of ATR, XRCC1, and the MRN complex components (MRE11/RAD50). After dismissal of TOP1, ATR and the MRN, additional components of DNA repair machinery recruit to the activated enhancer. The “thin blue line” indicates the presence of low levels of residual eRNA, not totally eliminated by hormone starvation, whereas the “thick blue line” represents induced bidirectional eRNA produced by the transcription unit.

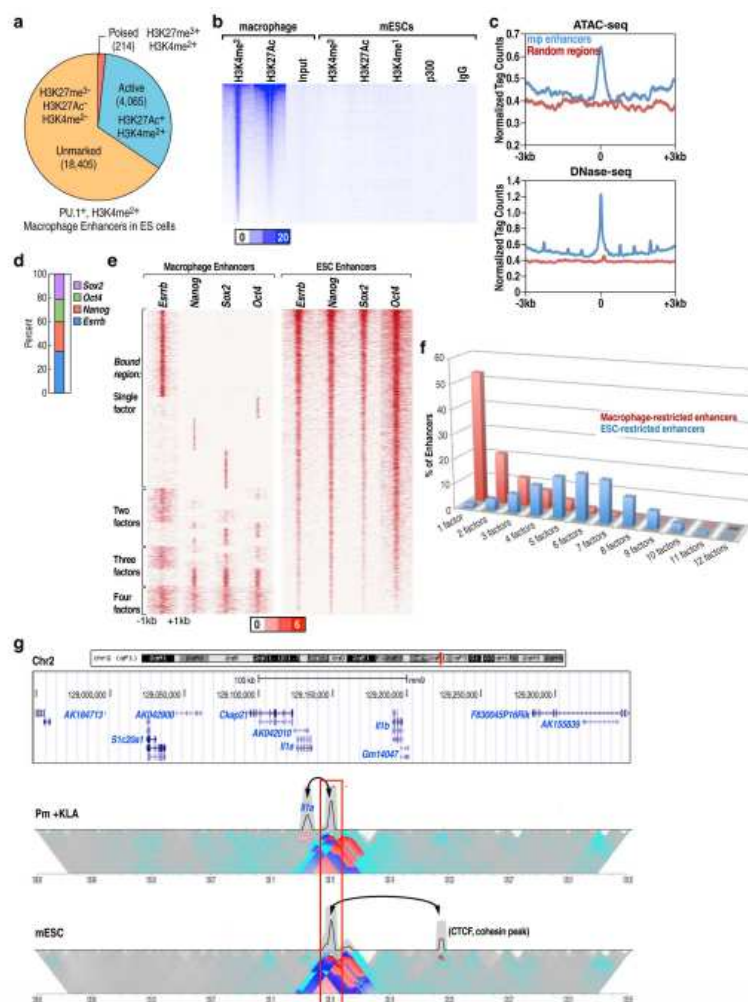


Figure 57. Features of macrophage enhancers in ESCs. a, Pu.1+, H3K4me2+ macrophage enhancer profiles in murine ESCs. Premarking is defined in panels d-f. b, Heatmap of H3K4me1, H3K4me2 and H3K27Ac, and p300 with -3kb ~ +3kb window centered on Pu.1 in 18,405 macrophage restricted enhancers in ESCs, indicating no H3K4me1, H3K4me2, H3K27Ac and p300 in these enhancers in ESCs. c, Tag density of ATAC-seq and DNase-seq in 18,405 macrophage restricted enhancer and random region in mESC, showing small degree of chromatin openness of macrophage restricted enhancer in ESCs. d, ChIP-seq data showing target percentage of ESC transcription factors-Esrrb, Nanog, Oct4, Sox2- in macrophage-restricted enhancers in a -1kb/+1kb from macrophage restricted enhancer. e, ENOS factor binding centered on each ESC factors in 6,775 premarked macrophage restricted enhancer (left) and 28,450 active murine ESC restricted enhancers (right) in -1kb ~ +1kb. f, 12 ESC transcription factors (Oct4, Sox2, Nanog, Esrrb, Smad1, E2f1, Tcfcp2l1, Zfx, Stat3, Klf4, c-myc and n-myc) binding in 6,809 active macrophage restricted enhancer and 8,209 active ESC restricted enhancers defined based on H3K27Ac (over 100 tags) in a -1kb/+1kb window, showing different binding pattern of ESC transcription factors in ESC enhancers and macrophage enhancers. g, 4C-seq in ESCs and macrophage. Black arrow represents the interaction frequency based on the trunc mean in 400kb window. Coordinate of macrophage putative enhancer in 5' of IL1a is indicated with red box.

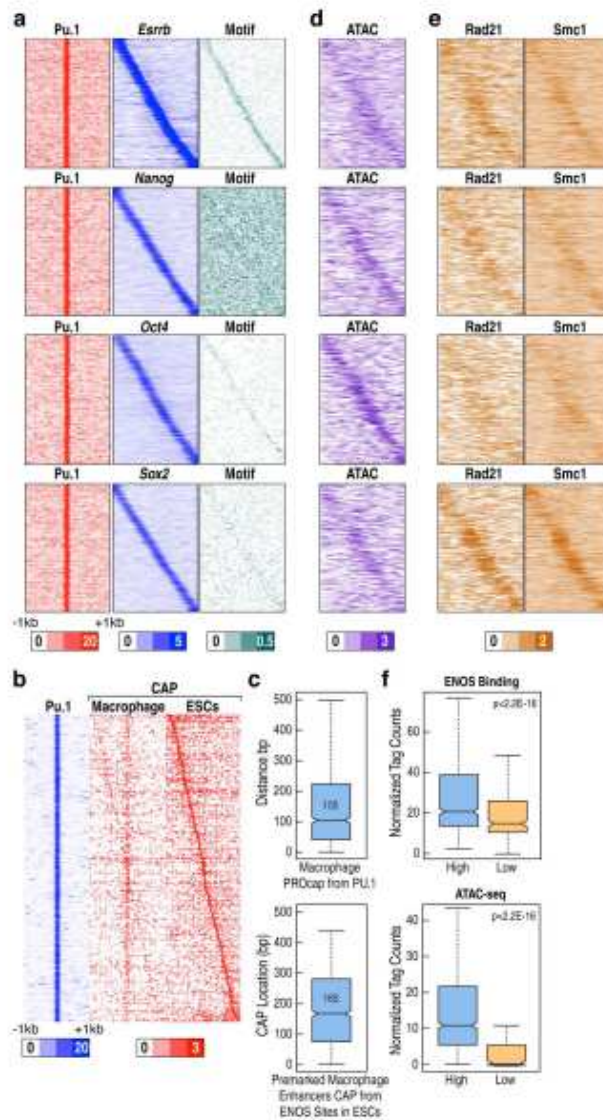


Figure 58. ENOS binding in macrophage restricted enhancers. a, ENOS binding in -1kb ~ +1kb window from 6,775 premarked macrophage enhancer, plotted relative to the location of the Pu.1 binding sites in the enhancers. Last panel shows corresponding binding sites of ENOS to the presence of the ENOS binding. b, 2345 PRO-cap peak identified in macrophages or ESCs in -1kb ~ +1kb window from 6,775 premarked enhancers is plotted by heatmap centered on Pu.1. c, Location of PRO-cap peak sites from Pu.1 binding in macrophage (top) or PRO-cap peak sites from the ENOS binding in ESCs (bottom) is calculated in 638 premarked macrophage enhancers, which has PRO-cap peaks in a -500bp/+500bp window from Pu.1 binding sites. d, ATAC-seq signal in ESCs in -1kb~+1kb from 6,775 macrophage enhancers is presented by heatmap by considering relative location from Pu.1. e, Relative location of Rad21 and Smc1 binding from Pu.1 binding sites in 6,775 ENOS bound macrophage enhancer in ESCs. f, ENOS binding intensity (top) and ATAC-seq signal intensity (bottom) in ~40% of highly enriched- vs. less enriched- Rad21 bound macrophage enhancers in ESCs.

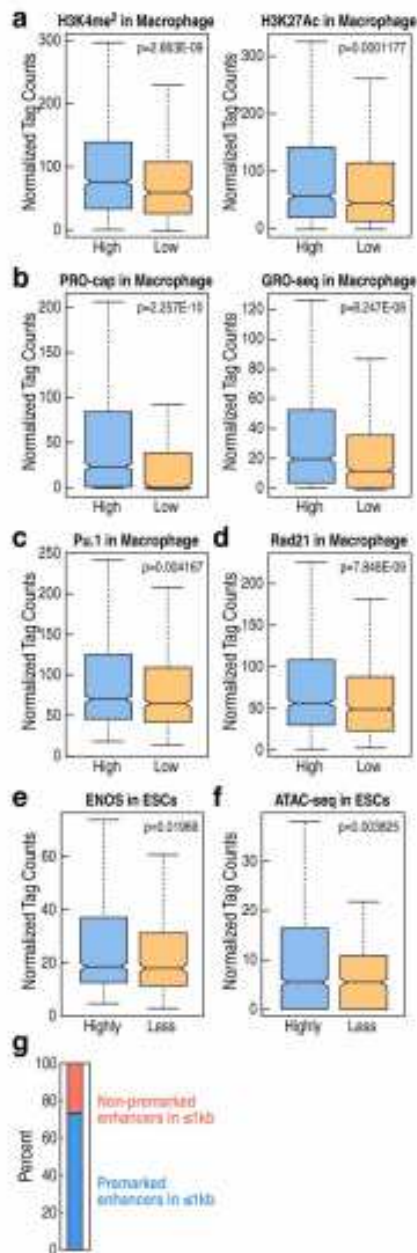


Figure 59. The association of ENOS binding to macrophage restricted enhancer activities. a, b, c, d, Comparison of the ~20% highest or lowest enhancers selected based on ENOS binding and PRO-cap signal in ESCs, and the level of (a), H3K4me₂ and H3K27Ac (b), PRO-cap and GRO-seq (c), Pu.1 (d), Rad21 in the mature macrophage. e, f, Comparison of the ~20% highly or less active macrophage enhancers selected based on GRO-seq signal of premarked macrophage enhancers in macrophage, and the level of (e), ENOS (f), ATAC-seq in ESCs. g, The number of functional macrophage enhancers in 6,775 enhancers which has ENOS in a -1kb/+1kb window (premarked enhancers) vs. 11,630 enhancers which doesn't have ENOS in a -1kb/+1kb window (non-premarked enhancers).

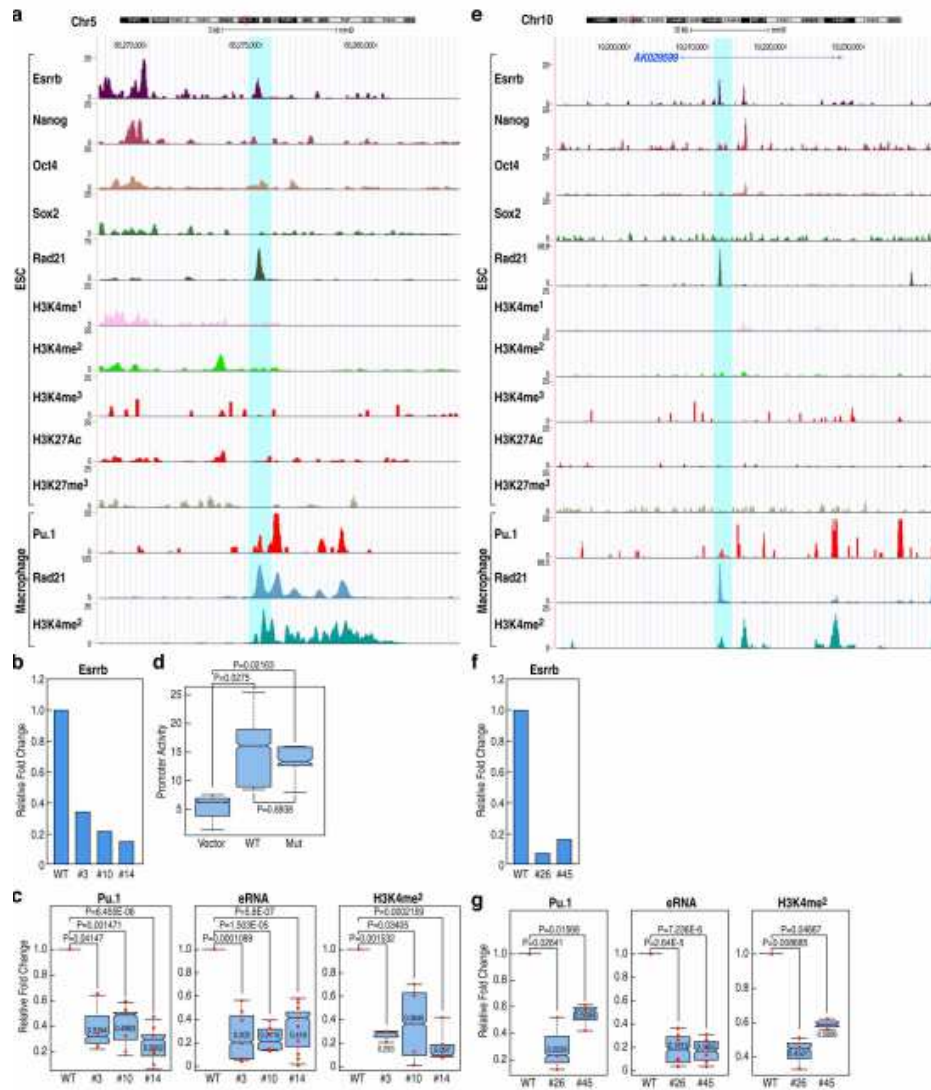


Figure 60. The functional role of Esrrb binding in macrophage restricted enhancer. a, Screenshot of the *Tlr1* locus. The blue box corresponds to CRISPR/Cas9 target region. b, ChIP-qPCR of Esrrb in WT vs. #3, #10 and #14 mutant clone in mESCs. One representative data is added and measured as a control. c, Pu.1 binding (left), TLR1 enhancer RNA (eRNA) transcription level (middle) and H3K4me2 level (right) in WT vs. #3, #10 and #14 mutant clones in ESDM. Each dot indicates each biological experiment ($n \geq 3$ biological repeats from two pooled different experiments). P-values are calculated using Welch's two t-test. d, Promoter activities in native full length *Tlr1* enhancer response to WT vs. Esrrb deleted mutation in Raw264.7 cells ($n=5$ biological repeats). e, Screenshot of the *Tnfaip3* locus. The blue box corresponds to CRISPR/Cas9 target region. f, ChIP-qPCR of Esrrb in WT vs. #26 and #45 mutant clone in mESC. One representative data is added and measured as a control. g, Pu.1 binding (left), *Tnfaip3* enhancer RNA (eRNA) transcription level (middle) and H3K4me2 level (right) in WT vs. #26 and #45 mutant clones in ESDM. Each dot indicates each biological experiment ($n \geq 3$ biological repeats from two pooled different experiments). P-values are calculated using Welch's two t-test.

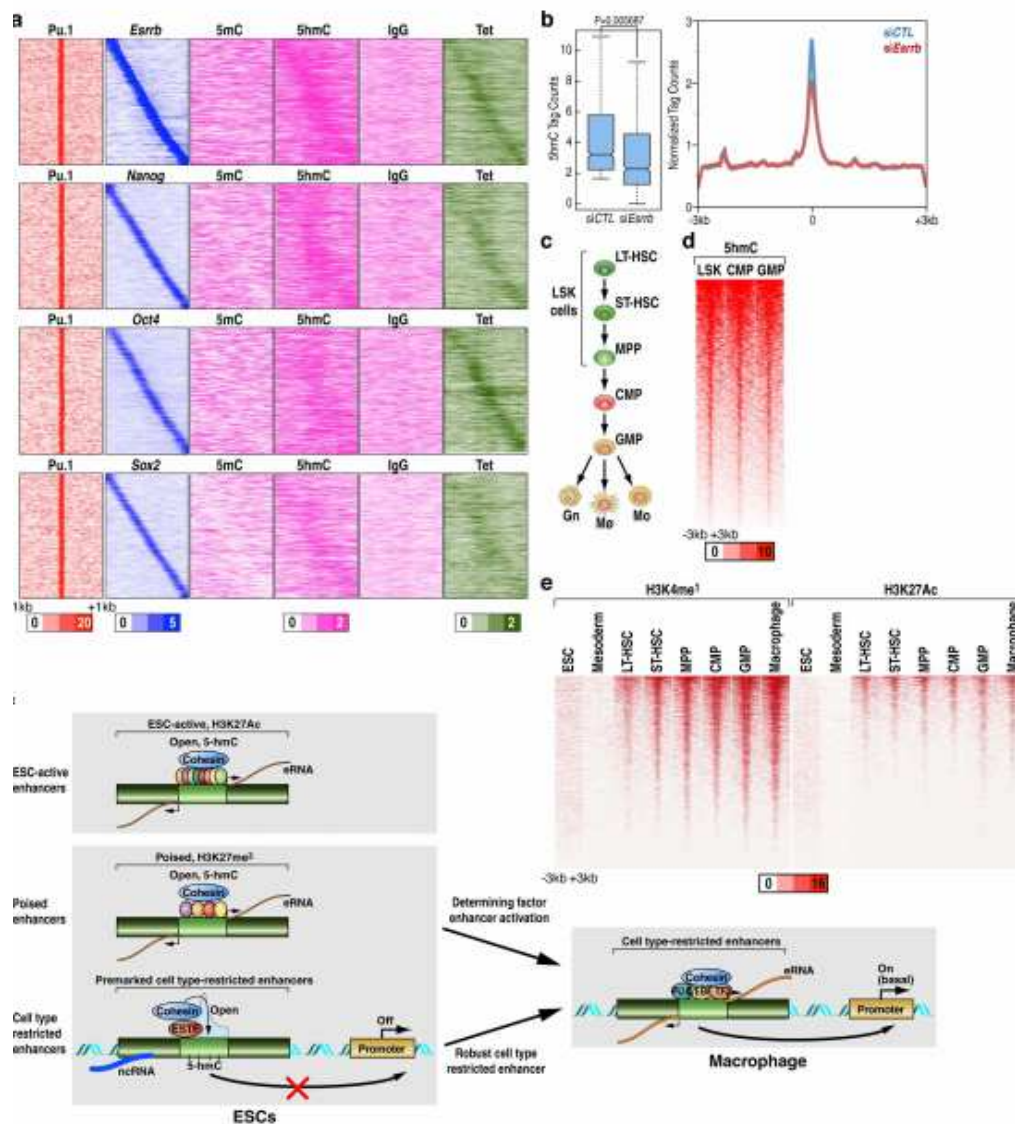


Figure 61. DNA Methylation modifications in macrophage restricted enhancer. a, Mapping of DNA methylation modification (5-mC and 5-hmC) and binding of Tet1 in 6,775 premarked macrophage enhancer in a -1kb/+1kb window and centered on Pu.1. b, Effects of siCtrl vs. siEsrrb on 5-hmC in 6,775 premarked enhancers in ESCs. P-values are calculated using Welch's two t-test. c, Schematic of the hematopoietic differentiation stages. d, 5hmC in LSK, CMP and GMP in 6,775 premarked enhancers in -3kb ~ +3kb window centered on Pu.1. e, H3K4me1 and H3K27Ac in ESC, mesoderm, LT-HSC, ST-HSC, MPP, CMP, GMP and macrophage in 6,775 premarked enhancers in -3kb ~ +3kb window centered on Pu.1. f, Model of "premarked" lineage determining and terminal differentiation enhancer indicating that poised enhancers bind several ENOS transcription factors, while the late-activated cell-specific enhancers are premarked based on binding of a single ESC transcription factor, causing chromatin opening, transcription of a ncRNA, and appearance of a 5-hmC mark in the area of the enhancer that may provide the molecular memory for what ultimately will be the core Pu.1/C/EBP α core from which eRNAs will be transcribed.

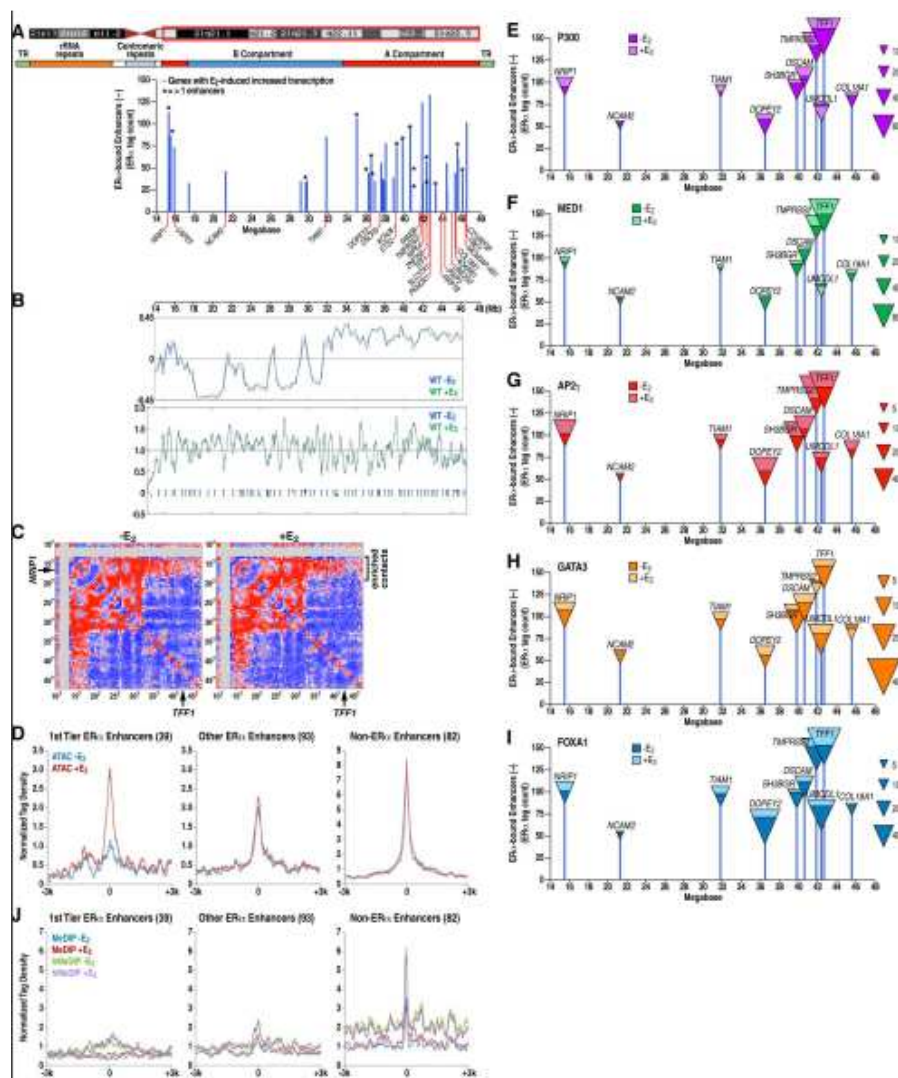


Figure 62. Identification of the ER α -bound first tier enhancers. A. Map of Chr.21 from UCSC genome browser. Cartoon below annotate the major structural features of the chromosome, telomeric repeats (TR), rDNA repeats, the centromeric repeats, A (Red) and B (Blue) compartments determined from data in panel B. The length of the line correspond to the level of ER α binding measured as ChIP-seq tag count. (*) Indicate clustered enhancers. The red bars diagram the E2 -upregulated coding transcription units. B. Hi-C analysis permitted identification of the A/B compartments, and calculation of the insulation scores to identify the larger putative chromosomal contact domains (n=67). C. Contact map from Hi-C data, analyzed at a resolution of 1Mb, detected long distance interactions across Chr.21, indicating the presence of very long distance interactions as well as the expected short-range interactions. D. Quantitative representation of the ATAC-seq data for the 39 most robust ER α -bound enhancers, compared to 93 weaker ER α -bound enhancers and 82 non-ER α bound active enhancers, performed in the presence of E2 (1h.). Quantitative “triangle” plot of E2-induced (1hr) binding of p300 (E), MED1 (F), AP2 γ (G), GATA3(H), FOXA1(I) to the 10 most robust ER α -positive enhancers. J. Methylation Status of 3 categories of enhancers on Chr21 by MeDIP and hMeDIP under +/- E2 conditions.

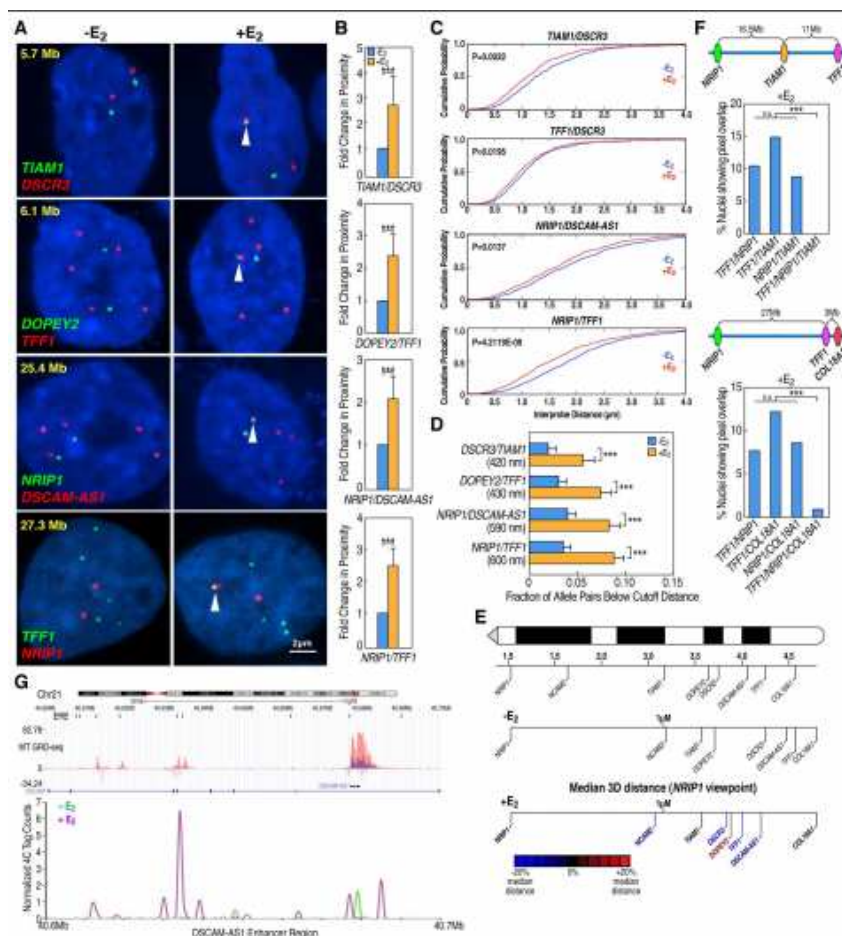


Figure 63. Estrogen-induced architectural and transcriptional dynamics in Chr21. A, DNA FISH images showing E2 induced spatial proximity between indicated genomic loci in MCF7 cells. Genomic distance between the probes is indicated at the top left corner of each image pairs. White triangle points to the pair of loci in proximity. TFF1 and DSCAM-AS1 loci show aneuploidy as evident by >2 FISH signal B. E2 induced fold change in proximity compared to the control condition. Fold change is calculated using data shown in panel (1. D). C. Comparison of cumulative distribution of distances between indicated “first tier” enhancers demonstrate that 3D distances between these loci reduces in response to E2 stimulation. D. Fraction of alleles coming below the indicated cut off distance. Determination of cut off distance value is explained in Figure S2.E E. Diagrammatic representation of E2 induced architectural changes in Chr.21. Diagram showing 1-dimensional view of q-arm of Chr.21 scaled to genomic distance (Top). Relative 3-D positions of indicated genomic loci as measured by DNA FISH from NRIP1 loci in un-stimulated (middle) and E2 stimulated conditions (bottom). F. Temporal change in E2-induced spatial proximity between NRIP1 and TFF1. Induced proximity is evident as early as 5 min after E2 treatment. It follows a cyclical pattern with a loss of E2 effect at 30 min and regaining proximity at 40 min time point G. 3 color DNA FISH analysis of NRIP1/TFF1/COL18A1 and NRIP1/DSCAM/COL18A1 genomic loci interactions, revealing the simultaneous co-localization of all 3 loci was a rare event under E2 stimulated conditions, compared to the induced proximity between any two of the component enhancers.

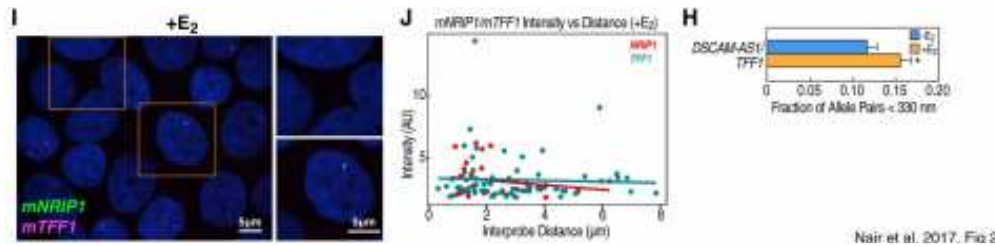


Figure 64: Functional roles of the first tier enhancer network on enhancer robustness. A, B. Q-PCR analysis indicating the reduced transcriptional output of indicated mRNAs in NRIP1e3 KO clones (A) and TFF1 KO clones (B). These are pooled data from 4 separate clones generated using different gRNAs for each locus deletion and 3 biological repeats for each clones. Total RNA was collected 3hrs. after addition of E2. C. Q-PCR analysis of effects of NRIP1e3 deletion on the TFF1 eRNA 1hr following E2 treatment. D. Meta analysis of Gro-seq data showing impact of tier1 enhancer deletion on eRNA transcription of the 39 most robust and 93 less robust ER α -bound enhancers. Minimal transcriptional impact of the deletion of robust ER α enhancers on 82 active non-ER α enhancers on Chr21 demonstrate the specific disruption of E2 enhancer network E,F. Visual representation of 1hr E2 stimulated eRNA induction in 10 most robust tier1 enhancers on Chr21. Area of the circle is derived from the total GRO-seq tag counts of the eRNA (Raw data used to generate the panels E-J are given in Table 3). G,H. Effect of deletion of TFF1e1 (shown in red) on the extent of eRNA induction in 10 most robust first tier enhancers. I,J. Effect of deletion of NRIP1e3 (shown in green) on the extent of eRNA induction in 10 most robust first tier enhancers.

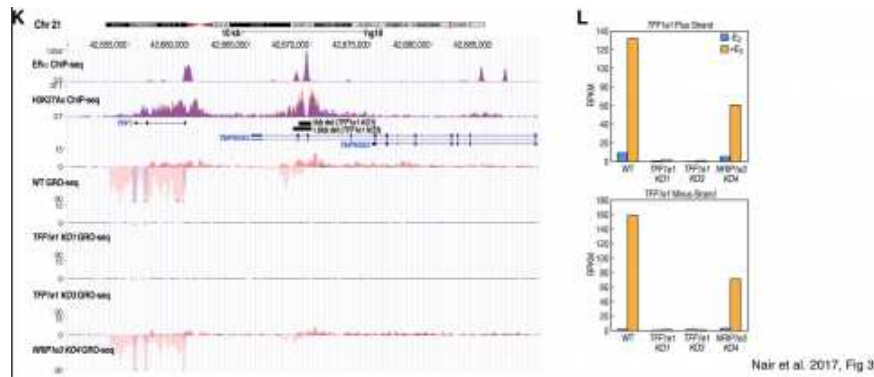


Figure 64: Continued. K, Browser image of TFF1 genomic locus from ER α ChIP-seq, H3K27Ac ChIP-seq and GRO-seq of WT and indicated tier1 enhancer knockout clones before (red) after (purple) E2 stimulation. TFF1e deletions abolishes the both TFF1 eRNA and mRNA transcription. Importantly, NRIP1e3 deletion significantly compromises the transcription from both TFF1 enhancer and coding region. L. RPKM value of TFF1eRNA from GRO-seq experiment showing the effect of TFF1e1 deletion and NRIP1e3 deletion of TFF1 enhancer transcription.

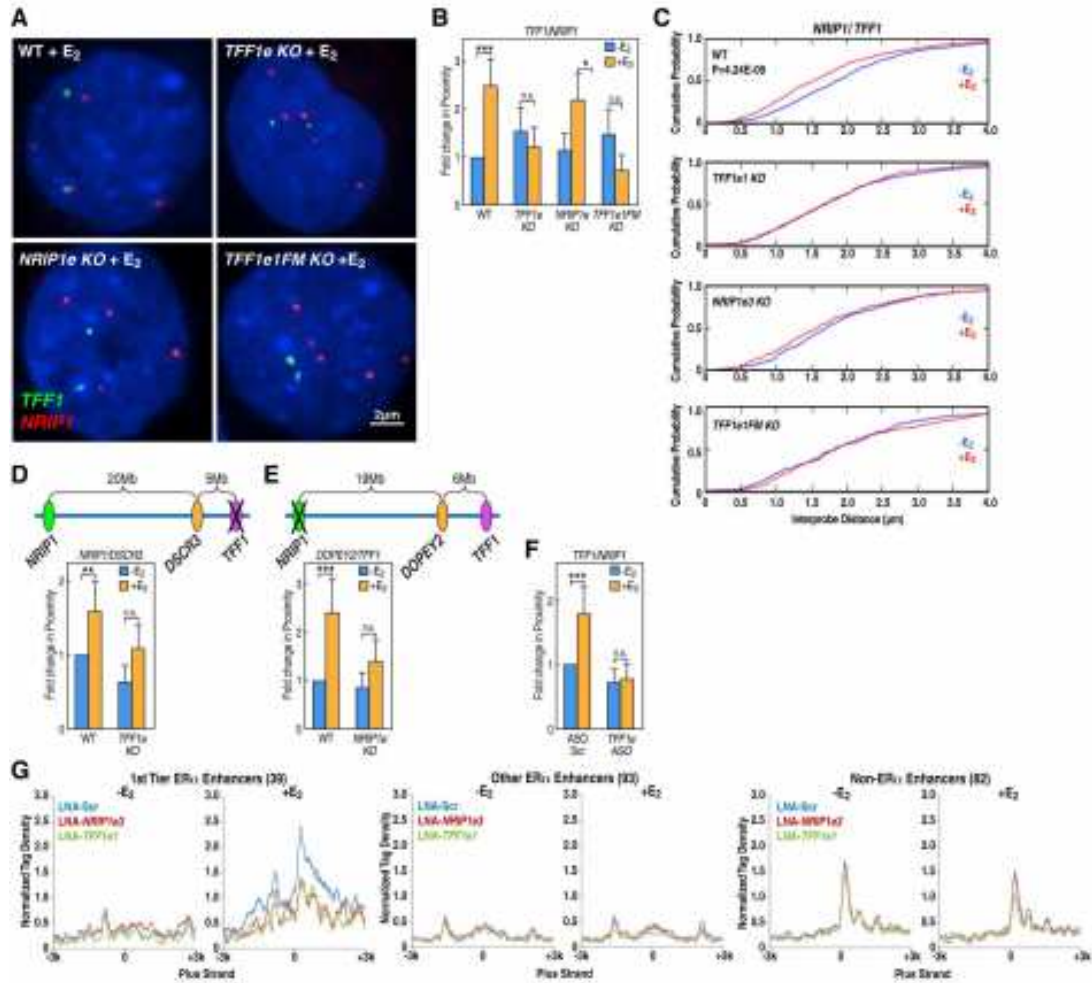


Figure 65. Effects of enhancer deletion and eRNA depletion on chromosomal dynamics. A. DNA FISH image from E2 treated WT showing E2 induced proximity between TFF1 and NRIP1 loci. This effect is absent in TFF1e1 KO, NRIP1e3 KO and TFF1e1FM KO, where a 51 nucleotide deletion remove the FOXA1 motif present on TFF1e. B. Quantitation of DNA FISH data from WT and mutant clones demonstrated the reduced E2 induced proximity of TFF1 and NRIP1 loci C. Cumulative distribution of TFF1/NRIP1 distances showing reduced spatial distance between NRIP1 and TFF1 in WT cells, but not in tier1 enhancer mutants D. Quantitation of DNA FISH data in WT and TFF1e1 KO cells reveal a compromised induced proximity between two other tier 1 enhancers regions (NRIP1e and DSCR3e) E. ASO mediated TFF1e1 knockdown significantly reduces E2 induced proximity between TFF1e1 and NRIP1e3 F. Meta analysis of GRO-seq data from TFF1e1 and NRIP1e3 eRNA knockdown experiment shows strikingly similar pattern to transcriptional impact of CRISPR deletion of respective enhancers (Compare to Figure 3.D). Data are pooled from at least two biological replicates analyzing at least 120 nuclei in each replicates. Statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$) was calculated with Wilcoxon Rank Sum test. P-value of Cumulative Frequency Distribution was calculated using Kolmogorov Smirnov test.

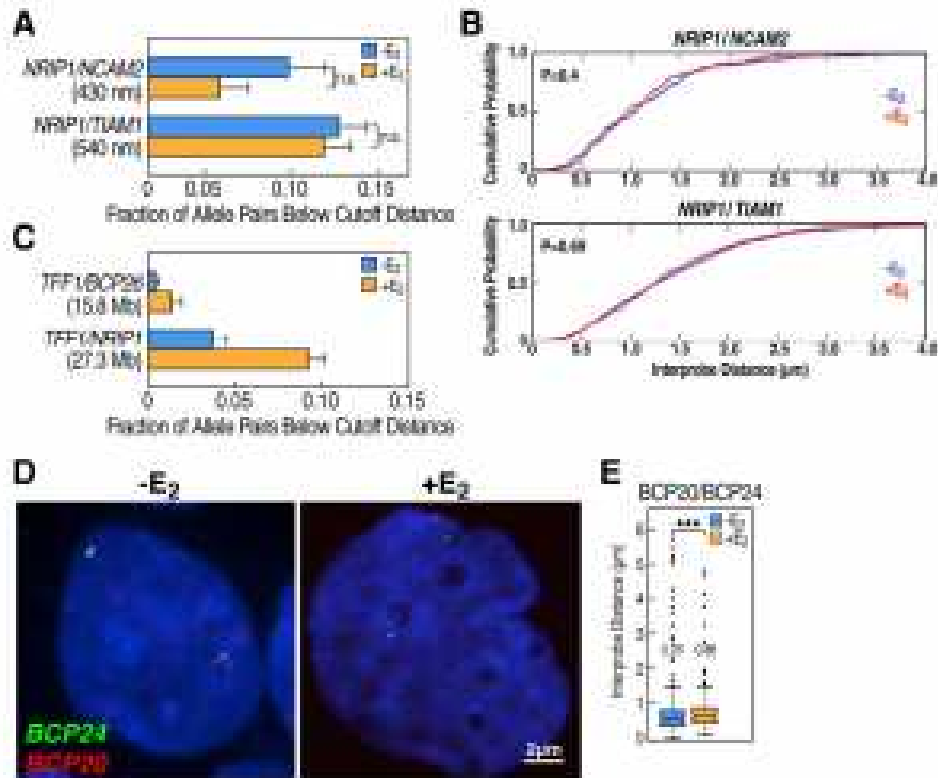


Figure 66. Analysis of interactions in the B compartment of Chr.21. A. DNA FISH analysis of proximity between E2 responsive genomic loci (NRIP1/NCAM2, NRIP1/TIAM1) that are located close to extended B-compartment on Chr.21 reveal an elevated basal interaction between these loci that does not change with E2 stimulation. B. Cumulative distribution of distances between NRIP1/NCAM2 and NRIP1/TIAM1 showing no change in spatial distance between these loci C. Low frequency of interaction between TFF1 loci and BCP26 (B-compartment probe located at Chr21 q-arm 26Mb) compared to TFF1/NRIP1 interaction (15.8x and 7.5x more relative interaction frequency for TFF1/NRIP1 in $-E_2$ and $+E_2$ conditions respectively) despite relatively smaller genomic distance between the former pairs (15.8 Mb vs 27.3 Mb) D. DNA FISH using probes located inside the extended B-compartment (BCP24 and BCP20, that at 4 Mb apart) E. Analysis of regions inside B-compartment reveal an E2 stimulus dependent increase in distance between these internally located probes suggesting an expansion. Data are pooled from at least two biological replicates analyzing at least 120 nuclei in each replicates. Statistical significance ($*p < 0.05$, $**p < 0.01$, $***p < 0.0001$) was calculated with Wilcoxon Rank Sum test. P-value of Cumulative Frequency Distribution was calculated using Kolmogorov Smirnov test.

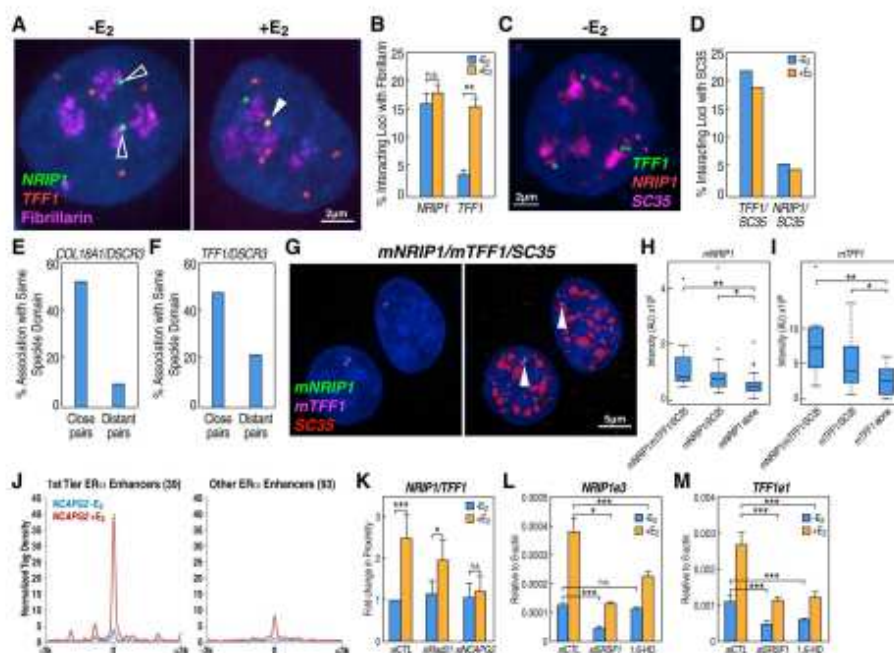


Figure 67: Sub-nuclear organelles and transcriptional robustness **A, B.** Immuno FISH using nucleolar marker (Fibrillarin), *TFF1* and *NRIP1* probes reveal positioning of NRIP1 in the close vicinity of nucleolar periphery. Less than 5% of *TFF1* locus are proximal to nucleolar surface in basal condition, but that frequency is increased 3 fold upon E₂ treatment indicating a E₂ stimulus dependent relocalization of *TFF1* **C, D.** Immuno FISH with Inter Chromatin Granule (ICG) marker (SC35), *TFF1* and *NRIP1* probes reveal significantly higher association of *TFF1* with SC35 surface compared to that of SC35 and *NRIP1*. **E,F.** Immuno FISH data using ICG marker (SON) with two different loci showing E₂ induced proximity (*COL18A1/DSCR3* and *TFF1/DSCR3*) reveal that in the same nucleus, those probe pairs that are positioned in closer spatial proximity associate with same speckle domain more frequently when compared to probe pairs that are positioned at a much larger spatial distance (52.5% vs 9.8% for *COL18A1/DSCR3* and 48% vs 22% for *TFF1/DSCR3*) **G.** Immuno RNA FISH using ICG marker (SC35) with *TFF1* and *NRIP1* intronic RNA probes. White triangle point to proximity of mTFF1 and mNRIP1 signal to same speckle domain **H, I.** RNA FISH signal intensity as a read out of transcriptional out put reveal that *NRIP1* (**H**) and *TFF1* (**I**) loci positioned on the same speckle domain has higher transcriptional out put compared to those loci that are associated with speckle individually. Loci that are not associated with any speckle domain have the least transcriptional activity. **J.** Meta analysis of ChIP-seq data using Condensin I subunit NCAPG shown robust E₂ mediated enrichment of NCAPG to tier1 enhancers compared to other less robust ERα-bound enhancers **K.** DNA FISH analysis reveals that siRNA mediated NCAPG knockdown, but not RAD21 (Cohesin subunit) knockdown, diminishes E₂ induced proximity between *NRIP1* and *TFF1*. **L,M.** Q-PCR analysis demonstrate the loss of E₂ induced eRNA expression from *NRIP1e3* (**L**) and *TFF1e1* (**M**) as a result of knockdown of SRSF1 (component of nuclear speckle) and treatment of cells with 1,6-Hexanediol (1,6-HD), an aliphatic alcohol that can melt membraneless cellular structures. Data is representative of 3 biological replicates. P-value was calculated using Paired Student's-t test

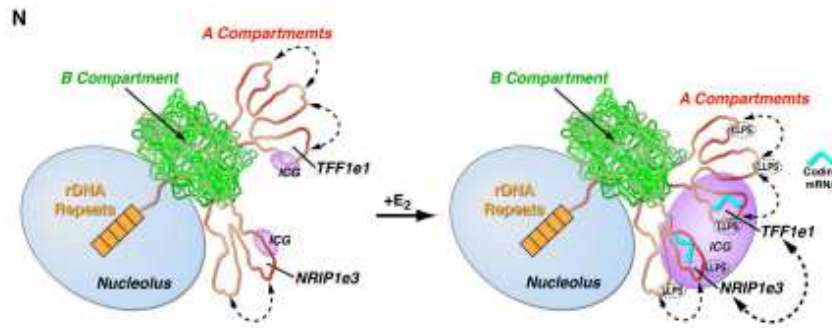


Figure 67: Continued. N. Model of basal and E₂-induced change in Chr.21 architecture. The nucleolar organizing region on the p-arm of Chr.21 tether Chr21 to nucleolus resulting in 'forced' proximity of *NRIP1* to nucleolar surface. The extended B compartment of Chr.21 is localized to nucleolar or nuclear periphery thus anchoring the chromosome to fixed cellular compartment and making the A compartment 'mobile'. Loci in the A compartment of Chr.21 is more frequently associated with speckle than region closer to B-compartment and nucleolus. E₂ dependent recruitment of Condensin along with increased production of eRNAs from tier1 enhancers further nucleate low complexity RNA binding proteins to the loci resulting LLPS and several fold increase in the local concentration of various components of co-factors and megatrans complex. This model predicts a random association of multiple robustly active loci to hydrogel like de novo assembled structures and mutually facilitates transcriptional activity. Immuno FISH data were obtained analyzing at least 120 nuclei. Statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$) was calculated with Wilcoxon Rank Sum test. P-value for comparison of RNA FISH intensity was calculated using Wilcoxon Rank Sum test.

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