The epigenetic regulation of cell cycle and chromatin dynamic by sirtuins

Paloma Martínez Redondo

Doctorat en Biomedicina

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

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Tutor: **Dr. Esteban Ballestar Tarín**

Director:
Dr. Alejandro Vaquero García

Tutor de tesis:
Esteban Ballestar Tarín

If you wou'd not be forgotten
As soon as you are dead and rotten,
Either write things worth reading
Or do things worth the writing

Poor Richard's Almanack, 1738

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Abstract

The chromatin consists of a hierarchical and dynamical structure that is modulated during the different cell cycle stages in order to maintain genome integrity and preserve the genetic information coded in the DNA. The dynamic structure of the chromatin depends on the coordination of the different chromatin remodeling processes: histone modifications, chromatin remodeling enzymes/complexes, DNA methylation and chromatin architectural proteins (CAPs). Within the chromatin, the histone-mediated regulation responds mainly to the modification of its N-terminal domain or "tail." Among the different histone modifications, acetylation at specific lysine residues (K) is one of the best characterized, and the acetylation of lysine 16 of histone H4 is the most frequently acetylated residue in eukaryotes. The acetylated form of H4K16 is an important mark of actively transcribed euchromatin from yeast to humans; whereas its non acetylated form is associated with gene silencing and heterochromatin regions. The dynamics of this histone modification is mainly governed by three enzymes: the histone acetyltransferases (HAT) MOF (males absent of the first), and the histone deacetylases (HDAC) SIRT1 and SIRT2. Therefore, both groups of enzymes are essential for the regulation of gene expression and chromatin organization in the nucleus, regulating the transition between transcriptionally active and inactive state of chromatin. SIRT1 and SIRT2 belong to the Class III of HDACs, termed as sirtuins, which are crucial for genomic integrity, adaptation to the environment and aging, among other functions. On one hand, SIRT2 is the only mammalian sirtuin located in the cytoplasm, which is known to shuttle to the nucleus during G2/M. Consistently, this HDAC has its main role in deacetylating H4K16Ac during G2-M. So far, the role of SIRT2 as the main H4K16Ac during mitosis has only been demonstrated by mammalian cell culture experiments or yeast studies. Therefore, for the first time, our study demonstrates the essential role of SIRT2 in regulating H4K16Ac levels during mitosis *in vivo*. As a matter of fact, our results support the function of SIRT2 in regulating chromatin dynamics by its involvement in the control not only of H4K16Ac levels, but also of H4K20me1-3 levels during the whole cell cycle. Notwithstanding, as happens with other sirtuin members, SIRT2 has also been shown to regulate and deacetylate non-histone substrates that govern cell cycle, stress response, cell survival and genome stability. Furthermore, one of the main roles of SIRT2 consists of modulating cell cycle progression and SIRT2 has been found to regulate diverse mitotic checkpoint proteins such as CDH1, CDC20, BubR1 and p53. Additionally, our results suggest that the chromatin histone patterns generated by SIRT2 during mitosis are essential in the control of cell cycle progression and attend to two complementary mechanisms: the deacetylation of both H4K16Ac and PR-Set7, the monomethyltrasferase of H4K20. We have found that SIRT2 is clearly involved in a mitotic checkpoint and regulate H4K20me1 deposition under stressful conditions, in order to preserve genome integrity. On the other hand, SIRT1 has been mainly involved in regulating heterochomatin formation and gene silencing by deacetylating histone and non-histone

substrates. In fact, SIRT1 is involved in the maintenance of genome integrity due to its role in heterochromatin formation by deacetylating histone marks (H3K9Ac and H1K26Ac) and regulating heterochromatin related proteins such as HP1, Suv39h1 and Ezh2. In addition, SIRT1 also deacetylates H4K16Ac, H3K9Ac and H1K26Ac at specific promoters in order to control gene expression; and regulates non-histone proteins such as p53, FoxO factors, and Rb, among others, to specifically modulate the gene expression pattern. Nonetheless, SIRT1 has recently been implicated in cell cycle regulation throughout the control of Mcm10, the eukaryotic DNA initiation factor essential for S-phase progression. Accordingly, our study also demonstrate how SIRT1 may be involved in the regulation of cell cycle progression by modulating the expression of PR-Set7 and Suv4-20h2, the enzymes in charge of mono- and di-methylate H4K20, respectively. Altogether this evidences the role of sirtuins in preserving genome integrity by modulating chromatin dynamics and cell cycle progression from mitosis to S-phase.

Resumen

La cromatina consiste en una estructura jerárquica y dinámica que se regula durante el ciclo celular con el fin de mantener la integridad del genoma y preservar la información genética codificada en el ADN. Esta estructura dinámica depende de la coordinación de diferentes procesos: modificaciones histónicas, la actividad de enzimas/complejos remodeladores de la cromatina, metilación del ADN y la participación de proteínas estructurales de la cromatina. De entre estos procesos, las modificaciones histónicas tienen lugar en el dominio N-terminal o "cola" de las histonas. Entre las diferentes modificaciones que pueden sufrir, la acetilación de lisinas (K) es una de las mejor caracterizadas, y de entre ellas, la acetilación de la lisina 16 de la histona H4 es la más frecuente en eucariotas. La forma acetilada de H4K16 es una marca importante en eucromatina transcripcionalmente activa que se ha encontrado desde levaduras hasta el ser humano; mientras que su forma no acetilada se asocia con el silenciamiento de genes y regiones de heterocromatina. Esta dinámica de acetilación/desacetilación de este residuo histónico se rige principalmente por tres enzimas: la acetiltransferasa de histonas (HAT) MOF (varones ausentes de la primera), y las deacetilasas de histonas (HDAC) SIRT1 y SIRT2. Por lo tanto, los dos grupos de enzimas son esenciales para la regulación de la expresión de genes y la organización de la cromatina en el núcleo, regulando la transición entre el estado transcripcionalmente activo e inactivo de la cromatina. SIRT1 y SIRT2 pertenecen a la Clase III de las HDACs, denominado como sirtuinas, y son cruciales para el mantenimiento de la integridad genómica, la adaptación al entorno y el envejecimiento, entre otras funciones. Por un lado, SIRT2 es la única sirtuina de mamífero que se encuentra en el citoplasma, pero la cual pasa al núcleo durante G2/M. Como consecuencia, la principal función de esta HDAC es la desacetilación de H4K16Ac durante G2-M. Hasta el momento, el papel de SIRT2 como una de las principales desacetilasas de H4K16Ac durante mitosis sólo se ha demostrado por los experimentos realizados usando células de mamífero en cultivo o levaduras. Por lo tanto, nuestro estudio demuestra por primera vez el papel de SIRT2 en la regulación de los niveles de H4K16Ac en mitosis *in vivo*. De hecho, nuestros resultados apoyan la función de SIRT2 en la regulación de la cromatina por su participación en el control no sólo de los niveles de H4K16Ac, sino también los de H4K20me durante todo el ciclo celular. No obstante, como sucede con otros miembros de la familia de las sirtuinas, SIRT2 también regula y desacetila sustratos no histónicos que controlan el ciclo celular, la respuesta al estrés, la supervivencia celular y la estabilidad del genoma. Una de las principales funciones de SIRT2 consiste en su participación en el control del ciclo celular, habiéndose descrito diversas proteínas involucradas en el control mitótico que parecen estar reguladas por SIRT2, tales como CDH1, CDC20, BubR1 y p53. Además, nuestros resultados sugieren que los patrones de histonas generados por SIRT2 durante mitosis son esenciales en el control de la progresión del ciclo celular y se deben a dos mecanismos complementarios: la desacetilación tanto de H4K16Ac como de PR-Set7, la

enzima encargada de monometilar H4K20. En consonancia, nuestro estudio ha podido demostrar como SIRT2 está claramente implicada en un punto de control de mitosis, y regula la deposición de H4K20me1 en condiciones de estrés, con el fin de preservar la integridad genómica. Por otro lado, SIRT1 ha sido principalmente descrita en la regulación de la formación de heterecromatina y silenciamiento génico por desacetilación de histonas y de sustratos no histónicos. De hecho, SIRT1 participa en el mantenimiento de la integridad del genómica a través de su función en la formación de heterocromatina, desacetilando marcas histonicas (H3K9ac y H1K26Ac) y regulando otras proteínas como HP1, Suv39h1 y Ezh2. Además, SIRT1 también desacetila H4K16Ac, H3K9Ac y H1K26Ac en promotores específicos con el fin de controlar la expresión génica; y regula proteínas no histónicas tales como Suv39h1, p53, factores FoxO, y Rb, entre otros, con el fin de modular específicamente el patrón de expresión. Además, SIRT1 ha sido recientemente implicada en el control del ciclo celular a través de la regulación de Mcm10, el factor eucariota esencial para la iniciación de la replicación del ADN durante la fase-S. Sin embargo, nuestros estudios demuestran cómo SIRT1 puede estar implicada en la regulación del ciclo celular modulando de la expresión de PR-Set7 y Suv4-20h2, las enzimas responsables de mono- y di-metilar H4K20, respectivamente. En conjunto, nuestros resultados evidencian el papel de las sirtuinas en la preservación de la integridad genomica mediante la modulación de la cromatina y de la progresión del ciclo celular desde mitosis a fase-S.

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Después de casi seis años de tesis es lógico que tenga mucho que agradecer a muchas personas que han pasado por mi vida, muchas de las cuales aun están en ella, y que espero permanezcan en ella el mayor tiempo posible. Por ello esta tesis no se la dedico a nadie en particular, sino a todos los que han estado a mi lado en los buenos y malos momentos, a todos los que han compartido conmigo horas interminables de trabajo riendo y/o llorando, a aquellos que han soportado con resignación mi horario de trabajo sin exigir y con toneladas de comprensión,…a todos vosotros.

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Venirme a Barcelona fue mi “salida del nido”, ya que sería la primera vez que viviría sin mis padres, en una ciudad diferente y sin tener a nadie conocido a mi lado. Sin embargo, tuve la suerte de toparme con gente increíble tanto dentro como fuera del laboratorio. Y es que de la vida de un pre-doc se habla así: “fuera y dentro del lab”, verdad? Porque las horas con pipetas, células, bacterias, ratones…se hacen a veces interminables, y acabas llegando a casa a horas intempestivas. Afortunadamente durante los primeros años conté con una “pequeña familia” al llegar a casa (Nahuel, Juli, Alex, Cristina, Diego…), que me hizo toda esta transición mucho más llevadera y divertida, entre asados, empanadas, exámenes de selectividad, fiestas, amigos invisibles… A ellos les debo muchos de los buenos recuerdos que guardaré de Barcelona.

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nuestras noches de pizzas a “domicilio”, en la “terraza” del PEBC, con Martis, Jóse, Vicky… gracias a las cuales se han creado algunas de las amistades más importantes de mi vida. Y a esas amistades también debo agradecer el estar hoy aquí, el haber podido llegar hasta el final sin abandonar, con una sonrisa en la cara y con ganas de más. Martis, Henar, Lauris y Javi han sido para mí los grandes pilares de estos casi seis años. Gracias a Javi y Lauris, por todos los viajes, las risas, las confesiones, el apoyo… porque con vosotros no solo me he divertido, también he aprendido a exigirme más y a querer saber más sobre el mundo. A Marta y Henar debo agradecerles el estar siempre a mi lado, pendientes de mí, personal y científicamente, sacándome una sonrisa en los malos momentos y compartiendo las sonrisas en los buenos. Porque ambas desprendéis felicidad, haciendo que te sientas feliz solo con estar a vuestro lado, aunque llevéis 12h trabajando como monos y los experimentos hayan ido fatal. Además, junto a Vicky fuimos las cuatro aventureras que viajamos de voluntariado a Kenia, compartiendo un viaje inolvidable, que nunca hubiese sido tan increíble sin vosotras tres! (Allí vimos enfadarse a Marta por primera vez!!! Jejeje).

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MUCHAS GRACIAS A TODOS,

Paloma

Abbreviations

53BP1	p53-binding protein 1
Ac	Acetylation
ACS	Acetyl-CoA synthetase
APC/C	Anaphase-promoting complex/cyclosome
ATM	Ataxia telangiectasia mutated
ATR	AMT- and Rad3-related
BAX	Bcl-2-associated X protein
BER	Base excision repair
BSA	Bovine serum albumin
BubR1	budding uninhibited by benzimidazoles related 1
CAPs	Chromatin architectural proteins
CDC14	Cell division cycle 14
CDC20	Cell division cycle 20
CDH1	E-cadherine
CDK	Cyclin-dependent kinases
CENP	Centromere protein
CHD	Chromodomain helicase/ATPase DNA binding protein
CHFR	Checkpoint With Forkhead-associated (FHA) And RING Finger Domains
ChIP-seq	Chromatin immunoprecipitation-sequencing
Chr	Chromatin
CRL4	cullin-ring finger ligase-4
CT	Chromosome territory
CTCF	CCCTC-binding factor
CtIP	CtBP-interacting protein
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA-damage response
DN	Double knockout
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-Protein kinase catalytic subunit
DNMT	DNA methylatransferase
dNTP	Deoxyribonucleotide
DSB	Double strand breaks
EDTA	Ethylenediaminetetraacetic acid
Ezh2	Enhancer of zeste 2
FBS	Fetal bovine serum
FOXO	Forkhead box class O
G2/M	The border between G2 and mitosis
G2-M	Both G2 and mitosis phases
GFP	Green fluorescent protein

HA Hemaglutinin A
HATs Histone acetylases
HDACs Histone deacetylases
HMTs Histone methyltransferases
HP1 Heterochromatin protein 1
HR Homologous recombination
HSF Heat shock response regulator
HU Hydroxyurea
ICD Interchromosome domain compartment
IF Immunofluorescence
IgG Immunigñobulin
IHC Immunohistochemistry
INO Inositol
IP Immunioprecipitation
IR Ionizing radiation
ISWI Imitation of switch
KDMs Lysine demethylases
KMTs Lysine methyltransferases
KO Knockout
Ku70 70k autoantigen
MBT Malignant brain tumor
MDC1 Mediator of DNA damage checkpoint 1
me Methylation
MMR DNA mismatch repair
MOF Males absent On the First
MRE11 Meiotic recombination 11
NAD+ nicotinamide adenine dinucleotide
NBS1 Nijmegen breakage syndrome 1
NES Nuclear exporting sequences
NF-KB Nuclear factor-KB
NHEJ Non-homologous end joining
NLS Nuclear localization signals
ORC Origin recognition complex
p Phosphorylation
PARPs Poly(ADP-ribose) polymerase
PcG Polycomb group
PCNA Proliferating cell nuclear antigen
PCR Polymerase chain reaction
PHD Plant homeodomain
Plk1 Polo kinase 1
PMSF Phenylmethylsulfonyl fluoride
Pol I RNA polymerase I
PP2A Protein phosphatase 2ª
PRC Polycomb repressive complex
pre-RC pre-replication complex
PRMTs Protein arginine methyltransferases

Rb	Retinoblastoma protein
RIP	RNA immunoprecipitation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
RSR	Replication stress response
RZZ	Rod, Zwilch and ZW10 complex
SAGA	Spt-Ada-Gcn5 acetyltransferase
SINE	Short interspersed nuclear domain
siRNA	Small interfering RNA
SIRT1-7	Sitent mating type information regulation 2 homolog 1-7
Skp2	S-phase kinase-associated protein 2
SMC	Structural maintenance of chromosomes
SRC	Steroid receptor coactivator
SSA	Single-strand annealing
SSB	Single strand breaks
Suv39h1	Supressor of variegation 3-9 homolog
Suv4-20h	Supressor of variegation 4-20 homolog
SWI/SNF	Switch/sucrose nonfermenting
TADs	Topologically associated domains
Tao1	thousand-and-one amino acid
TIM-Tipin	Timeless-Tipin
TSA	Trichostatin A
TSS	Transcriptional start site
UV	Ultraviolet
WB	Western blot
WRN	Gene associated with Werner's syndrome
WT	Wild-type
XPA	xeroderma pigmentosum group A

F	Phe	Phenylalanine	**S**	Ser	Serine	**Y**	Tyr	Tyrosin
K	Lys	Lysine	**W**	Trp	Tryptophan	**L**	Leu	Leucine
P	Pro	Proline	**H**	His	Histidine	**D**	Asp	Aspartic acid
R	Arg	Arginine	**I**	Ile	Isoleucine	**T**	Thr	Threonine
Q	Gln	Glutamine	**E**	Glu	Glutamic acid	**G**	Gly	Glycine
M	Met	Methionine	**A**	Ala	Alanine	**N**	Asn	Asparagine
C	Cys	Cysteine	**V**	Val	Valine			

Introduction

Introduction

1. Chromatin structure and organization

In Eukaryotes, the DNA is tightly wound by DNA binding proteins, the histones, forming what is termed as chromatin. This establishes a dynamic structure whose level of compaction is heterogeneous throughout its length and during the different cellular stages. The DNA association of these histone proteins establishes a hierarchical structure of successive levels of compaction, which ranges from the basic unit of chromatin, the nucleosome, to the highest degree of compaction, the metaphase chromosomes.

The nucleosome, as the basic repeating structural unit, is formed by eighth histone proteins and about 147 base pairs of DNA (Kornberg & Lorch, 1999). Studies during the 1970's identified this chromatin unit as two turns of DNA wrapped around a histone octamer of H2A-H2B dimer, a $(H3)_2(H4)_2$ tetramere, and a DNA linker where the ninth histone unit, H1, binds (Kornberg & Lorch, 1999; Olins & Olins, 1974; Thomas & Kornberg, 1975; Woodcock, Sweetman & Frado, 1976). Therefore, every genome contains hundreds of thousands of nucleosomes that are joined by the linker DNA (an average of about 20 bp) and gives the appearance of a string of beads when viewed using an electron microscope (Olins & Olins, 1974; Olins & Olins, 2003). Interestingly, there are two noteworthy issues related to the nucleosomes that confers them functionality. First, the histone tails (N- terminal domains of the protein) are exposed outside the nucleosome, and do not form any secondary structure, creating an ideal surface for covalent modifications by enzyme machineries. Second, the length of linker DNA varies between different species and tissues, in many cases randomly. But sometimes its length is the consequence of preferred positions of the nucleosomes that serve in the binding of regulatory elements (Lu, Wallrath & Elgin, 1995; Straka & Horz, 1991).

The nucleosome packing shortens the DNA fiber length about sevenfold, forming what is called the "11nm fiber". However, this "string-of-beads" is still too long to fit into the nucleus and it has to be further coiled into a thicker and shorter structure, termed as the "30nm fiber" by Finch and Klug (Finch & Klug, 1976) more than 35 years ago. In this model, although some variations exist, essentially nucleosomes are proposed to arrange into a two-start zigzag or into a one-start solenoid manner (Grigoryev et al., 2009; Kruithof et al., 2009; Routh, Sandin & Rhodes, 2008). But it was more than 25 years ago, when Dubochet and co-workers (Dubochet et al., 1988) pointed the absence of a 30nm fiber structure in vitrified sections of mammalian mitotic cells. That was the beginning of an important still open question: Does the 30nm fiber exist *in vivo*? The latest studies support what have been called the "polymer melt" model (Figure 1) (Eltsov et al., 2008; Stehr et al., 2008). In this model, the selective intra-fiber nucleosomal associations described for the 30nm fiber can only be accomplished under diluted conditions, as

in vitro systems. However, at the high nucleosome concentrations that occur *in vivo*, the nucleosome fibers are forced to interdigitate with one another, leading to the 'polymer melt' state. This model clearly changes the intra- to an inter-fiber nucleosome interaction model. However, DNA packaging in a eukaryotic must meet another requirement, apart from being sufficient to fit inside the nucleus; it must remain accessible to transcription factors and the RNA machinery when needed. These important requirements determine the mode of packaging and distinguish two chromatin domains in higher eukaryotes: Heterochromatin, which is more compacted and generally transcriptionally inactive; and euchromatin, which is less compacted and consider as transcriptionally active.

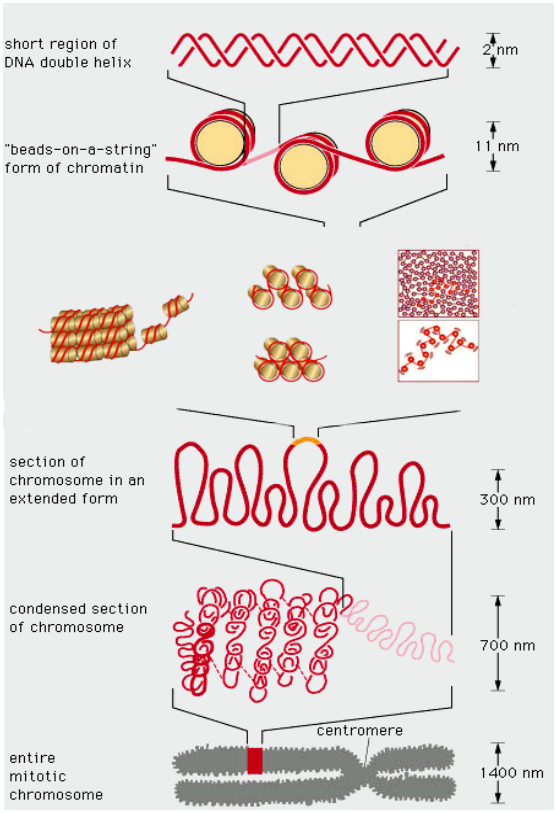


Figure 1. Chromatin organization of mammalian nucleus. Chromatin basic units are the nucleosomes, forming the "beads on a string". Chromatin increases its compaction level to form the 30nm fiber with a solenoid or zig-zag structure, or what has been described as the "polymer melt" organization. But the highest level of organization is due to inter- and intra-fiber interactions in rder to form the metaphase chromosomes.

Heterochromatin and euchromatin were described by the German botanist Emil Heitz (E. Heitz, 1928). Heterochromatin was initially described as the portion of genome that retains deep staining with specific DNA dyes when the dividing cells run from metaphase to interphase. Meanwhile, euchromatin was defined as the actively transcribed regions of the chromatin. The differences between heterochromatin and euchromatin are based on its accessibility by the transcription machinery. This fact conditioned the type of genes that can be found in one or another. Heterochromatin consists mainly of repetitive DNA sequences such us the highly repetitive pericentromeric DNA-satellite, whereas the transcriptionally active genes are located in the euchromatin. Consequently, the transition between these two levels of organization is vital for the control of transcription and replication, among other processes (Kouzarides, 2007a; Kouzarides, 2007b; Vaquero et al., 2004). The changes affecting the chromatin structure have a profound impact on gene expression and genome organization.

Besides the compartmentalization of genomes into heterochromatin and euchromatin, the distribution of DNA into different chromosomes has also been an important studied branch of the genome organization. During the 1980's it was determined that the chromosomes were maintained as individual entities in the nucleus instead of intermingeling spaghetti-like structures. Non-radioactive methods of *in situ* hybridization demonstrated unequivocally that the DNA of individual chromosomes is not disorderly distributed over the whole nucleus during interphase. The experiments showed how the chromosomes remained confined to a smaller subvolume, named as chromosome territory (CT) (Lichter et al., 1988; Schardin et al., 1985). From these observations what appeared was called the interchromosome domain compartment (ICD) model (Cremer et al., 2008; Cremer, Cremer & Lichter, 2014; Cremer & Cremer, 2010; Cremer et al., 1993). The model proposed that a three-dimensional channel network, the ICD-space, would surround and separate the individual CTs (Cremer et al., 2008; Cremer et al., 2014; Cremer & Cremer, 2010). This model has been reinforced according to the latest findings using newly developed molecular, genomic and computational approaches, such as, Chromosome Conformation Capture techniques (Hakim & Misteli, 2012; van Berkum et al., 2010). These territories are subsequently divided into two domains: a gene-rich domain that localize away from the nuclear periphery and are characterized by active gene expression and early replication; and a gene-poor domain that includes the compact repressive late-replicating heterochromatin fraction (Kalhor et al., 2012; Simonis et al., 2006). Additionally, the new higher resolution techniques demonstrated the presence of smaller large-scale structural units in the chromatin, known as topologically associated domains (TADs) (Dixon et al., 2012; Nora et al., 2012). These mega-base-sized chromatin domains, well conserved between mice and humans, divide the genome by the presence of boundary regions. Multiple factors have been associated with the boundary regions separating topological domains; those include the insulator binding factor CTCF, cohesin complexes, housekeeping genes, and SINE elements(Dixon et al., 2012; Seitan et al., 2013).

2. Chromatin dynamics

The existence of different modes of packing nucleosomes within fibers implies contacts between adjacent nucleosomes, which are dependent on histone tail modifications, chromatin remodeling enzymes, DNA methylation processes and chromatin architectural proteins (CAPs). The dynamic structure of the chromatin depends on the coordination of all these different chromatin remodeling processes in order to regulate the chromatin structure during the cell cycle and upon different stimuli to preserve genome integrity.

2.1. Histone modifications

The modifications of the histone tails include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation (Figure 2); and they do not only regulate chromatin structure by merely being there, they also recruit other enzymes or structural proteins ("readers") in order to regulate chromatin structure.

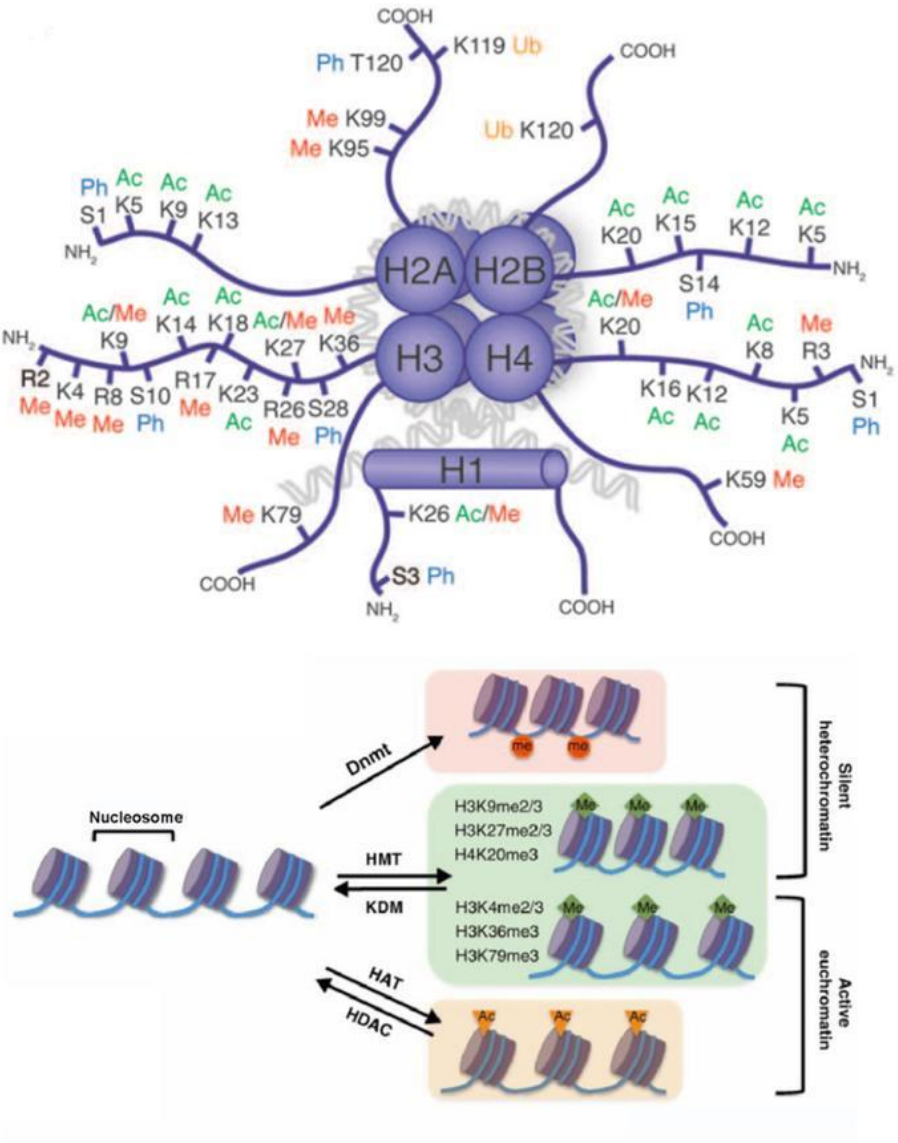


Figure 2. Visual scheme of histone modifications and their general effect on chromatin organization. A. The nucleosome histones and the histone linker H1 are substrates for several different enzymes that add or remove chrmichal groups or small proteins to their N-terminal domain. This general scheme includes the residues that are acetylated, methylated, phosphorylated and Ubiquitinated of histones H2A, H2B, H3, H4 and H1. B. Schematic representation of chromatin orgatization due to histone modifications: the heterochromatin formation is characterized by DNA methyñation and some histone methylation patherns; whereas the euchromatin presents acetylated histones and different methylation histone pattern.

It is now clear that some protein domains (also called as protein modules) are able to recognize and interact with chromatin and/or its modified components: bromodomains,

chromodomains, SANT domains, PHD fingers, Tudor domains and MBT domains (Figure 3). Bromodomains are small protein domains that are found in chromatin associated proteins such as HATs, KMTs and ATP-remodeling enzymes. Bromodomains bind to acetylated peptides and are highly conserved. However, despite their structural similarity, the overall bromodomain sequence is not highly conserved beyond the residues which are directly involved in acetyl-lysine binding. These differences in sequence may account for the differences in binding specificities seen between bromodomains; although a single bromodomain frequently displays affinity for multiple acetylated residues (Jeanmougin et al., 1997; Zhang et al., 2010). Therefore, the binding specificity of some bromodomain proteins comes from the association of a bromodomain with other binding module such as PHD finger motif; as happens in TIP5 of the NoRC remodeling complex (Zhou & Grummt, 2005). The PHD or plant homeodomain fingers are small protein domains found in nuclear factors, such as KMTs, ATP-remodeling complexes and transcription factors, that interact with histone H3 trimethylated at Lys 4 (H3K4me3) and the unmodified tail of histone H3 (unH3); or with histone H3 trimethylated at Lys 9 (H3K9me3) and histone H3 or H4 acetylated at various lysine residues (H3/H4Kac) (Musselman & Kutateladze, 2009).

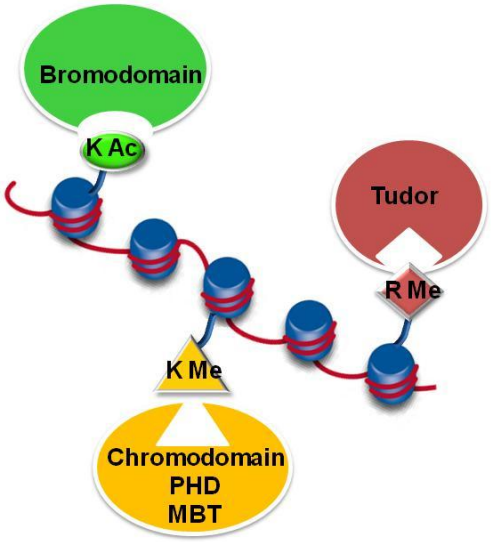


Figure 3. Histone-modifications binding modules. Lysine acetylation (K Ac) is recognized by proteins with bromodomains. The methylation of lysine residues (K Me) binds different modules according to their specificity: Chomodomains, PHD fingers and MBT domains. The methylated arginines (R Me) are read by proteins containing Tudos domains.

Chromodomains have been found in many other chromatin regulators (HATs, KMTs and ATP-remodeling enzymes) and structural proteins such as HP1; and are known to bind methyl-lysines, DNA and/or RNA. Contrary to bromodomains, these binding modules are highly specific as demonstrate by the specificity of Polycomb group protein binding, compared to HP1 (Fischle et al., 2003b; Jacobs et al., 2001). The SANT domain is a small motif present in some ATP-remodeling complexes which binds to non-modified histone tails; this may indicate a

mechanism to block histone modifications and maintain the unmodified state (Boyer, Latek & Peterson, 2004; de la Cruz et al., 2005). Tudor domains are commonly found in proteins in a wide range of organisms (Maurer-Stroh et al., 2003). It consist of a 60-aa hydrophobic pocket that constitutes a protein-protein interaction surface, which binds selectively to methylated lysine or methylated arginine residues (Jin et al., 2009; Selenko et al., 2001). The PHD or plant homeodomain fingers are small protein domains found in nuclear factors, such as KMTs, ATP-remodeling complexes and transcription factors, that interact with histone H3 trimethylated at Lys 4 (H3K4me3) and the unmodified tail of histone H3 (unH3); or with histone H3 trimethylated at Lys 9 (H3K9me3) and histone H3 or H4 acetylated at various lysine residues (H3/H4Kac) (Musselman & Kutateladze, 2009). And the MBT (malignant brain tumor) domains, which are putative methyl-lysine binding modules that bind methylated peptides from N-terminal tails of H3 and H4 histones; and are present in chromatin binding proteins involved in transcriptional repression such as some chromatin regulatory complexes (Wang et al., 2003).

The nature of histone modifications may determine the degree of chromatin compaction; indeed, historically, it was proposed that the histone modifications could constitute a code, subsequently termed as “the histone code” (Jenuwein & Allis, 2001) which could dictate biological outcomes according to its readers. For instance, histone acetylations have been interpreted as “open” chromatin marks, meanwhile some histone methylations were determined as “closed” chromatin marks which also recruit heterochromatin proteins. However, recent evidences suggest that the consequence of a specific modification on the histone tail depends on the context. This gave rise to a new term, “the chromatin signaling” (Lee, Smith & Shilatifard, 2010b; Sims & Reinberg, 2008). A remarkable example involves the trimethylation of H3 on lysine 4 (H3K4me3), usually associated with transcriptional activity, due to its presence at the transcriptional start site (TSS) of most expressed genes (Barski et al., 2007). Nevertheless, this same histone modification is considered as a silent mark in the context of DNA damage, when it is recognized by the plant homeodomain (PHD) of the inhibitor of growth 2 (ING2) tumor suppressor (Bua & Binda, 2009; Shi et al., 2006).

HAT family	HAT enzyme	Complex known	Histone specificity	Function
GNAT	Gcn5	STAGA, TFTC, ATAC	H2B, H3,	Coactivator, DNA replication
	PCAF	PCAF	H3, H4	Coactivator
	Hat1	HatB	H2B, H4 (K5, K12)	Histone deposition, silencing, DNA replication
	Elp3	Elongator	H3, H4	Transcriptional elongation
	Hpa2	n.d.	H3, H4	n.d.
	ATF-2	n.d.	H2B, H4	Sequence specific transcription factor
MYST	Sas2	SAS	H4K16	Silencing, DNA replication
	Sas3	NuA3	H2A, H3, H4	Silencing
	MORF	ING5	H3, H4	Transcriptional activation
	TIP60	TIP60	H2A, H3, H4	Transcriptional activation, DNA repair, apoptosis
	Esa1	NuA4	H4, H2A	Cell cycle progression, rDNA silencing
	MOF	MSL	H4K16	Transcriptional activation, DNA repair, X-chromosome hyperactivation
	HBO1	HBO1	H3, H4	DNA replication
	MOZ	AML1, ING5	H2A, H3, H4	Transcriptional activation
P300/CBP	P300	n.d.	H2A, H2B, H3, H4	Coactivator
	CBP	n.d.	H2A, H2B, H3, H4	Coactivator
SRC	ACTR	n.d.	H3, H4	Hormone receptor coactivator
	SRC-1	n.d.	H3, H4	Hormone receptor coactivator
	TIF2	n.d.	n.d.	Hormone receptor coactivator
HDAC family	**HDAC enzyme**	**Complex known**	**Histone specificity**	**Function**
Class I (Rpd3)	HDAC1	Sin3, NuRD, CoREST, NCoR/SMRT, PRC2	H3Ac, H4K16Ac	Transcriptional corepression
	HDAC2	Sin3, NuRD, CoREST, PRC2	H3(K56, K9)Ac, H4K16Ac	Transcriptional corepression
	HDAC3	NCoR/SMRT	H2AAc, H3 (K9, K14)Ac, H4(K5, K12)Ac	Heterochromatin, DNA repair, Transcriptional corepression, cell cycle
	HDAC8	n.d.	H2A, HAB, H3, H4	Transcriptional corepression, chromatin formation, cell cycle
ClassII (Hdal)	HDAC4	NCoR/SMRT	H3	Transcriptional corepression , DNA repair
	HDAC5	NCoR/SMRT	n.d.	Transcriptional corepressor, heterochromatin formation, replication
	HDAC6	n.d.	n.d.	DNA repair, cell migration
	HDAC7	NCoR/SMRT	n.d	Transcriptional corepression
	HDAC9	n.d.	H3, H4	Transcriptional corepression
	HDAC10	n.d.	H4	Transcriptional corepression
Class III (Sir2)	SIRT1	NCoR/SMRT	H1K26Ac, H3K9Ac, H4K16Ac	Transcriptional repression, heterochromatin formation, apoptosis, cell survival, cell cycle, DNA repair
	SIRT2	n.d.	H4K16Ac	Cell cycle, cell survival, DNA repair
	SIRT3	n.d.	H3K9Ac, H4K16Ac	Transcriptional repression, DNA repair, cell death
	SIRT4	n.d.	n.d.	Catabolism
	SIRT5	n.d.	n.d.	Catabolism
	SIRT6	n.d.	H3(K9,K56)Ac	DNA repair, Transcriptional repression, chromatin structure
	SIRT7	n.d.	H3K18Ac	rDNA regulation, Transcriptional repression
Class IV	HDAC11	n.d.	H3, H4	Replication, Transcriptional repression

Table1. Classification of HATs and HDACs into their respective families. The table includes their known complexes, their histone substrates and their functions

2.1.1. Acetylation

Histone acetylation consists of the addition of an acetyl group to the ε-amino group of a lysine side chain, forming an amide bond. Histone acetylation is known to be a potent regulator of chromatin compaction. When cells are treated with trichostatin A (TSA), an inhibitor of histone deacetylases, all the previously heterochromatic regions decondense to form structures that have the appearance of euchromatin (Gorisch et al., 2005). In general, hyperacetylation is known as a hallmark of active chromatin, whereas hypoacetylation is related to repressed chromatin. For instance, acetylation of histone 3 lysines 9 or 14 (H3K9Ac or H3K14Ac), and/or acetylation of histone 4 lysines 5, 8, 12 or 16 (H4K5Ac, H4K8Ac, H4K12Ac and H4K16Ac) are generally associated to active transcription regions (Allfrey, Faulkner & Mirsky, 1964; Allfrey & Mirsky, 1964; Fischle, Wang & Allis, 2003a; Pokholok et al., 2005; Shogren-Knaak et al., 2006; Shogren-Knaak & Peterson, 2006). The positive charge of the lysine side chain bind tightly to the negatively charge DNA forming a closed structure; thus, acetylation of these residues removes the positive change and attenuates the interaction. *In vitro* and *in vivo* studies have demonstrated how H4K16Ac stimulates (Akhtar & Becker, 2000; Hassan et al., 2007), and have critical roles in regulating gene expression, not only in *Drosophila*, but also in mammalian cells (Kapoor-Vazirani et al., 2008; Kapoor-Vazirani, Kagey & Vertino, 2011; Kind et al., 2008). In addition to their structural effect, this histone modification also acts as docking sites for readers with a bromodomain module. Bromodomain proteins are involved in a diverse range of functions, such as acetylating histones, remodeling chromatin, and recruiting other factors necessary for transcription. For example, the bromodomain of Gcn5 binds to acetylated H3 and anchors the histone acetyltransferase complex Spt-Ada-Gcn5 acetyltransferase (SAGA) to nucleosomes, allowing SAGA to acetylate adjacent nucleosomes (Hassan et al., 2007; Hassan et al., 2002; Sterner et al., 1999). And additionally ATP-dependent remodeling complexes, such as SWI/SNF, bind to the acetylated nucleosomes and remodel the promoter region to allow the formation of the pre-initation complex and ultimately promote transcription (Hassan, Neely & Workman, 2001).

The enzymes in charge of histone acetylation are the Histone acetyltransferases (HATs). These enzymes are involved in DNA repair mechanisms, cell cycle regulation, development and angiogenesis, among other processes (reviewed in Santos-Barriopedro et al, 2014). HATs are classified into four families, according to their homology (Table 1): GNAT, MYST, p300/CBP and SRC (steroid receptor coactivator), see the Table 1 (Roth, Denu & Allis, 2001; Sterner & Berger, 2000). Additionally, HATs are generally known to work in complexes in order to promote transcription, with the exception of p300 and CBP (Avvakumov et al., 2012; Doyon & Cote, 2004; Doyon et al., 2004; Huang et al., 2012; Vamos & Boros, 2012). On the other hand, the enzymes that catalyze the removal of these acetyl groups are the Histone deacetylases (HDACs). These histone modifiers are widely known to participate in processes such us heterochromatin formation, DNA repair, cell cycle regulation, differentiation and development (reviewed in Santos-Barriopedro et al, 2014). They are classified into four different

classes (Table 1) (Yuan & Marmorstein, 2012): Classes I, II and IV, that are known to required Zn^{+2} to catalyze the reaction, and to be inhibited by TSA treatment; and class III, also called Sirtuins, which require the cofactor NAD^{+} to deacetylase (Imai et al., 2000) and, interestingly, are not sensitive to TSA. Some HDACs are contained in multiprotein complexes together with a wide variety of other proteins that includes scaffold proteins and other enzymes such us the previously mentioned ATP-dependent remodelers (Allen, Wade & Kutateladze, 2013; Hayakawa & Nakayama, 2011; Kadamb et al., 2013).

2.1.1.1. H4K16Ac: a key histone modification

The main enzyme responsible for this histone modification in higher eukaryotes is the so-called Males absent On the First (MOF, also named as MYST1 or KAT8). It is a highly conserved member of the MYST histone acetyltransferase (HAT) family, originally discovered in *Drosophila* as an essential component of the complex involved in the X-chromosome dosage compensation complex (DCC), named as the male-specific lethal (MSL) complex. In mammals, MOF activity is tightly regulated in two distinct complexes: the MOF-MSL complex and the MOF-MSL1v1 complex (Li & Dou, 2010). Meanwhile, the removal of H4K16Ac depends on more than one enzyme. The main HDACs responsible for H4K16Ac deacetylation are SIRT1 and SIRT2. SIRT1 is claimed to be H4K16Ac deacetylase at specific regions, whereas SIRT2 seems to decrease H4K16Ac levels of the whole genome. In any case, the main difference is that both belong to different cellular locations; SIRT2 is a cytoplasmic protein which can only shuttle to the nucleus during G_2/M, where it can only deacetylate H4K16Ac during these cell cycle stages(Vaquero et al., 2006); and SIRT1, as a nuclear sirtuin may deacetylate H4K16Ac according to different stimuli, but independently on the cell cycle stage. Other HDACs that have been recently described to deacetylate this residue are HDAC1 and 2, during S-phase and DNA damage (Miller et al., 2010); and HDAC3 in *Drosophila* studies (Lv et al., 2012).

Hypoacetylation of H4K16Ac has been claimed to be a hallmark of cancer, and has been associated to defects on survival, differentiation, cell cycle and DNA repair (Fraga et al., 2005; Gupta et al., 2008; Kapoor-Vazirani et al., 2008; Li & Dou, 2010; Vaquero et al., 2006) (Gupta et al., 2005; Pushpavalli et al., 2013; Taipale et al., 2005; Zhao et al., 2013). The levels of H4K16ac dramatically affect embryogenesis and oncogenesis processes (Cao et al., 2014; Ma & Schultz, 2013). As a matter of fact, studies using MOF knockout mice show how MOF (and therefore H4K16Ac) absence leads to peri-implantation embryonic lethality in mice (Gupta et al., 2008; Thomas et al., 2008). Therefore, the cellular and chromatin roles of this histone modification are a key factor in cellular and genome integrity. One of the main roles of H4K16c is, as it has been already indicated, its capacity to inhibit the folding of higher orders of the chromatin structures (Shogren-Knaak et al., 2006; Shogren-Knaak & Peterson, 2006) creating an ‘open’ structure. Indeed, its role in inhibiting chromatin compaction may support the cell cycle regulation of H4K16Ac levels. In particular, H4K16Ac peaks during S-phase, when DNA

replication processes need an open chromatin state, and decrease at G2/M reaching the lowest levels during mitosis, concurrently with the methaphase chromosome formation(Vaquero et al., 2006). Recently, new *in vivo* data in yeast, demonstrated that H2A-H4 interaction showed a strong dependence on cell cycle stage, with a maximum binding in mitosis, correlating with low levels of H4K16Ac (Wilkins et al., 2014). Moreover, from the earliest studies, the acetylation state of H4K16 has been associated with epigenetic phenomena throughout evolution, from silencing in *S. cerevisiae*, male X-chromosome dosage compensation in *Drosophila*, to silencing in mammals (Alekseyenko et al., 2012; Thurtle & Rine, 2014; Vaquero, 2009). The accurate regulation of H4K16Ac levels is essential for the maintenance of the chromatin structure. Indeed, telomeres are generally maintained in a heterochromatic state, and the aging-associated decrease of Sir2p, together with an increase of Sas2p (H4K16 acetyltransferase in *S. cerevisiae*) activity, promote telomere structure disrupction (Dang et al., 2009; Kozak et al., 2010). All these facts confirmed that H4K16Ac inhibits higher order of chromatin organization and its deacetylation inhibits transcriptional activity. Nevertheless, as other histone marks, H4K16Ac serves also as a signal to promote or inhibit other protein binding to the chromatin, hence regulating other processes. In favor of this fact there is the role of H4K16 in DNA repair. The absence of this acetylation because of MOF depletion, leads to a complete abolishment of Mdc1 (mediator of DNA damage checkpoint protein 1) recruitment (Li et al., 2010), which is involved in the formation of DNA damage-induced 53BP1, BRCA1 and MRN foci (Stewart et al., 2003). This also suggests that inter- and intra-nucleosomal interaction between H4 tail and H2A is involved in the proper chromatin configuration for Mdc1 association. Furthermore, the role of H4K16Ac in the dosage compensation of the X-chromosome in *Drosophila* not only responds to an open chromatin state, it is also enhanced by its capacity to recruit RNA Polymerase-II to the acetylated promoters. The complete effect of H4K16Ac almost doubled the transcription capacity of the *Drosophila* male X-chromosome (Conrad et al., 2011). Moreover, studies in yeast showed how the presence of H4K16Ac inhibits the binding of Sir3 blocking the spread of heterochromatin in *S. cerevisiae* (Kimura, Umehara & Horikoshi, 2002; Suka, Luo & Grunstein, 2002). In addition, H4K16Ac has been reported to influence the activity of the ISWI family of ATP-dependent chromatin remodelers *in vitro* and *in vivo* (Clapier, Nightingale & Becker, 2002; Corona et al., 2002; Klinker et al., 2014; Shogren-Knaak & Peterson, 2006).

Taking all this into consideration, H4K16Ac is a key histone modification involved in several epigenetic processes that imply open chromatin states and active transcription, which should be differentially regulated throughout the cell cycle.

2.1.2. Methylation

In contrast to acetylation, histone methylation seems to be chemically more stable. This histone modification can occur in either lysines (K) or arginines (R). Specific methylation of lysine residues is known to occur on histone H1 at K26, on H3 at K4, K9, K27, K36, K79 and on

histone H4 at K20. Arginine methylation takes place within the tails of histone H3 at R2, R17, R26 and histone H4 at R3 (Ontoso et al., 2013; Zhang & Reinberg, 2001).

Arginine methylation is a frequent post-translational modification that is involved in a variety of cellular functions such as DNA repair, transcriptional regulation, RNA processing and signal transduction (Bedford & Clarke, 2009; Nicholson, Chen & Richard, 2009). In mammals there have been found nine protein arginine methyltransferases (PRMTs), PRMT1 to 9 (Yang & Bedford, 2013). These enzymes have been classified into type I, type II or type III, regarding their catalytic activities. Type I and type II enzymes catalyze the formation of MMA as an intermediate. Type I (PRMT1, 2, 3, 4, 6 and 8) catalyzes the production of asymmetric dimethylarginine (aDMA), whereas type II PRMTs (PRMT5 and 7) catalyze the formation of symmetric dimethylarginine (sDMA) (Yang & Bedford, 2013).

Lysine methyltransferases (KMTs) are essential in the regulation of chromatin signaling. This is corroborated by several studies where KMTs functional defects lead to cancer (Albert & Helin, 2010; Schneider, Bannister & Kouzarides, 2002) and neurological disorders (Ryu et al., 2006) among other human pathologies. Based on the sequence and structure of their catalytic domain, KMTs can be classified into two groups: DOT1 like (DOT1L) and SET-domain-containing lysine methyltransferases (Black & Whetstine).

However, histone methylation is functionally more complex than other histone modifications because of two main reasons: First, both lysines and arginines can be mono-, di- or tri- metylated; and second, because the methylation of a residue can have opposite consequences in gene expression. Among the methylation sites, H1K26, H3K9, H3K27 and H4K20 (Berger, 2007; Kouzarides, 2007a; Kouzarides, 2007b; Martin & Zhang, 2005) are related with transcriptional repression and heterochromatin structures formation. Accordingly, histone methylation is recognized by PHD finger proteins, chromodomain proteins and MBT domain proteins; such as HP1 that recognizes and binds to H3K9me3 allowing further H3K9 methylation by the HP1-associated methyltransferase SUV39H1 and promoting heterochromatin formation (Lachner et al., 2001); or PRC1 (Polycomb repressive complex 1) which recognizes and bind H3K27me3, promoting H2AK119 ubiquitination that is associated with gene repression (Wang et al., 2004a). On the other side, H3K4, H3K36 and H3K79 are usually active transcription sites (Berger, 2007; Kouzarides, 2007a; Kouzarides, 2007b; Shilatifard, 2006); although some methyl-lysine binding proteins can modify this fact.

The enzymes that eliminate methyl marks from the histone tails are the Lysine demethylases (KDM), which usually form part of a multiprotein complex that synergise with HDACs, KMTs and nuclear receptors. Two types of demethylases have been reported so far, depending on their catalytic reaction: the LSD1 and the Jumonji C (JmjC) (Cloos et al., 2008; Klose et al., 2007). In the first case, LSD1 can function KDM of mono- and di- methylation of H3K4 and H3K9 (Metzger et al., 2005; Shi et al., 2004), as well as for non-histone substrates such as p53 (Huang et al., 2007); and this specificity is mainly determined by its association with different complexes. In contrast, JmjC demethylases substrate consist of specific trimethylated histone tails (Takeuchi et al., 2006). The JmjC protein family contains the

conserved JmjC domain that was first identified in the Jumonji protein (JARID2) and there are 27 different JmjC domain proteins within the human genome, of which 15 have been published to demethylate specific lysines or arginines in the H3 tail (Cloos et al., 2008).

2.1.2.1. H4K20me: a key histone methylation

Another important histone modification that also takes place in the N-terminal domain of the histone H4 is the methylation of the lysine 20, which can be mono-, di- and tri-methylated (H4K20me1-3). This methylation marks are evolutionarily conserved from yeast to humans (Sanders et al., 2004; Schotta et al., 2004), with an important role on cell cycle regulation, DNA damage and repair, and chromatin structure (Botuyan et al., 2006; Oda et al., 2009; Sanders et al., 2004; Schotta et al., 2004; Tardat et al., 2007). In higher organisms, multiple enzymes have evolved to control histone H4K20 methylation. H4K20-specific methyltransferases include PR-Set7 and the two Set9 orthologs Suv4-20h1 and Suv4-20h2, meanwhile histone H4K20me1 demethylase activity has been demonstrated for PHF8 (Fang et al., 2002; Liu et al., 2010; Nishioka et al., 2002; Qi et al., 2010; Schotta et al., 2004). Structural, biochemical and cellular studies have established that PR-Set7 activity is limited to H4K20 monomethylation (H4K20me1) (Couture et al., 2005; Oda et al., 2009; Xiao et al., 2005); and therefore, Suv4-20h1 and Suv4-20h2 are in charge of di- and tri-methylation respectively (Schotta et al., 2004).

The monomethylation of this histone lysine has been widely related to higher order of chromatin structure; indeed, H4K20me1 establishes inter-nucleosomal contacts with the H2A histone of adjacent nucleosomes, possibly to promote compaction(Lu et al., 2008). In addition, in vivo experiments with decreased levels of PR-Set7 resulted in chromatin decondensation in interphase nucleus and expanded chromosome territory, which refers to a less compacted chromatin structure (Oda et al., 2009). Several additional studies support the role of H4K20me1 in chromatin compaction, and thus, in gene repression. From *Drosophila*, where H4K20me1 localizes exclusively to regions on polytene chromosomes that are transcriptionally inactive (Nishioka et al., 2002); to mammals where different studies have localized this histone monomethylation primarily within gene bodies and repressed genes; and also on the inactive female X-chromosome (Abbas et al., 2010; Congdon et al., 2010; Kohlmaier et al., 2004). In the same vein, trimethylation of this residue has been clearly associated to heterochromatin formation. The different studies have shown how the absence of Suv4-20h2 promotes decondensation (Evertts et al., 2013), and how H4K20me3 is highly enriched at pericentric heterochromatin, telomeres, imprinted regions and repetitive elements, suggesting that this modification is also involved in transcriptional silencing (Gonzalo et al., 2005; Regha et al., 2007; Schotta et al., 2004). Therefore, these studies can also support the H4K20me1/PR-Set7 role in chromatin compaction, due to its subsequent use as a Suv4-20h2 substrate. In contrast, H4K20me2 is broadly distributed across the genome (Schotta G et al., 2004), what may indicate

that this residue is not implicated in any kind of large-scale chromatin conformation. Meanwhile, some ChIP assays have related the presence of this histone methylation with replication origins (Kuo et al., 2012).

Nevertheless, the H4K20 methylation functions are the result not only of its activity within the chromatin structure, but also the modulation by the proteins that bind to it. The three levels of methylation serve as a binding site for different proteins that contribute to distinct cellular functions. Due to its function in recruiting other proteins and complexes, these histone modifications are involved in DNA repair pathways, DNA replication process, and genome integrity maintenance. Some of these "readers" are 53BP1 that recognize mono- and dimethylated H4K20, and functions as a signal in the DNA damage response pathway (Botuyan et al., 2006; Sanders et al., 2004; Oda et al., 2009); L3MBTL1, that binds to both mono- and dimethylated residues and works as a transcriptional repressor (Kalakonda et al., 2008; Sakaguchi et al., 2012; Trojer et al., 2007) or as a required factor for normal replication fork progression (Gurvich et al., 2010); Condensin II that recognizes H4K20me1, being involved in the maintenance of the genome integrity during the mitotic progression; and proteins of the replication machinery that have been nearly recently linked to H4K20me3, rather than H4K20me2 (Beck et al., 2012; Shen et al., 2010; Vermeulen et al., 2010).

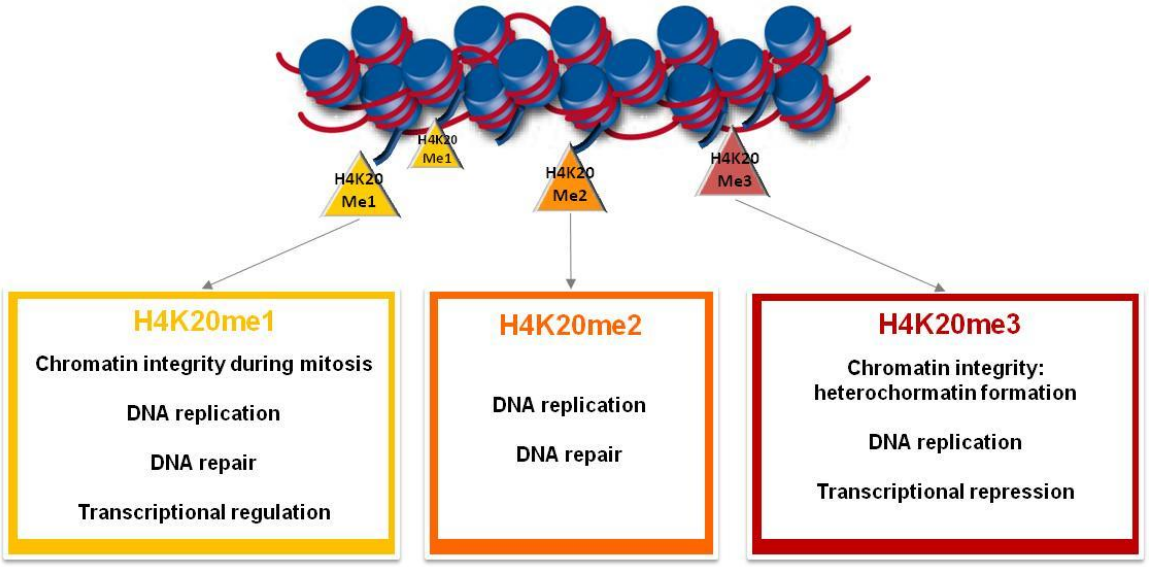


Figure 4. Roles of H4K20me1-3. Mono-, di- and tri- methylation of H4K20 have different and/or complementary roles in chromatin integrity, DNA replication, DNA repair, and transcriptional regulation.

Finally, an important characteristic of the H4K20me1, that differs from the di- and tri-methylation, is the fact that this histone mark is drastically regulated throughout the cell cycle. Another report has stated that H3K9 trimethylation is a cell cycle regulated mark, rising and falling during mitosis (McManus & Hendzel, 2006); but H4K20 monomethylation undergoes a much more dramatic cell-cycle regulated profile. It is practically absent at G1 and rises through

S-phase to get its higher levels at G2/M. Meanwhile, H4K20me2/3 are practically stable during the whole cell cycle (Rice et al., 2002).

Altogether, these previous studies imply that H4K20 methylation plays a role in transcriptional regulation and genome stability maintenance through the cell cycle (Figure 4).

2.1.3. Phosphorylation

All four nucleosomal histone tails contain acceptor sites that can be phosphorylated by a number of protein kinases and dephosphorylated by phosphatases. Histone phosphorylation can occur on serine, threonine and tyrosine residues, thereby adding a negative charge what may favor chromatin decondensation (Roth & Allis, 1992), although it has not been clearly demonstrated. Nevertheless, some histone phosphorylation marks such as histone H3 serines 10 or 28 have been demonstrated to increase during G2-M phases and regulate some chromatin condensation factors (Wilkins et al., 2014). Additionally, external stimuli also increase histone phosphorylation, as it happens with many signaling pathways, in order to control gene expression (Metzger et al., 2008; Nowak & Corces, 2004). For instance, phosphorylation of H2A.X (γ-H2AX) is an important histone modification that plays a major role in DNA damage response. In mammalian cells, this modification takes place on serine 139 of the H2A.X and is commonly referred to as γ-H2AX. This phosphorylation occurs in all phases of the cell cycle and is involved in diverse DNA-damage response (DDR), acting as a recruitment site for several DNA damage repair proteins and other chromatin-modifying complexes (Downs et al., 2004). Additionally, other histone phosphorylations such as Serine 36 of H2B or serine 1 of H4 have been linked to gene expression under stress conditions (Bungard et al., 2010; Govin et al., 2010).

Moreover, a number of proteins containing phospho-binding modules such as 14-3-3 and BRCT domains that can recognize phosphorylated histones have been identified and characterized as downstream effectors (Yun et al., 2011).

2.1.4. Ubiquitination

This is a very large modification that has been found on H2A and H2B. Ubiquitination refers to the post-translational modification of proteins by covalent attachment of one or more ubiquitin residues, leading to mono- or poly- ubiquitination. This ubiquitin residue consists on a small protein of about 9 KDa and can be added to histone and non-histone proteins (Ye & Rape, 2009). These modifications are not defined as activators or repressors; in fact the literature described opposite roles in regulating gene expression to each histone ubiquitination found. Whereas ubiquitination of H2AK119 is mediated by the Bm1/RingA protein of Polycomb complex PRC1 in order to repress transcription (Wang et al., 2004); H2BK120 ubiquitination is mediated by RNF20/RNF40 and UbcH6 to promote transcription (Zhu et al., 2005). Therefore,

there is still not enough information to establish the ubiquitination mechanism, although it is likely to be governed by the recruitment of other factors. Furthermore, the works from several laboratories have demonstrated the existence of cross-talks between histone H2B ubiquitination and histone H3 lysine 4/79 methylation. They described how the absence of H2B ubiquitination does not allowed the methylation of H3 in both residues (Shukla, Chaurasia & Bhaumik, 2009).

2.1.5. Sumoylation

This is another large modification and shows similarity with the ubiqutination. Accordingly, this modification adds an approximately 10 kDa small ubiquitin-like modifier (SUMO) polypeptide to the ε-amino group of certain lysine residues. Both ubiquitination and sumoylation attach small proteins to targets, and these small proteins serve as platforms for interaction with other proteins. This is quite different from other post-translational modifications (PTMs), such as phosphorylation, acetylation, and methylation, which add small chemical groups. Whereas phosphorylation, acetylation, and methylation are recognized by modification-specific protein domains, sumoylation is identified by SUMO polypeptides known as SUMO-interaction motifs, which conform to a well-conserved consensus sequence and can thus be used to predict and identify SUMO interaction sites on binding partners (Geiss-Friedlander & Melchior, 2007). Additionally, sumoylation is often coordinated with other histone posttranslational modifications occurring at regions flanking the sumoylated site. For instance, the sumoylation of Lys-386 of the tumor suppressor p53 is known to enhance Ser-392 phosphorylation by the protein kinase PKR (Bennett et al., 2012). In contrast, sumoylation of Lys-386 inhibits Lys-382 acetylation by p300/CBP, inhibiting its DNA binding capacity (Wu & Chiang, 2009). According to different studies, it became clear that the posttranslational modification occurring at the flanking regions of a SUMO-conjugation site dictate the efficiency of sumoylation and its functional outcomes.

2.1.6. ADP-ribosylation

ADP-ribosylation is a reversible post-translational protein modification (PTM) that happens in histone and non-histone substrates. Mono- ADP-ribosylation consists of the transfer of one ADP-ribose from NAD+ to specific amino acid residues of substrate proteins by releasing nicotinamide. In addition, the ADP-ribose units in the polymer can be linked by glycosidic ribose-ribose 1′-2′ bonds in order to poly- ADP-ribosylate. The chain length of the polymers is heterogeneous and can reach 200 to 400 units *in vitro* and *in vivo*. Currently, there are two families of mammalian proteins that mono- and or poly-ADP-ribosylate histones: First, a superfamily of eighteen proteins containing a conserved catalytic domain, termed poly(ADP-ribose) polymerase (PARPs), which use NAD^+ as their substrate to modify acceptor proteins with ADP-ribose modifications; and second, the NAD+-dependent protein deacetylases sirtuins.

Based on structural aspects and the enzymatic reactions, sirtuins are thought to mediate mono-ADPribosylation, whereas among the PARPs there are both mono- and poly-ADP-ribosyltransferases (Hassa et al., 2006).

The core histones and the linker histone H1 can potentially be ADP-ribosylated during or shortly after their synthesis in the cytoplasm and during transport into the nucleus, while bound to chaperones, or after their incorporation into chromatin. Several studies have shown that ADP-ribosylation of nucleosomes is involved in the regulation of the higher order chromatin structure, in repression and activation. In the absence of NAD+ (unphysiological conditions), saturated binding of PARP1 to nucleosomes promotes chromatin compaction as visualized by electron microscopy (Kim et al., 2004). By contrast, hyper-ADP-ribosylation of H1 has been associated with polynucleosome relaxation and increased DNA polymerase α activity (Niedergang et al., 1985). The histone ADP-ribosylation should be considered as a canonical histone modification that takes place during nuclear processes, such as DNA-replication, transcription and chromatin remodeling, and thereby affects chromatin function.

Information concerning the 'readers' of ADP-ribosylated histones is still very limited. However, several reports document the recruitment of specific proteins to sites of DNA damage in a PAR-dependent manner. Among the proteins whose recruitment to the sites of DNA damage depends on the enzymatic function of PARP1 are included the polycomb protein MEL-18 of the polycomb complex 1 (PRC1) and CHD4 (chromodomain helicase DNA-binding protein 4) (Chou et al., 2010; Polo et al., 2010). Although such binding proteins probably recognize the PAR structure rather than the histone modified site.

2.2. DNA methylation

In addition to histone modifications, DNA methylation is one of the main epigenetic mechanisms in eukaryotic cells linked to heterochromatin formation. It is a covalent modification that occurs in the fifth carbon of the cytosine base. The DNA methylation is mainly found in the CpG dinucleotides, and it is associated with stable long-term repression such as genomic imprinting and X-chromosome activation in mammals (Kim, Samaranayake & Pradhan, 2009). Moreover, CpG methylation induces compaction and rigidity in the structure of nucleosomes (Derreumaux et al., 2001; Geahigan et al., 2000), justifiying its location at centromeric and telomeric regions of many chromosomes (Brero et al. 2005; Barbin et al. 1994; Montpellier et al. 1994). In addition, as histone marks, the methylated CpGs also represent a recognition site for methyl-CpG binding domain (MBD) proteins, that recruit co-repressor complexes in order to alter chromatin structure (Zou et al., 2011); including the MeCP2 (methyl-CpG binding protein 2) protein, which represents a major mechanism by which DNA methylation can repress transcription in a coordinated response with HDAC activity (Jorgensen & Bird, 2002; Nan, Cross & Bird, 1998). Although DNA methylation does not seem to be a dynamic epigenetic modification, this DNA modification can also be removed in order to favor chromatin dynamic.

DNA demethylation can be achieved either passively, by simply not methylating the new DNA strand after replication, or actively, by a replication-independent process by enzymatic mechanisms (Chen & Riggs, 2011). However, the mechanism(s) of active demethylation remain poorly understood. In addition, the dynamic regulation of DNA methylation is also associated to its relation and influence on histone modifications create different repressing patterns with several repressed chromatin states. For this reason, mutant mouse embryonic stem cells that completely lack DNA methylation have alterations in the overall levels of histone H3 methylation and acetylation, decreased mobility of linker histones, and increased chromocenter clustering (Gilbert et al., 2007; Lee & Lee, 2012).

The “writers” of these modifications are the members of the DNA methyltransferase (DNMT) family of enzymes. In vertebrates, there are five known members of the Dnmt family that differ in structure and function and apart from DNMT2, all Dnmts comprise an N-terminal regulatory domain in addition to the C-terminal catalytic domain. Established DNA methylation patterns are maintained during DNA replication and DNA repair by the ubiquitously expressed DNMT1, due to its strong preference for hemimethylated CpG sites (Qin, Leonhardt & Pichler, 2011).

2.3. ATP-remodeling machines

The chromatin remodeling complexes refers to ATP-dependent enzymatic complexes (e.g., SWI/SNF, ISWI, NURD, etc.) that slide, replace, or remove nucleosomes to regulate the accessibility of the genomic DNA (Saha et al. 2006; Varga-Weisz P, 2001). Histone variant incorporation can be used to replace the canonical histones in order to regulate chromatin structure (e.g., H3.3, macroH2A, H2A.Z, etc.) (Ausio, 2006). Thus, they make chromatin available to proteins that need to access DNA or histones directly during cellular processes; for that reason they are required for processes such as transcription, DNA replication, recombination and repair (Hargreaves & Crabtree, 2011). Recently, the ATP-dependent chromatin remodeling factors have been classified into four families according to their ATPase subunit: the ISWI (imitation of switch), SWI/SNF (switch/sucrose nonfermenting), INO (inositol), and CHD (chromodomain helicase/ATPase DNA binding protein) families (Flaus & Owen-Hughes, 2011).

Importantly, chromatin remodeling factors are associated with histone modifiers. There are several studies that corroborate how some histone marks or their enzymes are required for chromatin-remodelers recruitment. For instance, acetylation of histone lysines and/or some HATs have been described in the recruitment of hSWI/SNF to the chromatin, regulating gene expression (Agalioti, Chen & Thanos, 2002). And additionally, the NuRD complex contains the HDAC1 and 2 (Varga-Weisz, 2014; Zhang et al., 1999). Therefore, the dominant mechanism to regulate chromatin structure consists of the recruitment of ATP-dependent chromatin remodeling enzymes by different histone marks. For that reason, some remodeling enzymes includes bromodomains (acetyl-lysine recognition domains) (Horn & Peterson, 2001), or plant

homeodomain (PHD) fingers or chromodomains (both to recognize methyl-lysines) (Patel & Wang, 2013) in their protein structure.

3. Sirtuins

During the last decade, the members of the Sir2 family, also known as sirtuins, have become firmly established as key regulators of the cellular response upon a variety of stresses, from metabolic to genotoxic stress. The ability of sirtuins to sense energy fluctuations in the cell is linked to their requirement of NAD^+ as a cofactor for their enzymatic activity (Figure 5) (Bosch-Presegue & Vaquero, 2011).

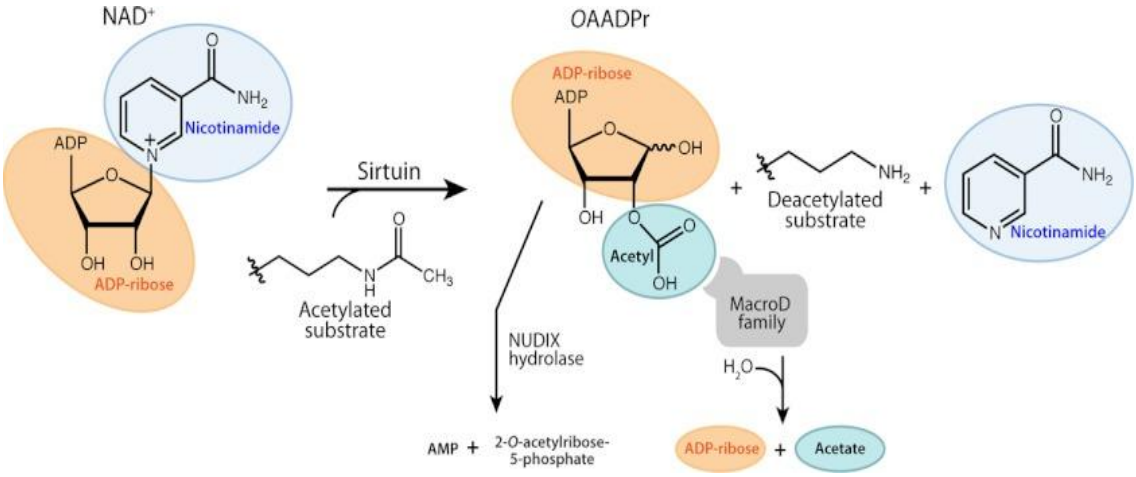


Figure 5. Sirtuin deacetylase activity reaction. The sirtuins activity is caharacterize for its NAD+ dependence. The deacetylase activity removes the acetyl group from the substrate creating two new products, the OAADPr and the Nicotinamide. The OAADPr is known to participate in signaling pathways, and the use of nicotinamide inhibits sirtuin activity.

As it has already been mentioned, sirtuins belong to the class III of the HDACs and their process of deacetylation differs markedly from other HDACs. While class I, II, and IV HDACs transfer the final acetyl group to the aqueous solution, sirtuins transfer the acetyl group from the substrate to an ADP-ribose molecule (Imai et al., 2000; Yuan & Marmorstein, 2012). Interestingly, ADP-ribosyltransferase and deacylase activity are also known in sirtuins, although our knowledge about these two processes is currently very limited (Hawse & Wolberger, 2009; Jiang et al., 2013).

As an almost universally conserved family, Sir2 has a long evolutionary history and its members encompass all the main life domains: prokaryotes, archaea and eukaryotes(Frye, 2000). A few prokaryotes do not appear to have sirtuins, but all eukaryotes examined thus far do have Sir2 homologs. The degree of conservation among Sir2 family members is restricted to

their catalytic domain, a region of approximately 250 residues (Frye, 1999) (Figure 6). In bacteria, the enzymatic domain consists only in a conserved enzymatic core domain whereas homologs in higher eukaryotes have amino(N) and carboxy(C)-terminal extension that are divergent among the members of the family (Mead et al., 2007) those differential terminal regions have been postulated to be responsible for Sir2 homologs regulation, recruitment and differential activity. Their ability to deacetylate proteins may have first appeared in bacteria as a mechanism to catabolize acetate and other acyl groups before adapting specifically to perform regulatory functions (Gardner & Escalante-Semerena, 2009; Starai et al., 2002). They have subsequently undergone considerable functional diversification during the course of evolution in order to adapt to increased complexities. For instance, mammals harbor 7 different sirtuins (SIRT1-SIRT7) that differ in their cellular location, substrate specificity, and functions (Figure 6) (North & Verdin, 2004).

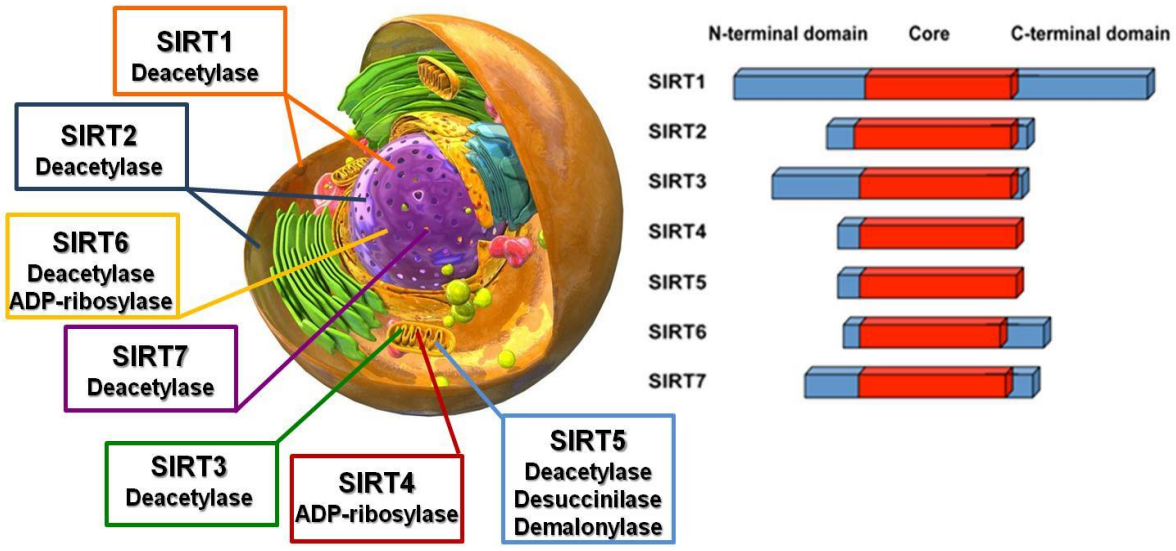


Figure 6. Sirtuins: cellular localization, enzymatic activity and structure. The main histone deacetylases of this family are SIRT1, SIRT2 and SIRT3; but SIRT6 and SIRT7 were also detected as histone deacetylases during the last years. These five histones are characterize for performing at least part of their catalytic activity in the nucleus: SIRT1 is nuclear, although shuttles to the cytoplasm under certain conditions; SIRT2 is cytoplasmic but it goes to the nucleos during G2-M phases; a small amount of a SIRT3 isoform is located in the nucleus, although it is a mitochondrial sirtuin; SIRT6 is clearly a nuclear protein, and SIRT7 has been found in the nucleolus. The rest of the sirtuins, SIRT4 and SIRT5, are the mitochondrial sirtuins.

Based on their phylogenetic relationship, sirtuins were classified from bacteria to humans into five types according to Frye (2000): Type I, includes members clearly linked to chromatin regulation, among other functions, that show true histone deacetylase activity (all yeast sirtuins, SIRT1-3, and others). Sirtuins of prokaryote origin involved in metabolic control constitute the Type II, which seems to have a primary ADP-ribosyltransferase activity and are mainly located in mitochondria (SIRT4 and others). Type III consist of another prokaryote-

related group of sirtuins-associated including most of the eubacterial and archeal Sirtuins, but also eukaryotic members located in mitochondria (SIRT5). Eukaryotic sirtuins also related to chromatin are included in type IV (such as SIRT6 and SIRT7), which was previously suggested to only behave as ADP-ribosyltransferases; however, it changed according to the very recent discovery of H3K9Ac- and H3K18Ac- specific deacetylase activity of SIRT6 in telomeres and SIRT7 in gene expression control, respectively (Barber et al., 2012; Michishita et al., 2008). Finally, Type U encompasses other uncharacterized prokaryotic sirtuins.

All these sirtuin functions have helped organisms to adapt and to survive oxidative, metabolic, or genotoxic stress by activating the stress response pathway in order to control genome integrity, cell cycle and chromatin expression, as well as signaling DNA damage and repair. However, under certain extreme conditions, such as chronic stress, some sirtuins, such as SIRT1, SIRT2, and SIRT3, can protect the organism by inducing cell senescence or apoptosis (Allison & Milner, 2007; Chua et al., 2005; Wang et al., 2006a). One of the best conserved sirtuin functions is the regulation of the metabolism homeostasis through control of the key enzyme acetyl-CoA synthetase (ACS), ranging from the bacterial CobB, to the mammalian SIRT1 and SIRT3 deacetylases (Chan et al., 2011; Hirschey et al., 2010; Starai et al., 2002; Starai et al., 2003). Nevertheless, throughout the evolution, sirtuins seem to have evolved in the crosstalk between genome and environment. Accordingly, Sir2 family NAD+-dependent deacetylase activity has been involved in chromatin structure from the very beginning in evolution. Sir2p was first found in a genetic screen of transcriptional silencing genes of the mating type loci in budding yeast, known as *mar1* (Ivy, Hicks & Klar, 1985; Klar, Fogel & Macleod, 1979). It was clearly shown to be required for the transcriptional mating type loci and subsequently, this protein was also implicated in transcriptional silencing at telomere proximal sites (Aparicio, Billington & Gottschling, 1991) and ribosomal repeats (Bryk et al, 1997; Fritze et al, 1997; Gottlieb and Esposito, 1989). However, no clear enzymatic activity was associated to this function until 2000, when several labs simultenously discovered the NAD+-dependent deacetylase activity of Sir2p (Imai et al., 2000)

Sirtuins have diversified and acquired novel functions along with evolution. Many of these new acquisitions are directly related to an increase in complexity and directly or indirectly related to maintenance of genome stability. Thus, mammalian sirtuins are known to protect the genome largely by different approaches: influencing chromatin structure, expression and repair through deacetylation of histones and other chromatin-factors; and regulating non-chromatin related proteins, involved in different cellular processes, including the stress response (Bosch-Presegue & Vaquero, 2013; Vaquero et al., 2007). The mammalian sirtuins, SIRT1, 2, 6, and 7 mainly exert their function via chromatin regulation. In past years, studies in knockout mice for each Sirtuin have provided important information about their respective roles in genome stability and tumorigenesis. Surprisingly, knockout mice of five sirtuins (SIRT1–4 and 6) clearly show increased levels of genome instability, as indicated by high levels of DNA damage, defects on DNA repair and the presence of chromosomal aberrations. Moreover, most of them undergo spontaneous tumorigenesis (Bosch-Presegue & Vaquero, 2013).

The case of SIRT3 is more complex, because only a small subpopulation localizes in the nucleus. Nevertheless, it participates in the repression of key stress-related genes through deacetylation of H3K9Ac and H4K16Ac in their promoters (Iwahara et al., 2012; Scher, Vaquero & Reinberg, 2007). The main fuctions of SIRT1 and SIRT2 in genome stability seem to be mainly related to cellular integrity, chromatin-associated functions, cell cycle and transcription regulation. SIRT1, as the closest *S.cerevesiae* Sir2p homolog in humans, is widely known as the NAD^+-dependent deacetylase of H1K26Ac, H3K9Ac and H4K16Ac (Jones, 2011; Vaquero et al., 2007; Vaquero et al., 2004); and complementary, SIRT1 function is associated to its non-histone substrates, most of them also histone modifiying enzymes, transcription factors, cell cycle related proteins and others, such as Suv39h1, p300, PARP1, or MOF. In addition, SIRT2 is also a crucial H4K16Ac deacetylase during G_2-M, and has also been related with other histone and non-histone proteins, such as H3K18Ac, H3K56Ac, p300 or tubulin. Meanwhile, SIRT6 has been mainly related to DNA repair since 2006 (Mostoslavsky et al., 2006) and according to its non-histone substrates such as PARP1, which is involved in BER (Base excision repair) and HR (Homologous recombination) (Mao et al., 2011); and the C-terminal binding protein interacting protein (CtIP), which participates in DNA double-strand break (DSB) repair process (Kaidi et al., 2010), among others. Indeed, it was not until 2008, when Michishita et al related specific SIRT6 deacetylase activity to H3K9Ac in telomeric regions in order to modulate cellular senescence and DNA repair (McCord et al., 2009). As it happened with SIRT6, SIRT7 histone deacetylase activity was recently reported by Barber et al (Barber et al., 2012) with a high specificity for H3K18Ac. The deacetylation of H3K18Ac at gene promoters required SIRT7 and, as a consequence, this chromatin modification helps in maintaining the transformed phenotype of cancer cells (Barber et al., 2012). Until then, SIRT7 mainly was related with rDNA transcription, being involved in positive regulation of the RNA polymerase I (Pol I) (Ford et al., 2006).

Altogether this indicates that under stress conditions, sirtuins work to maintain cellular integrity and genome stability; however, when the situation cannot be solved, the sirtuins help to regulate the cell death program in order to avoid negative stress consequences.

3.1. SIRT1

SIRT1 is the best studied sirtuin, and it has been reported to play important roles related to genomic stability maintenance, DNA repair, stress modulation, differentiation and development processes, among other functions. Interestingly, all these functions respond to its capacity to interact and/or deacetylate histone and non histone substrates (Figure 7). The phenotype of SIRT1 knockout mice is a clear reflection of the importance of this sirtuin in the regulation of a plethora of cellular functions. For that reason, independently from the mouse strain, a great percentage of the SIRT1 knockout mice died at early postnatal stages and

developed several abnormalities in different organs such as heart, lung, hematopoietic system, or pancreas, among others (reviewed by Bosch-Presegue & Vaquero, 2013).

SIRT1 was considered as an exclusive nuclear sirtuin for a long time; however, it was in 2006 when this sirtuin was described to shuttle between nucleus and cytoplasm due to at least two nuclear exporting sequences (NES) and two nuclear localization signals (NLS) (Tanno et al., 2007). The subcellular localization of SIRT1 seem to differ among different cell types and stimuli; in fact, cytoplasmic SIRT1 has been described in embryonic and adult neural precursor cells (NPCs), and it transiently localizes in the nucleus in response to different stimulus (Hisahara et al., 2008). As p53 and FoxO factors, which change their cellular locations according to their phosphorylation state (Huang & Tindall, 2007; Jimenez et al., 1999), some of the SIRT1 phosphorylations may be responsible of that shuttle (Revollo & Li, 2013; Tanno et al., 2007).

One of the best characterized functions of SIRT1 is its role in the formation and maintenance of heterochromatin. Accordingly, its counterpart in yeast, Hst1p, is also essential for heterochromatin repression by deacetylating histones H3 and H4 (Xie et al., 1999). The histone deacetylation capacity of SIRT1 is specific for some important histone residues such as H4K16Ac, H3K9Ac, and H1K26Ac. In turn, SIRT1 histone deacetylase activity is coordinated with subsequent “readers” and other histone modifiers in order to assure higher order of chromatin formation. H4K16Ac deacetylation favors chromatin compaction (Shogren-Knaak et al., 2006), but in order to promote heterochromatin formation, SIRT1 also participates in H3K9 trimethylation and H1K26 dimethylation. The role of SIRT1 in heterochromatin distinguished two fuctions: manteinance of constitutive heterochromatin, and formation of facultative heterochromatin. SIRT1 controls constitutive heterochromatin through deacetylation of H3K9Ac, and subsequent stabilization and recruitment of the H3K9 methyltrasferase, Suv39h1, in order to increase H3K9me3 levels (Bosch-Presegue et al., 2011; Vaquero et al., 2007). Moreover, it has been widely studied that H3K9me3 recruits the heterochromatin protein 1 (HP1) to the chromatin, and thereby helps maintaining heterochromatin structure (Bosch-Presegue & Vaquero, 2013; Vaquero, 2009). In addition to this structural function of SIRT1, histone modification by this sirtuin also regulates specific genes and cell fates in response to environmental changes. When the duration or intensity of an external aggressive factor, such as stress or fasting, are significant, SIRT1 is recruited to an euchromatin region in order to promote facultative heterochromatin formation. Upon SIRT1 arrival there is a reduction in H4K16Ac and H3K9Ac, together with the recruitment of the linker histone H1. Moreover, SIRT1 also deacetylates H1K26Ac, which would favor a more compact chromatin structure. The removal of these histone marks are accompanied by the deposition of H3K9me3 by Suv39h1 and the decrease of the levels of H3K79me2 as an active chromatin mark Therefore, this SIRT1 mechanism regulates the expression of a plethora of genes involved in stress response and survival mechanism (Bosch-Presegue & Vaquero, 2013). In addition, the heterochromatin regulation by SIRT1 has also been found as an important process in development in order to control gene expression patterns through the regulation of chromatin structure. As part of the

PRC4 complex, SIRT1 recruits histone H1 to the chromatin and deacetylates it, favoring its subsequent dimethylation by Ezh2, which creates a more compacted chromatin structure (Vaquero et al., 2004). Importantly, H1K26me2 is recognized by HP1, favoring facultative heterochromatin and thereby promoting chromatin compaction. In addition, SIRT1 association with the H3K4 demethylase LSD1 cooperates to regulate genes governed by the Notch signaling pathway. The H4K16Ac deacetylation activity, together with the demethylation of H3K4me1/2, collaborates in transcriptional repression (Mulligan et al., 2011). In agreement with this role of SIRT1, its knockout cells show defects on chromosome condensation in metaphase, interfering in mitotic progression and chromosome segregation (Boily et al., 2008; Bordone et al., 2006; McBurney et al., 2003a; McBurney et al., 2003b); as well as general heterochromatin deregulation (Bosch-Presegue et al., 2011). All in all, this shows how SIRT1 participates in the regulation of chromatin structure and organization in order to maintain genome stability.

In addition, SIRT1 also functions maintaining the genome integrity by regulating DNA damage signaling, DNA repair or even cell cycle arrest, through chromatin remodeling and other non-histone proteins. SIRT1 has been described to deacetylate H3K56Ac, which is associated with DNA damage signaling during S-phase (Yuan et al., 2009); nevertheless, SIRT1 role in damage response (DDR) goes beyond single histone and non-histone substrates; indeed, the involvement of SIRT1 in cell cycle checkpoints and DNA damage repair is the result of a complicated network that interconnects several SIRT1 substrates. Some of these substrates are NBS1 of the MRE11-RAD50-NBS1 (MRN) nuclease complex that activates the ATM-dependent DNA repair pathway (Yuan & Seto, 2007); the DNA repair factor Ku (Ku70 in mammals), which is involved in the NHEJ (non-homologous end joining) (Jeong et al., 2007; Thrower & Bloom, 2001); p53, the essential transcription factor involved in cell cycle arrest and apoptosis upon DNA damage (Cheng et al., 2003; Peck et al., 2010); and XPA, which is implicated in positioning the repair machinery around the injury in NER (nucleotide excision repair) lesions(Fan & Luo, 2010). But importantly, these substrates and pathways are in turn connected together with other SIRT1 substrates in order to equilibrate a complex reciprocal network. For instance, in order to orchestrate the DNA repair process and avoid cell death, SIRT1 negatively regulates the activity of both, HATs, Tip60 and MOF, which are involved in DDR together with the MRN complex, H4K16Ac and p53. Tip60 and MOF deacetylation by SIRT1 seems to be a transient process that may inhibit the apoptosis activation by p53 after their first DDR (Peng et al, 2012). In addition, SIRT1 deacetylates p53 in order to promote cell survival by reducing its activity as a pro-apoptotic transcription factor (Cheng et al., 2003; Peck et al., 2010)). Moreover, in order to be more accurate with the DDR regulation, SIRT1 subsequently deacetylases and regulates p300, which in turn acetylates XPA, p53, NBS1, and itself (Fan & Luo, 2010; Kasper et al., 2011; Yuan & Seto, 2007).

Altogether indicate the great implication of SIRT1 in maintaining genome integrity throughout chromatin remodeling and DNA repair. Indeed, these evidences strongly support the role of SIRT1 as a cytoprotector under stress conditions. According to that role, moderate stress conditions as well as caloric restriction increase SIRT1 expression and enhance its activity in

order to promote cell survival. SIRT1 inhibits apoptosis and favors cell survival pathways by activating FoxO transcription factors (Motta et al., 2004; Yang et al., 2005), the heat shock response regulator HSF1 (Westerheide et al., 2009), the RecQ DNA helicase member WRN (Digweed & Sperling, 2004) and the already mentioned Ku70 and its ability to sequester the pro-apoptotic BAX protein (Cohen et al., 2004); and by inhibiting the p53 family member p73 (Dai JM et al, 2007). However, in some studies, SIRT1 has been also described to participate in pro-apoptotic pathways under severe stress. For that reason SIRT1 deacetylates and inhibit ROS detoxification enzymes through Nrf2 (Kawai et al., 2011), the transcriptional activity of NF-κB by its RelA/p65 subunit (Rothgiesser, Fey & Hottiger, 2010b); and also activates cytosolic p53 implying a mitochondria-dependent response (Daitoku et al., 2004; Moll et al., 2005; van der Horst et al., 2004), and FoxO transcription factors (Daitoku et al., 2004; van der Horst et al., 2004). Therefore, SIRT1 function as a cytoprotector or a pro-apoptotic sirtuin according to the kind of stress and its intensity. Moreover, the effect of some of its substrates such as p53 and FoxO are controversial if we consider the different publications; however, both proteins are highly conserved through evolution, mainly FoxO family members whose acetylated lysine residues have been also found in yeast (Hcm1) and C. elegans (Daf-16) orthologs (Chiang et al., 2012; Daitoku et al., 2004); so its regulation by SIRT1 might differ according to different stimuli and other not-yet identified posttranslational modifications.

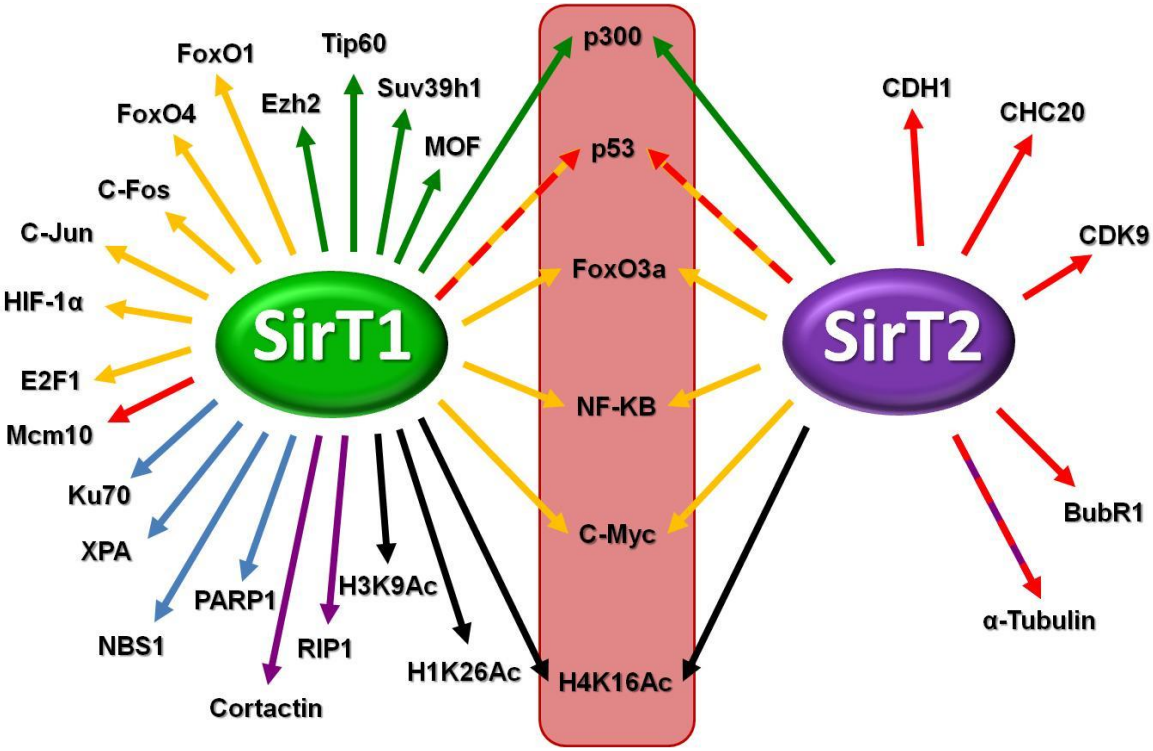


Figure 7. SIRT2 and SIRT1 histone and non histone substrates. The figure include substrates of both sirtuins and highlight the common substrates (red-pink box). The colour of the arrows grouped the type and function of the substrates. Green: Chromatin related substrates; Yelow: stress related susbtrates; Blue: DNA repair substrates; Red: proteins involved in cell cycle progression; and Purple: substrates involved in non-related functions.

The effort of SIRT1 to avoid genome instability and modulate the different cellular processes includes an important role in regulating S-phase progression. Its role as an important modulator of G1/S checkpoint is patent according to its effect on p53 and Rb (retinoblastoma); nevertheless, some of the newest data includes an important role in DNA and histone replication. Both replications are tightly regulated and coupled processes in order to maintain genome integrity. The inhibiting role of SIRT1 in both processes seems to be required for the maintenance of a proper S-phase progression; indeed, yeast Sir2p has been also involved in the control of replication origins (Fox & Weinreich, 2008; Pappas, Frisch & Weinreich, 2004; Zappulla, Sternglanz & Leatherwood, 2002). SIRT1 has been described to repress the transcription of histone genes when cells progress into late S-phase, correlating with increasing NAD^+ levels (He et al., 2011). Moreover, the positive transcription of those genes is regulated by other SIRT1 substrates, p300 and Tip60. This may indicate that the control of this process by SIRT1 is coordinated by the deacetylation of histone and non-histone substrates (He et al., 2011). The regulation of DNA replication is due to the deacetylation of other p300 substrate involved in promoting DNA replication initiation, Mcm10. SIRT1 seems to be essential in Mcm10 DNA binding capacity and degradation. Therefore, Mcm10 deacetylation has been described to reduce the amount of protein bound to the chromatin and control the S-phase moment when it should happen (Fatoba et al., 2013). By these two processes, SIRT1 may control the S-phase progression avoiding re-replication processes, favoring a moderate replication velocity, and inhibiting energy wasting. Accordingly, SIRT1 depletion increases replication velocity without decreasing S-phase duration (Fatoba et al., 2013) and SIRT1 KO in mice results in accumulation of cells in early mitosis (Wang et al., 2008), what may suggest also a checkpoint arrest due to the genomic instability accumulated during S-phase. The inhibition of DNA and histone replication upon stress conditions supports the role of SIRT1 in the negative regulation of these processes.

All this data demonstrated the role of SIRT1 in genome stability and cellular integrity maintenance through a complex network. SIRT1 coordinates the heterochromatin formation, the proper DNA damage response and the cell cycle progression; although its decisions and pathways depend on the stimuli and the cell type, among other factors.

3.2. SIRT2

SIRT2 is the only cytoplasmic sirtuin (Afshar & Murnane, 1999; Perrod et al., 2001) whose structure consists of a 304aa catalytic core and a 19-aa -residue in the N-terminal tail. Its catalytic core contains two domains: a larger domain that is a variant of the Rossmann fold, present in many diverse NAD(H)/NADP(H) binding enzymes; and a smaller domain that contains a structural zinc atom. At the interface of both domains, there is a large groove that includes the NAD^+ binding site and contains residues conserved across the Sir2 family (Finnin,

Donigian & Pavletich, 2001). Additionaly, North and Verdin (North & Verdin, 2007a) also identified a nuclear export signal (NES) between amino acids 41 and 51 of SIRT2 that was consistent with the canonical sequence of Crm1-dependent NES. Their findings explained how SIRT2 has been also localized in the nucleus as well as pointed out some possible nuclear roles (Figure 7) (North & Verdin, 2007a; Vaquero et al., 2006). Indeed, the same authors (North & Verdin, 2007b) identified CDK1/Cyclin B1, an important CDK/Cyclin complex expressed and activated during G2-M phases, as one of the kinases responsible for SIRT2 phosphorylation. Therefore, SIRT2 phosphorylation, also identified for other several residues (Dryden et al, 2003; North & Verdin, 2007) may be implicated in SIRT2 shuttling from the cytoplasm to the nucleus during G_2/M. Moreover, it seems that a proper balance of phosphorylated form of SIRT2 is crucial for the mitosis. For instance, its dephosphorylation by CDC14 at the end of mitosis has been described as a pre-requisite for mitotic exit. Consequently, overexpression of SIRT2 and also its phosphorylated form delays cell cycle progression throughout mitosis (Dryden et al., 2003; North & Verdin, 2007b). This fact clearly demonstrates an important role of SIRT2 in mitotic progression.

Accordingly, the role of SIRT2 in mitosis has been attributed to different substrates that are deacetylated by this enzyme. One of the first substrates characterized was α-Tubulin, which was identified as SIRT2 substrate in 2003 by Dr. Verdin's Lab. Although there is some data that does not recognize α-Tubulin as SIRT2 substrate (Bobrowska et al., 2012), the major part of the publications support that the acetylation level of this microtubular component is controlled by this sirtuin (Inoue et al., 2007b; Maxwell et al., 2011; Suzuki & Koike, 2007; Zhang et al., 2013). Despite the fact that HDAC6, another histone deacetylase, has also been described as α-Tubulin deacetylase (Hubbert et al., 2002), the first report of tubulin deacetylation by SIRT2 (North et al., 2003) has been reproduced by several research groups including a recent publication that demonstrated an NAD^+-dependent regulation of tubulin acetylation by SIRT2 (Skoge, Dolle & Ziegler, 2014). How this role of SIRT2 could be involved in mitosis responds to the microtubules dynamic during cell cycle. The acetylation/deacetylation pattern of the tubulin in the microtubules is associated with its polymeryzation/depolymerization process (Suzuki & Koike, 2007). Notwithstanding, one of the main SIRT2 substrates that reinforce its role in mitotic regulation is H4K16Ac. This function is highly conserved from the yeast Hst2p, which shows a very strong preference for H4K16Ac (Vaquero et al., 2006). The decrease of this histone acetylation occuring during mitosis was linked to its capacity of inhibiting the folding of higher orders of the chromatin fiber *in vitro (*Shogren-Knaak et al., 2006), being an obstacle for the interaction between the H4 tail of one mononucleosome and the H2A/H2B dimer of an adjacent mononucleosome (Robinson et al., 2008; Shogren-Knaak et al., 2006). Therefore, the entrance of SIRT2 to the nucleus in G2/M and the subsequent deacetylation of H4K16Ac is essential for the formation of metaphase chromosome structure what can affect the genome stability.

The involvement of SIRT2 in the control of cell cycle progression extends further by the regulation of signalling pathways leading to either cell cycle arrest and parallel DNA repair, or permanent cell cycle arrest and cell death. SIRT2 has been described in the regulation of few

mitotic checkpoints, such as the antephase (Inoue et al., 2007a) or the spindle checkpoints (Kim et al., 2011; North et al., 2014). The role of SIRT2 in controlling the entrance into mitosis appeared from its possible participation in the antephase checkpoint regulated by CHFR and p38 (Inoue et al, 2007); indeed, SIRT2 has also been related to p38 in controlling p53 accumulation (Li et al, 2010). In addition, one of the most important checkpoint regulators is the p53 tumor suppressor protein, which initiates expression of those genes that ultimately govern cell cycle arrest, DNA damage repair or apoptosis, controlling G_1/S and G_2/M transition. SIRT2 may be also involved in the G_2/M checkpoint by deacetylating p53 and thereby promoting cell survival (Jin et al., 2008; Peck et al., 2010). This function, in fact, seems to be conserved throughout evolution. In *C. elegans*, SIR-2.1, a SIRT2 ortholog, was shown to interact with PAR-5/FTT-2, a 14-3-3 homolog, promoting p53 accesibility and subsequently increasing the longevity (Wang et al., 2006b). As it happens with SIRT1, SIRT2-dependent p53 deacetylation seems to be necessary to decrease p53 activity under moderate stress conditions, in order to enhance cell survival (Peck et al., 2010). However, it is important to consider the different cellular localizations of both sirtuins. Even considering their respective shuttling between nucleus and cytoplasm (North & Verdin, 2007a; Tanno et al., 2007), their p53 regulation may be compromised with this situation, intriguing complementary functions according to different cell cycle stages, cell type or stimuli.

Notwithstanding, SIRT2 has also been related to the control of mitotic exit . The most recent study has been published this year by Dr. Sinclair's lab (North et al., 2014). This study tried to explain the SIRT2-dependent regulation of BubR1 in the aging process; nevertheless, in addition to its role in aging, BubR1 is a well-characterized component of the spindle checkpoint, whose inactivation and degradation is needed for mitotic exit (Elowe, 2011). According to their conclusions, SIRT2 deacetylase BubR1 inhibiting its degradation (North et al., 2014), which would in turn favor cell cycle arrest. This new data supports the role of SIRT2 as a tumor suppressor that participates in the anaphase-promoting complex/cyclosome (APC/C). However, Kim et al 2011 (Kim et al., 2011), which also corroborates the involvement of SIRT2 in the spindle checkpoint, underlined the role of SIRT2 in favoring the APC/C-mediated ubiquitination by the deacetylation of other two proteins involved in the spindle checkpoint, CDH1 and CDC20. These findings (Kim et al., 2011) contradict other studies and do not correlate with the role of SIRT2 as a tumor suppressor in mice, demonstrated by the same authors. Therefore, additional studies are needed to clarify the role of SIRT2 in the control of mitotic progression.

Beyond its function as a cell cycle regulator during mitosis, SIRT2 also participate in the regulation of the stress response. Accordingly, SIRT2 has been described as part of the replication stress response (RSR). RSR consists of a subset of DNA damage response (DDR) signaling networks, which recognize challenges to DNA replication and mobilize diverse DNA repair and cell cycle checkpoint pathways. SIRT2 seems to be involved in an ATR-dependent CDK9 deacetylation in order to promote its kinase activity, which is required for its functions in the RSR (Zhang et al., 2013). In addition, SIRT2 has also been shown to regulate different transcriptional factors upon stressful conditions. For instance, SIRT2 has been described to

deacetylase FoxO factors during differentiation, response to oxidative stress, and caloric restriction (Jing, Gesta & Kahn, 2007; Liu et al., 2012; Wang et al., 2007; Wang & Tong, 2009). However, its consequences are controversial. On one hand, FoxO3a deacetylation by SIRT2 can mediate FoxO3 ubiquitination and degradation enhancing life-span and tumorogenesis (Wang et al., 2012), but on the other it can also promote FoxO target genes expression enhancing apoptosis events in response to stress and damage (Liu et al., 2012; Wang et al., 2007). Moreover, SIRT2 also participates in the regulation of NF-κB, another transcription factor that controls important cellular processes, such as cell cycle, angiogenesis, adhesion and apoptosis (Karin & Lin, 2002; Mayo & Baldwin, 2000). Indeed, its p65 subunit was reported to be deacetylated by SIRT2 in the cytoplasm (Rothgiesser et al., 2010a). Notwithstanding, the role of SIRT2 in the stress response includes some histone substrates, and compromises processes such as DNA repair and transcription regulation. Within its histone substrates, SIRT2 has been shown to deacetylate H3K56Ac in order to favor DNA damage repair during S-phase (Vempati et al., 2010; Yuan et al., 2009); and it has been recently described as a H3K18Ac deacetylase in response to *Lysteria monocytogenes* infection, mainly favoring gene repression and cell survival (Eskandarian et al., 2013).

Complementary to its role in regulating several effectors by deacetylation, SIRT2 subsequentely regulates the activity of p300, an important HAT involved in H3K9Ac, H3K27Ac, H3K36Ac, H3K37Ac and H3K56Ac; and which acts as a transcriptional coactivator required for a wide range of important cellular processes which in turn are regulated by other SIRT2 substrates, such as FoxO factors, p53, and NF-KB (Das et al., 2009; Hassa et al., 2003; Liu et al., 1999; Szerlong et al., 2010). As SIRT1, SIRT2 deacetylases p300 (Black et al., 2008; Jiang et al., 2011); thus, sirtuin family members regulate both p300 histone and non-histone substrates, and p300 enzymatic activity, through deacetylation.

All the above findings point out the role of SIRT2 as a regulator of a network of proteins involved in genome stability maintenance and cell survival. SIRT2 participates in the maintenance of a proper chromatin structure, normal chromosome segregation, and stable cell cycle progression; but under stress conditions SIRT2 favors different molecular pathways that depend on the stimuli, cell cycle stage and intensity, among others factors, to preserve genome integrity and cell identity.

4. Interplay between H4K16 and H4K20

One important aspect that appeared within the histone code theory was the idea that modifications on the same or different histone tail may be interdependent. Therefore the modification in one residue can determine that of another either in cis or, more surprisingly, in trans. For instance, in the first case, it has been shown that methylation of H3K4 blocks both the binding of the remodeling deacetylation complex NURD and the methylation of H3K9

(Zegerman et al., 2002). As an example of trans effect, we can mention the ubiquitination of H2B in lysine 123, which is required for a subsequent and efficient methylation of H3K4 (Sun & Allis, 2002).

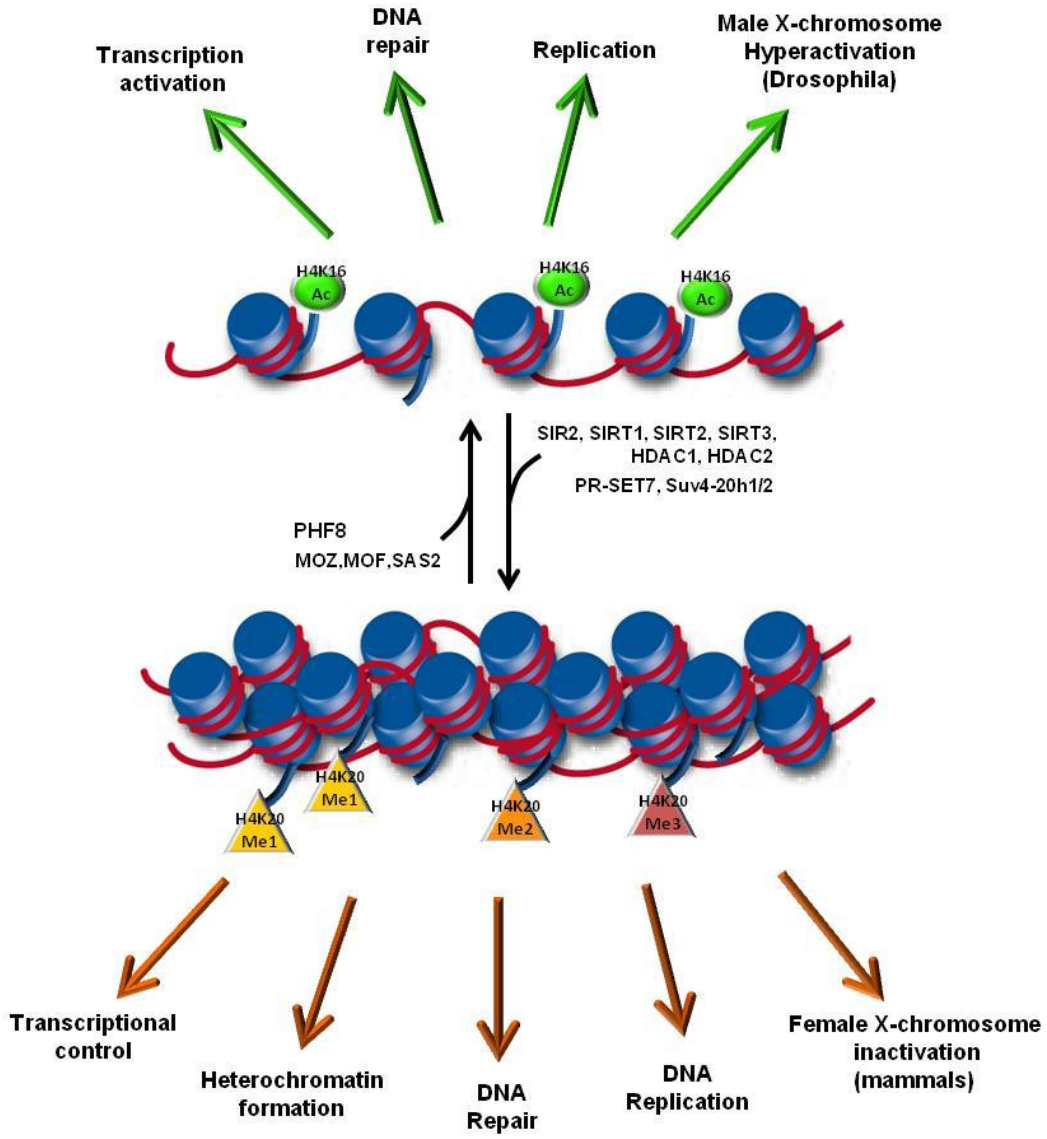


Figure 8. H4K16Acversus H4K20me1. The acetylation status of lysine 16 in histone H4 (represented as a green circle) performes critical roles favoring transcription and open chromatin state. Whereas methylation of K20 (represented by triangles) is involved in such an important roles through some opposite fuctions.

In the case of the histone H4, the acetylation of the lysine 16 has been clearly demonstrated to inhibit higher orders of chromatin condensation (Robinson et al., 2008; Shogren-Knaak et al., 2006); whereas the monomethylation of an adjacent residue, the lysine 20, was described as an important mark for metaphase chromosomes formation (Oda et al., 2009; Rice et al., 2002). Interestingly, both residues have been described to have opposite roles in X chromosome compensation. In one hand, acetylation of lysine 16 served in the

transcriptionally hyperactive male X-chromosome in *Drosophila* (Turner, Birley & Lavender, 1992)and on the other hand, the monomethylation of H4K20 participates in the female X chromosome inactivation in mammals (Schotta et al., 2008). Considering these facts, it would be obvious to hypothesize that as both histone marks present opposite functions, both should be mutually exclusive. Indeed, that idea was proposed in 2002 by Dr. Reinberg's lab (Nishioka et al, 2002), while studying H4K16ac levels in Drosophila X chromosome. Their data suggested a negative interplay between monomethylation of H4K20 and acetylation of H4K16 (Figure 8). In addition, both histone modification levels inversely correlate during cell cycle progression in human cells (Figure 9) (Rice et al., 2002; Vaquero et al., 2006).

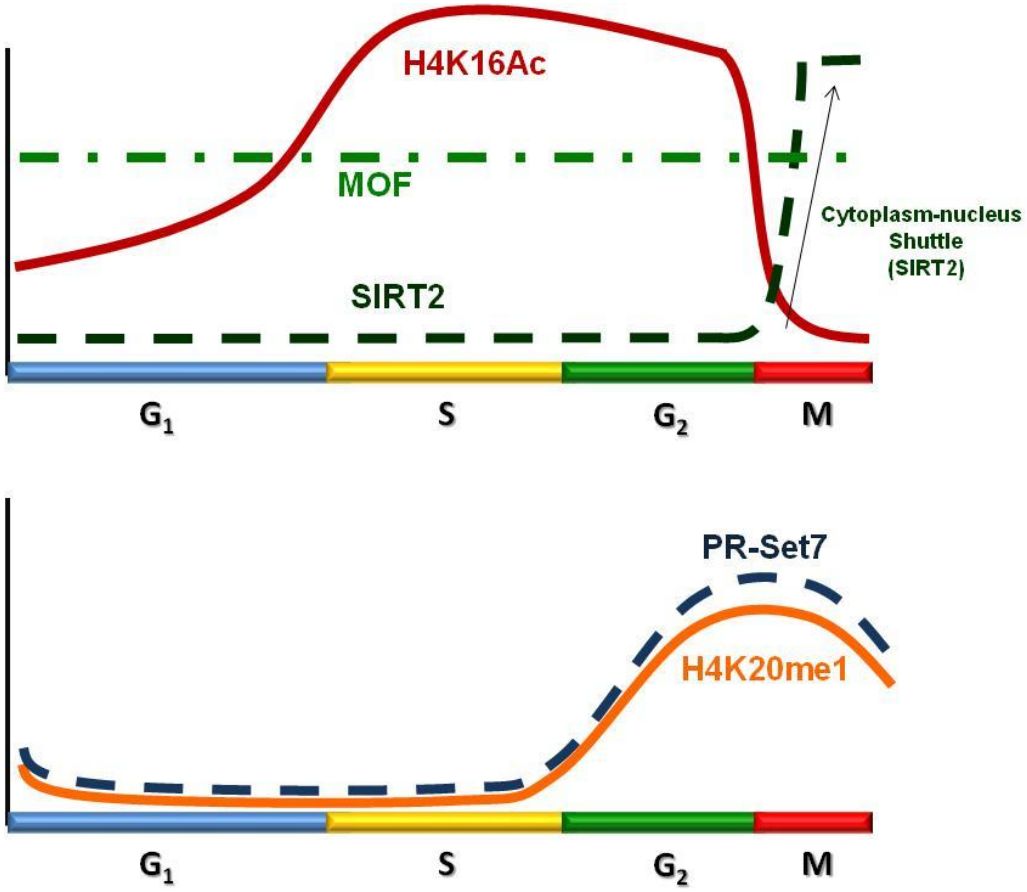


Figure 9. Schematic representation of H4K16Ac and H4K20me1 levels and deposition during cell cycle. The scheme includes two graphs with the levels of these histone modifications during the cell cycle, which are represented by red and orange lines (H4K16Ac and H4K20me1, respectively). The expression pattern of their respective enzymes are included as pointed green and blue lines (Light green: MOF; dark green: SIRT2; blue: PR-Set7). On the left side of the graphs are depicted the enzymatic reactions controlled by the enzymes.

Different features of H4K20me (H4K20me1/2/3) and H4K16Ac support this possible exclusion between both histone marks. For instance, H4K16ac and H4K20me have been shown to inversely regulate RNA polymerase II activity (Braunstein et al., 1993; Imai et al., 2000;

Kapoor-Vazirani et al., 2011). The H4K16Ac role in gene expression has widely been studied, mainly in yeast, pointing out how this histone mark enhances transcription. In mammalian cells H4K16Ac seems to be enriched around the transcriptionally active sites (Taylor et al., 2013). Accordingly, in yeast, work from many laboratories has identified the Sir2p histone deacetylases as the core components of silent chromatin at telomeres and silent mating type loci, together with Sir3 and Sir4 (Rusche, Kirchmaier & Rine, 2003). Sir2p of *S. cerevisiae* is absolutely required for transcriptional silencing in this organism, due to its histone NAD^+-dependent deacetylase activity on H4K16Ac, which limits access of RNA Pol II to the transcription sites (Braunstein et al., 1993; Gottlieb & Esposito, 1989; Imai et al., 2000; Tanny et al., 2004). On the other hand, methylated H4K20 has been found at transcriptionally inactive regions, such as mouse major and minor satellite repeats, and DNA tranposons, when using MEFs (Martens et al, 2005; Karachentsev D et al, 2005). Indeed, *Drosophila* studies demonstrated trimethylation of H4K20 in pericentric heterochromatin (Schotta et al., 2004). Moreover, mechanistic studies also support H4K20me as a repressive mark. First, H4K20me3 seems to block hMOF recruitment and acetylation of H4K16 at transcription promoters (Kapoor-Vazirani et al., 2011); and second, L3MBTL1, a MBT domain protein which is known to negatively regulate the expression of a subset of genes, seems to bind to H4K20me1 (Trojer et al., 2007), probably mediating down-stream silencing functions of this histone mark. In agreement with these findings, the recruitment of the 53BP1 to the DNA damage sites is mediated by direct interaction between the 53BP1 tandem Tudor repeat and histone H4 methylated on lysine 20 (H4K20me) (Hartlerode et al., 2012; Pei et al., 2011; Sanders et al., 2004); and it is interferes by H4K16Ac (Hsiao & Mizzen, 2013; Tang et al., 2013) right after DSBs (Hsiao & Mizzen, 2013).

However, there is also experimental data that does not support interdependence between H4K16Ac and H4K20me. Some publications demonstrated that in mammals, when using T-cells or HeLa cells (cervical cancer cells), H4K20me1 is present in active gene promoters and transcribed regions (Barski et al., 2007; Vakoc et al., 2006), and even coexists with H4K16Ac in MEL (line F4N, mouse erythroleukemia cell line) cells (Talasz et al., 2005). In addition, other experiments with *Drosophila* also found that the KMT Ash1 (ASH1L in humans), activates transcription by methylation of H3K4, H3K9 and H4K20 at the promoter of target genes (Beisel et al., 2002). Therefore, these findings positively related H4K20me1 with transcription, as happens with H4K16Ac. However, it is important to notice that only in one occasion (Talasz et al., 2005) the levels of H4K16Ac were also studied. It is also important to consider that loss of H4K16Ac as well as H4K20me3 is a hallmark in cancer cells compared to normal cells (Fraga et al., 2005). With respect to DNA repair, the acetylation of H4K16 has been associated with DDR by different HATs such as MOF and Tip60. Indeed, the abrogation of either of both enzymes abrogates damage-induced γ-H2AX (Kusch et al., 2004; Li et al., 2010; Sharma et al., 2010). Therefore, this may indicate coexistence between H4K20me and H4K16Ac at H4 tail during the DDR process, unless the two modifications may appear/disappear in a sequential progression. Interestingly, HDAC1 and HDAC2 localize to sites of DNA damage promoting deacetylatio of

H4K16Ac (Miller et al., 2010); and H4 cetylation by Tip60 has been described as a requirement for γ-H2AX dephosphorylation (Jha, Shibata & Dutta, 2008).

4.1. H4K20 methyltransferases: PR-Set7 and Suv4-20h

PR-Set7 protein levels oscillate during the cell cycle. Different studies showed how this enzyme is more abundant between late G_2 and early M phase, where it associates with mitotic chromosomes to create H4K20me1 marks (Oda et al., 2009; Rice et al., 2002). The protein levels decline as cells progress through G_1, being almost undetectable in S-phase. According to the enzyme, H4K20me1 levels peak during mitosis, and the decrease of this histone mark after mitosis may be due to two different processes: first, to the deposition of H4K20me2 and H4K20me3 using the monomethylation as substrate by the Su4-20h enzymes (Yang & Mizzen, 2009) (Figure 10); and second, due to the demethylation by PHF8 that occurs mainly at promoters and whose binding to chromatin peaks at G1/S border (Asensio-Juan, Gallego & Martinez-Balbas, 2012; Liu et al., 2010). Importantly, the levels of both Suv4-20h and PHF8 remain stable during the cell cycle, or at least there is not data demonstrating a protein level regulation pattern.

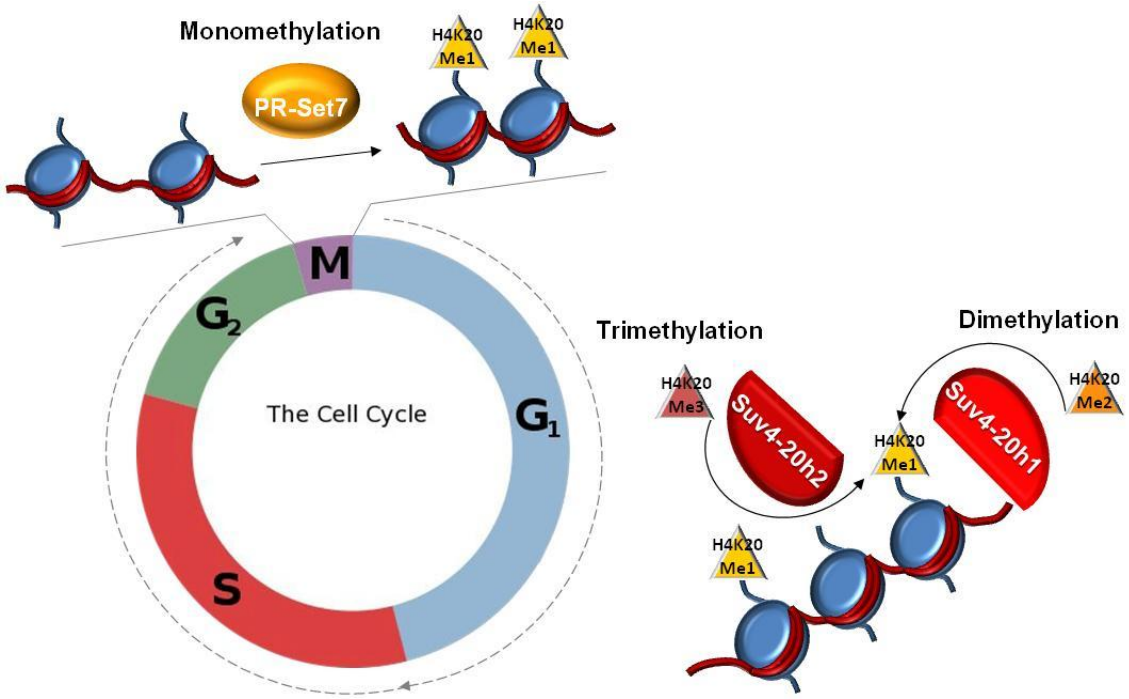


Figure 10. Representation H4K20 methylation dynamic during the cell cycle. PR-Set7 monomethylates H4K20 during mitosis and the Suv4-20h enzymes are in charge of di- and tri-methylate H4K20me1 during the whole cell cycle.

The oscillation of PR-Set7 protein levels (Figure 9) is primarily achieved by the concerted action of several ubiquitin E3 ligases, such as PCNA-coupled CRL4(Cdt2), APC/C (anaphase-promoting complex-cyclosome) complex and Skp2. This regulation is essential for both, the compaction of mitotic chromosomes and for the control of replication origins (Abbas et al., 2010; Jorgensen et al., 2007; Oda et al., 2010; Tardat et al., 2010). The degradation mechanism that has been clearly related to PR-Set7 downregulation levels right at the exit of mitosis, is its regulation by the APC/C ubiquitin E3 complex. PR-Set7 is phosphorylated by CDK1-cyclinB1 at the beginning of mitosis, and dephosphorylated by CDC14 at the end of anaphase. That dephosphorylation is what leads to APC/C-dependent degradation at the end of mitosis. Indeed, constitutive PR-Set7 phosphorylation impedes mitotic progression (Wu et al, 2010), what indicates that this is a necessary event. Additionally, degradation by the PCNA-coupled CRL4(Cdt2) mechanism seems to be involved in maintaining low levels of PR-Set7 protein during S-phase, in order to regulate DNA replication. This mechanism depends on the two PIP domains of PR-Set7 protein, which confer binding to the replication factor PCNA (Huen et al., 2008; Jørgensen et al.,2007), which is involved in DNA synthesis during S phase. That binding to PCNA is what has been reported to strongly stimulate PR-Set7 ubiquitylation and its proteasome-dependent degradation (Abbas et al., 2010; Jorgensen et al., 2007; Oda et al., 2010; Tardat et al., 2010). Additionaly, this degradation mechanism has been related to PR-Set7 protein degradation under stress conditions, which means that PCNA serves as a cofactor to promote PR-Set7 degradation in response to DNA damage (Oda, H. 2010). Furthermore, several groups have also reported that other ubiquitin E3 ligase, CRL1Skp2, also serves as a minor mechanism for fine-tuning PR-Set7 protein levels during the G_1/S transition, by targeting PR-Set7 outside the chromatin context (Brustel et al., 2011; Oda et al., 2010).

The accurate regulation of PR-Set7 during cell cycle clearly indicates the importance of this enzyme in controlling cycle progression. Indeed, the loss of PR-Set7 drastically affects cell cycle S-phase and mitosis progression. PR-Set7 knockout mouse embryos are not viable, displaying improper S- to mitosis progression and failing to develop beyond the four-cell stage (Oda et al., 2009). In Drosophila the absence of the PR-Set7 ortholog causes a reduction in both S-phase and mitotic indices in rapidly dividing larval tissues (Karachentsev et al., 2005; Sakaguchi & Steward, 2007); and accordingly, PR-Set7 deficiency in human cells using siRNA display improper S-phase entry and progression, as well as G_2 arrest (Jorgensen et al., 2007; Tardat et al., 2007). In addition, Suv4-20h doble-knockout (DN) mice, also show S-phase defects. Suv4-20h-dn mice are perinatally lethal and have lost nearly all H4K20me3 and H4K20me2 sites (Schotta et al., 2008). Detailed analysis of cell cycle stages revealed a reduction of S-phase cells with a concomitant increase of G_1-phase cells, indicating a partial block in G_1/S transition. A deeper analysis of the S-phase showed a significant delay for Suv4-20h-DN cells in S-phase entry as compared with wild-type cells (Schotta et al, 2008).

PR-Set7 activity during mitosis is essential for the proper condensation of metaphase chromosomes (Nishioka et al., 2002; Oda et al., 2009), in order to ensure the correct chromatin segregation; therefore, PR-Set7 absence caused chromosome decondensation and thus,

promote G_2 arrest (Oda et al, 2009). Additionally, PR-Set7 activity during mitosis is also indispensable for establishing of H4K20me1 at the replication origins that will be used in the following cell cycle (Tardat et al., 2010). As a matter of fact, in replication origins H4K20me1 is subsequently used by Suv4-20h enzymes to form H4K20me2/3 binding sites for the pre-RC complex (Beck et al., 2012; Shen et al., 2010). Therefore, the degradation of PR-Set7 by PCNA during S-phase assures that only one DNA-replication event takes during a cell cycle. For that reason, expression of a PR-Set7 mutant insensitive to this degradation causes the re-deposition of H4K20me1 and repeated DNA replication at origins in non specific sites (Tardat et al, 2010).

All things considered, the G_2 arrest after PR-Set7 deletion was caused by both, the decrease of H4K20me1 levels, as well as by the replicative stress caused during the preceding S-phase (Jorgensen et al., 2007; Tardat et al., 2007). This statement is supported by the studies of Jorgensen et al 2007, where the DNA damage enhanced by PR-Set7 loss is decreased by DNA-replication blocking. In other words, PR-Set7 activity regulates mitotic chromatin events that are also important for proper chromatin condensation in mitosis and DNA replication during S-phase. These events are also related with the deposition of H4K20me2 and H4K20me3 by their respective enzymes Suv4-20h1 and Suv4-20h2 using H4K20me1 as substrate; indeed, di- and tri- methylation levels of these two residues where severely reduced in PR-Set7 knockout mice (Oda et al, 2009).

In addition to the establishment of DNA replication origins by PR-Set7 together with Suv4-20h enzymes, H4K20me1 deposition also participates in heterochromatin formation by Suv4-20h2-H4K20me3 (Gonzalo et al., 2005; Schotta et al., 2004). Indeed, Suv4-20h2 is recruited by HP1 to H3K9me3 sites in order to favor heterochromatin formation by H4K20 trimethylation from H4K20me1 sites (Schotta et al, 2004); and in turn Suv4-20h2 recruits cohesin to favors pericentric heterochromatin structure (Hahn et al., 2013). For that reason, Suv4-20h deficiency results in telomere defects by derepressing recombination events (Benetti et al., 2007), as well as favors reduced levels of heterochromatin-associated cohesion (Hahn et al, 2013), which in turn contributes to the genomic instability. As a result, the mitotic H4K20me1 deposition is not only important for S-phase regulation by Suv4-20h, but also for Suv4-20h2-dependent genome integrity maintenance.

The loss of Suv4-20h2 and H4K20me3 has been identified in the context of cancer in several cancer models (Fraga et al., 2005). Furthermore, H4K20me3 levels were measured during the progression of skin cancer in a mouse model, and were shown to decrease steadily as the cells transitioned from normal to cancerous (Fraga et al., 2005). In bladder cancer, H4K20me3 levels were shown to decrease with increasing tumor grade (Schneider et al., 2011). Indeed, dysregulation of pericentric heterochromatin has been suggested to play important roles in cancer development and progression (Hahn, Dambacher & Schotta, 2010; Zhu et al., 2011); and cancer cells are frequently characterized by genomic instability and cohesion defects (Thompson, Bakhoum & Compton, 2010). However, it is unclear whether the relationship between low H4K20me3/Suv4-20h2 and tumor cells is causative or simply correlative.

To sum up, PR-Set7 activity during mitosis, together with Suv4-20h1 and Suv4-20h2 enzymes participate in the maintenance of genome stability during the cell cycle, and in the regulation of S-phase and mitosis progression.

5. CELL CYCLE

Cell cycle results in the duplication of each chromosome to form closely adjacent sister chromatids, which separate from each other to become daughter chromosomes. The molecular mechanisms underlying the cell cycle are highly conserved in all organisms with nucleated cells. Many of the genes and proteins involved in the mitotic cell cycle have been identified because of their high degree of similarity to homologous genes and proteins in the more extensively studied and understood organism, Saccharomyces cerevisiae.

All phases of the cell cycle are marked by an orderly progression of processes and its progression is regulated at several checkpoints which are necessary to prevent cells with damaged or missing chromosomes from proliferating.

5.1. Chromatin regulation

The structure of the chromatin differs during the different stages of the cell cycle, depending on the DNA amount and its function (Figure 11). During the G1-phase, cells are just beginning to synthesize proteins necessary for DNA replication, and thus the DNA at this stage is less compacted. During the S-phase there is replication of DNA without much alteration in the chromatin structure apart from the replication forks. However, not only is the DNA replicated, but the structure of chromatin as well, as a requirement to ensure that epigenetic information is also passed on to the daughter cells. Then the chromatin begins condensing during the G2-phase. The chromatin condensation increases as it nears Mitosis. Further condensation of the chromatin occurs during mitosis and the chromosomes are complete condensed at metaphase. The condensed chromosomes split into two equal parts at their centromere/kinetochore at anaphase and no major morphological changes occur during this phase in the chromosome structure. Chromosomes slowly revert to the uncondensed state at telophase as the cell completes its division.

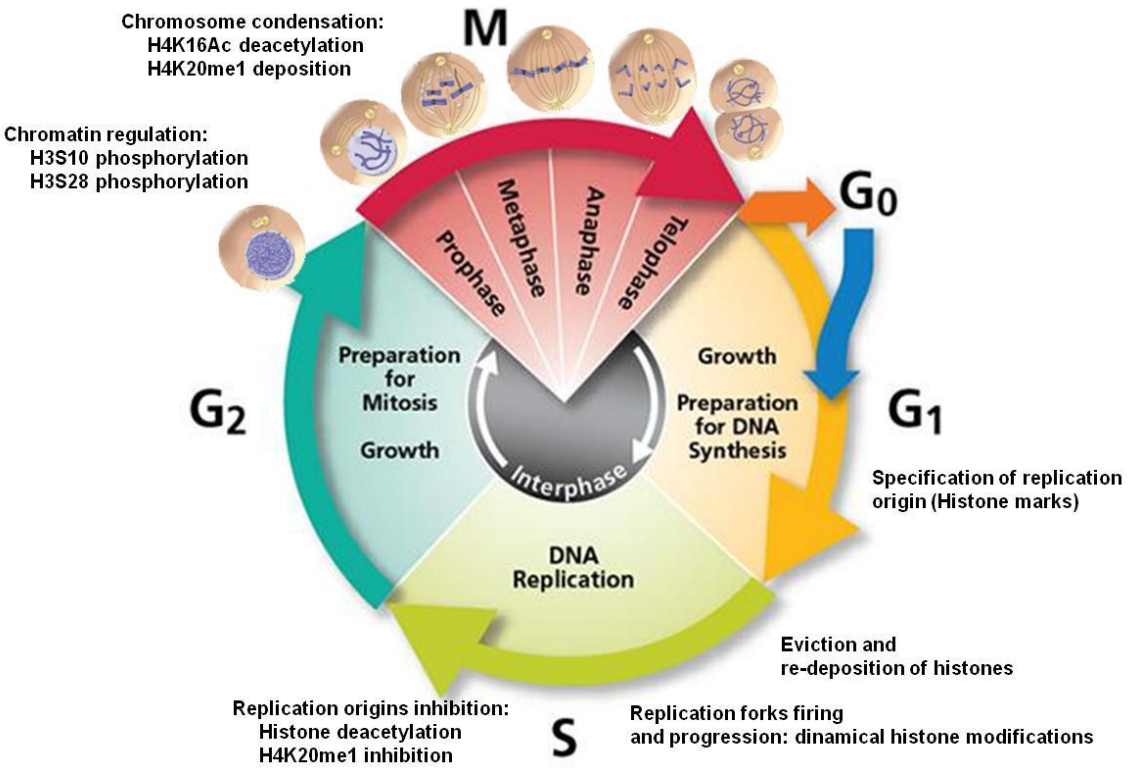


Figure 11. Interplay between chromatin structure and cell cycle regulation. All steps of cell cycle progression from the initiation of DNA replication to mitosis depend on chromatin modifications. Indeed, the deposition of several histone marks governs replication timing of each region of the genome. At the G_1/S transition, histone modifications are also required for the specification and activation of replication origins. During S phase, the chromatin structure has to be loosened to allow fork progression, and to be reconstructed behind the fork. This implies nucleosome dynamics as well as reproduction of pre-existing chromatin marks. During the Mitosis, chromosome formation is mediated by histone modifications, mainly phosphorylation, deacetylation and methylation.

5.1.1. S-phase: replication

One major feat is the duplication of the DNA, where the template for the replication machinery is chromatin. This is a highly regulated process requiring precise control. Eukaryotic cells have evolved molecular mechanisms that ensure a close coordination between the machineries involved in DNA replication and those effecting different levels of chromatin assembly. Conceptually, both histone modifications as well as nucleosome remodeling may aid in replication progression, facilitating the partial disassembly of chromatin before progress of the replication fork. In all eukaryotes, DNA replication begins at precise positions of the genome, which are termed as replication origins. The eukaryotic DNA is replicated from multiple sites, which are defined by the binding of the origin recognition complex (ORC).

It is during late G_1 when the pre-replication complex (pre-RC) starts to assemble. CDT1 and CDC6 are recruited to ORCs, allowing the subsequent loading of mini-chromosome maintenance (MCM) proteins (MCM2-7), which are considered as DNA helicases. Subsequent association of Cdc45, Mcm10 and GINS complex (consisting of Psf1,Psf2, Psf3 and Sld5) form the CMG complex (Cdc45-MCM-GINS). This complex creates the replication bubble and recruits DNA polymerases in order to form the replisome. Mcm10 plays an important role both in replication initiation and elongation. It interacts with the ORC components and binds to and recruits the DNA polymerase α-primase complex to replication origins (Ravi, Nivedita & Pai, 2013; Raynaud et al., 2014).

Chromatin modifications such as histone modifications and chromatin remodeling play an important role throughout the S-phase, because both loading of the pre-RC and progression of the replication fork require local loosening of chromatin structure. At the same time, chromatin marks play critical roles in the positioning of replication origins and the timing of replication (Ravi et al., 2013; Raynaud et al., 2014). Indeed, treatment of HeLa cells with TSA, leads to a more dispersive pattern of initiation site selection as well as an earlier activation of the late-firing β-globin origin (Kemp et al., 2005). Additionally, Sir2p inhibits activation of some origins, but not others (Pappas et al., 2004), by promoting an unfavorable chromatin structure for pre-RC assembly (Crampton et al., 2008). Consistent with this idea, it is likely that multiple histone modifications collaborate to specify the replication program. This hypothesis is supported by evidence demonstrating that a high number of chromatin modifying enzymes associate with the DNA polymerase processivity factor PCNA (Moldovan, Pfander & Jentsch, 2007). However, the chromatin events regulating the initiation of DNA replication are not well understood.

A great number of histone modifications have been reported to be accumulated at metazoan or yeast origins (Dorn & Cook, 2011), distinguishing between early- and late-firing origins. Different studies show how early-firing origins present open chromatin marks while late-firing origins have a compact chromatin structure during S-phase, which opens only transiently during their activation, reverting rapidly to its initial status. Therefore, early-firing origins have been described to present high levels of H3K9Ac, H3K14Ac, H4K16Ac, H3K18Ac, H3K27Ac and H3K4me3 and low levels of the silencing H3K9me3 mark (Eaton et al., 2011; Rampakakis et al., 2009). On the other hand, late-firing origins are detectable by a low level of H3K9Ac, H3K14Ac and H3K4me3, but increased H3K9me3. Other histone marks have been also related with replication origins, being involved in ORC components recruitment. That is the case of H4K20me2/3, as well as linker histone H1. The relationship of H4K20me and replication has been widely studied. At the beginning it was H4K20me1 the important histone mark whose deposition on mitosis would determine the S-phase progression (Jorgensen et al., 2007; Tardat et al., 2007), but subsequent studies demonstrate that H4K20me2/3 are the real histone marks that serves as binding site for ORCs components (Beck et al., 2012; Kuo et al., 2012). In any case, $CRL4^{Cdt2}$ –dependent PR-SET7 degradation prevents from re-replication events, as well as from premature condensation (Centore et al., 2010; Schotta et al., 2008). Histone H1 has been related to late-firing origin, and while its dephosphorylated state blocks replication, its

phosphorylation stimulates late-origins to fire (Dorn & Cook, 2011; Rampakakis et al., 2009); moreover, this phosphorylation seems to be essential for loosening the chromatin structure during the replication fork progression (Alexandrow & Hamlin, 2005). Moreover, several histone acetylation have been related to chromatin accessibility during S-phase progression. For example, some plant's nuclei in S-phase display increased levels of H3K18Ac, H4K5Ac, H4K8Ac, H4K12Ac and H4K16Ac (Costas et al., 2011). This last histone mark in an important histone mark that, indeed, peaks in S-phase (Vaquero et al., 2006) leading to a more open chromatin state.

As a complement to the histone marks, chromatin remodeling machinery may help to strategically position nucleosomes close or far from the ORCs. Indeed, a higher density of nucleosomes reduces the binding of ORC as well as other replication factors. Different studies demonstrated how replication origins tend to be located in nucleosome-free regions and enriched in the histone variants H3.3 and H2A.Z (Leonard & Mechali, 2013). Interestingly, origins of replication in larger eukaryotes are often found close to transcriptional promoter regions, and regulatory elements for transcription (Ehrenhofer-Murray, 2004). Moreover, two chromatin remodeling complexes, WSTF-ISWI and ACF1-ISWI, have been implicated in heterochromatin replication. Both complexes are targeted to heterochromatic replication foci, and may facilitate the movement of the replication fork through heterochromatin domains (Ehrenhofer-Murray, 2004).

Regulation of the replication of histone preoteins during this cell cycle stage should be also considered. Early studies showed how histone promoters were enriched in transcription factors, histone modifiers and chromatin remodelers upon S-phase entry. This is the case of CBP, p300, Tip60, and RNA polymerase II (He et al., 2011). This transcriptional activation at histone promoters during late G1 and S-phase is required for the coordination between DNA replication and histone replication.

At the end of S-phase, chromatin transcriptional activation (for histone replication) and DNA replication should be repressed, in order to assure that replication is not initiated more than once. Due to the important role of histone acetylation in S-phase progression, their deacetylation might happen at the end of S-phase. Similar to yeast, the mammalian metabolic cycle implies the oscillation of NAD^+/NADH ratio during the cell cycle. In particular, this ratio is lower in S-phase, especially in G_1/S border and S-phase entrance; but when cells progress into late S-phase the NAD^+/NADH ratio increase (Yu et al., 2009). This metabolic pattern activates the sirtuins, which therefore may be able to decrease the acetylation level of the chromatin, promoting chromatin compaction (He et al., 2011). In addition, other HDACs are known to associate with chromatin during late S-phase, such as HDAC3, or HDAC1 and HDAC2 as part of Mi-2/NuRD (nucleosome-remodeling deacetylase complex) and/or the Sin3/HDAC chromatin-modifying complex (Li et al., 2002; Peixoto et al., 2012). Whereas some reports pointed to the role of HDAC2 in the rearrangement of the nucleosomes during the formation of heterochromatin in late S phase, as well as the implication for HDAC3 in replication fork progression (Rountree, Bachman & Baylin, 2000; Sims & Wade, 2011). And other histone

marks related with chromatin condensation seem to increase throughout S-phase progression; for instance, the role played by H3K79me2 in limiting re-replication is vital for cell survival and coordinated progression through the cell cycle. This histone mark is present from G_1 phase in regions proximal to replication initiation sites, but then H3K79me2 expands to adjacent regions during S-phase (Fu et al., 2013).

Together, this data indicates that the initiation and progression of S-phase are regulated by a plethora of chromatin remodeler complexes and histone-modifiers enzymes, in order to maintain genome integrity (Figure 11).

5.1.2. G_2-M phases

Chromatin remodeling is a constant process that accompanies the cell cycle and it is especially dynamic during mitosis, when duplicated chromatin should acquire the highest compaction grade in preparation for nuclear division.The forces that shape the structure of the highly condensed metaphase chromosomes are still largely unknown. In vitro evidence suggests that the amino-terminal tails of the histones play an important role in chromosome hypercondensation. As it was already described, the modifications of the histone tails directly influence the biophysical properties of nucleosomes and can recruit or exclude proteins from chromatin. For that reason, H4K16Ac is highly reduced during mitosis (Vaquero et al., 2006). Accordingly, crosstalk between marks located on the same or different histone molecules can influence the writing or reading of these modifications (Kouzarides, 2007a).

One really important histone mark, which correlates with mitosis in all organisms, is the phosphorylation of histone H3 (H3S10p) by Aurora B (Fischle et al., 2005; Hendzel et al., 1997; Hirota et al., 2005) (Figure 11). This modification has different consequences that affect chromatin structure (Figure 12). The phosphorylation of this H3 residue regulates some H3-binding proteins that dissociate from chromatin in mitosis, such as SRp20 and ASF/SF2, which have been shown to be critical for maintenance of genome stability and cell cycle progression(Loomis et al., 2009). Furthermore, this histone mark seems to be involved in repression of general transcription during mitosis, due to its incompatibility with H3K4me, and thus, its negative effect on TAF3 (a subunit of the transcription factor complex TFIID) binding (Southall et al., 2009). Complementary, in yeast, it has been described that H3S10p allows the recruitment of the deacetylase Hst2p to nucleosomes. Therefore, Hst2p drives the deacetylation of H4K16Ac promoting chromatin condensation (Wilkins et al., 2014).These results clearly support the removal of H4K16Ac during mitosis by the mammalian SIRT2, described in Vaquero et al (Vaquero et al., 2006). Furthermore, an important consequence of H3S10p is its effect on heterochromatin structure. This phosphorylation weakens the binding of HP1 to the key heterochromatin marks, H3K9me2 or H3K9me3 (Kouzarides, 2007a). Its removal may facilitate mitotic chromosome condensation, but the specific reason why HP1 is removed is uncertain.

Nevertheless, some HP1 still remains bound to the chromatin at centromeres. There it seems to promote Sgo1 (shugoshin) binding to the centromeres (Yamagishi et al., 2008). It has been proposed that Sgo1 may function as a protector for centromeric cohesion before anaphase entry (Lee et al., 2008; McGuinness et al., 2005). Therefore, in mitosis, HP1 may play several roles including gene silencing, loading and retaining cohesin, promoting kinetochore assembly and preventing erroneous microtubule attachment to the kinetochores. Additionally, Shugoshin recruitment has been also related to H2A phosphorylation by Bub1 and H3K9me3 by Suv39h1 (Koch et al., 2008; Perera & Taylor, 2010).

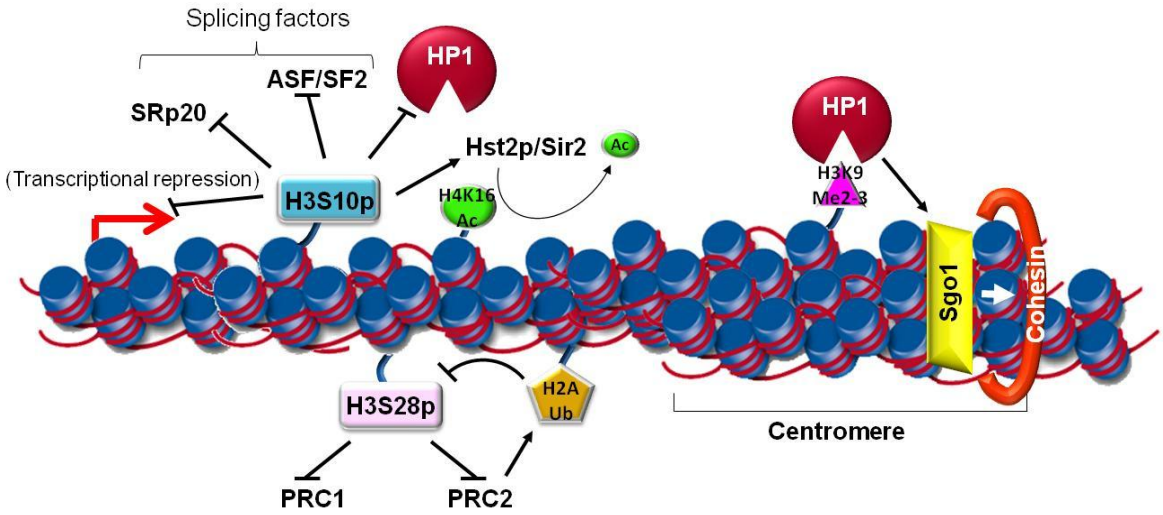


Figure 12. Chromatin regulation during mitosis. Some of the main histone marks that appear during mitosis are H3S10p and H3S28p. The phosphorylation of H3S10 promote the dissociation of SRp20, ASF/SF2 and HP1 from the chromatin; but do not disturb the heterochromatin structure of the centromeres. Moreover, H3S10p allows the recruitment of Hst2p to the chromatin in order to deacetylate H4K16Ac. In addition, H3S28p displace PRC1 and PRC2 from the chromatin favouring the decrease of H2A ubiquitination, which inhibits the phosphorylation of H3S28 by Aurora B.

Another mitotic specific histone phosphorylation might be responsible for displacing chromatin-modifiers complexes, the H3S28p by Aurora B. The phosphorylation of this residue seems to displace Polycomb PRC1 and PRC2 complexes from their binding sites on H3K27me3 in mitosis (Lau & Cheung, 2011; Perez-Cadahia, Drobic & Davie, 2009) (Figure 12). In the case of PRC1, its displacement might contribute to decrease H2A ubuquitinylation (Wang et al., 2004b). However, some data suggest that persistent sites of PcG remain on the genome during mitosis; and its function may be the re-establishment of PcG protein binding after mitosis (Follmer, Wani & Francis, 2012). In addition, loss of H2A ubiquitinylation in mitosis is complemented by the deubiquitinase USP16/Ubp-M. This deubiquitinilation might be needed for efficient phosphorylation of nucleosomal histones by Aurora B. The mechanism is still unknown, but interestingly the major ubiquitinylation site in H2A, H2AK119, is adjacent to H2AT120, which is implicated in Aurora B recruitment to centromeres (Joo et al., 2007).

Interestingly, some chromatin events occurring during mitosis appear to be important not only for this cell cycle stage, but also for the next S-phase: the activity of the histone methyltransferase PR-Set7 responsible for H4K20me1, which peaks in late G2 and early Mitosis (Nishioka et al., 2002; Oda et al., 2009). Deposition of H4K20me1 increase chromatin condensation during mitosis, and Suv4-20h2 recruits cohesin to methylated marks to favor pericentric heterochromatin structure (Hahn et al., 2013). This recruitment is essential for a proper chromosome segregation during mitosis, but its timing has not been determined. Additionally, H4K20 monomethylation allows the formation of the pre-RC during the next S phase (Beck et al., 2012; Kuo et al., 2012; Oda et al., 2009).

Other conserved effectors of chromosome condensation, outside the histone modifications, are condensins. These multisubunit complexes confer the ability to supercoil DNA positively via an ATP-dependent mechanism (Thadani, Uhlmann & Heeger, 2012). They are regulated by various mechanisms including phosphorylation by CDKs, Aurora kinase, and Polo kinase (Thadani *et al.*, 2012). Finally, a particular chromatin organization is required for centromere function and, thus, for sister chromatid segregation. Typical epigenetic marks of functional centromeres include the binding of a conserved variant of the conventional histone H3, termed CENP. Phosphorylation of CENP-A is required for the localization of CENP-C, a key mediator between centromeric chromatin and the outer kinetochore components (Goutte-Gattat et al., 2013).

Thus, histone modifications and specific proteins governing chromatin organization cooperate to allow chromosome condensation during mitosis in all eukaryotes, although the complete molecular mechanism is still unknown (Figure 11).

5.1.3. G_1-phase

Little is known about chromatin conformation during G1-phase. Indeed, it is considered a transition phase of those cells that either decide to continue cycling or choose quiescence. Nevertheless, the specification of the DNA replication origins by histone modifications is known to happen during late G_1, because it should be ready for S-phase entry. The mechanism of the histone deposition on the next firing origins is still unknown (Raynaud et al., 2014). Indeed, during this phase the Suv4-20h enzymes may be able to di- and tri-methylate H4K20me1, the histone modifications that will serve to the recruitment of proteins from the pre-RC (Tardat et al., 2010). Although Suv4-20h2 seems to be also involved in the initial recruitment of cohesin to heterochromatin during G1 phase (Hahn et al., 2013).

In addition, this phase is when the PRC2 complex has been described to increase its binding to the chromatin, after mitosis, binding to the H3K27me3 mark and colocalizing with sites of ongoing DNA replication (Aoto et al., 2008; Hansen et al., 2008). This recruitment is dependent on PRC1 binding and its enzymatic activity to form a higher order of chromatin structure, intensively, during middle and late G_1 (Aoto T et al., 2008). And according to this

repressing signaling, SIRT1 also seems to play a role in G_1 chromatin remodeling due to its association with histone H2B and H4 promoters together with NPAT, as happened at late S-phase. The H2B expression is low at the G_2/M and G_1 phases, and it is very likely that SIRT1 also associates with other histone promoters and plays a role in the coordinated regulation of different core histone genes in response to cellular redox status (He et al., 2011). However, it is intriguing that the INO80 chromatin remodeling complex has been also found, in yeast, at unfired origins in G_1 phase cells (Shimada et al., 2008).

Finally, the major contribution to chromatin repression during G_0 and G_1 phases of the cell cycle seems to be the Rb regulation. Hypophosphorylated Rb binds to and inhibits the transcriptional activity of E2F-regulated promoters, including the CDKs and cyclins. This inhibition is associated with histone modifier enzymes and CAPs that bind and collaborate with Rb, such as HDACs, BRM/BRG1, Suv39H1 and 2, and HP1 (Dunaief et al., 1994; Magnaghi-Jaulin et al., 1998; Nielsen et al., 2001; Vandel et al., 2001). Contrary, and at late G_1, in order to enter into S-phase, the E2F factor is able to bind to its promoters and it is known to recruit different HATs, such as Tip60, in order to promote gene expression. (Taubert et al., 2004).

5.2. Checkpoints

The stability of the genome is under constant threat from chemicals, radiation, and normal DNA metabolism. Therefore, in order to maintain genome stability during cell division, eukaryotic cells have evolved a number of surveillance mechanisms termed checkpoints. These checkpoints make sure the completion of essential molecular and cellular processes of each cell cycle stage before entering another or sometimes proceeding with the following step. If the checkpoints are not properly controlled, the cells may suffer catastrophic DNA damage that may lead to higher mutation rates, chromosome instability, and aneuploidy.

The coordinated and sequential progression of the different cell cycle stages is regulated by specific proteins, the called CDKs (Cyclins-dependent Kinases) (Morgan, 1997). The enzymatic activation of these kinases is due to its binding to other proteins termed Cyclins, which fluctuate during the cell cycle and are specific to the different stages (Figure 13) (Sher, et al 2004). This association allows a sequential activation of the different CDK/Cyclin complexes, key for a coordinated cell cycle progression. Accordingly, the regulation of these CDK-Cyclin complexes, as well as other cell cycle proteins, responds to different checkpoints that appear at different cell cycle moments. The checkpoints described until the moment includes: G1/S transition, intra-S-phase, G2/M transition, and Metaphase/Anaphase (Figure 13) (Spindle checkpoint) (Kastan & Bartek, 2004).

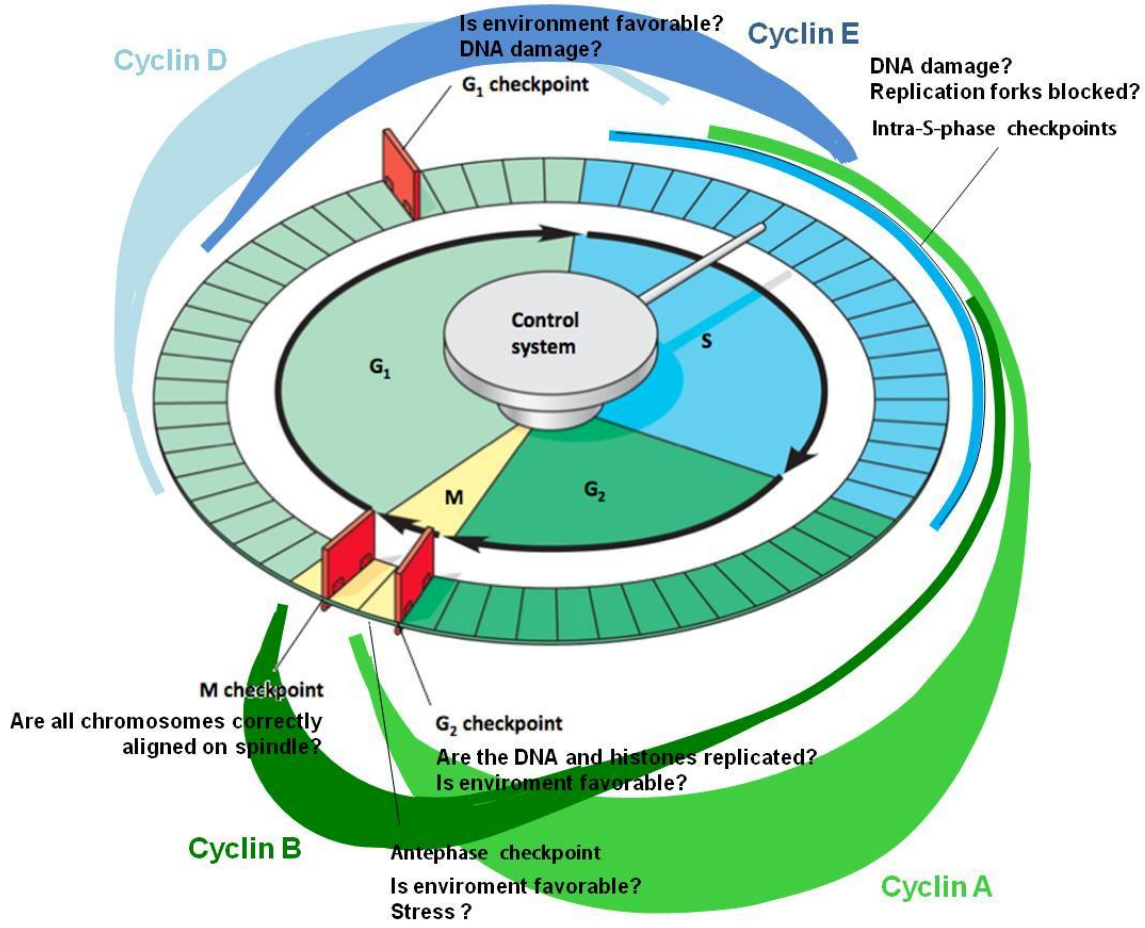


Figure 13. Schematic representation of cell cycle checkpoints . The depiction of the cell cycle includes the G_1/S checkpoint, (named as G_1 checkpoint) the intra S-phase checkpoint, the G_2/M checkpoint,(termed as G_2 checkpoint), the Antephase checkpoint and the Spindle checkpint (written as M checkpoint). The scheme also includes the expression of the cyclins during the cell cycle, which are important regulators of the different checkpoints.

5.2.1. G_1/S checkpoint

In order to prevent the entrance into S-phase after DNA damage, the response of the cell consists in two simultaneous pathways governed by ATM (ataxia telangiectasia mutated)/ATR (AMT- and Rad3-related) phosphorylation (Lukas, Lukas & Bartek, 2004):

- Cell cycle arrest throughout Cdc25A: In response to DNA damage, Cdc25A is phosphorylated by ATR/CHK1, activating its proteosome-mediated proteolysis. Thus, this process avoids the dephosphorylation of CDK2, and the subsequent activation of cyclin E/CDK2 and cyclin A/Cdk2

complexes (Kastan & Bartek, 2004). These two complexes are essential in promoting S-phase (Woo & Poon, 2003); hence, their inhibition blocks the cell cycle in G_1/S border.

- Arrest maintenance by p53: Upon DNA damage, both ATM and ATR directly phosphorylate p53 and MDM2 (he ubiquitin ligase that normally binds p53 and ensures its rapid turnover) (Ishikawa, Ishii & Saito, 2006; Kastan & Bartek, 2004). ATM and ATR also phosphorylate and activate CHK2 and CHK1, which in turn phosphorylate p53 and MDM2. These modifications contribute to the stabilization and accumulation of p53 protein, as well as favor its transcriptional activity for target genes (Dumaz & Meek, 1999). Among its regulated genes, p53 induce the expression of p21CIP1/WAF1, which inhibits the cyclin E/CDK2 kinase complex, leading to G_1 arrest (Wahl & Carr, 2001).

Although both phosphorylation pathways occur simultaneously, the Cdc25A pathway does not require protein synthesis, being the faster response (Mailand et al., 2000). However, the real control of this checkpoint is due to the accumulation of p53; and consequently, its loss suppress this checkpoint (Lukas et al., 2004).

The cyclin E/CDK2, together with the cyclin D/CDK4, works in conjunction to relieve inhibition of the retinoblastoma protein (Rb) and E2F complex. In G_1-phase uncommitted cells, hypophosphorylated Rb binds to the E2F-DP1 transcription factors forming an inhibitory complex. Upon DNA damage, E2F1 becomes phosphorylated by ATM and increasing its binding to Rb (Retinoblastoma) family members (Powers et al., 2004). Commitment to enter S-phase occurs through sequential phosphorylation of Rb by Cyclin D-CDK4/6 and Cyclin E-CDK2 that dissociates repressor complex, permitting transcription of genes required for DNA replication (van den Heuvel & Dyson, 2008).

5.2.2. Intra S-phase checkpoints

Some authors determine the existence of three different checkpoints inside the S-phase (Figure 13), which are mutually closely coordinated, and share some components (Bartek, Lukas & Lukas, 2004):

- The S-M checkpoint (also known as new origins of DNA replication checkpoint): This checkpoint is a reversible inhibition of the firing from those origins of DNA replication that have not yet been initiated. This happens through the phosphorylation of Cdc25A by CHK1 and CHK2, its subsequent degradation and the inactivation of cyclin E/CDK2 ((Bartek et al., 2004; Bartek & Lukas, 2003). Failure of the S-M checkpoint results in cells with incompletely duplicated DNA leading to mitotic catastrophe; it ensures that cells do not attempt to divide before the entire genome has been faithfully replicated (Bartek et al., 2004).

- The replication-dependent intra-S-phase checkpoint (commonly referred to as replication forks checkpoint): It is initiated when the progression of replication forks becomes stalled in response to stresses such as the depletion of deoxyribonucleotide (dNTP) pools, chemical inhibition of DNA polymerases, or as a consequence of the collision of replication forks with damaged DNA and/or aberrant DNA structures (Bartek et al., 2004). Its function consist of protecting the integrity of the replication forks and allow the recovery of cell-cycle progression after DNA repair and/or restoration of the dNTP pool (Tercero & Diffley, 2001). ATR-mediated phosphorylation and activation of CHK1 kinase leads to Cdc25A is rapidly degradation, and therefore to arrest the cell cycle (Bartek et al, 2003, 2004; Sorensen CS et al, 2004). Simultaneously, ATR phosphorylates the TIM-Tipin (Timeless-Tipin) complex and Claspin in order to promote replication fork stability, together with RPA (replication protein A) (Kemp et al., 2010).

- The replication-independent intra-S-phase checkpoint (generally referred to as intra-S-phase checkpoint), which can be induced by DSB: It does not require an active replication fork for the initiation; indeed, it is a DNA damage-induced checkpoint (Bartek et al., 2004; Merrick, Jackson & Diffley, 2004). Nevertheless, the intra-S-phase checkpoint might overlap with the replication-forks checkpoint in order to maintain of chromosome stability. In response to DNA damage, such as IR, ATM is also in charge of phosphorylating NBS1 to activate the S-phase checkpoint (Gatei et al., 2000; Wu et al., 2000; Yazdi et al., 2002), but the downstream effectors are not clear. BRCA1, which function in DNA damage response and transcription regulation; and SMC1, a component of the cohesin complex which is necessary for sister chromatid cohesion (Guacci, Koshland & Strunnikov, 1997), are also required for activation of the intra-S-phase checkpoint (Yazdi et al., 2002), and both are also phosphorylated by ATM in response to IR (Cortez et al., 1999; Yazdi et al., 2002).

It also seems clear that chromatin may be another important player on S-phase checkpoint. However, there is not much information about how chromatin influences or participates into any of these checkpoints. Some studies have shown how the histone H2A (or the related H2A.X) is phosphorylated by ATR/Mec1 and ATM/Tel1 in human cells and yeast (Downs, Lowndes & Jackson, 2000; Rogakou et al., 1998), and serves to recruit other checkpoint proteins as well as chromatin-remodelling factors such as the Ino80 complex (van Attikum & Gasser, 2009).

5.2.3. G2/M checkpoint

This checkpoint appeared in order to avoid the entrance into mitosis when the cells had not complete DNA replication or had suffered any kind of DNA damage (Figure 13). This damage might have happened during G_2 phase or the DNA damage that they acquired during

G_1 or S phase was not repaired. However, the accumulation of cells in G_2 may be also due to the replication checkpoint.

The G_2/M checkpoint acts through two simultaneous pathways in order to regulate the principal component of this checkpoint, the cyclin B/CDK1 complex:

- Cell cycle arrest by Cdc25A and Cdc25C: CHK1 and CHK2 are activated by ATR/ATM phosphorylation after DNA damage (Bartek et al., 2004; Bartek & Lukas, 2003). Then, both phosphorylate Cdc25A/C leading to their subcellular sequestration and degradation. This effect avoids the activation of CDK1 by Cdc25 dephosphorylation. Therefore, Wee1 and Myt1 maintain the inactivation of cyclin B/CDK1 complex by phosphorylation, blocking mitotic entrance (Donzelli & Draetta, 2003; Mailand et al., 2002).

- Arrest maintenance by p53: As happens in G_1/S border, the p53-p21CIP1/WAF1 pathway is activated. In this case ATR is the kinase in charge of this checkpoint (Nyberg et al., 2002; Taylor & Stark, 2001) and the p53 activation promote the expression of other genes such as GADD45, which inhibits the cyclin B/CDK1 complex; and the protein 14-3-3 that sequestrates cyclin B/CDK1 complex favoring its degradation (Taylor & Stark, 2001).

5.2.4. Mitotic checkpoints

5.2.4.1. Spindle checkpoint

The spindle checkpoint supervises the bi-orientation attachment of spindle microtubules to all condensed chromosomes before the initiation of anaphase division during mitosis (Figure 13). Therefore, at the heart of the spindle assembly checkpoint is the kinetochore, a multi-protein complex that assembles on the centromeric DNA of each chromosome (Rieder & Salmon, 1998). During mitosis, all the chromosomes get attached by their kinetochores from two opposite spindle poles and are aligned at the equatorial plane, at a stage referred to as metaphase. Only when all the chromosomes are properly attached and aligned at the equatorial plane, anaphase onset is triggered, allowing the splitting of sister chromatids and their delivery to each spindle pole (Zhou, Yao & Joshi, 2002).

Importantly, this checkpoint does not permanently arrest cells in mitosis. Rather, it delays mitotic progression until all kinetochores are attached (Musacchio & Hardwick, 2002; Musacchio & Salmon, 2007). The duration of a spindle checkpoint-mediated arrest is highly variable and appears to be cell type and organism dependent. Indeed, the activity of certain checkpoint kinases may modulate the length of a checkpoint-mediated arrest. Cells that do not satisfy the checkpoint often die or exit mitosis into the next G1 phase as single tetraploid cells via poorly understood “slippage” or “adaptation” pathways (Gascoigne & Taylor, 2008; Rieder & Maiato, 2004).

Briefly, the signal of unattached or improperly attached kinetochores is detected, transduced by the different checkpoint components, and ultimately inhibits the activity of the Anaphase-promoting complex (APC/C), the multi-subunit E3 ubiquitin ligase required for anaphase entry (Zachariae & Nasmyth, 1999). APC/C activation and substrate selectivity are carefully controlled at different stages of the cell cycle via its co-activator subunits CDC20 and CDH1, which recruit substrates to the APC/C and are essential for its enzymatic activity. CDK1 is one of the major regulators of co-activator binding to APC/C. In particular, during early mitosis, CDC20 is phosphorylated by CDK1, thereby allowing APC/C-CDC20 to be activated once the spindle assembly checkpoint (SAC) has been satisfied. At the same time, CDK1 phosphorylation of CDH1 prevents association of the APC/C with CDH1. Until anaphase onset, the spindle checkpoint prevents activation of the APC/C-CDC20 complex. Under proper conditions, APC/C-CDC20 is allowed to target its substrates, such as cyclin B and Securin for proteosome degradation, and thereby allows anaphase onset. Once cyclin B is degraded, CDK1 is inactivated and CDH1 is allowed to associate with the APC/C and direct its activity until late G1 phase of the cell cycle (Elowe, 2011; Pines, 2011). Loss of securin releases active separase, which cleaves the cohesin rings holding sister centromeres together (Hauf, Waizenegger & Peters, 2001; Uhlmann et al., 2000). Therefore, APC/C inhibition blocks sister chromatids separation by arresting the cell cycle in metaphase.

Well-characterized checkpoint components include the kinases Bub1, BubR1, Mps1, Prp4, Chk1 and Tao1. Other checkpoint components are Mad1, Mad2, Shugoshin, Aurora B, Plk1 and PP2A; and the three subunits of the Rod, Zwilch and ZW10 (RZZ) complex. Together, the function of each of these checkpoint proteins is needed to prevent anaphase entry when the spindle has a defect or when chromosomes are not properly attached (Yao & Dai, 2012; Zhou et al., 2002).

5.2.4.2. Antephase checkpoint

The DNA damage and spindle assembly checkpoints ensure genomic integrity by delaying cell cycle progression in the presence of DNA or spindle damage, respectively. Meanwhile, this checkpoint, named the “antephase checkpoint” by Matsusaka and Pines A (Matsusaka & Pines, 2004), acts to prevent cells from entering mitosis in response to a range of stress agents (Figure 13). Cells in antephase appear able to reversibly delay mitotic entry when exposed to certain stress conditions, and then are subsequently able to resume progression into mitosis.

Initial visual observations showed how after X-ray irradiation, grasshopper neuroblasts at early or mid-prophase decrease with time (Carlson, 1969a; Carlson, 1969b). Accordingly, avian and mammalian cells similarly exposed in early prophase also showed a reduction in the number of cells progressing into mitosis (Carlson, 1969a; Carlson, 1969b). This fact could mean that irradiated cells proceeded throughout the whole mitotic process but were subsequently

blocked at the next interphase. Alternatively, the irradiation treatment could have delayed entry into mitosis, reverting to an earlier phase of the cell division cycle. By careful observations of living neuroblasts, Dr.Carlson noted that the irradiated cells did revert from prophase to an earlier stage where the chromosomes were less compacted (Carlson, 1969a; Carlson, 1969b).

This finding supports the idea of a new checkpoint mechanism in normal cells to prevent entry into mitosis in the presence of stress agents or a suboptimal condition. And the window for mitotic cells to delayed mitotic entry and revert chromosome condensation appears from late G2 to prometaphase, as cells are no longer able to respond to mitotic stresses after prometaphase (Carlson, 1969a; Carlson, 1969b; Rieder & Cole, 1998).

This checkpoint is mainly mediated by CHFR (E3-ubiquitin ligase) and the kinase p38 (Matsusaka & Pines, 2004). Early studies on CHFR implicated it in delaying mitotic entry in cells exposed to various stress agents; however, its mode of action at the antephase checkpoint remains to be elucidated (Scolnick & Halazonetis, 2000). CHFR has been shown to interact with Aurora A and be required for its ubiquitination (Yu et al., 2005). Indeed, several other studies showed that downregulation or loss of CHFR in cells is closely associated with elevated Aurora A expression (Tomita M et al, 2009). But no evidence showing CHFR ubiquitination of Aurora A directly leading to instability was demonstrated. In addition, p38 is a key kinase of the stress pathway that has been previously implicated in the response to a variety of stress agents in G_2/M (Zhou & Elledge, 2000). Nevertheless, Mikhailov and colleagues found that treating various cell lines in culture with topoisomerase II inhibitors, which produce changes in chromatin structure, led to antephase delay in a ATM-independent manner (Mikhailov, Shinohara & Rieder, 2004). More importantly, enhanced p38 activity resulted in an antephase delay (Matsusaka & Pines, 2004).

6. DNA damage response: Chromatin dynamics

DNA damage can occur at any stage of the cell cycle and can be caused by both endogenous and exogenous sources. Accordingly, the response to damage depends on the cell cycle stage and the kind of damage.

Endogenous sources of DNA damage refers to the lesions caused by the DNA replication process, because of nucleotide errors; by reactive molecules produced by the cellular metabolism, such as reactive oxygen species (ROS); by recombination processes. Meanwhile, the exogenous sources include the damage caused by external substances and different kinds of radiation (for instance, ultra-violet radiation and ionizing radiation). As a result, the different DNA damage sources can generate double-strand breaks (DSB), single-strand breaks (SSB), excision of nucleotides or covalent unions DNA-protein.

To repair the variety of DNA lesions, cells have developed a complex DNA damage response (DDR) that can act by different DNA repair pathways: nucleotide excision repair (NER), base excision repair (BER), DNA mismatch repair (MMR), single-strand annealing

(SSA), nonhomologous end joining (NHEJ), and homologous recombination (HR). Therefore, depending on the kind and importance of the lesion, the repair mechanism will be different; and moreover, the cell may have to activate the cell cycle checkpoints or apoptosis mechanism. Usually the lesions are repaired with no consequences, but the DSBs can activate the checkpoints. If this kind of lesion is not correctly repair, it can cause translocations, deletions and chromosome fusions.

The DNA damage response (DDR) consists on a hierarchical pathway. The DNA damage is detected by a sensor and the lesion is processed and marked by the repair machinery; meanwhile the signal is transduced to the effectors: proteins and enzymes that will be in charge of the repair process (Harper & Elledge, 2007; Shiloh, 2003). The recognition and orchestrated repair of DSBs requires the dynamism of chromatin structure; it should be capable of rapid unfolding, disassembly, assembly, and refolding. Currently, we are focusing on two of the major mechanisms that control the dynamics of chromatin structure: ATP-dependent chromatin remodeling enzymes, and histone modifications.

Because of the importance of DSBs, the explanation will be focused on the context of DSBs repair.

6.1. Histone posttranslational modifications

According to what has been already described, histone modifications can regulate chromatin dynamics by either creating/eliminating binding sites for non-histone proteins that influence the structure and function of the chromatin; or affecting the compaction of chromatin fiber. During recent years, it has been clear that formation of a DSB induces several histone marks in order to regulate the chromatin platform.

One of the most intensively studied DSB-induced histone modifications is the phosphorylation of the histone variant H2A.X. The γ-H2AX in mammalian histones has also been described in yeast. This phosphorylation occurs at the earliest events of DSBs detection, and depends on ATM, ATR, and DNA-dependent protein kinase (DNA-PK). In mammalian cells, this mark spreads over at least one megabase of chromatin surrounding the DSB; mechanism that has been also found in yeast (Rogakou et al., 1999; Shroff et al., 2004). This large domain of γ-H2AX coordinates repair of damage with cell cycle checkpoints, acting as a binding site for repair and checkpoint proteins. For instance, in mammalian cells γ-H2AX provides binding site for MDC1, which works with γ-H2AX to promote recruitment of repair proteins to the sites of DNA breaks and which, in addition, controls damage-induced cell cycle arrest checkpoints (Stucki et al., 2005; Xie et al., 2007). MDC1 also participates in the intra-S phase and G2/M phase cell cycle checkpoints after exposure to ionizing radiation, regulating a properly CHK1 activation (Stewart et al., 2003). In addition, other histone modifications and histone-modifying enzymes play a role in promoting this MDC1-mediated checkpoint pathway. For instance,

acetylation of H4K16 by the human MOF has been suggested to regulate binding of MDC1 to γ-H2AX domains, presumably due to its chromatin unfolding function (Li et al., 2010; Sharma et al., 2010). γ-H2AX also acts recruiting other DNA repair proteins such as 53BP1 and BRCA1, and the role of MDC1 in this process is still unclear (Eliezer et al., 2009; Xie et al., 2007). Interestingly, mono- and di-methylation of histone H4 (H4K20me1-2), which have been shown to increase after laser irradiation and DSBs, also facilitates recruitment of 53BP1 to DSB chromatin (Hartlerode et al., 2012; Pei et al., 2011; Sanders et al., 2004). In addition, gamma-H2AX modification also promotes the binding of histone modifiers, such as the human HAT Gcn5, whose H3 acetylation promotes SWI/SNF recruitment via its associated bromodomain (Lee et al., 2010a). Gcn5 recruitment to DSBs has also been described in yeast as a requirement for efficient DNA repair; and although this mechanism is not known, it suggests that the function of this HAT in the DDR is evolutionarily conserved (Tamburini & Tyler, 2005). However, H3K9Ac deacetylation by SIRT6 has also been shown to be crucial for efficient DSB repair, in response to chronic stress. This sirtuin stabilize DNA-PKcs association with chromatin at DSBs (McCord et al., 2009).

Therefore, some DDR mechanisms are independent from γ-H2AX. In fact, histone modifications such as ubiquitination have been shown to increase after DNA damage. For instance, H2B monoubiquitination by RNF20 happens at DSBs sites (Wang & Elledge, 2007) and facilitates the local chromatin reorganization so that repair proteins, such as RAD51, BRCA1, and CtIP, can access the DNA (Price & D'Andrea, 2013). Also H2A, H3 and H4 ubiquitination seem to increase after UV-irradiation, reducing nucleosomal stability. This regulation has been suggested to facilitate the assembly of DNA repair complexes (Zhu & Wani, 2010). Another histone mark that has been shown to increase upon DNA damage is the acetylation. The function of this histone modification in chromatin relaxation has been claimed as an essential tool in the DNA repair process. The HATs Tip60, p300, MOF and Gcn5 are known to be important in DDR, regulating specific gene expression and favoring an open chromatin state (Narlikar, Fan & Kingston, 2002; Sterner & Berger, 2000). However, the DNA repair regulation by acetylated residues needs to be clarified in the DDR pathway timing, because some histone acetylations seem to interfere in other DNA repair signaling. For example, H3K56Ac has also been considered as a histone mark for DNA repair. This histone mark may not act as a recruitment site, but it seems to be required to facilitate final DNA repair processes and drive chromatin assembly after DNA repair (Chen et al., 2008; Munoz-Galvan et al., 2013); however, recent studies demonstrate how this histone mark needs to be subsequently deacetylated by some sirtuin family members and other HDACs (Michishita et al., 2009; Munoz-Galvan et al., 2013; Vempati et al., 2010; Yuan et al., 2009).

Methylation is another abundant epigenetic histone mark. Arginine methylation is less studied than lysine methylation, and no connection of this modification with DDR has been found so far. In response to DNA damage, two lysine methylations have been identified: H3K79me and H4K20me seem to be required for efficient UV-damage response (Bostelman et al., 2007; Sanders et al., 2004). Unlike γ-H2AX, these histone methylations are not induced by

DNA damage and are constitutively present on euchromatin (H4K20me1/2 but not H4K20me3). Both have been proposed as binding sites for 53BP1 and may act at early sensing step of the DDR (Huyen et al., 2004; Sanders et al., 2004). However, some data related to H4K20me1 indicated an increase of this mark after DSBs by the enzyme MMSET. This process seems to be regulated by γ-H2A.X-MDC1 pathway (Pei et al., 2011).

An important role in DDR is attributable to poly-ADP-ribosylation by PARP enzymes (Krishnakumar & Kraus, 2010; Polo & Jackson, 2011). PARP1 ADP-ribosylates H2A, H2B and H3 tails and ATP-remodeling complexes such as NuRD and Polycomb group proteins, which are therefore recruited to the chromatin. These histone modifications may then facilitate DNA repair by transcriptional silencing of the chromatin flanking the damaged sites, thereby minimizing the likelihood of DNA breakage caused by collisions of advancing RNA polymerases with repair reactions (Lukas, Lukas & Bartek, 2011). Nevertheless, PAR formation also can trigger chromatin relaxation depending on location and/or timing (Krishnakumar & Kraus, 2010).

Thus, multiple histone marks play key roles within DSB chromatin to recruit several mediators of the DDR and also other chromatin remodelers, orchestrating their functions with the repair process.

6.2. ATP-dependent chromatin remodeling enzymes

Besides the covalent histone modifications, another important mechanism involved in chromatin modulation participates in DDR. The ATP-dependent remodelers are rapidly recruited to DSBs in order to destabilize or remove a small number of nucleosomes from surrounding chromatin. This chromatin remodeling event facilitates the recruitment and function of DNA damage sensors and effectors, such as the yeast DNA repair complex Mre11/Rad50/Xrs2 (Mre11/Rad50/Nbs1 in mammals) (Shim et al., 2005).

Many ATP-dependent remodelers have been described to be recruited at DNA damage sites from yeast to humans. Indeed, the INO80 remodeler complex seems to be recruited by γ-H2A.X to favor DDR by chromatin remodeling and subsequent DNA repair proteins recruitment (Kadamb et al., 2013). The same remodeler complex has been implicated in HR repair mechanism, together with others such as SWI/SNF (Symington & Gautier, 2011) hSNF2H, and CHD4 remodeling enzymes (Papamichos-Chronakis & Peterson, 2012). In addition, in budding yeast, also the nucleosome remodeler RSC (p400 in mammals), member of the SWR-C family (Xu et al., 2010), govern the incorporation of the H2A.Z variant to DSBs. This process seems to be required for an efficient NHEJ (Shim et al., 2005; Xu et al., 2012). Recently another histone remodeling mechanism has been described as ensuring efficient DSB processing. It is the displacement of H2A-H2B dimers by the yeast Fun30 (mammalian Etl1) (Costelloe et al., 2012; Chen et al., 2012) in order to facilitate DNA processing.

Thus, a plethora of ATP-dependent remodeling enzymes are recruited to DSBs in order to mobilize, evict, or disrupt nucleosomes and other histone proteins, facilitating the DDR.

Additionaly, some of these complexes also recruit DNA repair proteins and complexes, whose activity may in turn be favored by the remodeled chromatin.

Objectives

Objectives

When we started this project, the role of SIRT2 in genome integrity and cell cycle progression was unknown from a chromatin point of view. The main role that related SIRT2 with chromatin regulation and cell cycle progression was the deacetylation of H4K16Ac during mitosis (Vaquero et al, 2006); however, those previous studies were performed in cell culture and *in vitro*, but there was not *in vivo* data that correlate SIRT2 with H4K16Ac, and no evidence of the functional implications of this activity. Therefore, our main objective was to understand the role of SIRT2 and H4K16Ac deacetylation in the control of cell cycle and genome stability.

In order to address this objective, we aimed to develop the following issues:

1. Confirm SIRT2 as the main histone deacetylase of H4K16Ac during mitosis *in vivo.*

2. Characterize the functional relationship between SIRT2 and H4K16Ac in G_2-M.

3. Determine the implication of SIRT2 in the maintenance of genome integrity and its possible consequences in tumorigenesis.

4. Study the redundancy between the two H4K16Ac-specific sirtuins, SIRT1 and SIRT2, in chromatin and the cell cycle regulation.

Materials & Methods

Materials & Methods

1. MICE

1.1. SIRT2 knockout mice

The original ES cells and mice were generated by D. Alt's lab; nevertheless, the SIRT2 $^{-/-}$ C57-BL6 mice used in these experiments were obtained by D. Tong's lab using the original ES cells. The murine SirT2 gene was cloned from a 129 mouse genomic DNA library. A 2.7 kb SacI-SacI fragment containing exons 3 and 4 was cloned into the pLNTK vector as a 5' arm, and a 3.8 kb SacI-SacI fragment was inserted into the opposite side of the pGK-Neo cassette as a 3' arm. By homologous recombination exons 5, 6, and a part of exon 7 were deleted. The targeting construct was electroporated into TC1 embryonic stem cells, and correctly targeted clones were isolated via positive and negative selection followed by Southern blotting. The targeted clones were further transiently transfected with pMC Cre recombinase to remove the Neo gene. For mice genotyping, DNA was extracted from ear punches according to standard methods, and standard PCR was performed as described later.

1.2. MOF conditional knockout mice

The mice were obtained from Dr. Voss lab. The transgenic conditional MOF knockout mice consist on mice with MOF gene flanked by two *lox* sequences, which allow the recombination by the activity of a Cre recombinase, and therefore the loss of MOF gene. Accordingly, the mice have not only the MOF-*lox* flanked gene, but also the cre recombinase gene inserted randomly in the genome. We generated the MOF defeficient/CRE strain by breeding a Mof(lox/+) male (described in Thomas T et al, 2008) into a CAGG-cre-ERT(T/+) transgenic female, as described by Hayashi & McMahon 2002. Dev. Biol. 244: 305-318. Both mice were 100% C57-BL6, and therefore, the resultant mice are also C57-BL6. For mice genotyping, DNA was extracted from ear punches according to standard methods, and standard PCR was performed as described later.

According to the CAGG-cre-ERT recombinase, it is conditionally expressed, so the MOF gene is only deleted under certain conditions. The CRE expression is dependent on Tamoxifen treatment, and it consists on a 96h of treatment with 1uM of 4-OH-Tamoxifen (SIGMA). The medium is changed every 48h and the treatment is added every 24h until the 96h are completed. The genotyping also allows detecting MOF deletion using the same standard PCR. In this case the resultant fragments are of a different size (Fig).

1.3. SIRT2/MOF conditional knockout mice

The SIRT2 and the MOFdef/CRE strains are both C57-BL6, so it was easier and less time consuming to create a double knock out strain. We bred $SIRT2^{-/-}$ mice with (lox/lox-CRE) mice. The difficulty resided in maintain the CRE recombinase gene, because the CRE/CRE (homozygous) females were incapable of raising their babies, and the genotyping does not allow the distinction between homozygous and heterozygous for this gene.

We considered that we got a stable ne strain stain after 5 generations of retro-breeding. Nevertheless, the mice obtained were really heterogeneous, and difficult to maintain between generations: ($SIRT2^{-/-}$-lox/lox-CRE), ($SIRT2^{+/-}$-lox/lox-CRE), ($SIRT2^{-/-}$-lox/lox), ($SIRT2^{-/-}$-lox/+), ($SIRT2^{+/-}$-lox/lox), ($SIRT2^{+/-}$-lox/+), ($SIRT2^{-/-}$-lox/+-CRE), ($SIRT2^{+/-}$-lox/+-CRE), and ($SIRT2^{+/+}$-+/+-CRE) stain after 5 generations of retro-breeding. The ($SIRT2^{+/-}$-lox/lox), ($SIRT2^{+/-}$-lox/+), and ($SIRT2^{+/+}$-+/+-CRE) mice were used as Tamoxifen controls, so in our experiments we are able to check the effect of the treatment without the MOF deletion.

2. CELLULAR CULTURE

2.1. Primary fibrobrasts

- Mouse embryonic fibroblasts (MEFs) derived from WT, SIRT2 knockout mice, and the conditional knock-out mice (MOF and MOF/SIRT2) were generated from day 13.5 embryos by standard methods.
- Primary Ear Fibroblasts were obtained from adult (8-16 wks) WT and SIRT2 knockout mice by standard methods.

Both were grown and maintained in Dulbecco's Eagle's medium (GIBCO) supplemented with 100 units/ml of penicillin/stremtomycin, 2mM of glutamine, non-essential aminoacids [Glycine, L.Alanine, L-Asparagine, L-Aspartic Acid, L-Glutamic Acid, L-Prolina, L-serine] (GIBCO), 1mM Sodium Pyruvate (GIBCO) and 10% FBS (GIBCO); cultured at 37º in 5% CO_2.

2.2. Immortalized fibroblasts

Immortalized MEFs from SIRT1 knockout mice were obtained from Dr. Alt's lab (Cheng HL, et al, 2003; Planavila A et al, 2012) and grown and maintained in Dulbecco's Eagle's medium (GIBCO) supplemented with 100 units/ml of penicillin/stremtomycin, 2mM of glutamine, and 10% FBS (GIBCO); cultured at 37º in 5% CO_2.

2.3. Cell lines

- HeLa: The line was derived from cervical cancer cells.
- HEK293F: cell line originally derived from human embryonic kidney cells.
- Platinum-A retroviral cells: a potent retrovirus packaging cell line based on the 293T cell line.

These cell lines were grown and maintained in Dulbecco's Eagle's medium (GIBCO) supplemented with 100 units/ml of penicillin/stremtomycin, 2mM of glutamine, and 10% FBS (GIBCO); cultured at 37º in 5% CO_2.

3. CELLULAR PROCEDURES

3.1. Transfection and retroviral infection

For co-immunoprecipitation experiments the different expression vectors were transfected into HeLa and HEK293F cells using polyethylene (PEI)

For retroviral generation, Platinum-A cells were transfected using TransIT-LT1 Transfection Reagent (Mirus). The transfection medium was collected 24h after transfection, replaced with fresh media for 24h extra, and collected again by then. The two collected viral suspension (culture media) were filtered using a 0.45um filter in order to avoid the presence of any Platinum-A cell.

The retroviruses were used to infect primary MEFs and Fibroblasts. The collected media was used to infect adding 8 ug/ml polybrene, and incubated for 48h. Then the medium was removed and replace for fresh media. MEFs or Fibroblasts were then visualized by immunofluorescence or FACs.

Plasmids used for transfection: pCDNA4/T0Flag-SIRT1, pCDNA4/T0-SIRT1-HA, pCMV4FLAG-SIRT2, pCDNA4/T0SIRT2-HA, pCDNA3PR-Set7-myc, pCMV4FLAG-PR-Set7, pCDNA4/T0Suv4-20h1.1-HA, pCDNA4/T0Suv4-20h1.2-HA, and pCDNA4/T0Suv4-20h2-HA. The plasmids pCDNA4/T0Suv4-20h1.1-HA, pCDNA4/T0Suv4-20h1.2-HA, and pCDNA4/T0Suv4-20h2-HA were subcloned from pCISuv4-20h1.1-HA, pCISuv4-20h1.2-HA and pCISuv4-20h2-HA, a gift from Dr. D. Alan Underhill (Tsang et al., 2010).

Plasmids used for infection pMSCV-Flag-SIRT2, pMSCV-Flag-SIRT2(H150Y), pMSCV-SIRT2-GFP, pMSCV-SIRT2(H150Y)-GFP, pMSCV-Flag-PRSet7, pMCSV-Flag-PRSet7K90Q and pMCSV-Flag-PRSet7K90R

3.2. Synchronization procedure

HeLa cells were synchronized using the double thymidine block: Firs block with 4 mM thymidine for 14h, then the cells are released for 10h in fresh medium, and blocked again also with 4 mM thymidine for 14h. Finally the cells were release with fresh media.

The cells were harvested at different times in order to obtain cells in S-phase, G2/M and G1-phase. The timing corresponded to 3.5h, 8h and 14h after release, and the cell cycle stage was determined using flow cytometry. The samples were analyze using Propidium Iodide, following standard protocols.

3.3. Cellular treatments

For Mitosis block, HeLa cells were treated 24h with 4mM Thymidine, released for 3h with fresh medium and then treated for 12h with 250ng/ml Nocodazole.

MEFs mitosis block was performed for 4h using 0.1 ug/ml KaryoMAX Colcemide (Life Technologies).

For oxidative stress treatments cells were treated with H_2O_2 for 1h at different concentrations: 2mM for HeLa cells and 200uM for MEFs and Adult fibroblasts.

Gamma radiation was delivered using an aluminum filter at 100 kVp (Faxitron Cabinet X-ray System, Faxitron X-ray Corp., Wheeling, IL).

For the detection of PR-Set7 foci, the cells (fibroblasts) were first treated with 15Um of MG132 according to Tartadt et al, 2007.

The Nicotinamide and TSA treatment consisted of 24h of treatment with 2mM of Nicotinamide, adding 1 µM *TSA during the last 2h.*

4. PROTEIN PROCEDURES

4.1. Cellular fractionation

a. For total protein extraction, the cell pellet is directly resuspended into *Laemmli buffer by sonication in water bath.*

b. To determine the amount of protein in the tight chromatin fraction: after the Dignam protocol extraction (considered as the soluble fraction), the chromatin pellet was washed once with BC-500+0.05%NP40 and then solubilized in *Laemmli buffer* by *sonication* in a water bath (considered as tight chromatin fraction).

4.2. Western blot

Western blot technique was performed according to standardized protocols. Briefly, the protein samples were loaded into a sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) of different polyacrylamide percentages depending on the protein size I wanted to detect. The electrophoresis and transference were performed at denaturalized conditions in presence of SDS. For membrane blocking we used 5% not-fat milk in PBS-0.1%Triton. The primary and secondary antibodies were diluted into PBS-0.2% Tween and incubated for 1h and 30min repectively. The secondary antibodies are antibodies peroxidase-combined (HRP) ahn therefore the result was developed using Luminol or HRP Enhanced Chemiluminiscence (ECL) as substrates.

Densitometric analysis of the western blots was performed with Quantity One software (Biorad).

The samples were used as whole-cell extracts using Dignam protocol, chromatin fraction or total extraction resuspended into *Laemmli* buffer according to the "Chromatin fractionation" section.

4.3. Protein degradation assay

HeLa cells, transfected with Suv4-20h2 or PR-Set7 +/- SIRT1, were treated with 5uM of MG132 (Proteosome inhibitor), or 25uM of Amonium chloride (Lysosome inhibitor) 4h after transfection, and incubated for 24h. Also non-treated cells were used as controls.

The cells were then harvested and resuspended in *Laemmli buffer in order to analyze by western blot the total amount of protein.*

4.4. Protein co-immunoprecipitations and pull downs

The co-immunoprecipitations were performed using whole-cell extracts prepared according to the Dignam protocol[24]. Co-immunoprecipitations were performed using FLAG-agarose (Sigma), HA-agarose (Sigma), Myc antibody (Cell Signaling), and Protein G Agarose (Millipore). The protocol consist in co-transfect the two specific plasmids of the proteins with different tags to HeLa or 293F cells. 48h after transfection, the cells are harvested, washed with PBS and used for protein extraction.

Once the whole-cell extract is obtained, each sample is incubated with agarose beads conjugated with the specific antibody (anti-Flag, anti-HA or anti-Myc) for at least 4h at 4ºC rotating. After the incubation, the beads are washed several times using BC-100 and BC-500 (with or without 0.05%NP40) and then the proteins are eluted using 0.2M Glycin pH2.3. The result is loaded in a polyacrilamide electrophoresis gel to detect both proteins.

For in vitro PR-Set7 and SIRT2; PR-Set7 and SIRT1 pull-downs, Glutathione sepharose beads (GE Healthcare) were pre-incubated with or without PR-Set7-GST purified from bacteria. After washing, the same beads were incubated with SIRT2-HA or recombinant SIRT2 purified from bacteria or SIRT1-HA. The samples were eluted, after several washes with BC500, with 20mM Glutathione.

For the Peptide pulldown assay, the tandem tudor domain of human 53BP1 (amino acids 1220–1711) was purified from *E.coli*. Biotinylated peptides spanning residues 1–23 (1–25 for H4K16acK20me1 peptide) of H4 were obtained from Anaspec (Fremont, CA). 400 pmol of peptides were pre-bound to Strep-Tactin macroprep beads (IBA) and then incubated with purified wild-type or binding-deficient 53BP1 tudor construct (400 pmol or varying amounts from from 150–1200 pmol) for 4 h at 30 ºC in 20 mM HEPES pH 7.9, 50 mM KCl, 0.01% NP-40. After washes, bound material was eluted and analyzed by Western blotting.

5. *PURIFICATION PROCEDURES*

5.1. Oligonucleosomes purification

HeLa oligonucleosomes purification was performed as described (Schnitzler, 2001). Briefly, the nucleus were extracted using RSB Buffer (Tris 10mM pH 7.5, NaCl 10mM, $MgCl_2$ 3mM) and 1% NP40. Then, the MNase digestion was performed using a ratio of 1 units/5*10^4cells at 37ºC for specific times depending on the efficiency of the reaction. The result of the digestion is clarified and purified using a sacarose gradient (5%-30%). The fractions containing mononucleosomes or oligonucleosomes are dyalized against the N-buffer (10mM Hepes pH7.5, 1mM EDTA, 10mM KCl, 10% Glycerol and 0.2mM PMSF). The nucleosomes or oligonucleosomes are kept at -80ºC.

5.2. PR-Set7 and SIRT2 purification from mammalian cells

Both proteins were purified from HeLa cells transfected with either pCMV4Flag-PR-Set7 or pCDNA/4T0-SIRT2-HA. The whole cell extracts, including the chromatin bound fraction were obtained by resuspending the cells into RIPA Buffer (150 mM NaCl, 50 mM HEPES pH 7.4, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) and Benzonase (SIGMA) (25 to 50 U/mL per 5*10^6 cells), and incubated for 4h to O/N in agitation at 4ºC.

The final sample was incubated with Flag or HA resins (SIGMA) for 4h in agitation at 4ºC, and the purified proteins was obtained after several washes using BC-100 (Tris pH 7.8, 0.5 M NaCl, 0.5 mM EDTA, 0.1 Mm PMSF, 0.1 Mm DTT, 25 mM NaCl, 10% glicerol) and BC-500

(Tris pH 7.8, 0.5 M NaCl, 0.5 mM EDTA, 0.1 Mm PMSF, 0.1 Mm DTT, 100 mM NaCl, 10% glycerol) bufferes. The proteis were eluted using 0.2ug/ml of Flag or HA peptide in BC-500.

5.3. Recombinant HIS purification

rPR-Set7 and rSIRT2 were purified by this method using: pET30Bpr-Set7 and pET30bSIRT2. The proteins were purified from bacteria using the following protocol: Bacteria pellet was resuspended and lysed using lysis buffer (20mM Tris pH8.0, 100mM NaCl, 10mM EDTA pH8, 0.1%NP40, 0.5% NP40 and 2% sarkosyl) and sonication. The extraction was then purified by incubation with NiNTA Agarose (Qiagen). After several washes with lysis buffer and BC-500, the proteins were obtained by elution with 100mM Imidazol in BC-500. Purified samples are kept at -80ºC.

5.4. Recombinant GST purification

GST-PR-Set7 was purified by this method using: pGEXPR-Set7-GST. The proteins were purified from bacteria using the following protocol: Bacteria pellet was resuspended and lysed using NTEN buffer (0.5M NaCl, 10% Glycerol, 20mM Hepes pH 7.9, 1mM EDTA pH8, 0.1%NP40, 20mM 2-mercaptoetanol and 1mMPMSF) and sonication. The extraction was then purified by incubation with Glutathione Sepharosa 4B (GE). After several washes with NTEN buffer, NTEN-0.7M NaCl buffer and TST buffer (50mM Tris pH 8.0, 150mM NaCl, 0.1% Triton and 5mM DTT), the proteins were obtained by elution with 20mM of reduced Glutatione (SIGMA) in TST buffer. Purified samples are kept at -80ºC.

6. ENZYMATIC ASSAYS

6.1. In vitro PR-Set7 deacetylation by SIRT2

Purified PR-Set7 after treatment with TSA and nicotinamide was used as substrate for SIRT2. The deacetylation assay protocol was modified from Vaquero et al. (2004), using PR-Set7 as substrate instead of core histones or oligonucleosomes (Vaquero et al., 2004), with or without NAD^+. The reaction was stopped by dyalizing the sample using BC100 at 4ºC.

6.2. *In vitro PR-Set7 HMT*

Histone methyltransferase assays were performed using purified Flag-PR-Set7 (normal conditions, N/T or after SIRT2 deacetylation). PR-Set7 was incubated in 50 mM Tris pH 8.5, 5 mM $MgCl_2$, 4 mM DTT, ^{3}H-labeled SAM and nucleosomes for 1 h at 30 ºC. Reactions were stopped by addition of SDS sample buffer and separated by SDS-PAGE. After transferring to PVDF membranes, incorporated ^{3}H was detected by fluorography with EN3HANCE spray (Perkin Elmer) and loading was assessed by coomassie staining.

6.3. *In vitro Nucleosome deacetylation previous to HMT*

The deacetylation assay protocol was modified from Vaquero et al. (2004), using 1ug oligonucleosomes (Vaquero et al., 2004), with or without NAD^+. Then, the samples were dialyzed against the HMT buffer (50 mM Tris pH 8.5, 5 mM $MgCl_2$, 4 mM DTT) to eliminate the NAD^+ from the reactions and then added to PR-Set7 HMT assays with ^{3}H-labeled SAM. Histone methyltransferase assays were performed with Flag-tagged full-length PR-Set7 purified from mammalian cells and incubated 1 h at 30 ºC. Reactions were stopped by addition of SDS sample buffer and separated by SDS-PAGE. After transferring to PVDF membranes, incorporated ^{3}H was detected by fluorography with EN3HANCE spray (Perkin Elmer) and loading was assessed by coomassie staining.

6.4. *In vitro HMT using recombinant nucleosomes*

Substrate chromatin was reconstituted from a plasmid containing 12 repeats of the 601 nucleosome positioning sequence and histone octamers by gradient dialysis from 2 M to 0.4 M NaCl overnight, followed by a step dialysis into TE. *Xenopus* core histones were purified from *E. coli* and reconstituted into octamers. H4K16ac-modified H4 was generated by chemical ligation of a synthetic H4(1–27) peptide acetylated at position 16 and a truncated human H4Δ(1-27)I28C purified from *E. coli*. Ligation under denaturing conditions omitting the final desulfurization steps was performed essentially as described previously (He et al., 2003). Ligated full-length product was separated from unligated peptide and H4 through cation exchange chromatography on a monoS column.

6.5. *In vitro SIRT2 dephosphorylation*

For the SirT2 dephosphorylation assay, 0.5 µg of Flag-SirT2 purified from HeLa cells using Flag resin was treated with 5 units of calf intestinal phosphatase (CIP) using the buffer specified for the commercial (NEB), at 37ºC for 3h.

7. CELL CYCLE ANALYSIS

7.1. FACS analysis and Sorting

Cell cycle and polyploidy analysis were analyzed using the Click-iT® EdU Flow Cytometry Assay Kit (Invitrogen, life Technologies) with some modifications. Briefly, cells were pulse labeled with 10mM EdU at 37^0C for 45 min, and fixed with 70% ethanol, permeabilized with 0.5% Triton-PBS and stained with the ClickEdu cocktail. Cells were finally resuspended in 1μg of 7AAD (Pharmingen) and run and analyzed in a FC500 FACS (Beckman Coulter) and with Flow Jo 7/8 program. To monitor mitosis, fibroblasts were fixed and permeabilized as described above and stained with 2.5□gr of anti-phospho Ser10 H3 antibody (Abcam) for 1h at room temperature. To monitor Cyclin B1 levels, fibroblasts were fixed with 4%PFA and permeabilized with ice-cold 100% methanol. Cells were then stained with either anti-CyclinB1-Alexa 488 (Cell Signaling Technologies) or an isotype matched control Alexa-488 antibody (eBiosciences), and resuspended in 1□g of 7AAD. For sorting, 2.10^6cells/ml primary fibroblasts were stained with 10□gr/ml Hoechst 33342 (Invitrogen, Life Tecnologies). Cells were then sorted using a Moflo XPD Cell Sorter (Beckam Coulter), plated and finally recollected at different time points. Prior to harvesting, cells were pulsed labeled with Edu and/or treated with H_2O_2.

8. MICROSCOPY TECHNIQUES

8.1. Immunohistochemistry (IHC)

Immunohistochemistry was performed on fresh frozen cryostat cut sections. 15-um OCT frozen sections were cryosectioned and fixed using methanol (-20º) or 4% of paraformaldehyde, for H2AX, H4K16Ac, and H4K12Ac, or H4K20me1 and H3K4me3 staining, respectively.

Briefly, after fixation, the tissues were washed with PBS and permeabilized with PBS-Triton 0.2% for 15min at RT. The following step was the blocking, using 10% of Normal goat serum-PBS, 1h incubation in a humid chamber at RT. Then, after washes, the tissues were incubated with the primary antibody diluted into 3% of Normal goat serum-PBS, in the humid chamber, at 4ºC, O/N. The following day, the slides are washed, and incubated with the secondary antibody (Alexa488 or Alexa568 purified from goat) diluted in 3% of Normal goat serum-PBS,1h at RT, in the humid chamber. Finally, after the respective washes, the tissues are incubated with 1ug/ml of DAPI for 10min and washed after mounting.

Antibodies used: anti-γH2AX rabbit, H4K12Ac, and H4K16Ac from Upstate; anti-H4K20me1 from Novus Biologicals; anti-H4K20me2 from Active Motif; anti-H3S28p from Sigma-Aldrich; and H3K4me3 from Cell signaling.

8.2. Immunofluorescence (IF)

For general immunofluorescence, the he cells were washed and fixed with 4% PFA (paraformaldehyde) for 7min at room temperature (RT). Then the cells were permeabilyze using Buffer B (3%BSA + 0.2% Triton in PBS) for 5min and subsequently blocked with 3% BSA-PBS for 1h. The primary antibody was incubated for 1h in Buffer B at RT, followed by 3 washes of 3% BSA-PBS and 30min of the secondary antibody incubation in Buffer B at RT. After 3 washes with PBS, the cells were stained with 1ug/ml of DAPI for 4min and subsequently washed with PBS.

In the case of PR-Set7 foci, the permeabilization step of the immunofluorescende precedes the fixation step; this difference is important to remove the soluble fraction. After the initial washes with ice-cold PBS, the cells are incubated with 0.5%Triton-PBS for 10min at 4ºC. Then after a single wash, the cells are fixed with 3%PFA for 15min. The following steps happen to be the same as the general protocol.

The acquired image stacks were processed by Huygens deconvolution software (Scientific Volume Imaging). Three-dimensional reconstructions, computer-generated visualization of the signal and image analysis were obtained using Imaris software (Bitplane, A.G.).

Antibodies used: anti-γH2AX and H4K16Ac from Upstate; anti-H4K20me2, anti-H4K20me3, anti-H3K9me3 and anti-SETD8 Cell Signaling; anti-53BP1, anti-H4K20me1 from Novus Biologicals; anti-H4K20me2 from Active Motif; anti-H3S28p from Sigma-Aldrich.

8.3. Fluorescent in situ hybridization (FISH)

For this technique we used chromosome spreads preparations obtained from MEFs cells cultured and blocked in mitosis with colcemid (already described). The cells were trypsinized, washed and resuspend in 0.056M KCl, for an hypotonic shock. Finally, the cells were stamped on frozen slides, and fixed using methanol:glacial acid (3:1).

The FISH was performed according with StarFISH Protocol N Pan Telomeric and Centromeric Chromosome Paints (Cambio).

8.4. Neutral comet assay

To detect Double Strand Breaks (DSB), Neutral comet assay was performed using a modified protocol from (Olive PL & Banath JP. 2006).

The comet slides were hand-made: To prepare the slides prior to include the cells, the frosted slides were dipped into 2% of regular agarose solution in PBS, and dried at RT. Then, after trypsinizing and washing the cells with cold PBS, 10.000 cells are mixed with 0.5% of Low melting agarose (LMA) in PBS and spread all over the slide using a coverslip. The layer is solidified incubating the slides horizontally at 4ºC for 10min. Finally, a new layer of 1% LMA is spread over the previous one and solidified also at 4ºC for 10min. During the process the cells and slides should be protected from light. Once we have the slides ready, the cells immersed into the agarose were lysed by a neutral solution containing 0.5M EDTA, 0.5mg/ml Proteinase K and 2% Sarkosyl (pH 8) for 18-20h. This step should be done at 37º and protected from light.

The following step consists on several washes with the neutral running buffer (90mM Tris, 90mM Boric Acid, 2 mM EDTA, pH8.5). Then, the slides were carefully positioned in an electrophoresis tank and run at 11V (3mA) for 25min. Finally the slides were stained with 10ug/ul Hoechst and washed carefully but intensively with H_2O previously to visualization.

More than 50 images per sample were taken using an Epifluorescence microscope (Leica AF5000) and analyzed and quantified using the Comet Score program (free downloaded application).

9. mRNA PROCEDURES

9.1. mRNA stability assay

HeLa cells were transfected with an expression vector containing SIRT1-HA or an empty vector. The cells were then treated with 5ug/ml of Actinomycin D (SIGMA) for the indicated times, and immediately resuspended in TRIZol reagent (SIGMA) for RNA extraction. Then the respective cDNA was used for RT-qPCR analysis.

9.2. mRNA immunoprecipitation (RIP)

HeLa cells were transfected with an expression vector containing SIRT1-HA or an empty vector, synchronized and removed at different times. The cell pellets were homogenized in RSB Buffer (Tris 10mM pH 7.5, NaCl 10mM, $MgCl_2$ 3mM) and 1% NP40, and incubated on ice for 10min in order to obtained the cytoplasmic fraction. Then, the nuclear pellets were

resuspended into Lysis buffer (), incubated for 10min on ice, and finally sonicated briefly at low intensity for 15seconds. The resultant supernatants were the nuclear fraction.

Both fractions for each sample were first precleared with 2ug of IgG rabbit (Cell signaling) and 20ul of magnetic beads, rotating O/N at 4ºC. Then, the samples were incubated with 2ug of anti-HA antibody (SIGMA) and 20ul of magnetic beads, rotating 4h at 4ºC. The beads were then washed three times with Lysis buffer (300mM NaCl instead of 100mM) and finally eluted adding TRIZol reagent. mRNA was extracted using SIGMA TRIZol protocol, and then treated with DNase (Promega) for 1h at 37ºC. Then, the final extraction was performed using Phenol Acid, followed by precipitation by 100% Ethanol with Sodium acetate (3M) and Glycogen, and washed with 80% Ethanol. The mRNA was then resuspended in RNase-free water and used for retrotrascription with the Transcriptor first strand cDNA synthesis kit (Roche) by duplicated, with and without the Taq polymerase as DNA control.

The cDNA was analyzed using RT-qPCR.

9.3. RNA purification, PCR and RT-qPCR

The RNA purification was performed using TRIZol reagent (SIGMA) according to its standardized protocol. The retro-trascription in order to generate cDNA was performed using the Transcriptor first strand cDNA synthesis kit (Roche), according to its specifications and using its Random hexaminer primers.

Real-time quantitative PCR analysis from isolated mRNA was performed using the Thermal Cycler 7900HT Fast Real-Time PCR System (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems). Relative gene expression was determined by ΔΔCT method.

In the case of SIRT2 knock out cells, to normalize the γ-satellites values obtained in the samples the housekeeping genes (HK) used were tubulin, EEF2 and RPL38. In the case of SIRT1 knockout cells, to normalize the PR-Set7 and Suv4-20h2 values obtained in the samples the housekeeping genes used were EEF2, RPL38, and HPRT1.For Actinomycin and synchronization studies in HeLa cells, to normalize the PR-Set7 and Suv4-20h2 values obtained in the samples the housekeeping genes used were NCL, RPL38, and HPRT1. NCL and RPL38 were also used as negative controls for the RIP assays.

In all the cases, except for the RIP assay, the CT values of the HK were substracted from the γ-Satellites, PR-Set7 or Suv4-20h2 CT values for each sample (ΔCT). Then, ΔCT of the control sample was substrated from ΔCT of others samples ΔΔCT. The relative levels of γ-Satellites were calculated as 2-ΔΔCT. Standard PCR was performed using GoTaq Mix (Promega).

Name	Forward primer 5'-3'	Reverse Primer 5'-3'
mTubulin	GCCAGAGTGGTGCAGGAAATA	TCACCACGTCCAGGACAGAGT
mRPL38	AGGATGCCAAGTCTGTCAAGA	TCCTTGTCTGTGATAACCAGG
mEE2F	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
mHPRT1	AGCTACTGTAATGATCAGTCGACG	AGAGGTCCTTTTCACCAGCA
mγ-Satellites	CATATTCCAGGTCCTTCAGTGTGC	GACGACTTGAAAAATGACGAAATC
mPR-Set7	AAGAAGCGGGAGGCTCTGTACG	TTGAGTGGCATCCACGCAGTAG
mSuv4-20h2	CGGTGAGAACGACTTCAGCA	CTCACAGGTATAGCATTCAC
hRPL38	TGGGTGAGAAAGGTCCTGGTCCG	CGTCGGGCTGTGAGCAGGAA
hHPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
hNCL	CTGCCTCAGAGGATGAGGAC	TCTGTTTGGCCATTTCCTTC
hPR-Set7	AAGAAACGGGAGGCTCTGTACG	TCTAGTTGCATCCACGCAGTAG
hSuv4-20h2	TATGGGCTGCCTTACGTGGTGCGTG	CGGGATCAGGATGGGGCCTGGGGTC
SIRT2 Genotyping	GACTGGAAGTGATCAAAGCTC	CAGGGTCTCACGAGTCTCATG
		CAAATCTGGCCAGAACTTATG
MOF Genotyping	TATCTGCCTTTCTCTGTCAATGGG	AGGTGAGCCAGGTTAGGACTTGG
		TGGCACACACCTTTAGATCCACC
CRE Genotyping	CTCTAGAGCCTCTGCTAACC	CCTGGCGATCCCTGAACATGTCC

10. Tumor induction experiments and histopathological analysis

Fourteen age-matched (8-12-wk-old) mice of each genotype, *SIRT2-/-* and wild type, respectively, were treated with DMBA and TPA according to Blanco et al. (2007)[26]. Briefly, it consist on a single dose of DMBA in the previously shaved back of the mice, followed by two dosis per week of TPA on the same area of the animal, for 15 weeks. The number and size of papillomas per mouse was weekly recorded. Skin samples and other mouse tissues were recovered after sacrifice and embedded in paraffin according to standard protocols. For histopathological analysis hematoxylin-eosin standings were used.

Results

Results

1. *SIRT2 participates in the maintenance of the genome integrity during the cell cycle in vivo.*

1.1. SIRT2 knockout mice

The SIRT2 knockout mice were originally generated by D. Alt's lab by deleting exons 5, 6, and a part of exon 7 using Homologous recombination (HR) (Figure R1.A); nevertheless, we obtained SIRT2 $^{-/-}$ C57-BL6 from D. Tong's lab, which used the original ES cells form Dr. Alt to generate the mice in their laboratory. In our lab we used the mice obtained from D. Tong's lab to create a significantly large colony of wild-type (WT) and SIRT2 knockout (KO) mice.

When we got the mice, the characterization of SIRT2 loss in mice was still not published; however, while working with this mice colony we were not able to recognize any characteristic phenotype in the SIRT2 knockout mice (Figure R1.B). The size of the SIRT2 KO mice was normal compare to wild-type, the number of knockout mice born was also similar to wild-type mice, the behavior was apparently normal after SIRT2 depletion, the size of the organs was also similar between wild-type and knockout mice, etc. Therefore, the effect of SIRT2 loss could not be demonstrated by the phenotype of the animal, but it might affect these mice in a cellular level. For that reason, we decided to investigate the effects of SIRT2 deficiency at a cellular level.

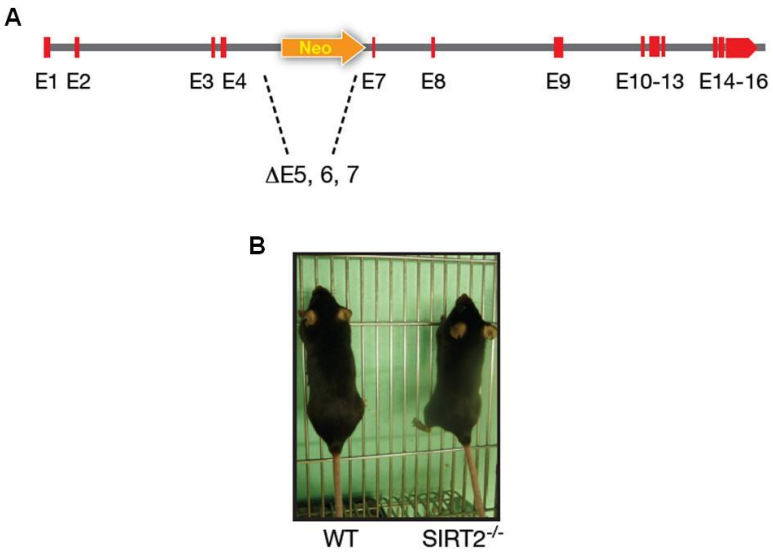


Figure R1. SIRT2 knockout mice. **A.** Schematic representation of the generation of SIRT2 knockout mice by Dr. Alt's lab, by deleting exons 5 and 6 and part of exon 7 using HR. **B.** Picture of SIRT2 knockout mice six weeks-old.

1.2. SIRT2 is responsible of H4K16 deacetylation during mitosis in vitro and in vivo.

Although the SIRT2 knockout mice did not show any characteristic phenotype, the relationship between SIRT2 and H4K16Ac was already described by Vaquero et al in 2006. They found higher levels of this histone acetylation in mitosis in absence of SIRT2 using MEFs derived from SIRT2 knockout mice. This fact supports the pattern of H4K16Ac levels during the cell cycle, which consists of high levels of this histone modification during S-phase that decrease at G_2/M border to reach the lowest levels during mitosis. Nevertheless, this previous study was proved using only cultured cells obtained from the mice or yeast (Vaquero et al., 2006); therefore, the implication of SIRT2 in the decrease of H4K16Ac during mitosis was still not clarified *in vivo*. For that reason, one of our main goals included the clarification of SIRT2 role on H4K16Ac deacetylation *in vivo*.

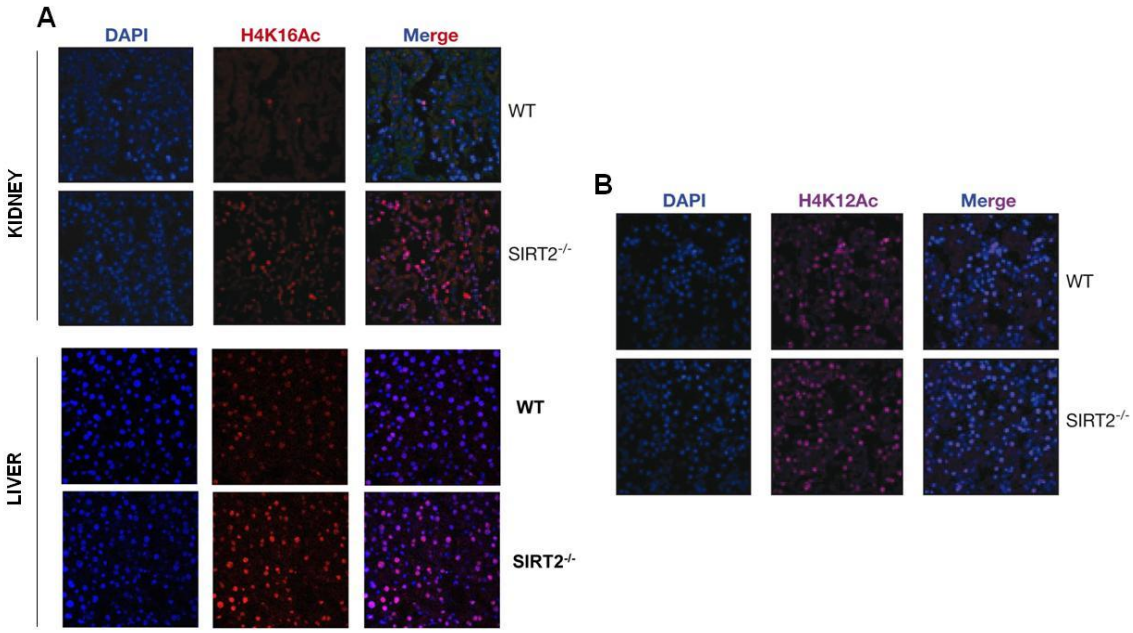


Figure R2. SIRT2 knockout mice show H4K16 hyperacetylation *in vivo*. A and B. H4K16Ac levels in Kidney and Liver cryosections derived from WT or SIRT2 knockout mice detected by IHC. **C.** H4K12Ac levels in Kidney derived from WT or SIRT2 knockout mice detected by IHC.

Therefore, immunohistochemistry experiments, using a specific antibody anti-H4K16Ac, were performed in kidney (Figure R2.A upper panel) and liver (Figure R2.A. lower panel) cryosections from the SIRT2 knockout C57-BL6 mice. These mice were created from Dr. Alt, and their MEFs were previously used by Vaquero et al (2006). Our results (Figure R2.A) showed how H4K16Ac levels were clearly increased in SIRT2 knockout mice kidney (figure R2.A, upper panel) and liver (figure R2.A, lower panel) tissue cryosections compared to the wild-type (WT). There is no need for any quantification to realize how the intensity of H4K16Ac is higher in knockout mice tissues in both, the kidney and the liver. To be sure that this was a specific event for H4K16Ac, and SIRT2 loss does not affect any other H4 acetylation, we also

performed H4K12Ac immunohistochemistry, which is not determined as SIRT2 substrate. We were not capable of seeing any difference between wild-type and knockout mice; which indicates that H4K16Ac deacetylation by SIRT2 is an *in vivo* specific function (Figure R2.B).

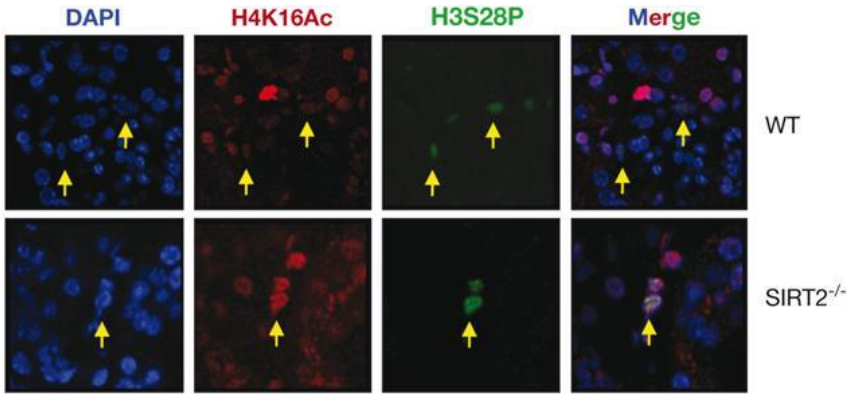


Figure R3. SIRT2 knockout mice show H4K16 hyperacetylation during mitosis *in vivo*. H4K16Ac levels in Kidney, including mitotic cells, identified by staining with the marker H3S28P, by IHC of cryosections.

In addition, to confirm the specific role of SIRT2 in the control of H4K16Ac levels during mitosis, we used the phosphorylation of H3S28p as a mitotic marker. The proliferation rate in kidney is of about 1-3%, so the use of H3S28p antibody allowed us to identify cells in mitosis. As it is shown in figure R3, the cells with the highest intensity of H3S28p staining in the SIRT2 knockout tissues corresponded to the cells with higher levels of H4K16Ac, indicating that H4K16Ac deacetylation by SIRT2 was happening in mitotic cells; which in turn corroborates that H4K16Ac deacetylation by SIRT2 is a specific function *in vivo*.

1.3. SIRT2 and H4K16Ac are involved in genome integrity maintenance.

Due to the fact that H4K16 has been clearly involved in high order of chromatin organization (Thurtle DM & Rine J, 2014; Alekseyenko AA et al, 2012; Vaquero A, 2009), which is an important process during mitosis in order to form the metaphase chromosome; we hypothesize that the absence of SIRT2 might negatively influence the chromatin compaction process. According to the role of chromatin structure in DNA protection, the consequences on its organization after SIRT2 loss raised an important question. Does it affect genome integrity? In fact, inactive/condensed chromatin is much less susceptible to DSB induction by γ-rays than expressed/decondensed domains (Falk, Lukasova & Kozubek, 2008). And upon oxidative stress, the DNA in decondensed chromatin regions were found to contain 14-fold fewer DNA strand breaks than naked and supercoiled DNA, whereas the DNA of “native” chromatin and “condensed” chromatin contained 100-fold and 300-fold fewer breaks, respectively (Ljungman &

Hanawalt, 1992). Therefore, in order to study the possible involvement of SIRT2 and its regulation of H4K16Ac levels in genome stability, we decided to investigate the levelS of DNA damage and genome instability in absence of SIRT2.

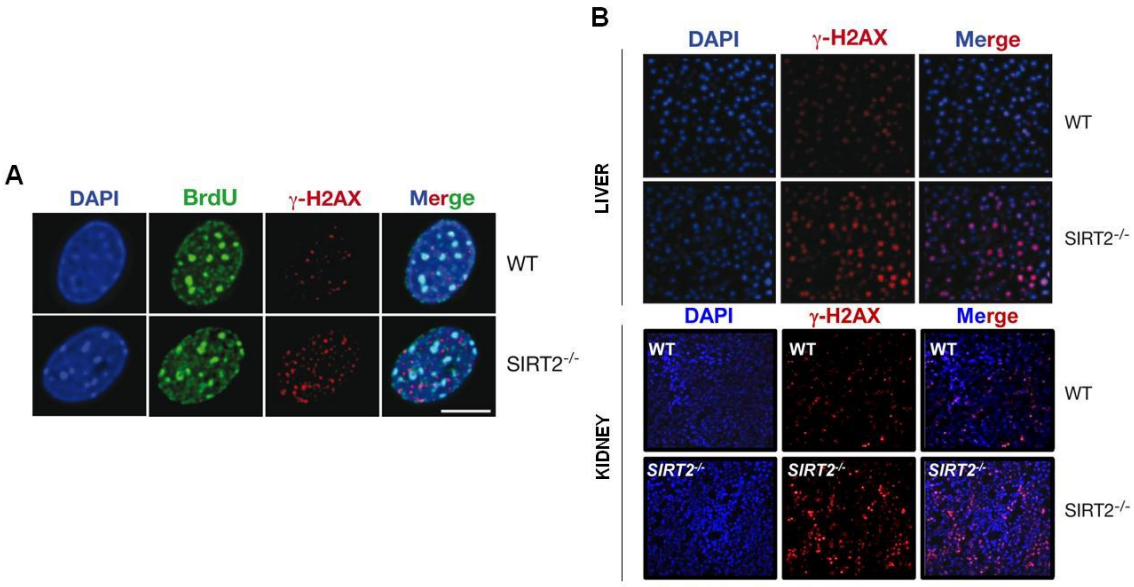


Figure R4. SIRT2 knockout mice harbor higher levels of DNA damage. A. IF experiments of WT and SIRT2 knockout MEFs of γ-H2AX and BrdU incorporation. DAPI staining of the cells are shown as a control for DNA. **B.** γ-H2AX levels in Kidney cryosections derived from WT or SIRT2 knockout mice detected by IHC.

We started using the best known method to detect DNA damage, which is based on the quantification of an important histone modification, the γ-H2A.X. This histone modification marks DSB and serves as a recruiting site for DNA repair proteins (Downs et al., 2004). For that reason, we performed immunofluorescence (IF) using γ-H2AX antibody in cultured cells of knockout and WT MEFs (n=3 WT cell lines and n=3 KO cell lines) (Figure R4.A). This technique also allowed us the distinction of S-, G_2-M and G_1-cells: S-phase was marked using BrdU, which detects replicative DNA; G_2-M cells, were recognized because of the double content of DNA, marked by DAPI staining; and G_1 cells, were determined because BrdU absence and non-duplicated DNA, measured by DAPI staining. According to the results SIRT2 KO cells showed an elevated number of γ-H2A.X foci, and a higher intensity, compared to wild-type MEFs (Figure R4.A). In order to demonstrate that the DNA damage levels were also increased *in vivo* upon SIRT2 depletion, we analyze γ-H2AX intensity using the immunohistochemistry (IHC) technique. Cryosections of liver and kidney tissues were obtained from different WT and SIRT2 KO mice and stained using γ-H2AX antibody. As was demonstrated by the IF results, the absence of SIRT2 produced higher levels of γ-H2AX also in tissues (liver and kidney), compared to the WT samples (Figure R4.B).

In addition, in order to confirm the IHC and IF results, we decided to use other techniques such as the detection of γ-H2AX levels by western blot analysis and the DSB quantification in wild-type and SIRT2 knockout cells using a well known technique termes as Neutral comet assay technique.

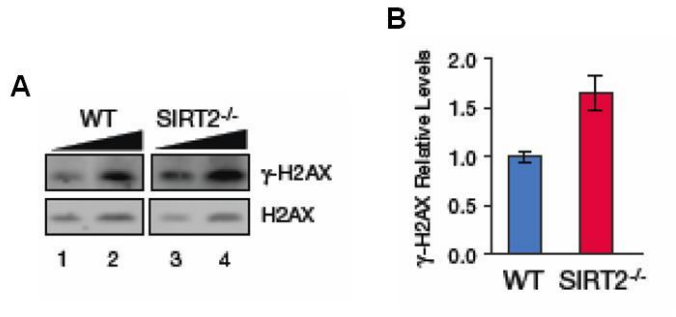


Figure R5. SIRT2 deficiency higher levels of γ-H2AX. A. Western blot analysis of γ-H2AX levels, increasing the amount of sample loaded (lanes 2 and 4 contain 3-fold more sample than lanes 1 and 3). **B.** Quantification by densitometry analysis of experiments in A.

In the first case, the western blot analysis of γ-H2AX levels confirmed previous results obtained by IF and IHC. The figure shows a higher intensity of the γ-H2AX band in SIRT2 KO MEFs compared to the WT (Figure R5.A); in fact, the quantification by densitometry analysis shows 1.6-fold more γ-H2AX levels in SIRT2 KO cells than in WT cells (n=3 WT cell lines and n=3 KO cell lines) (Figure R5.B).

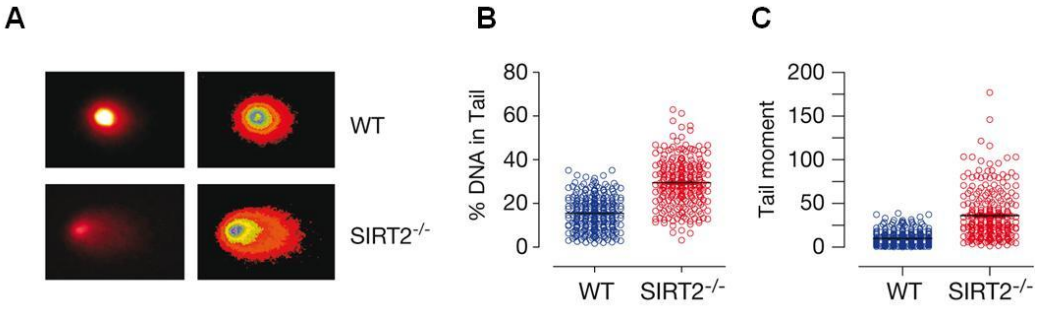
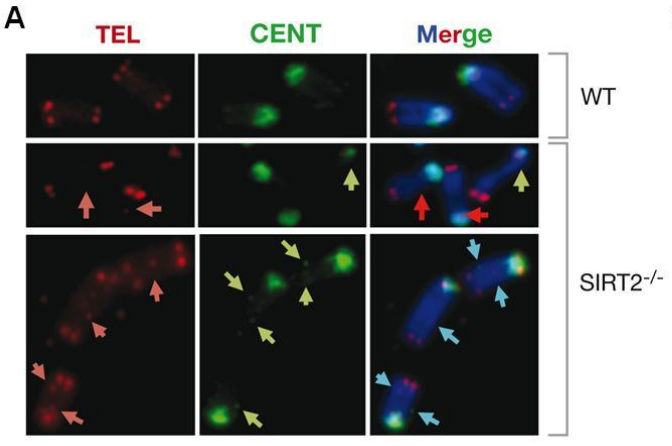


Figure R6. SIRT2 deficiency causes DSB. A. Image of DNA damage quantification in WT and SIRT2 knockout MEFs by neutral comet assay. First column shows the real images taken during the experiments and the second column is a representation of the images using the Comet Score program. **B and C.** Quantification of experiments in C. Data represented corresponds to the percentage of DNA present in comet tail and Tail moment quantification.

Additionally, the Neutral comet assay also demonstrated higher DNA damage levels in SIRT2 KO cells compare to the WT (Figure R6). The comet neutral assay technique was created by the idea of combining DNA gel electrophoresis with fluorescence microscopy to visualize the migration of DNA strands from individual agarose-embebed cells (Olive & Banath, 2006). In our case, the use of neutral buffers allowed us the quantification of DSB in single cells when using primary fibroblasts (Figure R6). The quantification of the “Tail moment” (Figure

R6.B) determines the product of relative DNA content of the tail and the distance between the center of the mass of the DNA in tail and that in the head. In addition, the “Percentage of DNA in tail” (Figure R6.C) determines just that, the percentage of DNA accumulated in the tail. These results are not expressed as a mean average because of DSB heterogeneity; as a matter of fact, not all the cells show the same DNA damage levels, and the cells behave differently according to their cell cycle stage and the moment inside each stage. Therefore, an increase of DNA damage using this technique is demonstrated by the distribution of the individual DNA damage level. In our analysis, the quantification of both, “Tail moment” and “Percentage of DNA in tail”, supports the increased DNA damage by DSB in the absence of SIRT2, because the distribution analysis showed in the graphs is clearly higher in KO MEFs (Figure R6.B-C).

The increasing amount of DNA damage, as well as the negative effect on chromatin compaction, in absence of SIRT2, made us hypothesize that there was a direct effect on chromosome formation during mitosis. Therefore, the final analysis that confirmed the involvement of SIRT2 into the genomic stability maintenance was the chromosome analysis using FISH technique. This analysis is based on the observation of several metaphase spreads obtained from WT and SIRT2 KO MEFs blocked with colcemid, and stained with DAPI and probes that hybridized with centromeres and telomeres (n=3 WT lines and n=3 KO lines) (Figure R7). As we expected, the centromeric and telomeric probes used revealed how SIRT2 deficiency mainly leads to several chromosome aberrations that involved a high order of chromatin compaction structures (Figure R7.B). These aberrations include centromeric duplications or deletions, or duplicated and faint telomeres; which may be the result of DSB with or without subsequent DNA re-ligation, and unusual decondensation processes, among others. Therefore, the above results suggest a clear involvement of SIRT2 in the maintenance of genome stability.



B

ABERRATION TYPE	WT	KO
Telomeric Duplication (DTM)	53	163
Centromeric Duplication (DCM)	57	167
No Proximal Telomere (NPT)	10	14
1 Distal Telomere (1PT)	6	10
No Distal Telomere (NDT)	6	14
1 Distal Telomere (1DT)	2	5
No Telomeres (NT)	0	1
Faint Proximal Telomere (FPT)	14	41
Faint Distal Telomere (FDT)	7	14
Circular Chromosome (CC)	0	1
Small Centromere (SC)	11	59
# Aberrations	166	489
# Chromosomes	1404	1882
% Aberrations	11,8%	25,98%
Total Aberrant Fold	**2,2**	

Figure R7. SIRT2 knockout mice harbor genomic instability. A. IF of metaphase chromosome spreads form WT and SIRT2 knockout primary fibroblasts stained with FISH chromosome paints for centromeres (CENT) and telomeres (TEL), and counterstained with DAPI. Arrows indicate chromosomal aberrations relative to loss or duplicated centromeres and telomeres. B. Quantification of observed chromosomal aberrations in A.

Nevertheless, to assess the direct contribution of SIRT2 to the higher genome instability found in SIRT2 knockout cells, we performed the γ-H2AX analysis again, but using FACS technique, and after re-expressing SIRT2 protein by retrovirus infection (Figure R8.A-B). Briefly, expression vectors containing the coding sequence for SIRT2, SIRT2 catalytic mutant (H150Y) or the empty vector were infected into SIRT2 KO primary fibroblasts by retrovirus. After 48h of infection, the cells were stained using γ-H2AX antibody and analyzed by FACs. This technique allowed us to pinpoint if the DNA damage can be avoid by the presence of SIRT2. The wild-type cells showed around 11.9% of cells marked with the γ-H2AX antibody (Figure R8.A), whereas the SIRT2 knockout mice doubled this percentage to a 23.3% of cells with DSB. However, the re-expression on the active SIRT2 reduced the knockout gamma-H2AX percentage to a 9.6%; meanwhile, when SIRT2 was catalytically inactive (H150Y), it was not able to decrease the DNA damage levels (33%). Therefore, our results confirmed how the re-expression of SIRT2 helps in the recovery of basal DNA damage according to the wild-type cells. This demonstrates, together with the results above, that SIRT2 is the specific H4K16Ac deacetyltransferase *in vivo* during mitosis, and that its activity is important for genome stability maintenance.

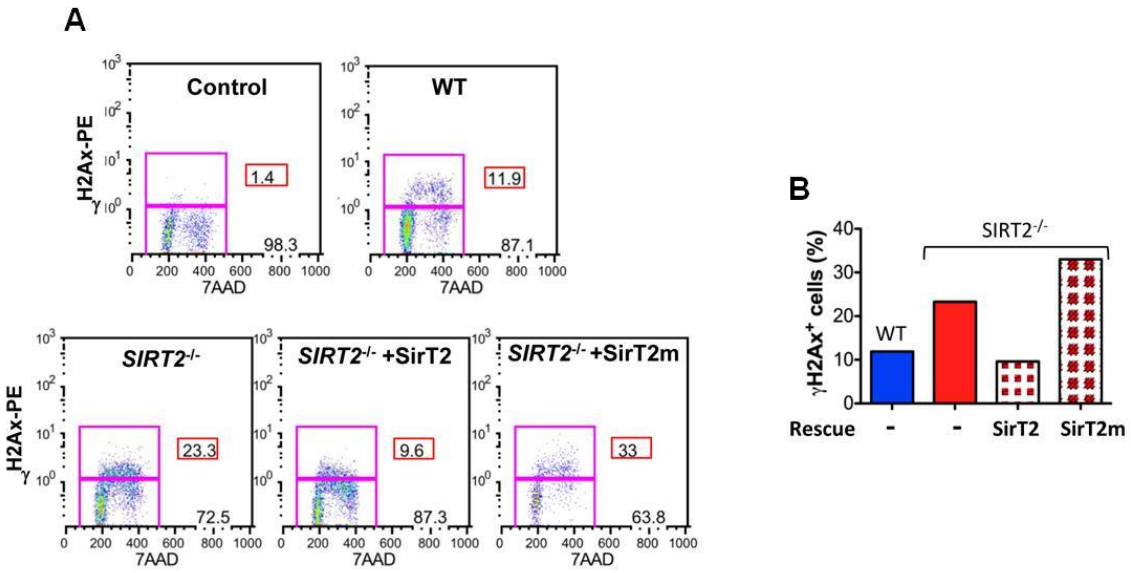


Figure R8. SIRT2 recovery reduces DNA damage levels. A. Rescue experiment of the levels of -H2AX analyzed by FACS, of SIRT2 knockout cells with GFP-tagged empty vector, or SIRT2 either WT form (SirT2) or the catalytic mutant H150Y (SirT2m). A negative control (control) and WT cells (WT) were included in the analysis. **B.** Quantification of experiment in A.

2. SIRT2 is essential for chromatin dynamics during the cell cycle

2.1. H4K16Ac levels determine H4K20me1 deposition

The clear involvement of SIRT2 in genome stability arose from our studies of γ-H2AX foci, DSB levels and chromosome aberrations. Nevertheless, only one of the techniques used allowed us the distinction of the different cell cycle stages, the immunofluorescence. The staining of S-phase cells using BrdU, and the DAPI quantification to distinguish between G1 and G_2-M cells, gave us a more complete vision of when DNA damage was being accumulated due to SIRT2 absence. It was interesting to realize that the γ-H2AX quantification showed the maximum accumulation of DNA damage in S-phase, where the absence of SIRT2 increases the DNA damage detection in about 1.34-fold more than in WT MEFs (Figure R9), when SIRT2 was apparently performing its activity during mitosis.

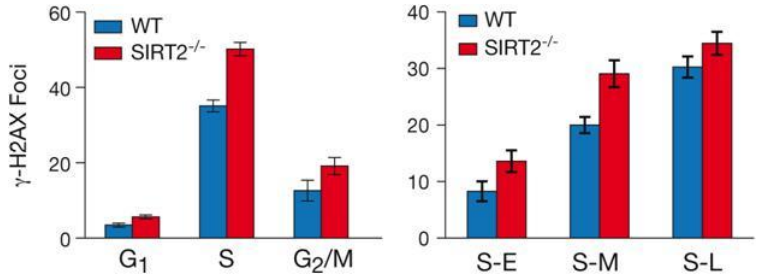


Figure R9. SIRT2 knockout mice harbor higher levels of DNA damage. Quantitative analysis of γ-H2AX foci formation in WT and SIRT2 knockout MEFs throughout cell cycle progression (N>20 for each time point).

Therefore, these results might indicate that the influence of SIRT2 on chromatin structure during mitosis compromises genome stability during S-phase. In other words, the regulation of H4K16Ac levels during mitosis might be essential for specific functions that take place during S-phase. Interestingly, the monomethylation of H4K20me1 happens during mitosis, and its downregulation causes genome instability mainly during S-phase due to several functions associated with this cell cycle stage (Jorgensen et al., 2007). Moreover, the possible interplay between H4K20me1 and H4K16Ac was still under debate. Previous publications suggested an interplay between these two histone modifications, that happen in the H4 histone tail, mainly because of their opposite roles in regulating chromatin condensation and their cell cycle dependence; in reality, H4K16Ac favors open chromatin states and decreases during mitosis, whereas H4K20me1 has been shown to promote silencing and increase in metaphase chromosomes (Hsiao & Mizzen, 2013; Nishioka et al., 2002; Rice et al., 2002). Accordingly with these previous observations, SIRT2 deficiency with the increased H4K16Ac levels, might correlate with decreased levels of H4K20me1 in our knockout mice. Consequently, we

hypothesize that SIRT2 and its H4K16Ac deacetylase activity could be involved in H4K20 monomethylation deposition during mitosis.

Therefore, to study this hypothesis, we started analyzing the levels of H4K20me1 in absence of SIRT2 using our knockout MEFs by immunofluorescence. In order to distinguish the different cell cycle stages we stained the cells with EdU (analog of BrdU that mark replicating cells) and H3S28p antibody. The immunofluorescence images and the quantification of the H4K20me1 levels during the different cell cycle stages (Figure R10.A-C) showed how the loss of SIRT2 correlates with increased H4K16Ac levels and decreased H4K20me1 levels during mitosis. The quantification of the metaphase chromosomes images result in about 37% less H4K20me1 in absence of SIRT2 (Figure R10.B-C).

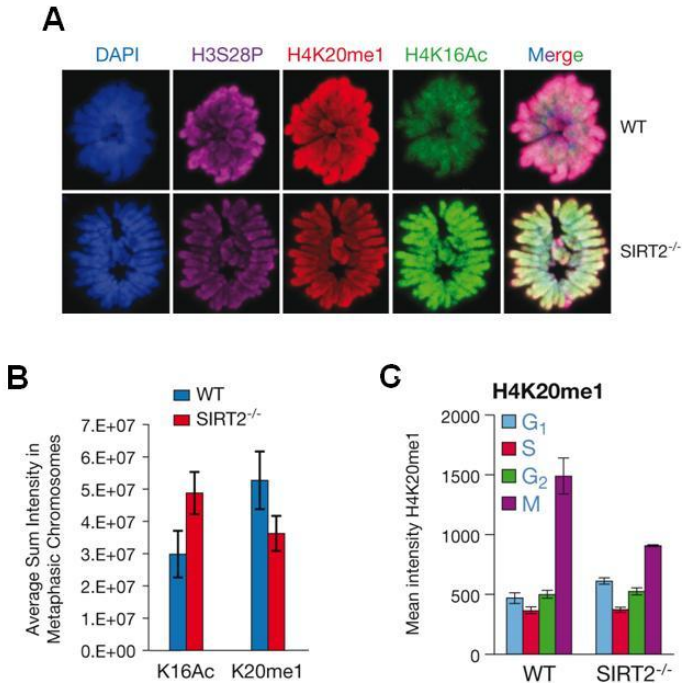


Figure R10. SIRT2 knockout cells show increased H4K20me1 levels in mitosis. A. IF of metaphase cells from wild-type (WT) or SIRT2 knockout MEFs. Cells were stained with antibodies against H3S28P, H4K20me1, and H4K16Ac and then counterstained with DAPI. **B.** Quantification of multiple experiments as in A measuring H4K20me1 levels versus H4K16Ac. **C.** Quantification of the mean intensity of H4K20me1 in IF assays from wild-type or SIRT2 knockout MEFs through the different stages of the cell cycle (n > 20 for each time point).

These results obtained using primary MEFs were confirmed *in vivo* with IHC analysis of H4K20me1 using tissue cryosections of WT and SIRT2 KO mice. For the study we used kidney cryosections of several WT and SIRT2 KO mice (n=6) and the levels of H4K20me1 were detected using the same antibody of the IF analysis. According to the previous result, the cryosections stained with H4K20me1 (Figure R11.D) clearly show higher levels of this histone

modification in SIRT2 knockout mice with respect to the wild-type; meanwhile, the immunohistochemistry of H3K4me3 (Figure R11.E) showed invariable levels despite SIRT2 loss. H3K4me3 was used as a control for IHC specificity, because this histone mark has never been related with SIRT2 before. Therefore, altogether this indicates that the decrease of H4K20me1 levels in absence of SIRT2 is a specific event.

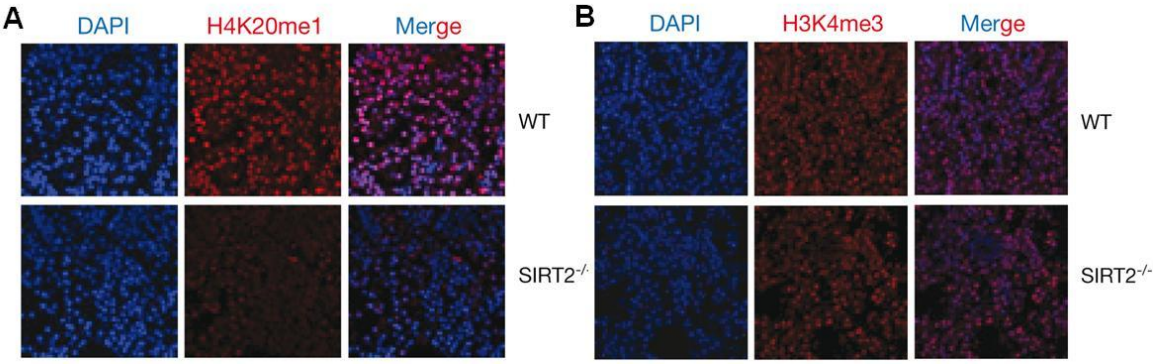


Figure R11. SIRT2 absence affects H4K20me1 levels *in vivo*. A. H4K20me1 levels in kidney cryosections prepared from wild-type or SIRT2 knockout mice. **B.** H3K4me3 levels in kidney cryosections prepared from wild-type or SIRT2 knockout mice.

Furthermore, to confirm that this effect on H4K20me1 was dependent on SIRT2 deacetylase activity and that it was not due to a whole developmental process of successive generations with higher H4K16Ac levels, we decided to perform a protein recovery experiment again. We re-introduced SIRT2 wild-type protein or SIRT2 catalytic point mutant protein (H105Y) into the SIRT2 knockout MEFs by retroviral infection. The results were also analyzed by immunofluorescence, and as it can be inferred from the quantification graph (Figure R12), SIRT2 catalytic activity is necessary for H4K20 monomethylation. When SIRT2 catalytically active was re-introduce to the SIRT2 KO cells, the levels of H4K20me1 increased up to 1.3-fold more than when using the empty vector. On the other hand, SIRT2 catalytically inactive did not recover or increase H4K20me1 levels.

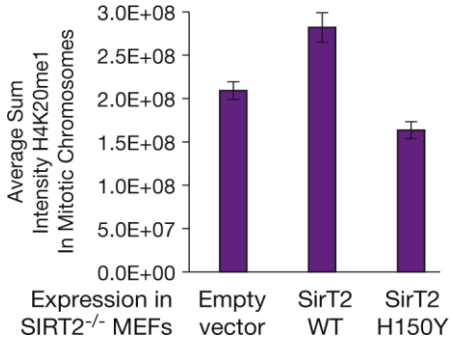


Figure R12. H4K20me1 levels depend on SIRT2 catalytic activity. Quantification of the levels of H4K20me1 in SIRT2 knockout MEFs upon overexpression of SIRT2 wild type or catalytically inactive point mutant H150Y.

According to our results and the previous findings by other authors, it seemed that the deposition of H4K20me1 is somehow inhibited by the only presence of H4K16Ac, outside other SIRT2 functions. To further characterize this effect, we performed an *in vitro* H4K20me1 deposition assay in collaboration with Dr. Reinberg's lab. We performed a PR-Set7 HMT assay using recombinant nucleosomes as substrates, containing an H4 N-terminal tail generated by chemical ligation to contain either unmodified or acetylated K16. As a control, the same methylation assay was performed using the catalytic SET domain of the H3K9me2-specific HMT G9a. The results included in Figure R13 (B-D) show how the activity of PR-Set7, the H4 monomethyltrasferase, but not G9a, the H3 mono- and di-methyltrasferase, is partially inhibited by the presence of H4K16Ac in recombinant nucleosomes. The upper panel of figure R13.B does not show differences between lanes 1-4 and 5-8, respectively On the other hand, in the upper panel of figure R10.A, the marks on lanes 5-8 are lighter than the marks on lanes 1-4, respectively, which indicate that the methylation of the nucleosomes in 5-8 by PR-Set7 was lower than in lanes 1-4. In addition, the quantification in C of PR-Set7 methyltransferase experiments shows a significant increase of H4 methylation in unmodified recombinant nucleosomes respect to the recombinant nucleosomes containing H4K16Ac. These results, together with the SIRT2 knockout results, support the role of H4K16Ac as an impediment for H4K20me1 deposition.

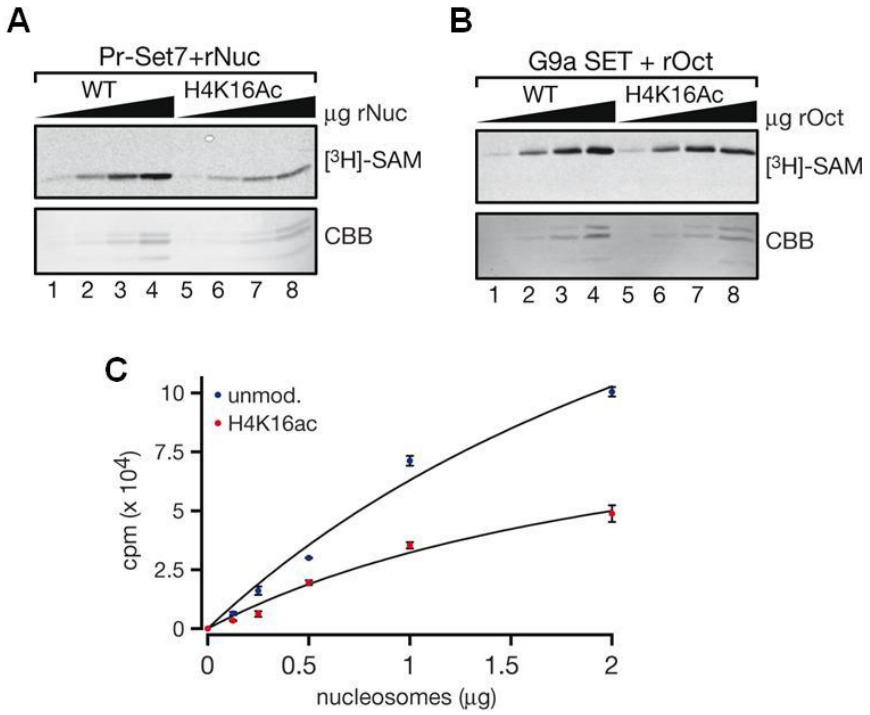


Figure R13. H4K16Ac interferes in H4K20me1 deposition. A. HMT assay of recombinant PR-Set7using as substrates increasing amounts (0.125, 0.25, 0.5, and 1 mg) of recombinant nucleosomes either unmodified (wild-type [WT]) or containing symmetrical H4K16Ac assembled by chemical ligation. **B.** As a control, 0.125, 0.25, 0.5, and 1 mg of recombinant octamers (rOct) (either wild type or acetylated in the H4K16A by chemical ligation) were tested in HMT assays as substrates of the recombinant catalytic SET domain of G9a. These octamers were previously used to reconstitute the recombinant nucleosomes used at top. In both, A and B HMTassays, histone proteins were stained with Coomassie blue (CBB). A representative experiment of a set of three assays is shown in each case. **C.** Quantification of similar experiments shown in A but using 200 ng instead of 50 ng of recombinant PR-Set7 was quantified by scintillation counting. Data shown are means and SEM of two experiments.

In addition, the role of SIRT1 as H4K16Ac deacetylase allowed us to used SIRT1 deficiency in order to demonstrate if the effect on H4K20me1 during mitosis due to SIRT2 absence was specific for the increased levels of H4K16Ac. SIRT2 has been claimed to generally deacetylate H4K16Ac during mitosis, and no patterns or specific regions have been determined (Vaquero et al, 2006); however, SIRT1 is known to deacetylase H4K16Ac in specific promoters, in order to promote silencing and favor heterochromatin formation, and its loss do not increase H4K16Ac levels during mitosis (Vaquero et al., 2004). Therefore, we decided to determine the behavior of H4K20me1 upon the loss of the other main H4K16Ac deacetylase, SIRT1. For that purpose we used the immortalized SIRT1 deficient MEFs obtained from the knockout mice created by Dr. Alt's lab (Cheng et al., 2003; Planavila et al., 2012). We performed immunofluorescence analysis of H4K20me1 levels in mitosis, using H3S28p as a mitotic marker (Figure R14). As we expected, the absence of SIRT1 did not alter H4K20me1 levels respect to wild-type cells. This result indicates that the deposition of H4K20me1 during mitosis is related to the levels of H4K16Ac during this cell cycle stage; in other words, this data demonstrate that the levels of H4K16Ac during mitosis determine H4K20me1 deposition during this cell cycle stage, and supports the antagonism between these two histone marks.

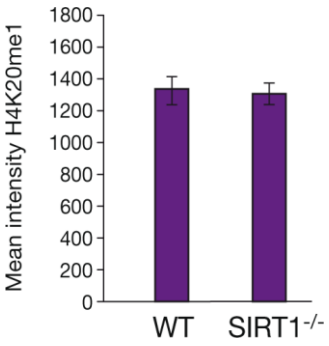


Figure R14. Regulation of H4K20me1 levels during mitosis is SIRT2 specific. Quantification of mean intensity of H4K20me1 in metaphase chromosomes of wild-type or SIRT1 knockout MEFs (n > 20 for each time point).

2.2. SIRT2 interacts with PR-Set7.

In agreement with the results above, SIRT2 could be also modulating H4K20me1 deposition during mitosis by a complementary mechanism, such as the modulation of its specific methyltransferase, PR-Set7. Indeed, a similar mechanism has been described for SIRT1, which not only deacetylates H3K9Ac, but also enhances its trimethylation by recruitment and stabilization of the enzyme Suv39h1 (Bosch-Presegue et al., 2011). We then hypothesize that as it happens with SIRT1, H3K9 and Suv39h1 (Vaquero et al., 2007), maybe not only SIRT2 histone deacetylase capacity influences H4K20me1 deposition, but also SIRT2 may contribute to PR-Set7 activity.

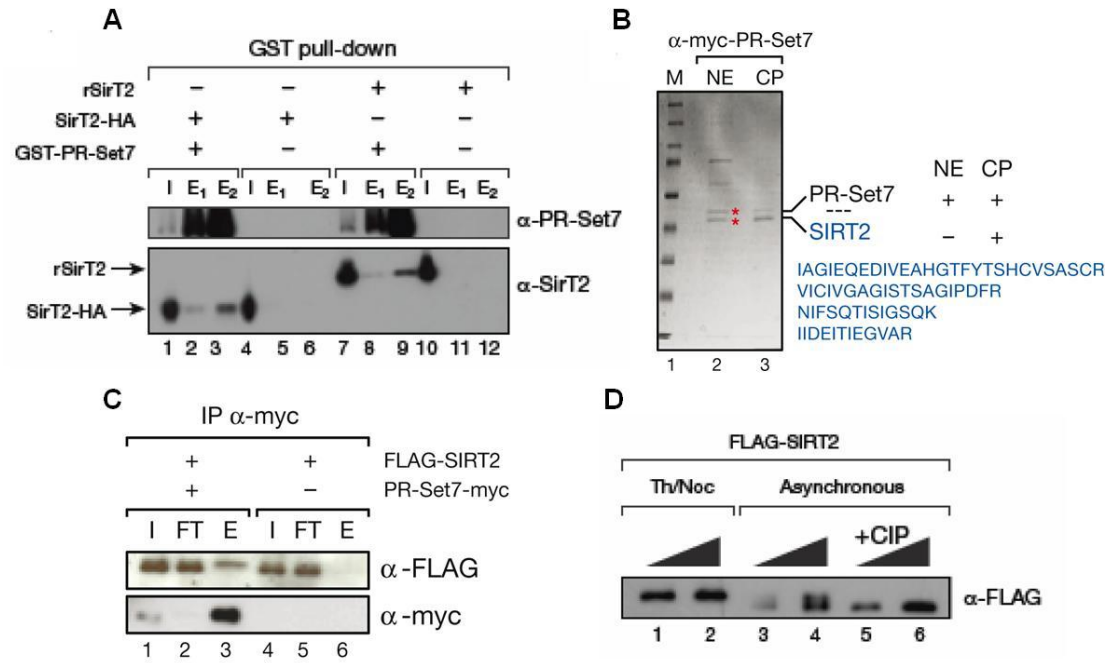


Figure R15. SIRT2 directly interacts with PR-Set7 in mitosis. A. *In vitro* pull-down of recombinant purified proteins PR-Set7 and SIRT2. The pull-down was against GST-PR-Set7 using Glutathione Sepharose 4B beads. **B.** Colloidal-stained gel of Myc-PR-Set7 purified with Myc resin from nuclear extract (NE) and chromatin pellet (CP) of Myc-PR-Set7-overexpressing cells. The band corresponding to PR-Set7 was cut and analyzed by mass spectrometry. Endogenous SirT2 peptide sequences identified only in chromatin pellet are shown in blue. **C.** Myc resin immunoprecipitation of extracts from HeLa cells previously transfected with Flag-SIRT2 and/or PR-Set7-myc as indicated. (I) Input; (FT) flow-through; (E) elutions. **D.** Western-blot of purified FLAG-SIRT2 (FLAG resin) from cells previously blocked in mitosis with sequential combined treatment of thymidine and nocodazole or unsynchronized (lanes 1-4). Purified FLAG-SIRT2 from asynchronous cells was incubated with calf intestine phosphatase (CIP) (lanes 5-6). Black triangles indicate two amounts of extract loaded (5 and 10 micrograms).

In order to study this hypothesis, we started with co-immunoprecipitation assays between SIRT2 and PR-Set7 in HeLa cells; as well as *in vitro* pull-downs of the recombinant proteins. According to our expectations, the HDAC SIRT2 and the KMT PR-Set7 directly interact, as it can be concluded by the co-immunoprecipitation and the *in vitro* pull-down experiments shown in Figure R15 (A-C). The pull-down assay (Figure R15.A) demonstrated how both proteins directly interact and do not need any other binding partner, because both SIRT2, purified from mammalian cells (SIRT2-HA) and recombinant SIRT2 purified from bacteria (rSIRT2), eluted together with the recombinant PR-Set7 purified from bacteria (GST-PR-Set7) (Figure R15.A, lanes 3 and 9). Moreover, the co-immunoprecipitation assays in HeLa cells demonstrated that this interaction happens *in vivo* (Figure R15.C lane 3), and it is not due to a forced mechanism *in vitro*. Thus, the Figure R12.B shows how endogenous SIRT2 protein was found by mass spectrometry analysis after the purification of PR-Set7-myc in the

chromatin-bound fraction. This data demonstrates that the interaction between these two proteins is stable *in vivo* and takes place in the chromatin context.

A deeper analysis of the co-immunoprecipitation assay in HeLa cells between Flag-SIRT2 and PR-Set7-myc (Figure R15.C) showed how only the upper band of Flag-SIRT2 interacts with PR-Set7-myc. As it was already described by other authors, the phosphorylation level of SIRT2 increases during mitosis (Dryden et al., 2003), so our data indicated that both proteins interact during this cell cycle stage. In order to ensure that the two Flag-SIRT2 bands that appear in the western blot were the result of a different SIRT2 phosphorylation level, and at the same time, this level depends on the cell cycle stage, we performed two different approximations. First, we blocked HeLa Flag-SIRT2 transfected cells in mitosis using thymidine/nocodazole treatment (Figure R15.C, lanes 1-2); and then, we treated Flag-SIRT2 obtained from asynchronous HeLa Flag-SIRT2 transfected cells with or without CIP phosphatase (Calf Intestinal Phosphatase) *in vitro* (Figure R15.C, lanes 3-6). The CIP is an alkaline phosphatase that nonspecifically catalizes the dephosphorylation of phosphorylated proteins, RNA and DNA (Dryden et al., 2003). The western blot analysis of the samples (Figure R15.D) show how the upper band of Flag-SIRT2 correspond to a high phosphorylated state of SIRT2, as a result of the CIP treatment eliminates the upper band (Figure R15.D, lanes 5-6). In addition, this phosphorylated state is characteristic of mitosis, according to lanes 1-2 of figure 12.D, owing to the fact that when cells were stopped in mitosis, the lower band disappeared. Our conclusions correlate with previous findings that identify SIRT2 as a substrate of CDK1/Cyclin B and other kinases during mitosis (Dryden et al., 2003; North & Verdin, 2007b). Altogether this indicates that SIRT2 and PR-Set7 interact *in vivo* during mitosis.

2.3. SIRT2 regulates PR-Set7 spreading.

Our previous results showed how SIRT2 might influence H4K20me1 deposition in an H4K16Ac- dependent and independent manner, as H4K16Ac inhibits H4K20me1 deposition and SIRT2 interacts with PR-Set7. Accordingly, SIRT2 could directly regulate PR-Set7 by different, but also complementary, mechanisms: First, due to PR-Set7 accurate protein level control during cell cycle (Abbas et al., 2010; Brustel et al., 2011; Jorgensen et al., 2011; Oda et al., 2010; Tardat et al., 2010; Wu et al., 2010), SIRT2 could participate in the regulation of PR-Set7 protein levels. Indeed, this mechanism has been already described for other SIRT1, which protects another histone methyltransferase, Suv39h1, from proteosome degradation (Bosch-Presegue et al., 2011). Second, PR-Set7 should bind to the chromatin in order to perform H4K20me1 deposition (Wu et al, 2010), so SIRT2 could regulate the chromatin-bound fraction of this enzyme. And finally, SIRT2 might deacetylate PR-Set7 in order to enhance its histone methyltransferase activity; furthermore, previous studies showed how deacetylation of non-histone proteins by different sirtuins, including SIRT2, leads to modified enzymatic activity ((Motta et al., 2004; Yang et al., 2005; Zhang et al., 2013).

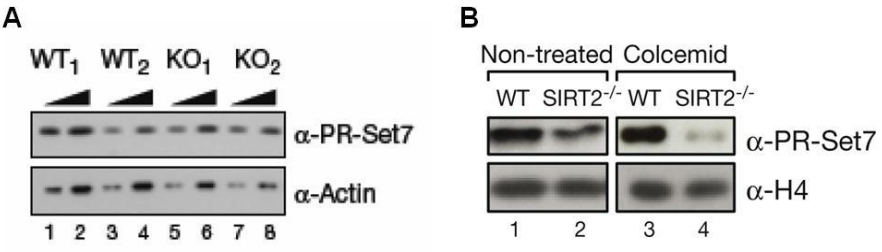


Figure R16. SIRT2 affects the chromatin bound fraction of PR-Set7. A. Western-blot of the levels of endogenous PR-Set7 in different WT or SIRT2 knockout MEFs. **B.** Levels of endogenous PR-Set7 in insoluble chromatin fractions of wild-type or SIRT2 knockout MEFs either asynchronous or blocked in mitosis with colcemid. Histone H4 Western blot is shown as a loading control.

Therefore, we started studying the first possibility: "SIRT2 might participate in the regulation of PR-Set7 protein levels". For that purpose we analyzed the total PR-Set7 levels in presence or absence of SIRT2, using SIRT2 knockout and wild-type MEFs. As it can be inferred from the western blot image (Figure R16.A), the SIRT2 absence did not affect the total amount of PR-Set7, because WT and SIRT2 KO MEFs showed the same amount of total endogenous PR-Set7 according to actin levels (actin was used as the housekeeping gene). Nevertheless, as we pointed out previously, PR-Set7 should be bound to the chromatin in order to perform its activity. Therefore, to study the second possibility: "SIRT2 might regulate the chromatin-bound fraction of this enzyme"; we determined the insoluble protein fraction of endogenous PR-Set7 in the SIRT2 absence using MEFs. We used western blot technique to analyze the tight chromatin fraction obtained according to "Material and Methods" section. Due to the dependence of SIRT2 and PR-Set7 interaction with the cell cycle, we used asynchronous MEFs, and also MEFs blocked in mitosis using Colcemid treatment for 4h to perform this analysis. This treatment allowed us to enrich the samples in mitotic cells. Our results (Figure R16.B) clearly demonstrated how SIRT2 absence decreases the amount of PR-Set7 bound to the chromatin, mostly in the cells enriched in the mitotic phase (Figure R16.B, lane 4).

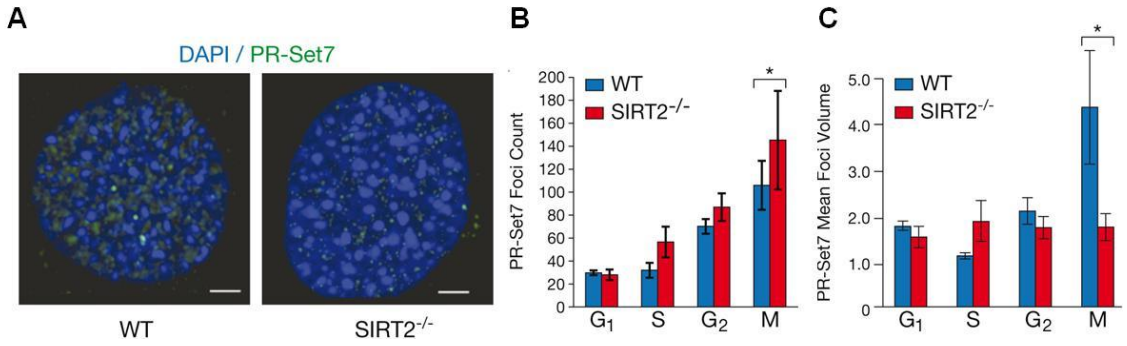


Figure R17. SIRT2 affects the chromatin bound fraction of PR-Set7. A. PR-Set7 foci in wild-type and SIRT2 knockout fibroblasts detected by IF experiments in G_2/M cells (bar, 5 mm) as described previously (Tardat et al. 2010). **B.** Quantification of the number of foci of PR-Set7 from cells at different stages of the cell cycle (n > 20 for each time point). **C.** The mean volume of PR-Set7 foci in the same experiment was quantified.

In addition to the western blot analysis, we used a special IF technique that only detects the chromatin-bound fraction. This technique, which was already used for detecting PR-Set7 foci by other authors (Tardat et al, 2007), permeabilizes the cells before fixation to remove the soluble fraction. However, due to the highest degradation rate of PR-Set7, the cells were previously treated with the proteosome inhibitor MG132. Using this technique, we were able to confirm the western blot results. The images allowed the quantification of the PR-Set7 foci number (Figure R17.B) as well as the foci volume (Figure R17.C) in presence or absence of SIRT2, and distinguishing the different cell cycle phases by EdU and H3S28p staining. The PR-Set7 foci analysis showed how in mitosis the volume of these foci were significantly higher, about 2.21-fold more in wild-type fibroblasts than in SIRT2 knockout fibroblasts (Figure R17.C); however, the number of foci seemed to significantly increase in absence of SIRT2, from a mean average of 107 in WT cells to 145 in KO cells (Figure R17.B). These results made us hypothesize that maybe SIRT2 does not affect the binding capacity of PR-Set7 to the chromatin, but it may influence PR-Set7 spreading throughout the chromatin. This hypothesis would explain why PR-Set7 is able to bind to the chromatin in absence of SIRT2, creating an even higher number of foci (Figure R17.B); but SIRT2 is essential to increase the volume of these foci (Figure R13.C). Therefore, PR-Set7 might not be recruited by SIRT2. Our results suggested that as SIRT2 is needed for PR-Set7 spreading; therefore, PR-Set7 might recruit SIRT2 to the chromatin in order to perform its activity.

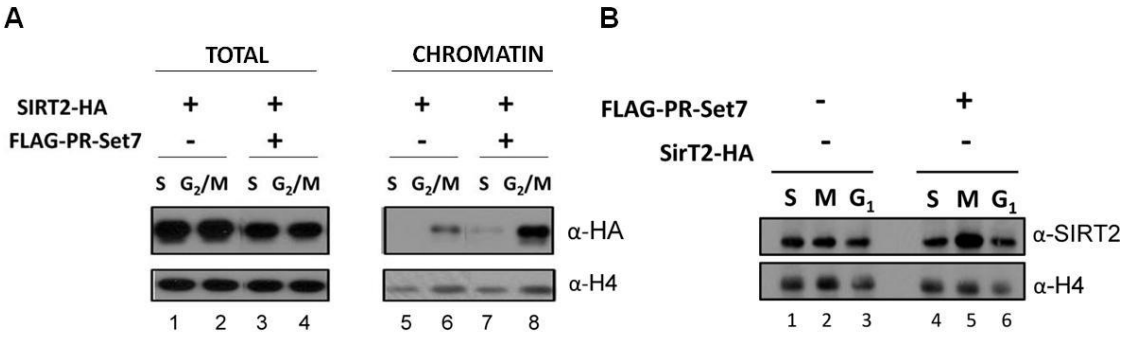


Figure R18. PR-Set7 recruits SIRT2 to the chromatin. A. Overexpression of Flag-PR-Set7 increases the levels of SIRT2 in chromatin insoluble fractions, but not the total amount of the protein. **B.** Overexpression of Flag-PR-Set7 increases the levels of their endogenous SIRT2 in chromatin insoluble fractions.

To clarify this possibility, we analyzed the SIRT2 chromatin-bound fraction. For these experiments we transfected HeLa cells with SIRT2-HA +/- Flag-PR-Set7 (Figure R18.A), and analyzed the total amount of both proteins and their chromatin-bound fractions. In addition, the transfected cells were synchronized using a double thymidine block. After the blockage, the cells were released and harvested at different times, getting cells in S- and G_2-M phases (determined by FACs analysis). Our western blot analysis confirmed two facts: first, that SIRT2-HA is bound to the chromatin during G_2-M phase (Figure R18.A, lanes 5-6); and second, that the levels of SIRT2-HA bound to the chromatin increased when we added Flag-PR-Set7 (Figure

R18.A, lanes 6 and 8). In order to confirm these results, we used HeLa cells to detect the SIRT2 endogenous protein, with an anti-SIRT2 antibody, in presence or absence of transfected Flag-PR-Set7 (Figure R18.B). For those experiments, we also synchronized the cells using a double thymidine block, and the cells were harvested at S-, G_2-M, and G_1- phases (determined by FACs analysis). This new approximation also demonstrated how PR-Set7 increased the chromatin bound fraction of SIRT2 during G_2-M phase (Figure R14.B, lanes 2 and 5). Therefore, both experiments support the role of PR-Set7 in recruiting SIRT2 to the chromatin.

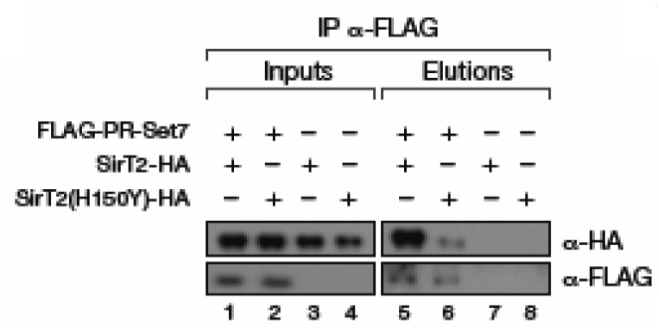
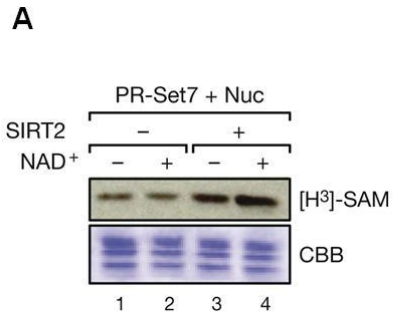


Figure R19. SIRT2 catalytic activity is essential for PR-Set7 interaction. FLAG Immunoprecipitation from HeLa whole cell extracts of FLAG-PR-Set7 either WT or the point mutant catalytically inactive (H150Y) HA-tagged SIRT2 as indicated.

At this point we had to fine-tune our third possibility: “SIRT2 might deacetylate PR-Set7 in order to enhance its histone methyltransferase activity”. Therefore we proposed two different, but complementary, possibilities: First, that PR-Set7 could need the histone deacetylase activity of SIRT2 in order to spread along the chromatin; and/or second, that PR-Set7 could need a post-translational modification (deacetylation) in order to promote its spreading. However, before studying either of the two hypotheses, it was essential to determine if the interaction, between both enzymes, was dependent on SIRT2 catalytic activity. For that reason we performed co-immunoprecipitation assays using Flag-PR-Set7 and SIRT2-HA or SIRT2(H150Y)-HA (catalytic point mutant). The western blot analysis of the Flag immunoprecipitation (Figure R19) showed how the binding between PR-Set7 and SIRT2 catalytic point mutant was significantly decreased compared to the wild-type form of SIRT2 (Figure R19, lanes 5-6). These results indicated that SIRT2 catalytic activity seemed to be important for the interaction between these two enzymes.

Therefore we continued with the study of one of our fine-tuned hypothesis: “PR-Set7 could need the histone deacetylase activity of SIRT2 in order to spread along the chromatin”. For that purpose, we performed a PR-Set7 nucleosome methylation assay preceded by SIRT2 nucleosome deacetylation *in vitro* (see “Material and Methods” section for the procedure). The deacetylation assays were performed in presence or absence of SIRT2, and in presence or absence of NAD^+, to ensure that the process or the NAD^+ was not interfering with the results.

The analysis of the radiography (Figure R20.A) showed how PR-Set7 was capable of adding methyl groups to the nucleosomes in all the cases, although its activity was enhanced when SIRT2 was capable of deacetylase H4K16Ac (Figure R20.A, lane 4). These results indicated that PR-Set7 needed SIRT2 histone deacetylase activity in order to favor its methyltransferase spreading throughout the chromatin.



B

K90Ac Detection	Yes/ No	Andromeda Score	Localization Probability	PEP
PR-Set7	–	–	–	–
PR-Set7 (N+TSA)	+	54,259	1	0,023125
PR-Set7 (N+TSA) +SIRT2	+	80,706	1	0,000611
PR-Set7 (N+TSA) +SIRT2 +NAD+	–	–	–	–

C

MERRGPGRPRTDGENVFTGQSKIYSYMSPNKCSGMRFPLQEENSVTHHEVKCQG
KPLAGIYRKREEKRNAGNAVRSAMKSEEQKIKDARKGPLVPFPNQKSEAAEPPKT
PPSSCDSTNAAIAKQALKKPIKGKQAPRKKAQGKTQQNRKLTDFYPVRRSSRKSK
AELQSEERKRIDELIESGKEEGMKIDLIDGKGRGVIATKQFSRGDFVVEYHGDLIEIT
DAKKREALYAQDPSTGCYMYYFQYLSKTYCVDATRETNRLGRLINHSKCGNCQTK
LHDIDGVPHLILIASRDIAAGEELLYDYGDRSKASIEAHPWLKH

K(Ac)GPLVPFPNQK

Figure R20. SIRT2 catalytic activity influences H4K20me1 deposition. A. HMTassay of PR-Set7 using oligonucleosomes purified from HeLa cells as substrates preincubated with or without SirT2 6 NAD+. Histone proteins were stained with Coomassie blue (CBB). **B.** Mass spectrometry analysis of PR-Set7 purified from cells in different conditions found an acetylation site in K90 of PR-Set7 (indicated in the PR-Set7 sequence shown in C) in cells previously treated with HDAC inhibitors N+TSA but not in untreated cells. Analysis of tandem mass spectrometry parameters in K90 identification indicates a high confidence in the detection. The table includes three parameters: andromeda score (peptide score), localization probability, and PEP (posterior error probability of the identification). (Rows 3,4) Purified PR-Set7 (N+TSA) was incubated with SIRT2 NAD+. **C.** Primary structure of PR-Set7. The sequence of one of the detected K90-acetylated peptides is indicated in yellow. The catalytic SET domain is shown in light yellow.

To analyze if the second hypothesis was part of the equation ("PR-Set7 could need a post-translational modification (deacetylation) in order to promote its spreading"), we analyzed by mass spectrometry the presence of any acetylated residue in PR-Set7 protein, and the possible SIRT2 deacetylase activity. For this part, we purified Flag-PR-Set7 from mammalian cells (HeLa) in two different conditions: one untreated, and the other treated with Nicotinamide and TSA (N+TSA). The Nicotinamide is a specific sirtuin inhibitor, and TSA is an HDAC class I, II and VI inhibitor. PR-Set7 purified in presence of N+TSA was used for *in vitro* deacetylation assays with SIRT2, in presence or absence of NAD^+. The results of the mass spectrometry analysis were obtained in collaboration with Dr. Krüger's lab (Figure R20.B). They found only one acetylated residue in the samples treated with N+TSA, which disappear when treated with SIRT2 only in presence of NAD^+. As you can see in Figure R20.C, this residue corresponds to

the Lysine 90 (K90) according to the Pubmed ID number AAL40879.1. This residue is found out of the SET domain which goes from the residue 188 to the 307, so it does not directly affect the catalytic domain.

Together these results indicate that PR-Set7 recruits SIRT2 in order to deacetylate H4K16Ac and itself favoring PR-Set7 spreading.

2.4. PR-Set7 is regulated by K90 acetylation/deacetylation.

The acetylation level of PR-Set7 could affect different behaviors of the protein:

1. The acetylation level of PR-Set7 could influence the interaction between SIRT2 and PR-Set7; indeed, our previous results demonstrated how the catalytic activity of SIRT2 is needed for the interaction.

2. PR-Set7 acetylation level could influence its capacity of binding to chromatin, because SIRT2 absence affects PR-Set7 spreading but do not alter its binding capacity.

3. PR-Set7 deacetylation could influence its catalytic activity; in fact, SIRT2 has been shown to increase the kinase activity of CDK9 by deacetylating the protein (Zhang et al., 2013).

To assess the contribution of PR-Set7 deacetylation by SIRT2 in their binding capacity, we obtained the point mutants of this lysine residue (K90). We mutated the Lysine 90 to an Arginine (R) to mimic the deacetylated lysine, or to a Glutamine (Q) to mimic the acetylated residue. After several co-immunoprecipitation assays using HeLa cells, we were able to confirm that SIRT2 bound strongly to the "deacetylated form" of PR-Set7 (K90R, Figure R17.A lane 2) than to the acetylated form (Figure R21.A, lane 3), and even more than to the WT (Figure R21.A, lane 1). These results were quantified by densitometry using the Quantity One program, and the graph (Figure R21.B) shows an increase of around 1.5 times more when using K90R, the deacetylated mimical form.

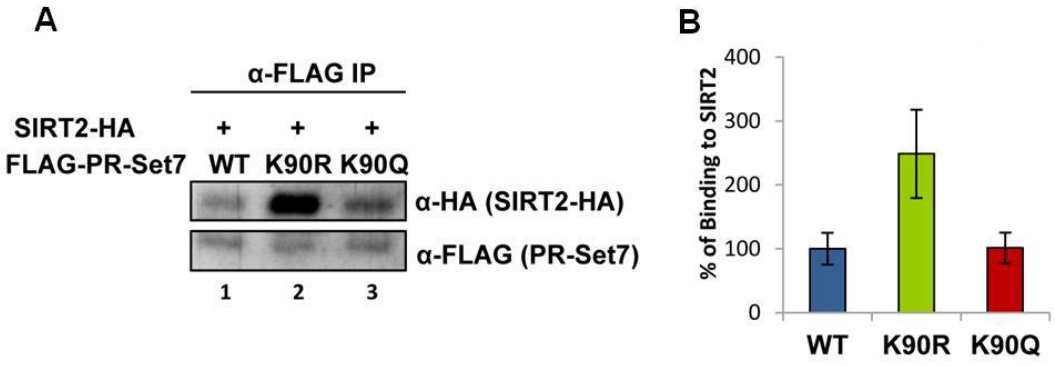


Figure R21. PR-Set7 and SIRT2 interaction is dependent on K90 acetylation. A *In vitro* anti-Flag pull-down of mammalian purified Flag-PR-Set7 (WT), Flag-PR-Set7K90R or Flag-PR-Set7K90Q with SIRT2-HA purified from HeLa transfected cells. **B.** Flag-PR-Set7 pull-down quantification (mean+/- standard error) of n=6 experiments.

This result might indicate that SIRT2 first deacetylates PR-Set7 and then its binding affinity will increase. Thus, we focused our attention in the next point: determine if PR-Set7 acetylation level could influence its capacity of binding to chromatin. To this end, we analyzed the tight chromatin fraction of the different mutants together with the wild-type form (WT) by western blot analysis and by IF (Figure R22), as was done in previous experiments. As a result, both techniques demonstrated that K90Q, the "acetylated form", was significantly more retained into the chromatin than the other two (K90R and the wild-type form of PR-Set7).

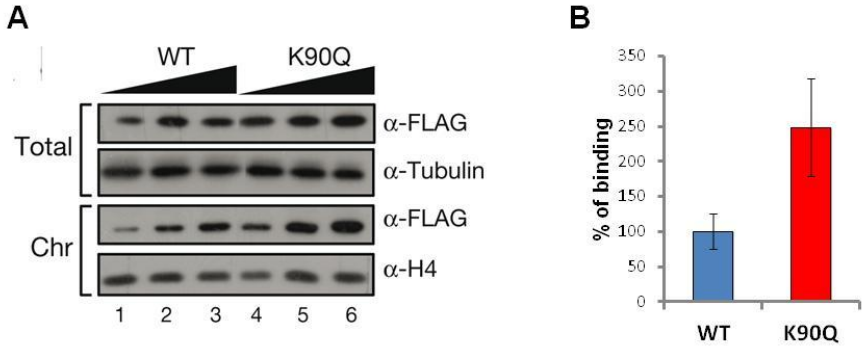


Figure R22. PR-Set7 acetylation in K90 affects its chromatin binding capacity. **A.** Western blot from whole-cell extract (Total) or chromatin pellet (Chr) prepared from HeLa cells previously transfected with an increased amount of expression vector encoding either Flag-tagged PR-Set7 wild type or K90Q. Tubulin was used as a control of whole-cell extract, and histone H4 was used as control of chromatin pellet. **B.** Quantification of the chromatin-bound fraction of PR-Set and the mutant K90Q (n=6, experiments like C). Data obtained according to the proportion of the chromatin fraction respect to H4 and the total amount of protein expressed.

For the western blot analysis, HeLa cells were transfected with the different point mutants (K90R and K90Q) or with the wild-type form of PR-Set7, and the tight chromatin was extracted according to the cellular fractionation protocol explained in "Material and Methods" section. This technique revealed higher levels of K90Q in the chromatin-bound fraction (Figure R22. A, third panel), as can clearly be inferred from the quantification analysis (Figure R22. B). The binding capacity of K90Q, according to the western blot technique, is increased up to 248%.

A

	WT	K90Q	K90R
PR-Set7 Mean Foci Intensity	399 ± 15	297 ± 10**	322 ± 16**
PR-Set7 Total Foci Intensity / Cell	1434 ± 111	1962 ± 204*	1071 ± 102*
Cell Population	N = 143	N = 127	N = 120

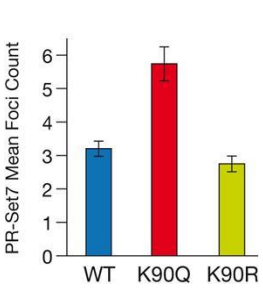


Figure R23. PR-Set7 acetylation in K90 affects its chromatin binding capacity. **A and B.** Quantification of the number of foci per cell (A) and the mean intensity of PR-Set7 per foci and per nucleus (B)of an experiment similar to that in Figure R17 but using primary MEFs overexpressing PR-Set7 wild-type or mutant proteins, as indicated.

In addition, the immunofluorescence analysis was performed using primary wild-type MEFs overexpressing K90R, K90Q or WT PR-Set7 tagged with Flag. This technique detected that the total intensity of foci (data expressed as "PR-Set7 Total foci intensity/cell" in Figure R23.A), as well as the number of foci (Figure R23.B), was higher for K90Q. This data indicates that the "acetylated" form of PR-Set7 increase its binding to the chromatin (the sum of the intensity of all K90Q foci in each cell was higher) by creating higher number of foci. However, the intensity of each focus (data expressed as "PR-Set7 Mean foci intensity" in Figure R23.A) appeared to be significantly lower in the case of K90Q compared to the wildtype, from 297±10 (K90Q) to 399 ±15 (WT) ($p<0.01$). This data demonstrates that although the "acetylated form" K90Q seems to bind better to the chromatin creating a higher number of foci, the size of each focus is smaller than when PR-Set7 is deacetylated (K90R) or in the WT form. Therefore, PR-Set7 acetylation may be related to an increased chromatin binding capacity and a lower spreading capacity throughout the chromatin. Consequently, these new results support the idea of an acetylated PR-Set7 form that binds to the chromatin, and spreads throughout it after being deacetylated in K90 by SIRT2.

We then considered the next step, studying the effect of PR-Set7 deacetylation on its catalytic activity. In order to avoid artefactual interference, we preferred to use the wild-type PR-Set7 form purified from mammalian cells in presence or absence of the HDACs inhibitors (N+TSA) and treated *in vitro* with SIRT2 +/- NAD^+. This experiment allowed us to investigate if the acetylation of PR-Set7 influences its capacity of methylating H4K20 *in vitro*, and at the same time we were able to discriminate if SIRT2 deacetylated residue was involved. Our results showed how the methyltransferase activity of PR-Set7 was not influenced by its acetylation state (Figure R24.A). Indeed, PR-Set7 was capable of methylating the nucleosomes in all cases with no difference (Figure R24.A, second panel).

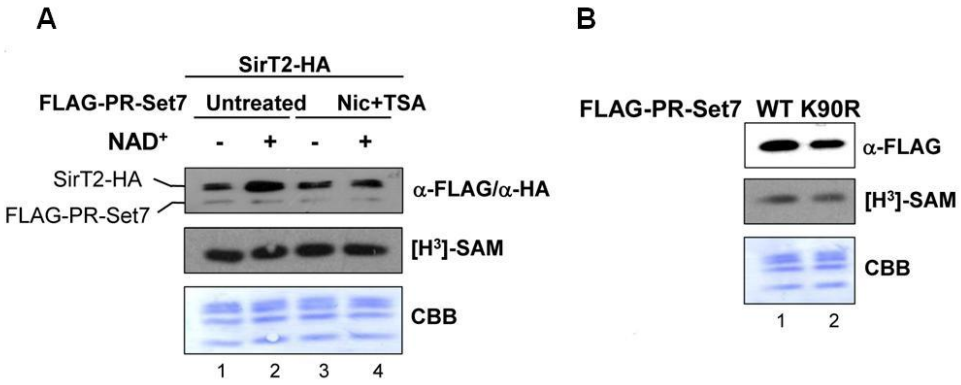


Figure R24. PR-Set7 deacetylation does not affect its enzymatic activity. A. Effect of SIRT2 deacetylation in PR-Set7 activity. HMT activity of PR-Set7 expressed in HeLa cells under normal conditions or treated with the HDAC inhibitors nicotinamide and TSA (Nic+TSA), and incubated with SIRT2 +/- NAD+. The assay was performed in two steps: First, purified PR-Set7 was incubated with or without purified SirT2 in presence or absence of NAD+. Second, the rest of the HMT assay including the reaction buffer and oligonucleosomes were added to the reaction and incubated as a regular HMT assay. Upper panel: Western-blot of the proteins. Middle panel: HMT assay; Lower panel: Coomassie Blue staining of the HMT assay. **B.** HMT assay of purified FLAG-PR-Set7 WT or K90R mutant. Upper panel: Western-blot of PR-Set7 with FLAG antibodies; Middle panel: HMT assay; Lower panel: coomassie-blue staining of the HMT assay

In support of these results, we also performed *in vitro* methyltransferase assays using PR-Set7 WT form and K90R form (the one whose spreading capacity was shown to be higher), and the final results also showed how PR-Set7 deacetylation in K90 do not affect its enzymatic activity. The radiography showed how the PR-Set7 activity measured by [H^3]-SAM, was proportional to the amount of protein used for the assay (Figure R24.B).

Thus, our results confirmed that SIRT2 is recruited by PR-Set7 to the chromatin in order to deacetylase both, H4K16Ac and PR-Set7, favoring H4K20me1 deposition and enhancing PR-Set7 spreading throughout the chromatin.

3. SIRT2 and PR-Set7 are involved in a mitotic checkpoint

Once we confirmed the mechanism of SIRT2-dependent PR-Set7 regulation, it was important to determine the circumstances under which this regulation was happening.

According to previous studies, SIRT2 is clearly involved in stress response regulation throughout their interaction with multiple stress-related transcription factors, such as FoxO, p53 or CDK9 (Chua et al., 2005; Liu et al., 2012; Wang et al., 2007; Wang & Tong, 2009; Zhang et al., 2013). In addition, SIRT2 was related to the regulation of important cell cycle checkpoints that ensures genome integrity upon different external factors including stress (Elowe, 2011; Kim et al., 2011; Zhang et al., 2013). Thus, PR-Set7 activity has been clearly associated with replicative stress and genome stability through its mitotic function(Jorgensen et al., 2011; Rice et al., 2002; Tardat et al., 2007). The stress response is an important cellular function that acts throughout different pathways including the chromatin regulation. H4K16Ac and H4K20me1 are clearly involved in the regulation of chromatin structure during cell cycle (Robinson et al., 2008; Schotta et al., 2008; Shogren-Knaak et al., 2006), and also at specific sites such as DNA replication origins, gene promoters, or heterochomatin regions (Beck et al., 2012; Braunstein et al., 1993; Imai et al., 2000; Kapoor-Vazirani et al., 2011; Shen et al., 2010; Vermeulen et al., 2010). Therefore, both histone marks may respond upon stressful conditions in order to regulate chromatin structure and transcription, to ensure genome integrity. For that reason it is possible that the interplay between these two enzymes, SIRT2 and PR-Set7, during mitosis could be related to stress response protection.

The study of the possible role of PR-Set7 in stress response together with SIRT2 raised several questions: 1. Does SIRT2 regulate PR-Set7 behavior under stress? In this sense, it was already described (Oda et al., 2010) that PR-Set7 levels decrease after stress induction by peroxide treatment in normal MEFs. 2. Does SIRT2 regulate H4K20me1 deposition under stress? 3. Does this interplay affect cell cycle regulation upon stressful conditions?

In order to answer our first question, we studied if the behavior of PR-Set7, under stressful conditions, was dependent on SIRT2. Therefore, we used our SIRT2 knockout and

wild-type MEFs, treated or and without peroxide (H_2O_2). According to what was published, total PR-Set7 levels decreased after hidrogen peroxide treatment in our wild-type MEFs, as well as upon SIRT2 depletion (Figure R25.A). These results support our previous findings where SIRT2 was not necessary for the regulation of PR-Set7 levels, in this case, even under stressful conditions. In addition, we analyze H4K20me1 levels by immunofluorescence, detecting H4K20me1, H3S28p and EdU staining. Contrary to what happened with PR-Set7 levels, H4K20me1 levels increased upon stress when analyzing wild-type primary fibroblasts in mitosis (Figure R25.B). Interestingly, the quantification analysis (Figure R25.B) showed how the absence of SIRT2 negatively affects the levels of H4K20me1 after DNA damage in mitosis and S-phase. Indeed, in mitosis, when this monomethylation seemed to increase under stress conditions (in wild-type cells), the loss of SIRT2 did not allow this positive regulation. These results made us hypothesize that the chromatin behavior of both histone modifiers (SIRT2 and PR-Set7) can be regulated by stress conditions.

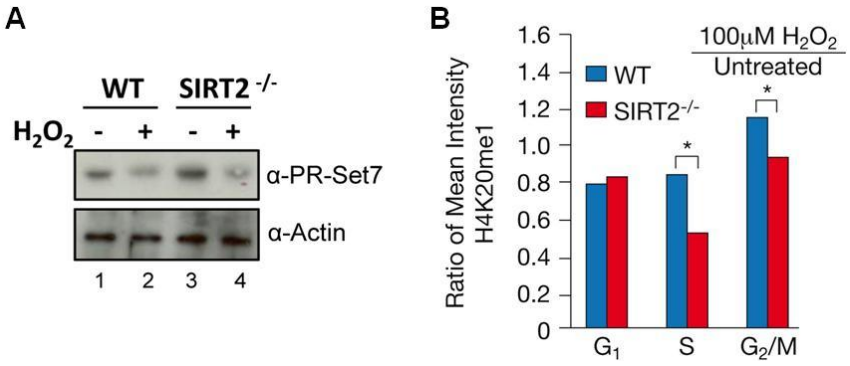


Figure R25. H4K20me1 levels are affected by stress conditions. A. Western-blot of the levels of endogenous PR-Set7 in WT or SIRT2 knockout MEFs under normal or oxidative stress conditions (H_2O_2). **B.** H4K20me1 levels in IF experiments from wild-type (WT) and SIRT2 knockout primary fibroblasts at different stages of the cell cycle, cultured under normal conditions or under oxidative stress. The values shown represent the ratio of H4K20me1 levels in treated cells (100 mM H2O2 for 1 h) to those of untreated cells. (*) The differences between wild-type and SIRT2 knockout cells in S phase and G2/M were significant ($P < 0.05$).

The mechanism of PR-Set7 in recruiting SIRT2 to the chromatin could be involved in the stress response, because PR-Set7 levels are not affected, but H4K20me1 levels increase upon peroxide treatment (Figure R25). For that reason, we analyzed the chromatin fraction of both enzymes upon peroxide treatment, using single (SIRT2 or PR-Set7) and double transfections (SIRT2 and PR-Set7) in HeLa cells. As expected, both proteins increased their chromatin-bound fraction in mitosis when the cells were treated with peroxide (lanes 1-3 and 7-9 of Figure R26.B). And this increase was even higher when both proteins were transfected together (lanes 4-6 of Figure R26.B). Hence, this data established a direct correlation between these two enzymes, chromatin recruitment and stress; indicating that SIRT2 recruitment by PR-Set7 to the chromatin is necessary for stress response. According to these results, our co-immunoprecipitation assays between Flag-SIRT2 and PR-Set7-myc (Figure R26.C), performed

in the presence of H_2O_2, demonstrated how the interaction between SIRT2 and PR-Set7 increase under stressful conditions, despite the lower levels of PR-Set7 (Figure R21.C, lanes 5 and 6).

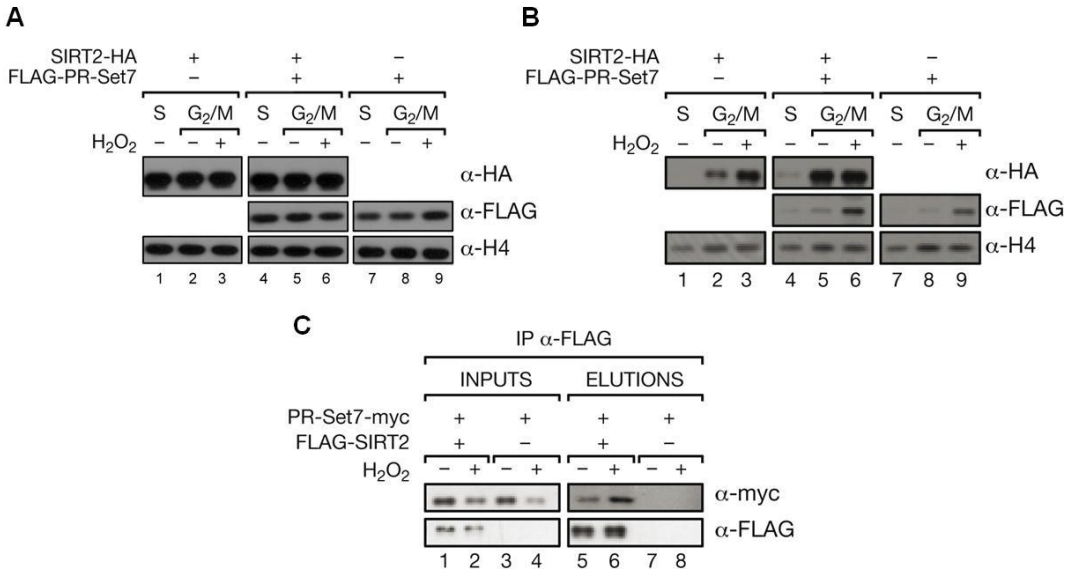


Figure R26. Stress conditions affects PR-Set7 and SIRT2 interplay. A and B. Experiment similar to that in Figure R18.A, but in this case, determining the levels of exogenous SIRT2-HA and Flag-PR-Set7 in insoluble chromatin fractions subjected to H_2O_2-induced oxidative stress (lanes 3,6,9). The cells were blocked in S-phase or G_2/M. The total amount of protein is showed in A, and the chromatin-bound fraction in B. **C.** Immunoprecipitation with Flag resin of extracts from HeLa cells previously transfected with Flag-SIRT2 and/or PR-Set7-myc under normal conditions or H2O2-induced oxidative stress.

As it was pointed out in the introduction, the regulation of stress or DNA damage response is critical for cell cycle progression, which may lead to cell cycle arrest at different cell cycle checkpoint. Consequently, in order to answer our third question ("Does this interplay affect cell cycle regulation upon stress conditions?") we hypothesized that the interplay between SIRT2 and PR-Set7 might be needed for cell cycle control and its regulation under stress conditions. In order to investigate this assumption, we first analyzed the cell cycle progression of SIRT2 knockout MEFs respect to the wild-type cells under stress conditions (H_2O_2) by FACs analysis. For the experiment, to distinguish between cell cycle phases, the DNA content of the cells was stained with 7AAD (a double-strand nucleic acid *intercalant)*, and mitotic cells were discriminated using anti-H3S28p. The results are collected in Figure R22.A-B. When the MEFs were treated with peroxide, the wild-type cells showed a decrease in mitotic cells of around 35%; whereas the absence of SIRT2 abrogates this decrease in about 30% (Figure R22.A-B). These results indicate that in absence of SIRT2 there is a missing checkpoint that allowed the entrance into mitosis despite the DNA damage state of the cells.

Additionally, WT and SIRT2 knockout MEFs were sorted in G_2-M, according to the DNA content measured by Hoescht staining. The sorted cells were re-grown under normal conditions or upon treatment with H_2O_2. The results (Figure R27.C) show how only wild-type MEFs were drastically arrested during G_2-M (it can refer to G_2/M checkpoint, Spindle checkpoint or Antephase checkpoint). The graph shows how, after 15h of release, the percentage of wild-type cells in G_2-M phases decreased only about 15.2%, whereas in knockout cells the decrease is almost doubled (28.5%). In addition, the loss of SIRT2 seemed to allow cell cycle progression because the percentage of cells in S-phase reached 22% when in WT MEFs the percentage was only 13%. In addition, the stress response upon SIRT2 loss leads to a higher number of apoptotic events (13.3% WT versus 37% KO). It appeared that in absence of SIRT2 there is problem with another checkpoint that allow the mitotic progression despite the DNA damage caused by the genotoxic agent.

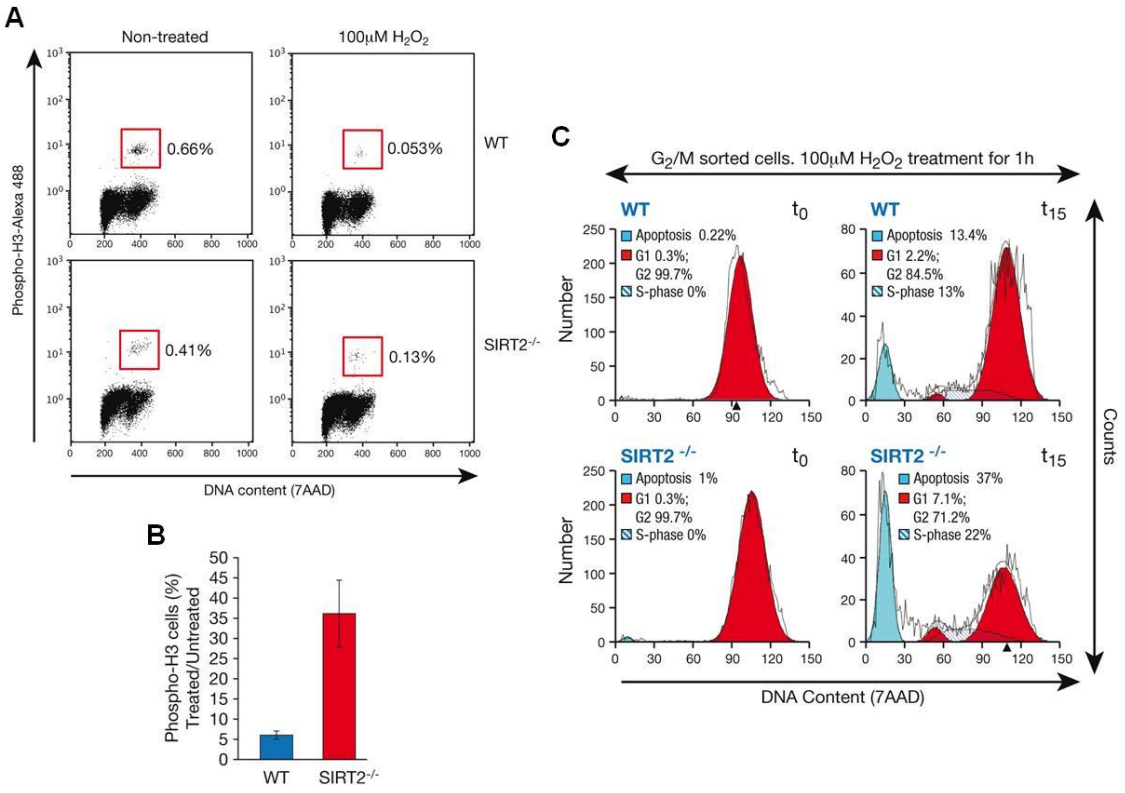


Figure R27. SIRT2 and PR-Set7 may participate in a mitotic checkpoint. A. FACS analysis of mitotic cells shown in D.Wild-type and SIRT2 knockout MEFs treated or untreated for 1 h with 100 mMH2O2 sorted by DNA content (7AAD) and a mitotic cell marker (H3 phosphorylation). **B.** Quantification of the percentage of mitotic cells in wild-type and SIRT2 knockout MEFs shown in A. **C.** The cells sorted at G2/M were incubated with 100 mMH2O2 for 1 h. Cells were harvested either immediately after 1 h of H2O2 treatment (t0) or at 15 h post-treatment (t15), fixed, and then analyzed by FACs. DNA was stained with 7AAD.

In order to ensure that SIRT2 is involved in a mitotic checkpoint at the entrance of mitosis, we performed the same experiment of non-sorted cells upon peroxide treatment, recovering SIRT2 expression in the knockout MEFs and Fibroblasts. Therefore, after re-introducing SIRT2 (SIRT2-EGFP) by retroviral infection, the cells where treated under normal

conditions or with peroxide and analyzed by FACs. As previously done, the cells were stained with 7AAD and H3S28p. The results (Figure R28) clearly show how SIRT2 is necessary for a checkpoint control during G_2-M, because the addition of the active SIRT2 protein allowed the decrease of the G_2-M population upon DNA damage. The re-expressed SIRT2 decrease the amount of mitotic cells after peroxide treatment in about 52% compared to KO cells, in both, MEFs and primary fibroblasts. Therefore, in conjunction with the previous results, this indicated that SIRT2 and PR-Set7, are involved in a checkpoint during G_2-M.

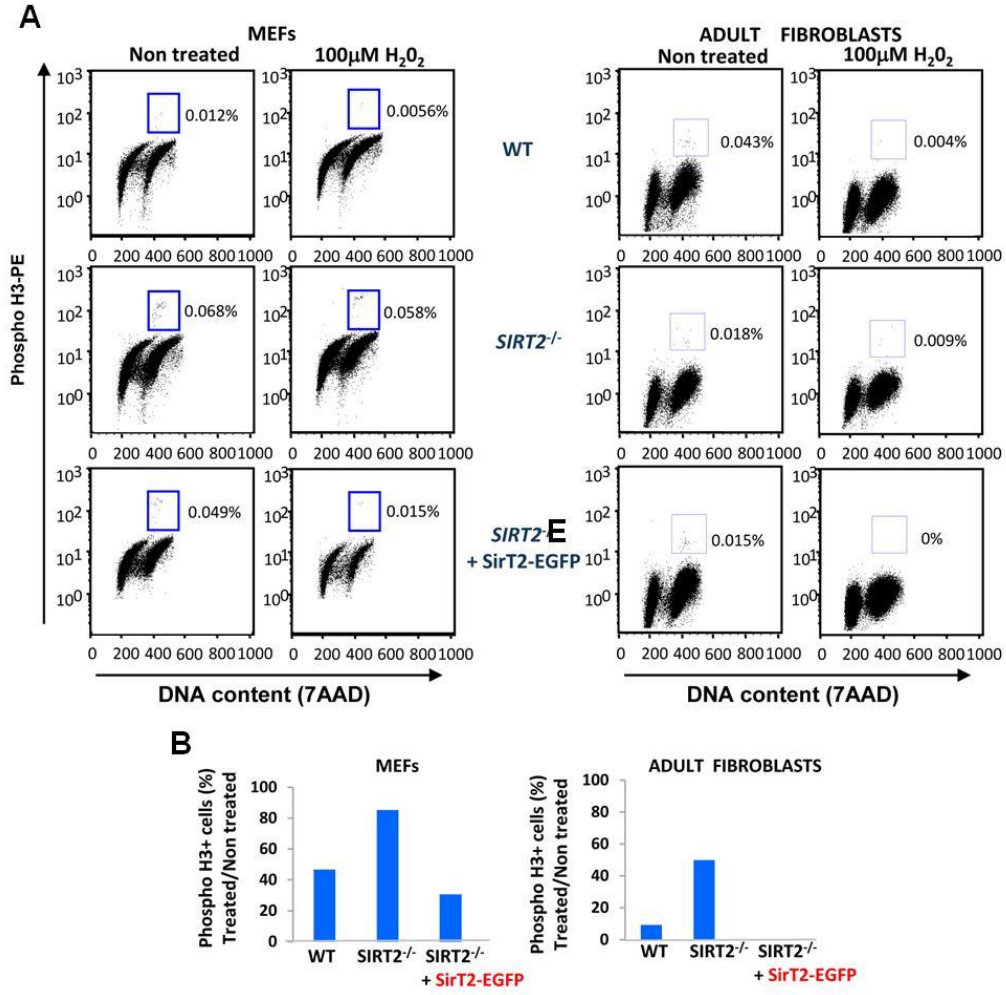


Figure R28. A. SIRT2 participates in a mitotic upon stress response. A. Rescue experiment of the mitotic arrest under oxidative conditions. WT and SIRT2 knockout adult MEFS (left) or fibroblasts (right) were infected with either control or a SIRT2-EGFP expressing retrovirus treated or untreated with 100µM H_20_2 treatment for 1h. Dot plots represent the FACS analysis of mitotic cells analyzed according to DNA content (7AAD) and a mitotic cell marker (H3S28 phosphorylation). **B.** Quantification of the percentage of mitotic cells in WT and SIRT2 knockout and rescued SIRT2 knockout MEFs in the rescue experiment shown in A.

In addition, in order to corroborate the important role of H4K16Ac in the control of mitotic progression we also used wild-type and MOF knockout or heterozygous MEFs. For that reason, we obtained the MOF conditional mice (*lox*-flanqued MOF gene) from Dr. Voss' lab, and the colony was amplified and crossed with the CRE recombinase transgenic mice obtained from

the same lab, in order to generate conditional KO and Heterozygous (He) mice (MOF deficient/CRE mice). These mice are “conditional knockout” because MOF depletion is lethal during the development; therefore, the complete MOF knockout or Heterozygous MEFs were obtained after 72-96h of 4-OH-Tamoxifen treatment, in order to activate the Cre recombinase activity and delete MOF gene (*lox* flanked).

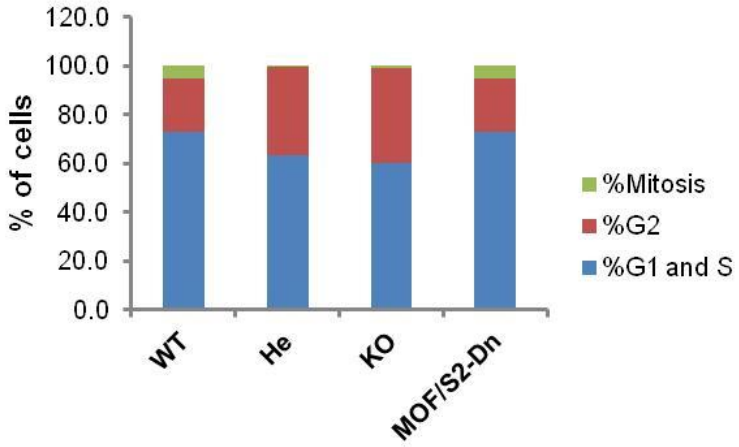


Figure R29. A. SIRT2 participates in a checkpoint of mitotic entrance. Mitotic cells (H3S28p and H3S10p staining), G_2 cells (H3S10p but not H3S28p staining) and G_1/S cells (only DAPI staining) were quantified (n>5000 for each cell line) in wild type (WT), MOF heterozygous (He), MOF knockout (KO) and double-knockout for MOF and SIRT2 (MOF/S2-Dn). WT mice refer to MEFs MOF deficient/CRE not treated and MEFs MOF non-deficient/CRE treated.

The WT and MOF knockout MEFs obtained after 4-OH-Tamoxifen treatment were analyzed by immunofluorescence in order to distinguish G_2 or mitotic cells from S and G_1 cells. For that reason, the cells were stained with the antibody against H3S10 phosphorylated (known to mark both, G_2 and mitotic cells), with H3S28p and DAPI. More than five thousand cells were analyzed in each case and the experiment was performed twice with three different MEF cell lines. The results (Figure R29, bars 1-3) clearly show how MOF knockout or knockdown (He) cells are arrested in G_2 and do not progress throughtout mitosis. The mean percentage of mitotic cells in WT MEFs was 5.3%, whereas it decreases in both KO and He MOF to 1%. These results demonstrated that upon MOF loss, when the levels of H4K16Ac decrease (Taipale et al, 2005), the cells are incapable of progress far from G_2. Therefore, suitable H4K16Ac levels may be needed for proper mitotic progression. In order to demonstrate that this G2/M checkpoint was due to H4K16Ac dynamic, we performed the same experiment including double knockout of MOF and SIRT2 MEFs. The double knockout mice of SIRT2 and MOF were obtained in our lab by crossing SIRT2 KO mice with MOF deficient/CRE mice. Several additional cross-overs were needed in order to generate MOF lox/lox-CRE mice together with $SIRT2^{-/-}$. Consequently, our double MOF/S2 knockout mice are both SIRT2 and MOF knockout after 4-OH-Tamoxifen treatment. Therefore, the immunofluorescence analysis was perfomed also using MOF/S2 KO MEFs upon 4-OH-Tamoxifen treatment. The results showed in Figure R29 demonstrate how the double knockout was able to recover the mitotic proportion of the

wild-type MEFs (5.0%). These results indicate that SIRT2 is clearly involved in a checkpoint at mitotic entrance (G2/M).

4. *Genomic integrity during cell cycle progression depends on H4K16Ac and H4K20me regulation.*

4.1. SIRT2 regulates H4K20me levels throughout the cell cycle.

The involvement of SIRT2 throughout PR-Set7 regulation into cell cycle progression became fully apparent when we realized that the cell cycle analysis of SIRT2 knockout MEFs, published in 2006 (Vaquero et al, 2006), was extremely similar to the Suv4-20h deficient MEFs, published by Schotta et al (2008) (see Figure R30). Both deficient cells showed a longer G_1 phase and a shorter S-phase, compared to the wild-type cells; meanwhile, the G_2-M phases showed just a small increase when the proteins were deleted.

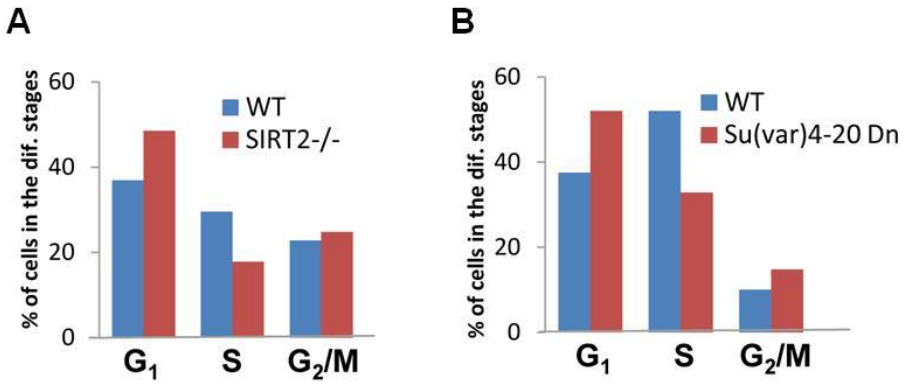


Figure R30. Cell cycle distribution upon SIRT2 deletion is similar to Suv4-20h deficiency. Distribution of primary MEFs through the cell cycle for SIRT2 knockout and Suv4-20DN (compared to their respective WT littermates). The data was reproduced from Vaquero *et al.*, 2006 (SIRT2) and Schotta *et al*., 2008 (Suv4-20hs). The latter is a courtesy from Dr Thomas Jenuwein (MPI, Freiburg) ,

The explanation of this fact attributed to H4K20me1 as the substrate of Suv4-20h enzymes (Oda et al, 2009), and accordingly, lower levels of this histone mark due to SIRT2 absence might also entail a decrease in H4K20me2/3. For that reason, we first decided to analyze the levels of these marks in our SIRT2 knockout MEFs using the immunofluorescence technique and distinguishing the different cell cycle phases according to EdU and DAPI staining (Figure R31.A-D). This technique demonstrated how the values of both histone modifications, which remain stable during the cell cycle, suffered a drastic reduction when SIRT2 protein was

depleted. The quantification of H4K20me2 immunofluorescence shows a strong decrease of this histone mark, which ranges between 40% (in G_1) and 72% (in mitosis) of reduction (Figure R31.B). Additionally, the absence of SIRT2 caused a severe diminution, of about 70% (in G_2) to 81% (in S-phase), in H4K20me3 levels (Figure R31.D). All in all this may indicate that the regulation of H4K20me1 deposition by SIRT2 is mainly affecting subsequent dimethylation and trimethylation H4K20 sites. To such a degree, these results generated an important question: Does SIRT2 also regulate Suv4-20h enzymes in order to control the whole H4K20me during the cell cycle? As a matter of fact, the levels of Suv4-20h enzymes are not cell cycle-dependent and could also contact SIRT2 during G_2-M.

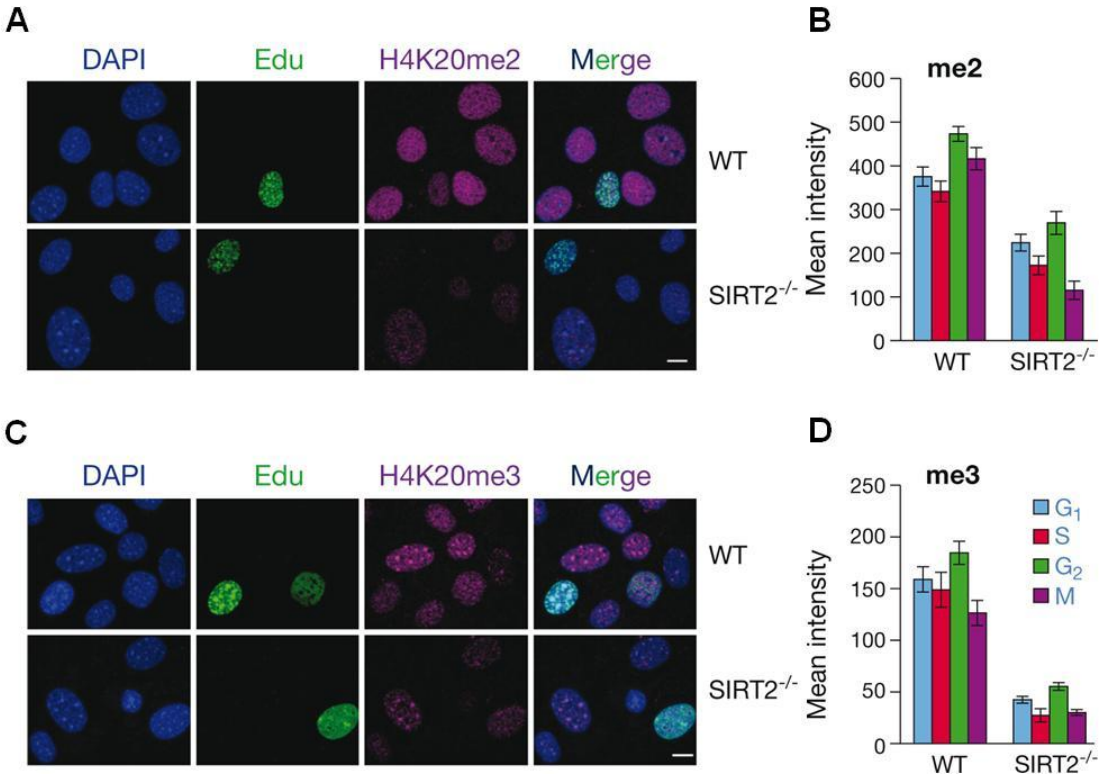


Figure R31. SIRT2 knockout cells suffer from drastically low levels of H4K20me2/3. A and C. Levels of H4K20me2 and H4K20me3 in wild-type (WT) or SIRT2 knockout MEFs determined by IF. S-phase cells were visualized by EdU staining. **B and D.** Quantification of experiment in A and C through the different stages of the cell cycle.

Therefore, in order to determine if Suv4-20h enzymes were regulated by SIRT2, we studied the possible interaction between Suv4-20h1/2 and SIRT2. We performed co-immunoprecipitation assays in 293F cells using the two isoforms of Suv4-20h1 (Suv4-20h1.1 and Suv4-20h1.2), Suv4-20h2 (Figure R31), and PR-Set7 as the positive control. HeLa cells were not used for these co-immunoprecipitation experiments because the expression of Suv4-20h proteins was abrogated in those cells. Our evidence strongly indicated that none of the Suv4-20h enzymes interact *in vivo* with SIRT2 (Figure R31, lanes 13-15); meanwhile the

positive control shows the interaction between SIRT2 and PR-Set7 (Figure R27, lane 16). These results support the role of SIRT2 in regulating H4K20me2-3 through H4K20me1 deposition during mitosis, and not due to Suv4-20h modulation.

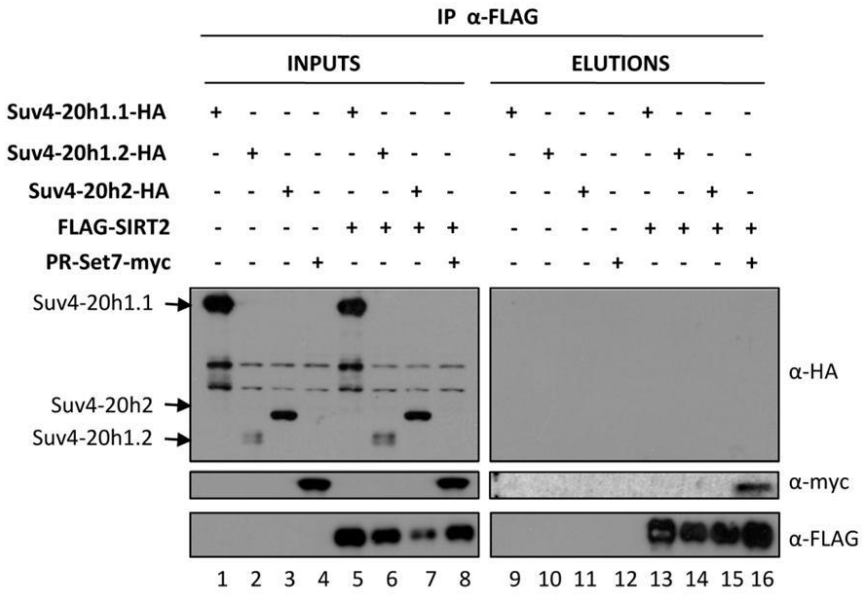


Figure R32. SIRT2 loss induces a cell cycle alteration that resembles loss of Su(var)4-20 enzymes. Immunoprecipitation of FLAG-SIRT2 (FLAG resin) with HA-tagged Suv4-20h1.1, h1.2 and h2 from whole cell extracts of 293F cells previous transfected. Myc-tagged PR-Set7 was also included as positive control (lanes 8 and 16).

4.2. SIRT2 loss affects cell cycle progression out of mitosis.

As it was described in the introduction, H4K20me1, H4K20me2 and H4K20me3 are involved in several processes that include regulation of cell cycle progression, genome integrity maintenance, DNA repair and replication among others (Beck et al., 2012; Oda et al., 2010; Oda et al., 2009; Rice et al., 2002; Schotta et al., 2004; Schotta et al., 2008; Tardat et al., 2010). Certainly, the depletion of any of these histone methyltransferases drastically affects genome integrity and cell cycle progression. Therefore, the above-described data indicate that SIRT2 might be important for all these processes by controlling H4K20me1 deposition and subsequent H4K20me2-3 levels. In order to confirm these possible functions of SIRT2, we first decided to determine whether SIRT2 loss is associated with H4K20me2-3 deficiency phenotype during S-phase.

Problems in S-phase have been detected due to low levels of H4K20me2/3 as well as aberrant monomethylation of H4K20. The loss of Suv4-20h in mice has been described as

delaying S-phase entry (Schotta et al., 2008), and non-degradable PR-Set7 causes re-initiation of DNA replication (Tardat et al., 2010). In line with these phenotypes, the decrease of H4K20me1/2/3 levels, due to SIRT2 deficiency, might entail problems in S-phase entry and progression. To address whether S-phase entry is delayed under SIRT2 loss, we sorted wild-type and SIRT2 knockout primary fibroblasts in G_1-phase in order to analyze their entrance to S-phase. The sorted cells were re-plated and incubated under normal conditions for 17h or 24h. We used FACS analysis to determine the S-phase progression using EdU as a replication-dependent marker, and 7AAD to determine the DNA content (Figure R33). The graph shows a significant delay for SIRT2 knockout cells in S-phase entry as compared with wild-type cells. Indeed, it was found that the percentage of cells that entered S-phase after 17h was decreased by about 28% upon SIRT2 depletion.

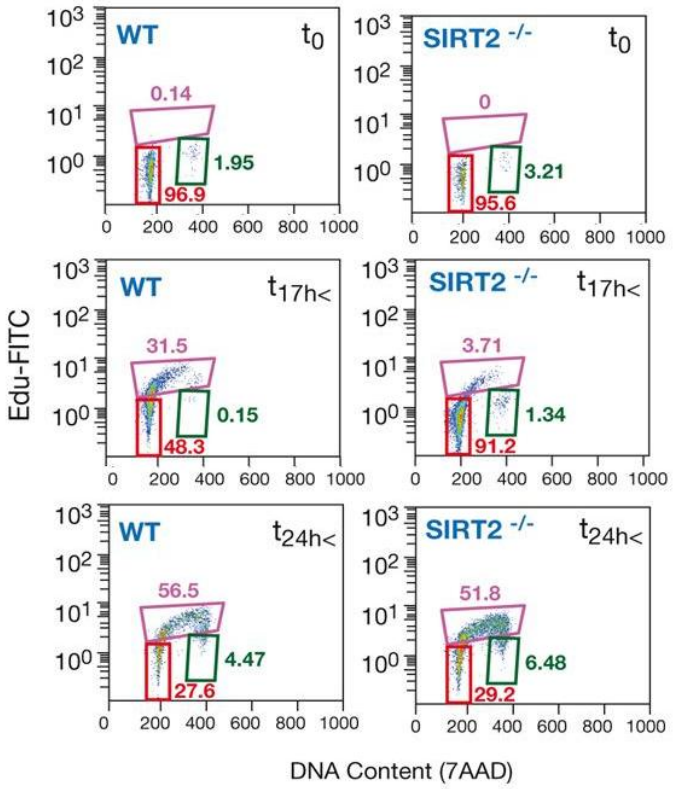


Figure R33. SIRT2 deficiency affects S-phase entry. Analysis of S-phase entrance of wild-type and SIRT2 knockout fibroblasts. Cells were sorted at G_1 and harvested at the indicated times. S-phase progression was measured by FACS using DNA content either alone (left panel) or in combination with EdU (right panel).

In addition, we investigated possible repeated DNA replication upon PR-Set7 deregulation due to SIRT2 loss. For this reason, we analyzed the cell cycle distribution of WT and SIRT2 KO primary fibroblasts by staining the DNA with 7AAD and the replicative cells with

EdU. The study of polyploidy cells was included in the analysis (Figure 34.A left panel), together with the detection of Cyclin B1. Both, the presence of Cyclin B1 in replicative cells (Figure 34.B) and EdU staining of 4N cells (Figure 34.A right panel) would indicate re-replication events after the original S-phase replication; because Cyclin B1 is not expressed in S-phase cells (mostly in mitosis), and 4N cells are suppose to have ended replication. In the left panel of Figure R29.A, the percentage of cells with 4N content increased in 6.53% upon SIRT2 loss; additionally, the percentage of these cells stained with EdU (Figure 34.A right panel) was 6.6% higher than in wild-type fibroblasts, and the Cyclin B1 increased 13% (Figure 34.B) when SIRT2 was absent. Then our results demonstrated increased re-replication events in SIRT2 deficient primary fibroblasts.

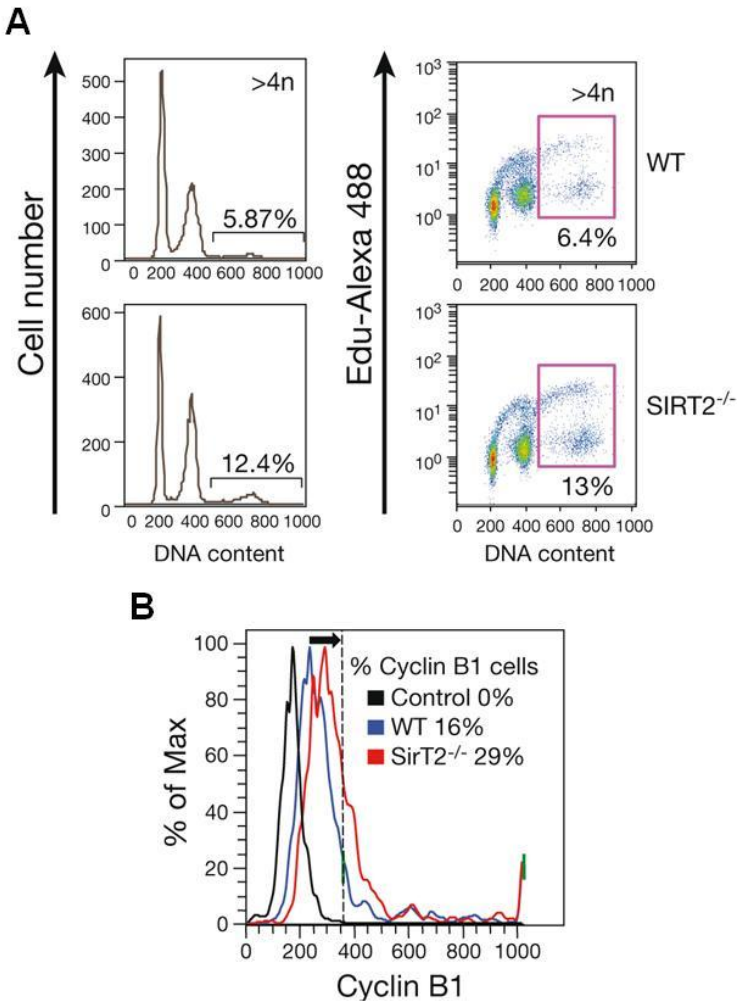


Figure R34. SIRT2 deficiency affects the DNA replication process. A. Cell cycle profiles of wild-type and SIRT2 knockout fibroblasts obtained by FACS analysis. The values shown are the percentage of polyploid cells (DNA content >4N). (Right panels) EdU staining denotes the presence of polyploid cells with rereplicated DNA. **B.** Overlay histograms showing expression of Cyclin B1 in polyploid cells from wild-type and SIRT2 knockout fibroblasts (A).

Together this data indicates proliferation defects in SIRT2 knockout cells that would be consistent with impaired cell cycle progression due to aberrant H4K20me1-3.

4.3. SIRT2 regulates heterochromatin formation and DNA repair associated with H4K20me.

Furthermore, H4K20me participates in heterochromatin formation and DNA repair signaling (Oda et al., 2010; Oda et al., 2009; Rice et al., 2002; Schotta et al., 2004; Schotta et al., 2008). On one side, H4K20me1 and H4K20me3 have been described to participate in facultative and constitutive heterochromatin formation, respectively (Gonzalo et al., 2005; Lu et al., 2008; Nishioka et al., 2002; Regha et al., 2007; Schotta et al., 2004); and on the other side, H4K20me1 and H4K20me2 have been involved in 53BP1 recruitment to DBSs in order to favor DNA repair (Botuyan et al., 2006; Oda et al., 2009; Sanders et al., 2004).

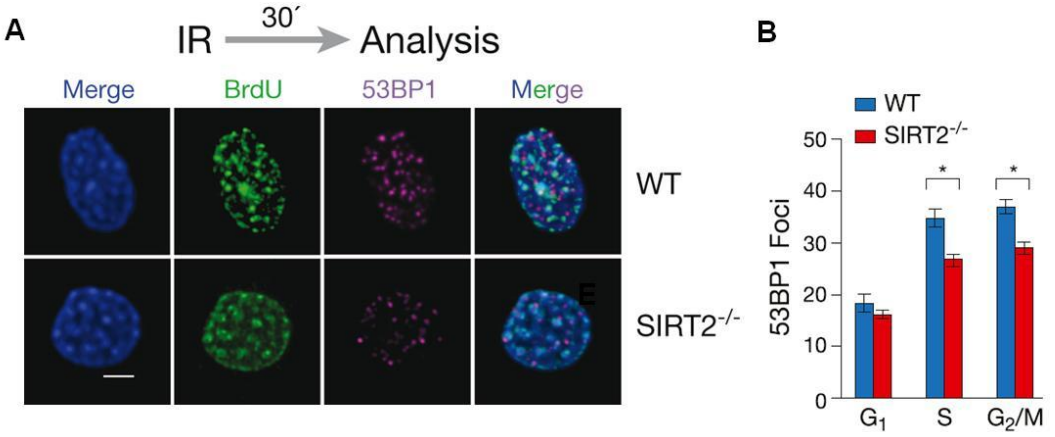


Figure R35. Loss of SIRT2 affects several functions of H4K20me2/3. A. Microscopic analyses of 53BP1 focus formation at X-ray (IR)-induced DSBs in wild-type and SIRT2 knockout MEFs. The S-phase cells were stained by EdU incorporation and counterstained with DAPI. Bar, 5mm. **B.** Quantitative analysis of the images (n>20 for each time point) showed an important decrease in 53BP1 upon SIRT2 deficiency, as shown in A, throughout progression of the cell cycle.

According to the capacity of H4K20me1-2 to recruit 53BP1, we tested if the loss of SIRT2 with the subsequent decrease of the three levels of H4K20 methylation affected 53BP1 recruitment to the chromatin after DNA damage. We performed immunofluorescence analysis of 53BP1, after X-ray radiation, in WT and SIRT2 KO MEFs; discriminating G_1-, S- and G_2-M phases by BrdU and DAPI staining. Our immunofluorescence images (Figure R35.A) show substantially lower levels of this scaffolding protein of the DNA repair pathway, 53BP1, in the absence of SIRT2. In fact, the quantification of the immunofluorescence analysis (Figure R35.B) demonstrated how the number of 53BP1 foci significantly decreased about 20% in S-phase and 24% in G_2-M-phases upon SIRT2 deficiency. This effect is clearly due to the H4K20me1-2 decrease upon SIRT2 loss, because the levels of DNA damage in these cells were significantly

higher, as was demonstrated by our previous experiments (Figures R4-6). In other words, according to the DNA damage levels, SIRT2 knockout cells would entail higher number of 53BP1 foci; however, our experiments showed lower levels of 53BP1 protein in absence of SIRT2, according to lower levels of H4K20me.

Finally, the involvement of SIRT2 throughout H4K20me3 regulation in the formation of higher order of chromatin compaction was addressed by the analysis of γ-satellite expression and the levels of H3K9me3. The analysis of the γ-satellite expression is the easiest way to indirectly detect the compaction level of heterochomatin in mice, and has already been used in previous studies (Bosch-Presegue et al., 2011). γ-satellite, also known as major satellite, in mice are AT-rich arrays up to several megabases in length, localized in pericentromeric regions. This region, together with the minor satellite, is generally transcriptionally inactive due to enrichment of repressive histone modifications, DNA methylation and repressive transcription factors (Lehnertz et al., 2003; Martens et al., 2005). Therefore, the analysis of the expression of these regions is an indirect method to determine the heterochromatin state in mice cells. In addition, the trimethylation of H3K9 is an important histone mark of heterochromatin (Bosch-Presegue et al., 2011), so its quantification is also important to determine the role of SIRT2 in heterochomatin.

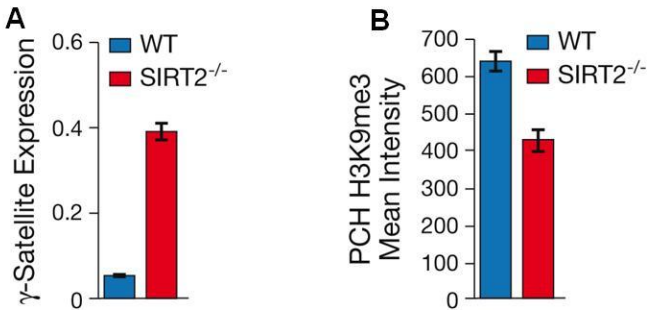


Figure R36. Loss of SIRT2 affects heterochromatin formation. A. mRNA levels of pericentromeric γ-satellite in WT and SIRT2$^{-/-}$ MEFs analyzed by Real-Time PCR. **B.** Average density of H3K9me3 in pericentromeric heterochromatin in wild-type and SIRT2 knockout MEFs.

In order to determine γ-satellite expression levels, we used the quantitative PCR technique to quantify its mRNA in WT and SIRT2 knockout MEFs (n=5 WT lines; and n=5 KO lines). The analysis by quantitative PCR (Figure R36.A) showed higher levels of γ-satellite expression in absence of SIRT2 (0.39+/-0.026) compared to the wild-type (0.05+/+0.009). More specifically, the expression of gamma-satellites was eight-fold higher in the absence of SIRT2 compared to WT, with a high significance ($p<0.001$). These results indicated how the chromatin organization was being compromised by increased levels of H4K16Ac in absence of SIRT2.

The levels of H3K9me3 were analyzed by IF analysis in WT and SIRT2 KO MEFs. Its quantification (Figure R36.B) shows a reduction of 35% in the intensity of H3K9me3 staining

when SIRT2 was depleted. Therefore, as was already described (Figure R2), SIRT2 is essential for the maintenance of genome integrity.

Overall, our data above suggests that SIRT2 is involved in the maintenance of the genome integrity in mitosis and S-phase, not only by deacetylating H4K16Ac and promoting H4K20me1 deposition, but also by influencing H4K20me2/3 levels during cell cycle.

5. SIRT2 is a tumor suppressor.

Taking all the results explained so far, we would have expected a characteristic phenotype in our SIRT2 knockout mice. However, as it was described above, the mice did not display any phenotypic difference with respect to wild-type animals, including new-born size, adult size, aging phenotype, mendelian problems, spontaneous tumor development, etc. How could that be possible if this protein seems to be involved in such an important processes? Certainly, the role of SIRT2 as tumor suppressor was already published in 2011 by Dr. Deng's lab. Their SIRT2 knockout mice developed spontaneous tumors within their first year. However, the mice strain that they used for their experiments were chimeras of a mixed background (NIH Black Swiss and C57B6), whereas our strain, C57-BL6, is known to be highly resistant to tumor development (DiGiovanni, Bhatt & Walker, 1993).

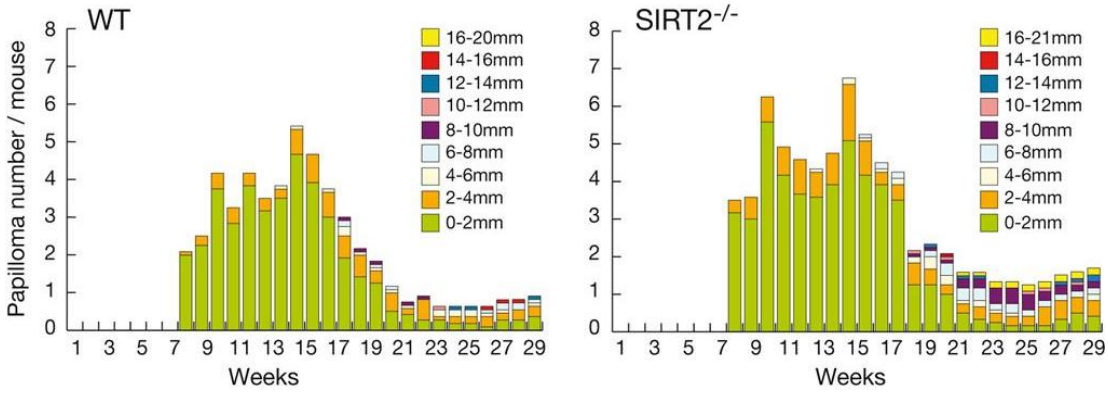


Figure R37. SIRT2 knockout mice are more prone to tumorigenesis. Number of papillomas of each size per mouse, at the indicated number of weeks after starting the DMBA/TPA treatment. The treatment was interrupted at week 15, but the papillomas analysis continued. **B.** Representative wild-type and SIRT2 knockout animals in week 25. **C.** Papilloma size in wild-type (WT) versus SIRT2 knockout mice at week 25 after starting the treatment.

Due to this, we decided to perform a well known classical skin tumorigenesis assay (Blanco et al., 2007), which would allow us to constrain the appearance of the possible phenotype. This assay consisted of a single dose of DMBA and fifteen weeks of treatment with

TPA, twice a week; where the DMBA consist of the mutagenic agent and the TPA is the proliferation-inducing agent. The size of the papillomas was measured each week and quantified according to the graphs in Figure R37.A. It can be inferred from Figures R37 and R33, that the main difference between wild-type and SIRT2 knockout mice was not the response to the tumors, but the size of the tumors themselves. The number of papillomas was not significantly higher in SIRT2 knockout with respect to wild-type mice, but the size of those papillomas was significantly higher from the 19th week. The size of the majority of the papillomas developed in SIRT2 knockout mice were over 8-10mm; whereas in wild-type mice the size of the papillomas was variable and mostly under 8-10 mm (Figure R37). For example, in the 25th week of the treatment, the media of the papilloma size was 1.7 times higher in SIRT2 knockout mice respect to the wild-type (Figure R38.B); as it can also be inferred from the pictures (Figure R38.A).

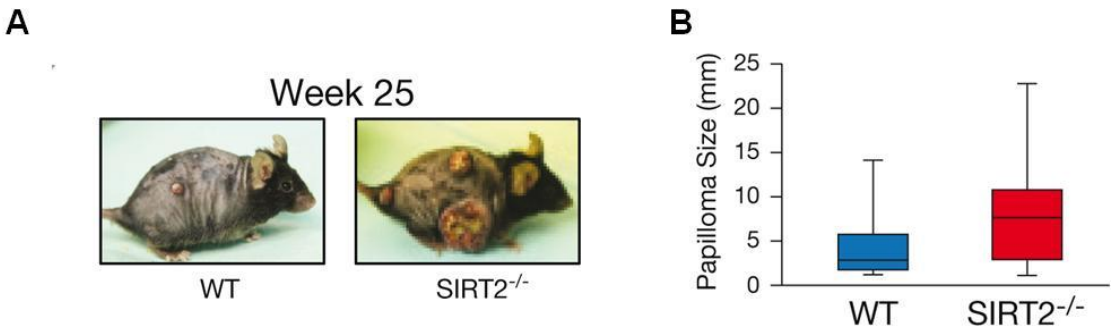


Figure R38. SIRT2 knockout mice are more prone to tumorigenesis. A. Representative wild-type and SIRT2 knockout animals at week 25. **B.** Papilloma size in wild-type (WT) versus SIRT2 knockout mice at week 25 after starting the treatment.

Notwithstanding, the most impressive result from this experiment resides in the survival rate (Figure R39). The percentage of survival showed in the graph for the wild-type mice was 100% during the whole treatment and after the end of the experiment; on the contrary, SIRT2 knockout mice died prematurely from the 21th week (Figure R39). Some of the SIRT2 KO mice deaths were due to the bad prognosis of their developed tumors; however, we were unable to determine the cause of death of all the KO mice due to their sudden death.

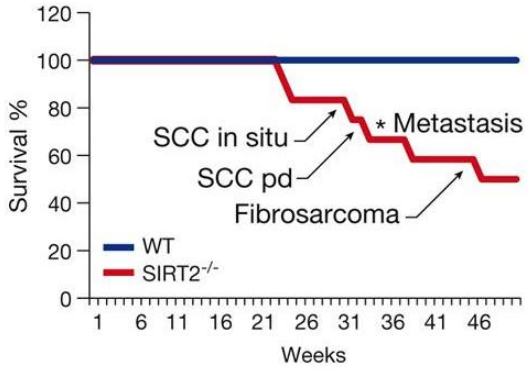


Figure R39. SIRT2 loss decreases the survival rate under tumorigenesis treatment. Survival curve of DMBA/TPA-treated wild-type and SIRT2 KO mice.

The loss of SIRT2 favored the appearance of metastatic events and aggressive tumors, such as squamous cell carcinoma and fibrosarcoma (Figure R40), diminishing the survival of the animal. In point of fact, squamous cell carcinoma (SCC) is a type of cancer that may occur in many different organs, including the skin; and it is considered a malignant tumor of squamous epithelium (epithelium that shows squamous cell differentiation) (Dallaglio et al., 2013). Fibrosarcoma (fibroblastic sarcoma) is a malignant mesenchymal tumor derived from fibrous connective tissue and characterized by the presence of immature proliferating fibroblasts or undifferentiated anaplastic spindle cells in a storiform pattern (Vascellari et al., 2003). Consequently, both types of tumor growth have been related to metastasis (Kim et al., 2008).

Therefore, these results confirm the role of SIRT2 as a tumor suppressor.

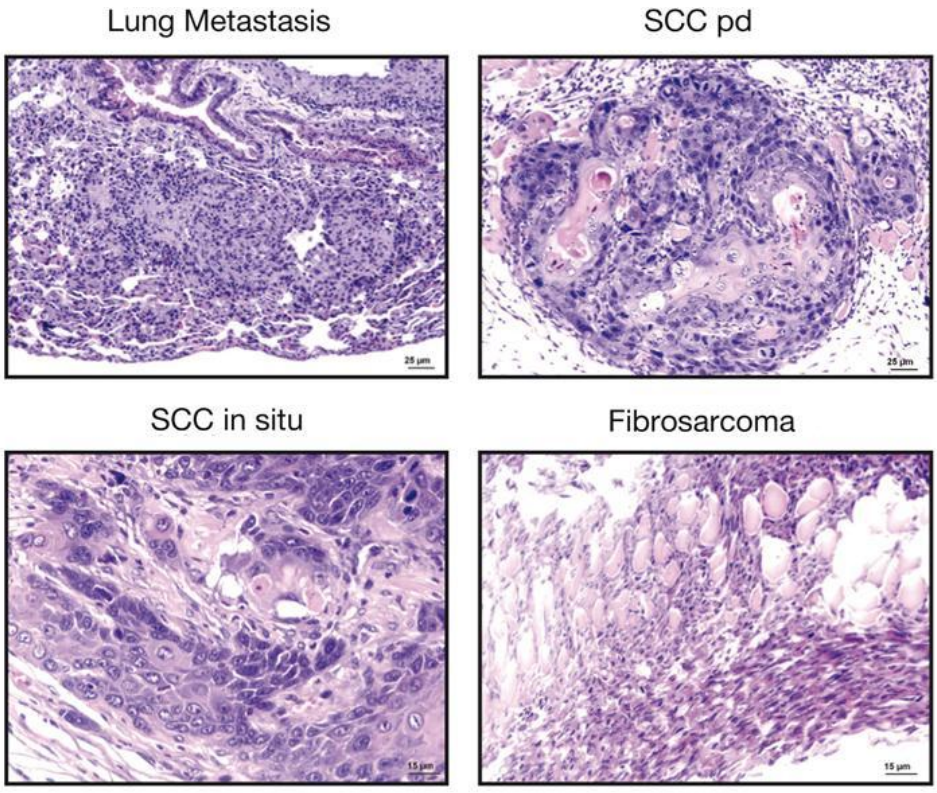


Figure R40. SIRT2 deficiency promotes tumor development. Representative examples of the neoplasias and metastases indicated in Figure R33 and developed by SIRT2 knockout mice**. Top left image:** Lung metastasis of a poorly differentiated skin squamous cell carcinoma, which shows lymphoplasmocytic infiltrate. **Top right image:** Poorly differentiated skin squamous cell carcinoma (SCC pd) with pleomorphism and numerous mitotic cells, clearly growing invasively into the dermis, subcutaneous tissue, and subcutaneous muscle. **Bottom left image:** In situ skin squamous cell carcinoma (SCC in situ) shows focal cellular atypia, high number of mitotic cells and basal membrane disruption. **Bottom right image:** Cutaneous fibrosarcoma that shows pleomorphic spindle cells, as well as invasion and destruction of dermis, subcutaneous tissue and skeletal muscle.

6. Opposite regulation of H4K20me enzymes by SIRT1.

While we were working with SIRT2 and PR-Set7, we realized that the other main H4K16Ac deacetylase, SIRT1, might be also involved in H4K20me1-3 deposition out of mitosis. The main difference between SIRT2 and SIRT1 resides in their cellular localization. While SIRT2 is only present within the nucleus from G_2/M transition to the end of mitosis; SIRT1 is localized in the nucleus constantly and could regulate H4K20me1-3 deposition out of mitosis.

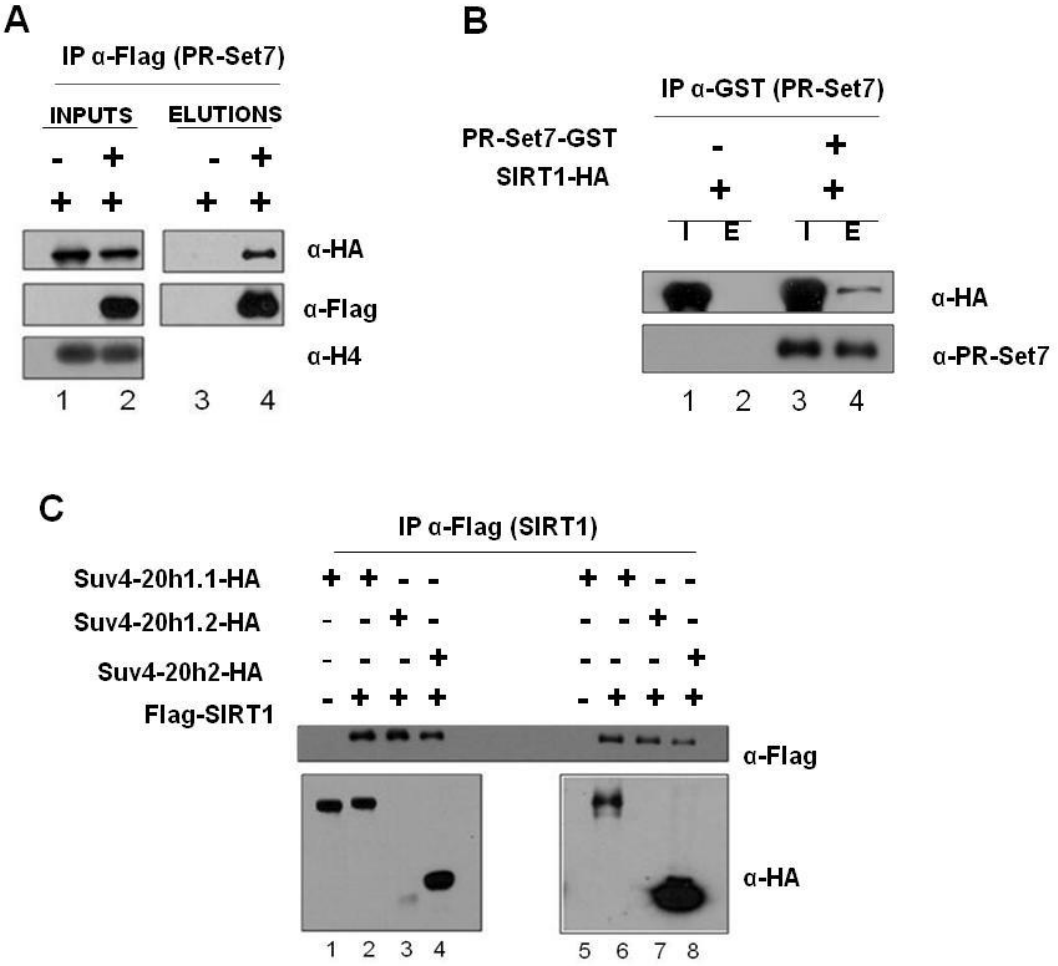


Figure R41. SIRT1 by directly interacts with PR-Set7. A. FLAG resin immunoprecipitation of extracts from HeLa cells previously transfected with Flag-PR-Set7 and/or SIRT1-HA as indicated. Inputs and elutions are indicated. **B.** *In vitro* pull-down of recombinant purified GST-PR-Set7 and SIRT1-HA purified from mammalian cells. The pull-down was against GST-PR-Set7 using Glutathione Sepharose 4B beads. (I) correspond to Inputs and (E) to elutions. **C.** FLAG resin immunoprecipitation of extracts from HeLa cells previously transfected with Flag-SIRT1 and/or Suv4-20h1.1-HA/Suv4-20h1.2-HA/Suv4-20h2-HA, as indicated. Inputs correspond to lanes 1-4 and elutions are lanes 5 to 8.

To confirm that PR-Set7 regulation may not be an exclusive function of SIRT2, we performed co-immunoprecipitation studies between SIRT1 and PR-Set7. In addition, due to the important role of Suv4-20h out of mitosis and the involvement of both SIRT1 and Suv4-20h2 in heterochromatin formation (Bosch-Presegue et al., 2011; Gonzalo et al., 2005; Regha et al.,

2007; Schotta et al., 2004; Vaquero et al., 2007), we also analyzed the possible interaction of SIRT1 with the three Suv4-20h enzymes. The study was performed by co-immunoprecipitation of Flag-PR-Set7 and SIRT1-HA in transfected HeLa cells; and Flag-SIRT1 and Suv4-20h1.1-HA, Suv4-20h1.2-HA and Suv4-20h2-HA in transfected 293F cells. Surprisingly, our results (Figure R41. A and C) confirm the interaction of SIRT1 with PR-Set7, Suv4-20h1.1 and Suv4-20h2. In addition, pull-down experiments were performed using recombinant GST-PR-Set7 purified from bacteria and SIRT1-HA purified from mammalian cells. This experiment (Figure R41.B) confirmed the direct interaction between SIRT1 and PR-Set7. The fact that this interaction is not reflected in the mitotic levels of H4K20me1 (Figure R14) may be due to the fact that contrary to SIRT2, SIRT1 could regulate PR-Set7 and H4K20me1 out of mitosis, and under specific circumstances.

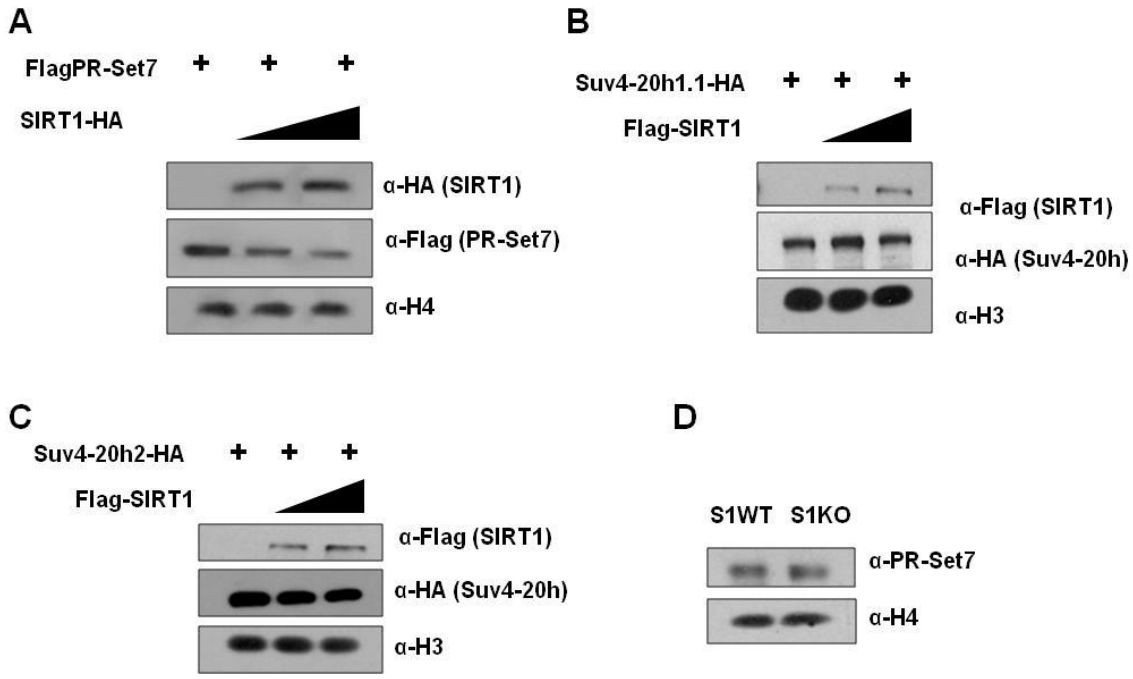


Figure R42. SIRT1 negatively regulates PR-Set7 and Suv4-20h2. A. Total extracts from HeLa cells previously transfected with Flag-PR-Set7 and increasing amounts of SIRT1-HA, as indicated. Histone H4 was used as a loading control. **B.** Total extracts from HeLa cells previously transfected with Suv4-20h1.1 and increasing amounts of Flag-SIRT1, as indicated. Histone H4 was used as a loading control. **C.** Total extracts from HeLa cells previously transfected with Suv4-20h2 and increasing amounts of Flag-SIRT1, as indicated. Histone H4 was used as a loading control. **D.** Detection of endogenous PR-Set7 levels when using total extracts from immortalized MEFs derived from wild-type and SIRT1 knockout MEFs.

Interestingly, while working with transfected SIRT1-HA and Flag-PR-Set7 proteins in HeLa cells, we realized that Flag-PR-Set7 levels were compromised when it was co-transfected with SIRT1-HA. Contrary to what we expected, as it can be inferred from Figure R42.A, increasing SIRT1 levels trigger a decrease of the total transfected PR-Set7 protein. We decided to investigate if this effect on PR-Set7 by SIRT1 was specific to this enzyme by determining if

SIRT1 had the same impact on Suv4-20h enzymes. Therefore, we co-transfected Suv4-20h1.1-HA or Suv4-20h2-HA with increasing levels of Flag-SIRT1 in 293F cells, and the total extracts were analyzed by western blot. We used Suv4-20h1.1 and Suv4-20h2 because both interacted with SIRT1 in previous experiments (Figure R42.C). The western blot analysis (Figure R42.B-C) shows how SIRT1 negatively affects Suv4-20h2 protein levels as well (Figure R42.C), but not Suv4-20h1.1 (Figure R42.B), indicating a specific down-regulation of PR-Set7 and Suv4-20h2 by SIRT1.

In order to confirm how SIRT1 negatively regulates PR-Set7 levels we used SIRT1 immortalized knockout MEFs to analyze the total amount of endogenous PR-Set7 protein in absence of SIRT1. The Suv4-20h1.1 or Suv4-20h2 levels were not analyzed because there was not any useful specific antibody available. However, the detection of endogenous PR-Set7 in absence or presence of SIRT1 did not show any difference (Figure R42.D); thus, the absence of SIRT1 did not seem to affect endogenous PR-Set7 levels. This fact made us look deeper into the PR-Set7 protein degradation process, which is a really complex proteosome-dependent mechanism, enhanced upon DNA damage or stress (Oda et al., 2010; Figure R30.B). In addition, from the earliest studies, SIRT1 has been widely involved in combating oxidative stress throughout different mechanism of cytoprotection or pro-apoptosis. This sirtuin mediates the stress response throughout the modulation of the chromatin (Bosch-Presegue & Vaquero, 2013; Vaquero, 2009) and the regulation of stress-related factors (Motta et al., 2004; Rothgiesser et al., 2010b; Yang et al., 2005). Accordingly, it would be possible that the relationship between these two proteins was happening under stress conditions; in fact, the transfection process is also considered as a stress stimulus to the cells. Then, we decided to analyze the DNA damage-dependent degradation of PR-Set7 in absence of SIRT1, as we previously performed with SIRT2 knockout cells (Figure R25). The levels of total endogenous PR-Set7 upon peroxide treatment, using the immortalized SIRT1 knockout and wild-type MEFs, were analyzed by western blot. The results (Figure R43) showed how PR-Set7 levels did not decrease under peroxide treatment when SIRT1 was absent (Figure R43, lane 4); meanwhile, in wild-type cells, PR-Set7 levels decreased drastically as it was already described by other authors (Oda et al., 2010). Ergo, SIRT1 absence seemed to inhibit PR-Set7 degradation upon DNA damage. This result indicates that SIRT1 regulates PR-Set7 levels under stress conditions, and maybe also Suv4-20h2.

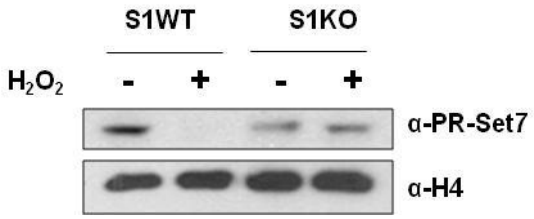


Figure R43. SIRT1 regulates PR-Set7 levels under stress conditions. Total extracts from immortalized MEFs derived from wild-type and KO SIRT1 mice, after peroxide treatment.

According to these results we hypothesized that the effect of SIRT1 might be favoring PR-Set7 and Suv4-20h2 protein degradation. According to the mechanism already described by Oda et al 2010, PR-Set7 is degraded by proteosome upon stress conditions. In order to investigate the degradation pathway, both co-transfections, (PR-Set7+/-SIRT1) and (Suv4-20h2+/-SIRT1), were treated with a proteosome inhibitor (MG132) or a lysosome inhibitor (Amonium chloride). Both pathways are involved in protein degradation, and MG132 was already used by other authors to inhibit proteosome-dependent PR-Set7 degradation (Oda et al., 2010, Tardat et al., 2007). However, contrary to our expectations, as the Figure R38 shows, the proteosome inhibitor MG132 did not change the negative effect of SIRT1 over both proteins (Figure R44.A, lanes 4-6; Figure R44.B, lanes 4-6); meanwhile, the Amoniun chloride treatment (lisosome inhibitor) seemed to reduce in some grade that effect for both proteins, PR-Set7 (Figure R44.A, lanes 7-9) and Suv4-20h2 (Figure R44.B, lanes 7-9). Consequently, the lysosome degradation pathway, and not the proteosome degradation pathway, should be somehow involved in the effect of SIRT1 on PR-Set7 and Suv4-20h2 levels.

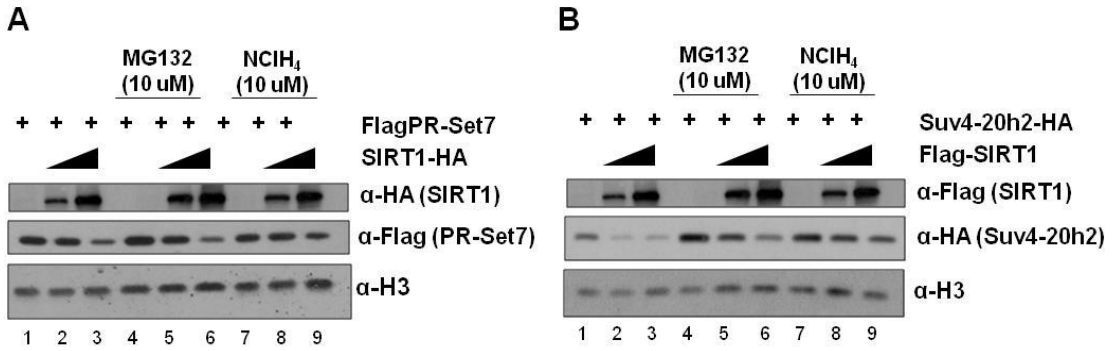


Figure R44. SIRT1 regulates PR-Set7 through a non proteosome-dependent pathway. A. Total extracts from HeLa cells transfected with Flag-PR-Set7 and increased levels of SIRT1-HA, and treated with MG132 (proteasome inhibitor) or $NCIH_4$ (lysosome inhibitor) as indicated. B. Total extracts from 293F cells transfected with Suv4-20h2-HA and increased levels of Flag-SIRT1, and treated with MG132 (proteasome inhibitor) or $NCIH_4$ (lysosome inhibitor) as indicated.

7. SIRT1 participates in mRNA regulation.

Together with proteosome, one of the major pathways of protein degradation in eukaryotic cells involves the uptake of proteins by lysosomes. Lysosomes are membrane-enclosed organelles that contain an array of digestive enzymes, including several proteases. They have several roles in cell metabolism, including the digestion of extracellular proteins taken up by endocytosis as well as the gradual turnover of cytoplasmic organelles and cytosolic proteins (Dice, 2000). According to the protein targeted by the several lysosomal pathways, that include cytosolic proteins, membrane receptor proteins, or cytosolic organelles proteins, neither PR-Set7 nor Suv4-20h2 seemed to be a possible target of this method of degradation. Both

proteins are nuclear and there is no evidence of any possible shuttle to the cytoplasm. Therefore, the lysosome degradation pathway should be affecting these two histone methyltransferases by a different mechanism. Indeed, Lysosomes contain various hydrolases that can degrade proteins, lipids, nucleic acids and carbohydrates. The lysosomes contain enzymes capable of degrading DNA and RNA completely to nucleosides and phosphate. For instance, in hepatocytes, lysosomal nucleic acid degradation may serve largely to support autophagy degradation of endogenous nucleic acids (John B. Lloyd, 1996). Therefore, this may indicate that SIRT1 might regulate PR-Set7 and Suv4-20h2 mRNA levels instead of the protein degradation.

In order to demonstrate this hypothesis, we decided to analyze the behavior of the mRNA of PR-Set7 and Suv4-20h2 in presence of SIRT1. Thus, HeLa cells were trasfected with SIRT1-HA or with the empty vector, and synchronized using a double thymidine block. The cells were harvested at S-, G_2-M and G1-phases and the mRNA levels were analyzed by quantitative PCR. The analysis (Figure R45) showed a cell cycle-dependent degradation of both PR-Set7 and Suv4-20h2 in presence of SIRT1. In the case of PR-Set7, the statistics analysis demonstrated that addition of SIRT1 significantly decreased mRNA levels in S-phase (Figure R45.A); whereas the mRNA degradation of Suv4-20h2 was significantly enhanced in G_1 phase (Figure R45.B). The data above pointed out a new role of SIRT1 in mRNA degradation using lysosomal machinery; and its involvement in regulating PR-Set7 and Suv4-20h2 levels throughout this mechanism.

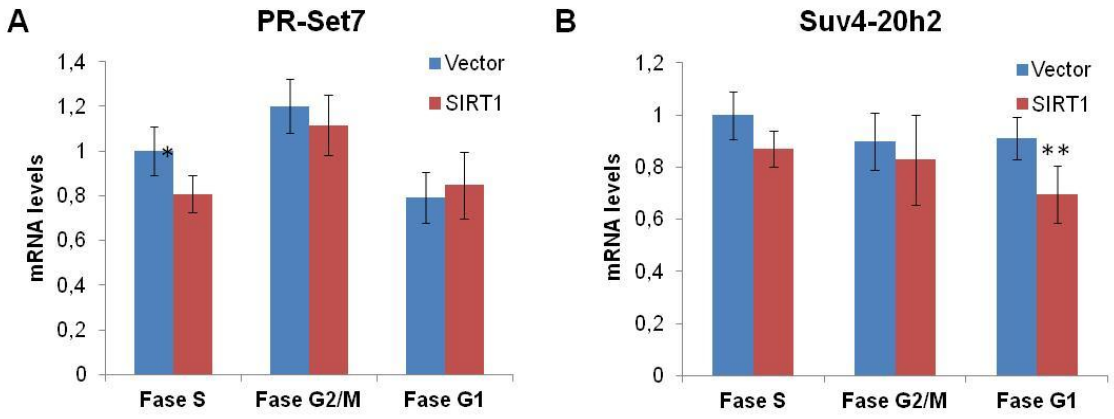


Figure R45. SIRT1 regulates PR-Set7 and Suv4-20h2 mRNA levels. A. Quantification of endogenous PR-Set7 mRNA levels by Real Time PCR. HeLa cells were transfected with or without SIRT1-HA, synchronized and harvested at indicated cell cycle stages. **B.** Quantification of endogenous Suv4-20h2 mRNA levels by Real Time PCR. HeLa cells were transfected with or without SIRT1-HA, synchronized and harvested at indicated cell cycle stages.

Nevertheless, in order to ensure that SIRT1 promotes the degradation of PR-Set7 and Suv4-20h2 mRNA, we decided to use a common mRNA degradation assay using Actinomycin D. This DNA intercalator is commonly used as inhibitor of transcription, because it can prevent the progression of RNA polymerases (Casse et al., 1999).

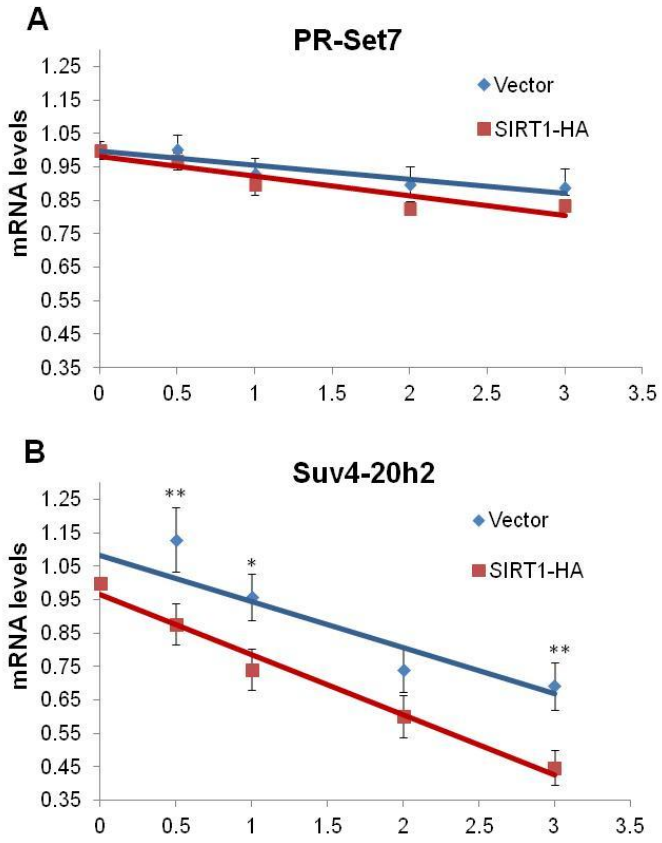


Figure R46. SIRT1 regulates Suv4-20h2 mRNA degradation. A. Quantification of endogenous PR-Set7 mRNA levels by Real Time PCR. HeLa or 293F cells were transfected with or without SIRT1-HA, treated with Actinomycin D and harvested at indicated times. The representation includes a tendency line according to the values **B.** Quantification of endogenous Suv4-20h2 mRNA levels by Real Time PCR. HeLa or 293F cells were transfected with or without SIRT1-HA, treated with Actinomycin D and harvested at indicated times. The representation includes a tendency line according to the values.

For the experiment, we transfected HeLa or 293F cells with SIRT1-HA or with the empty vector. Then the cells were treated with Actinomicyn D for 0.5, 1, 2 and 3 hours, according to "material and methods" section. The levels of mRNA levels were analyzed by quantitative PCR. Our final results (Figure R41) showed a clear involvement of SIRT1 in Suv4-20h2 degradation (Figure R41.A); meanwhile the same effect was not observed for PR-Set7 (Figure R41.B). The levels of PR-Set7 mRNA are barely affected by Actinomycin D, in presence or absence of SIRT1 (Figure R41.A), and the statistical analysis was not able to find any significance between both conditions. Contrary, the levels of Suv4-20h2 mRNA were clearly degraded upon Actinomycin D treatment without SIRT1 (Figure R41.B, blue line), and this degradation was significantly enhanced by SIRT1 addition (Figure R41.B, red line). This data supports the role of SIRT1 in Suv4-20h2 mRNA degradation by lysosomes, but it is not as clear for PR-Set7.

In order to investigate if the effect on Suv4-20h2 mRNA degradation was directly due to SIRT1 binding to the mRNA molecule, we performed RNA immunoprecipitation assays (RIP). For these experiments, HeLa cells were transfected with SIRT1-HA or with the empty vector,

and synchronized by a double thymidine block. As previously done, the cells were harvested at S-, G2-M and G1-phases. Cytoplasmic and Nuclear extracts were obtained and used to immunoprecipitate SIRT1-HA using anti-HA antibody and magnetic beads. The elutions were treated with DNase and the mRNA was analyzed by quantitative RNA. The results (data not shown) were not able to demonstrate the binding of SIRT1 to the mRNA of Suv4-20h2. This data may indicate that SIRT1 is regulating Suv4-20h2 mRNA levels indirectly. Further investigation is needed to determine the possible pathway.

Discussion

Discussion

When we started this study the role of SIRT2 in chromatin remodeling during the cell cycle was restricted to H4K16Ac deacetylation during mitosis according to *in vitro* assays (Vaquero et al, 2006); moreover, the consequences of this function were still unknown. Although the accurate regulation of H4K16Ac levels during the cell cycle insinuated a key role for this histone mark in the control of cell cycle progression, the previous studies of SIRT2 did not described any correlation between chromatin regulation by SIRT2 and cell cycle control. In addition, the interplay between H4K16Ac and H4K20me1 was also mainly associated to their role in the regulation of chromatin structure (Shogren-Knaak et al. 2006; Robinson PJ et al, 2008; Turner et al., 1992; Nishioka et al, 2002; Schotta et al, 2008), and was still under debate. Therefore, our study started in order to investigate the key role of sirtuins and H4K16Ac in the control of chromatin dynamics during the cell cycle. Accordingly, we have been able to demonstrate for the first time, the importance of H4K16Ac levels regulation during mitosis for the whole cell cycle progression and the essential role of sirtuins in controlling the H4K16Ac and H4K20me1-3 dynamics throughout the cell cycle.

This study corroborates the key role of SIRT2 as the main histone deacetylase of H4K16Ac during mitosis *in vivo*, establishing also a direct correlation between H4K20me1-3 and H4K16Ac levels throughout the cell cycle. Our data has demonstrated how the lack of SIRT2 affects not only H4K16Ac, but also H4K20me1-3 levels during mitosis and in other cell cycle stages. In addition, our analysis of SIRT2 KO MEFs upon genotoxic stress seems to support the key role of SIRT2 as tumor suppressor by controlling H4K16Ac and H4K20me1 levels under stressful conditions during mitosis. Notwithstanding, one of the most important results derived from our work supports an extremely complex regulation of H4K20me1 deposition by PR-Set7, according to the accurate regulation of this enzyme. In fact, our results described how the lack of SIRT2 negatively affects H4K20me1 levels during mitosis, how the deacetylation of PR-Set7 by SIRT2 affects its chromatin-bound fraction, and how the overexpression of SIRT1 decreases PR-Set7 mRNA stability. Accordingly, these results seem to evidenced that PR-Set7 is not only regulated by proteosome degradation (Tardat, M. et al. 2010; Abbas, T. et al 2010; Oda, H. 2010; Jørgensen, S. et al 2011).

To better understand the complexity of our study the different results obtained will be explained and discussed below.

1. SIRT2 regulates genome integrity by controlling H4K16Ac and H4K20me1 levels.

In spite of the fact that our SIRT2 knockout mice, obtained from Dr. Tong's lab and created by Dr. Alt's lab, did not show any characteristic phenotype; our analysis of their tissues and primary fibroblasts (MEFs) clearly demonstrated how SIRT2 deficiency increases H4K16Ac levels during mitosis (Figure R2-3). Our IHC analysis of SIRT2 knockout mice tissues showed a general increase of H4K16Ac levels enhanced in mitotic cells; indeed, kidney tissues, with relative low division rate (about 2-3%), also showed higher H4K16Ac levels in absence of SIRT2. These results may indicate that H4K16Ac deacetylation during mitosis is essential to maintain the H4K16Ac levels during the cells cycle; therefore, the absence of deacetylation in mitosis entails a general increase of H4K16Ac during the whole cell cycle. For the first time we were able to confirm the role of SIRT2 as the mitotic H4K16Ac deacetylase *in vivo*, as was claimed by Dr. Reinberg's lab eight years ago; and its role in the maintenance of proper H4K16Ac patterns during the whole cell cycle. In turn, the role of H4K16Ac deacetylation in higher order of chromatin formation might identify SIRT2 as an essential player in genomic integrity maintenance. Indeed, acetylation of H4K16 has been previously found to promote chromatin decompaction exposing naked DNA to exogenous and endogenous DNA damage sources (Falk et al., 2008; Robinson et al., 2008; Shogren-Knaak et al., 2006; Shogren-Knaak & Peterson, 2006). Accordingly, our experiments confirmed how SIRT2 deacetylase activity on H4K16Ac is needed to maintain a proper chromatin structure, reducing the effect of DNA damage agents. For that reason, increased levels of H4K16Ac in SIRT2 knockout mice correlate with less compacted heterochromatin, demonstrated by higher γ-satellite expression and lower H3K9me3 levels (Figure R36), together with significantly enhanced levels of DNA damage (Figure R4-6) and chromosome aberrations (Figure R7). All in all this demonstrates that proper levels of H4K16Ac are essential to avoid genome instability. For that reason, not only is SIRT2 absent, but MOF deletion also entails higher DNA damage and threatens genome integrity. Previous studies have shown how MOF absence decreases H4K16Ac levels, affecting gene expression, and DNA repair mechanisms (Deng et al., ; Li et al., 2010); whereas SIRT2 absence increase H4K16Ac levels in mitosis, affecting chromatin compaction, chromosome formation and subsequent DNA repair and gene expression mechanisms.

However, the only deacetylation of H4K16Ac during mitosis could not explain the severe effects on chromatin stability suffered upon SIRT2 deficiency, which mainly leads to DNA damage during S-phase. Acetylation of H4K16 inhibits higher order of chromatin formation, but extra histone marks have been described to be required for metaphase chromosome formation during mitosis; indeed, mitotic entrance correlates with the increase of specific mitotic histone marks, including heterochromatin histone modifications such as H4K20me1 (Fischle et al., 2005; Hendzel et al., 1997; Hirota et al., 2005; Lau & Cheung, 2011; Nishioka et al., 2002; Oda et al., 2009; Perez-Cadahia et al., 2009; Rice et al., 2002). Among the histone modifications that

participate in chromosome condensation, H4K20me1 deposition is involved in silencing, chromatin compaction, DNA replication and DNA damage (Beck et al., 2012; Nishioka et al., 2002; Oda et al., 2009; Sanders et al., 2004; Shen et al., 2010; Vermeulen et al., 2010). In fact, our study shows how the levels of H4K20me1 are affected in the absence of SIRT2 due to two complementary regulatory mechanisms: H4K16Ac levels and PR-Set7 modulation.

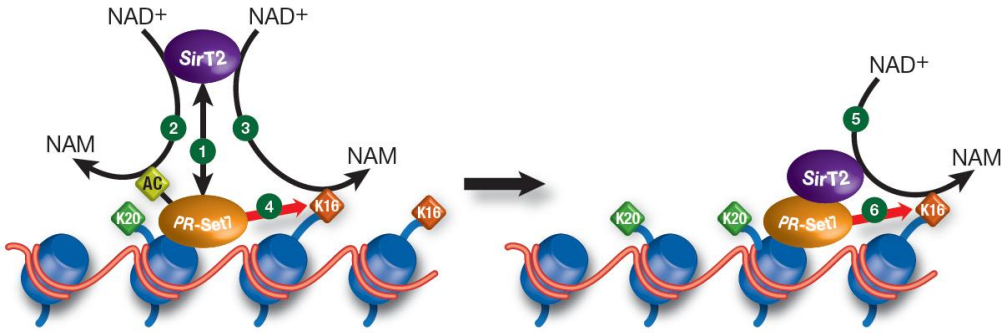


Figure D1. Proposed model of the regulation of H4K20me1 deposition by SIRT2 based in our results. (1) PR-Set7 recruits SIRT2 to specific chromatin regions. SIRT2 then deacetylates both PRSet7 (2) and H4K16Ac (3) from neighboring nucleosomes. PR-Set7 deacetylation in K90 induces its mobilization. (4) Then, SIRT2-bound PR-Set7 monomethylates H4K20 in the neighbor nucleosome. This is followed by translocation to the following nucleosome, where deacetylation of H4K16Ac (5) and H4K20me1 (6) take place again. Overall, we propose that SIRT2 regulates the activation and spreading of PR-Set7 in G2/M.

As it was demonstrated by SIRT1 and Suv39h1 (Bosch-Presegue et al., 2011), our findings determined how SIRT2 promotes PR-Set7 activity by a histone deacetylase activity complemented by a protein deacetylation mechanism. First, we found out how the deacetylation of H4K16Ac by SIRT2 is needed to allow a proper H4K20me1 deposition by PR-Set7 (Figure R10-11). For that reason, our experiments showed how PR-Set7 recruits SIRT2 to the chromatin (Figure R18) in order to favor its spreading, deacetylating the surrounding H4K16Ac (Figure D1). Nevertheless, the regulation of H4K20me1 by SIRT2 consists of a more complex mechanism that correlates with the complexity of PR-Set7 regulation already pointed out by other authors. In fact, as it was already described in the introduction, the protein levels of PR-Set7 are accurately regulated to determine the levels of H4K20me1 during the cell cycle (Tardat, et al., 2010; Abbas et al., 2010; Oda et al., 2010; Jørgensen., et al 2011), and the control of PR-Set7 binding to chromatin is also essential for H4K20me1deposition (Wu et al., 2010). Therefore, previous findings correlate H4K20me1 levels with two different regulation mechanism of PR-Set7: the phosphorylation of PR-Set7 in the Serine 29, which inhibits PRSet7 binding to the chromatin and blocks its degradation; and by several ubiquitination mechanisms during mitosis and S-phase, in order to control its protein levels throughout the cell cycle. Notwithstanding, in addition to these previous studies, our data demonstrates for the first time a direct interplay between SIRT2 and PR-Set7 in order to regulate H4K20me1 levels during

mitosis. Indeed, our results first report an acetylation/deacetylation mechanism that regulates PR-Set7 activity throughout its binding capacity and spreading throughout the chromatin. Our results describe how SIRT2 deacetylates PR-Set7 in K90 and the acetylation level of this residue seems to be essential for PR-Set7 chromatin foci formation (Figure R23 and D2), and the subsequent H4K20me1 deposition. This new mechanism does not invalidate any of the previous statements about PR-Set7 regulation; actually, our findings consist of a complementary mechanism in PR-Set7 fine-tuning.

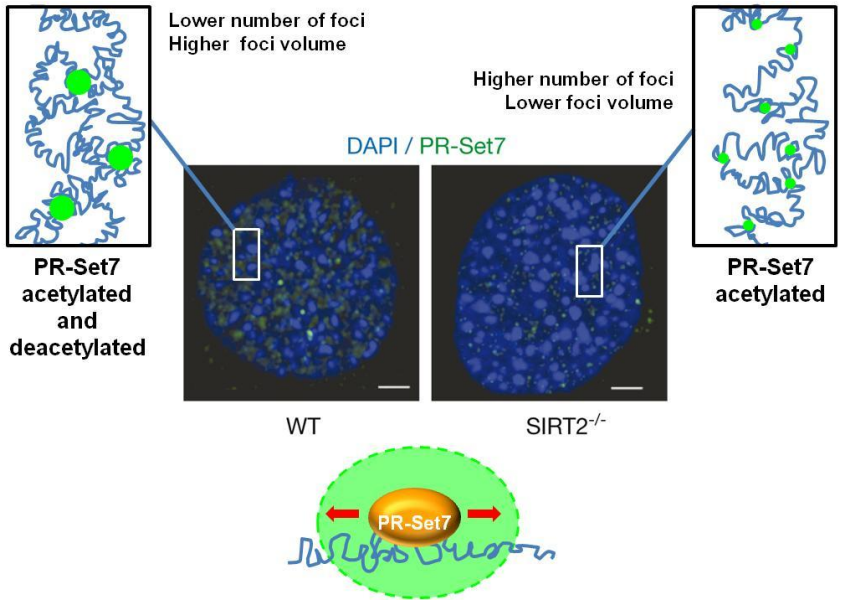


Figure D2. PR-Set7 foci formation according to K90 acetylation levels. The right part of the image shows how in the absence of SIRT2, correlating with acetylated K90, the foci of PR-Set7 are smaller and more numerous compare to the left part of the image, when SIRT2 is present. This description indicates that acetylated K90 has a greater binding capacity, but it is unable to create foci of the same extension than when it is deacetylated.

The histone methyltransferase activity of PR-Set7 is dependent on its binding to the chromatin, and according to Wu et al (2010) the phosphorylation of PR-Set7 by cyclin B1/CDK1 on Serine 29 participates in the control of its chromatin-bound fraction. Therefore, our SIRT2-dependent PR-Set7 regulation mechanism describes an additional posttranslational modification that helps in regulating the capacity of this enzyme to bind to the chromatin. Our findings, together with the previous results, may indicate that PR-Set7 is dephosphorylated in S29 (Wu et al., 2010) and acetylated in K90 in order to bind to the chromatin, meanwhile its spreading seems to be dependent on K90 deacetylation by SIRT2 (Figure D1). Nevertheless, the acetylation levels of K90 do not influence its degradation pattern by APC/C, because the total levels of PR-Set7 were not affected by SIRT2 deficiency; contrary to phosphorylation in S29, which was claimed as an important mechanism in the regulation of APC/C-dependent degradation (Wu et al., 2010).

Consistent with the importance of K90 acetylation in PR-Set7 regulation by SIRT2, our results show how the interaction of both enzymes depends on PR-Set7 acetylation levels (Figure R21), and altogether this allowed us to propose a model of the regulation of H4K20me1 deposition by SIRT2 (model in Figure D1). Based on our findings, acetylated PR-Set7 may recruit SIRT2 to the chromatin in order to deacetylate both, itself and H4K16Ac. Under those circumstances, SIRT2 may first deacetylase PR-Set7 enhancing the interaction between both enzymes and the subsequent PR-Set7 spreading. The increased interaction between both enzymes due to K90 deacetylation may favor the role of SIRT2 in deacetylating the surrounding H4K16Ac, favoring H4K20me1 deposition. However, how this interaction/interplay is lately abrogated, is still unknown; but interestingly, both histone-modifiers, PR-Set7 and SIRT2, are modulated by cyclin B1/CDK1 phosphorylation and CDC14 dephosphorylation (Dryden et al., 2003; North & Verdin, 2007b; Wu et al., 2010). Although the binding between SIRT2 and PR-Set7 was not inhibited using both recombinant proteins purified from bacteria, indicating non-dependence on their phosphorylation pattern, future studies will have to determine if CDC14 interaction with both enzymes would affect SIRT2-PR-Set7 binding integrity. In addition, it would also be important to determine the timing between phosphorylation and acetylation of PR-Set7 and the possible existence of additional marks in order to control its activity or the interplay between SIRT2 and PR-Set7. So far, our findings helped to complete the mechanism of H4K20me1 deposition during mitosis. Accordingly, mitotic H4K20me1 deposition may be governed by PR-Set7 phosphorylation/dephosphorylation and acetylation/deacetylation mechanisms during mitosis, and ubiquitination at mitotic exit; together with H4K16Ac deacetylation by SIRT2 (Figure D1).

In addition, the role of SIRT2 in regulating PR-Set7 supports the increased genome instability under SIRT2 depletion. The increased levels of H4K16Ac partially explained the effects observed in DSBs and chromosome aberrations; notwithstanding, the role of PR-Set7 and H4K20me1 in heterochromatin formation corroborate this phenotype. PR-Set7 has been described to favor heterochromatin formation by different mechanism, favoring chromatin compaction by monomethylation of H4K20 (Oda et al., 2009), or creating H4K20me1 as either recruitment site for chromatin repressors (Kalakonda et al., 2008; Sakaguchi et al., 2012; Trojer & Reinberg, 2007) or as substrate to allow H4K20me3 by Suv4-20h2 (Schotta G et al, 2004; Gonzalo S et al, 2005; Regha K et al, 2007). Accordingly, the importance of these marks in the maintenance of pericentric heterochromatin and telomeres (Benetti R et al., 2007; Schotta et al., 2008; Schotta et al., 2004) endorse the telomere and centromere aberrations that were demonstrated upon SIRT2 depletion when using the centromere and telomere probes (Figure R5). In addition, the abrogation of H4K20me1 or H4K20me3 leads to increased DNA damage sensitivity (Oda et al., 2009; Schotta et al., 2008) as happens with SIRT2 knockout mice (see Figure D3).

Taking all into consideration, SIRT2 participates in the maintenance of genome integrity throughout the regulation of H4K16Ac and H4K20me1 levels, together with the modulation of PR-Set7 behavior, during mitosis.

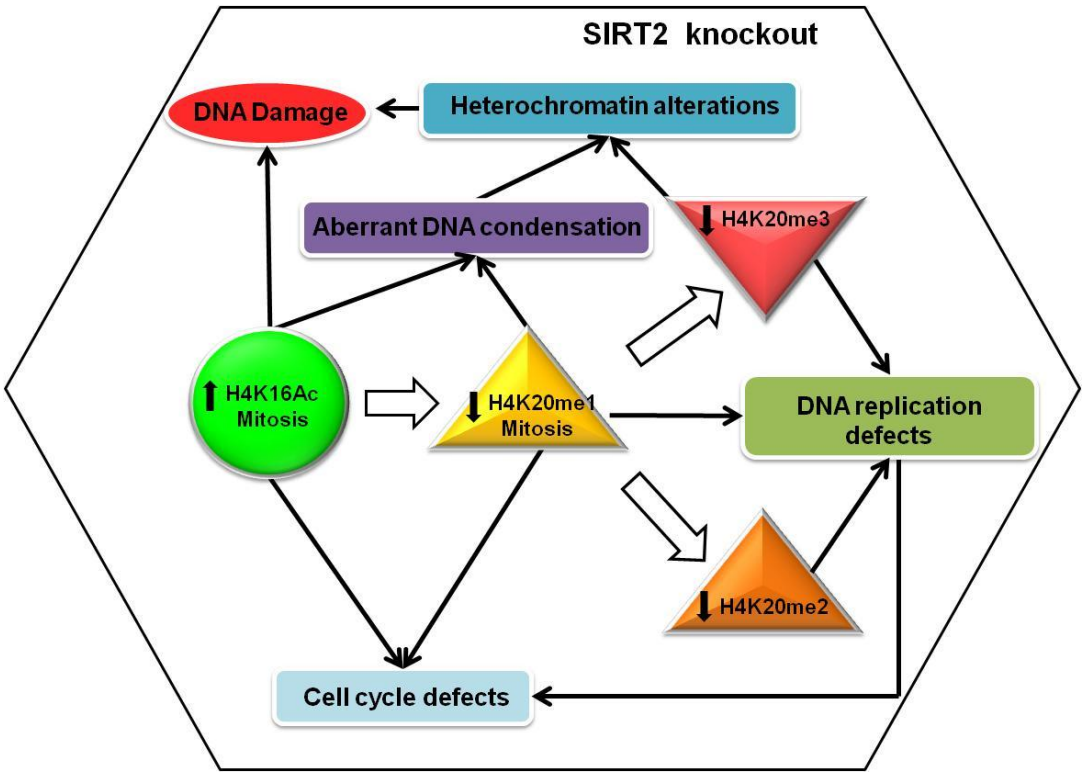


Figure D3. Scheme of the consequences of SIRT2 deficiency. SIRT2 absence causes increased H4K16Ac and decreased H4K20me1 levels in mitosis. The downregulation of H4K20me1 levels negatively affect H4K20me2/3 deposition. The altered levels of H4K16Ac, H4K20me1 and H4K20me3 favor DNA damage by mainly affecting the chromatin structure. In addition, severe alterations in cell cycle progression appear as a result of S-phase defects due to deficient H4K20me, together with the mitotic influence of H4K16Ac and H4K20me1.

2. *SIRT2 regulates cell cycle progression by controlling H4K20me levels: A new role for sirtuins*

Monomethylation of H4K20 has also been implicated in several functions outside mitosis; indeed, monomethylation of H4K20 is an important histone mark whose deposition happens during mitosis and impacts different mechanisms that take place in other cell cycle stages. The role of PR-Set7 in S-phase progression, DNA repair and heterochromatin formation is partially due to the dynamics of H4K20me1 outside mitosis, which consist of serving as substrate for other histone methyltransferases (Suv4-20h1 and Suv4-20h2) in order to di- and tri-methylate this residue (Oda et al., 2009). Mono-, di- and tri-methylated H4K20 have been demonstrated by previous studies to serve as recognition sites for DNA repair proteins (53BP1) (Botuyan et al., 2006; Sanders et al., 2004; Oda et al., 2009), pre-replication components (ORCs) (Beck et al., 2012; Shen et al., 2010; Vermeulen et al., 2010), and heterochromatin formation (Evertts et al., 2013; Oda et al., 2009; Benetti et al., 2007). Consistent with H4K20me

dynamic mechanism, our data showed how the levels of H4K20me2/3 were also compromised in absence of SIRT2 (Figure D3). Accordingly, not only were H4K20me2/3 levels decreased upon SIRT2 deficiency, but SIRT2 knockout cells also showed a characteristic profile associated with Suv4-20h1-2 loss (see Figure D3); such as a significant delay in S-phase entry (Figure R33), decreased 53BP1 binding (Figure R35), and defects in heterochromatin formation (Figure R36).

According to the role of H4K20me2 and H4K20me3 in recruiting ORC proteins (Beck et al., 2012; Shen et al., 2010; Vermeulen et al., 2010), SIRT2 knockout cells show a clear defect in cell cycle progression beyond mitosis. The importance of the pre-RC complex formation in S-phase entry and progression (Aparicio, Weinstein & Bell, 1997; Bicknell et al., 2011) supports the delay in S-phase entry due to SIRT2 loss, and was previously described in Suv4-20h2 DN (Schotta et al., 2008). Pre-RC complex assembly starts upon G1-phase entry, and S-phase entry is delayed until a critical level of licensed origins assembles (Ge & Blow, 2009). In fact, ORC1 deficiency delays S-phase entry (Bicknell et al., 2011), and among others, this ORC seems to be recruited by H4K20me2/3 (Beck et al., 2013).

Although it is known that only a part of H4K20me1 serves as substrate for Suv4-20h enzymes to allow di- and tri- methylation (Oda et al., 2009; Schotta el at., 2008), the levels of reduction in H4K20me2-3 upon SIRT2 deficiency (Table D1) strongly suggest that most of the H4K20me1 altered by SIRT2 loss is actively converted in di- and tri- methyl during the cell cycle. Indeed, these specific defects in Suv4-20h DN were described upon a global decrease in H4K20me2/3 (Schotta el at., 2008); whereas our study demonstrated how the partial loss of these histone marks by SIRT2 deficiency also entail such consequences. Furthermore, we discard a possible involvement of the demethylase PHF8 in this SIRT2-dependent H4K20me regulation, because as it was described for SIRT1, PHF8 has been described to exert its demethylase activity on H4K20me1 within specific promoters, and specifically, during G_1/S (Liu et al., 2010). Therefore, SIRT2-dependent regulation of PR-Set7 and H4K20me1 levels may affect specific regions of the genome that are in turn important for H4K20me2/3 deposition. Future experiments will have to confirm these specific regions regulated by SIRT2.

	% Reduction H4K20me		
	me1	me2	me3
G_1	–	40%	73%
S	–	50%	81%
G_2	–	43%	70%
M	40%	72%	75%

Table D1. Scheme of influence of SIRT2 loss on H4K20me1-3 levels. SIRT2 absence causes the reduction of H4K20me1 during mitosis, but it affects H4K20me2-3 levels during the rest of the cell cycle.

In addition to the S-phase entry defects due to reduced H4K20me2/3 levels, and consistent with the role of SIRT2 in regulating H4K16Ac and H4K20me1 levels during mitosis, our study also found other S-phase-related defects due to SIRT2 loss. According to our cell cycle analysis, SIRT2 absence favors re-replication events (Figure R34), which have previously been attributed to PCNA-insensitive PR-Set7 (Tardat et al., 2010). The literature explains how H4K20me1 deposition is negatively controlled during S-phase in order to inhibit the re-firing of replication origins, because H4K20me1 and H4K20me2 serves as a binding site for the components of the pre-replication complex (Beck et al., 2012; Shen et al., 2010; Tardat et al, 2010; Vermeulen et al., 2010). In agreement with this data, erroneous and uncontrolled H4K20me1 deposition could create extra DNA replication origins. Therefore, our results may indicate that the loss of SIRT2-dependent PR-Set7 regulation not only decreases H4K20me1 levels, but also allows aberrant deposition of H4K20me1 throughout the genome. In fact, our immunofluorescence experiments, to determine the chromatin-bound fraction of PR-Set7, showed how either SIRT2 deficiency or K90 hyperacetylation (K90Q) increased the number of PR-Set7 foci in the chromatin (Figure R17 and R23).

To sum up, SIRT2 is involved in the control of cell cycle progression by modulating S-phase entry and progression through H4K20me levels. The role of SIRT2 during G_2-M as the main deacetylase of H4K16Ac, together with the regulation of H4K20me1 deposition by PR-Set7 during this cell cycle stage, determines the following stages of the cell cycle.

3. SIRT2 and PR-Set7 participate in the regulation of mitotic progression.

Based in our findings, SIRT2 and PR-Set7 may function in a mitotic checkpoint in order to ensure genome integrity throughout mitosis. Accordingly, SIRT2 was already described by other authors in several mechanisms involved in different G_2-M checkpoints (Inoue et al, 2007; Kim et al, 2011; Noth et al, 2014). In addition, although previous studies focused on PR-Set7 influence in cell cycle on its relationship with S-phase progression (Tardat et al., 2007; Jorgensen et al., 2007), some authors determined general cell cycle defects upon PR-Set7 depletion by siRNA (Houston et al., 2008; Tardat et al., 2007) or using PR-Set7 conditional knockout MEFs (Oda et al., 2009). Briefly, the absence of PR-Set7 seems to drastically shorten the G_1-phase, rarely increase S-phase, and significantly augment G_2-M phases. Indeed, it is likely that the effect on G_2-M may be due to G_2 arrest because of the essential role of H4K20me1 in maintaining genome integrity during the cell cycle. This is supported by the observations that the cell cycle defects do not manifest until several cell cycles after depletion of PR-Set7 (Houston et al., 2008; Jorgensen et al., 2007; Tardat et al., 2007).

Our evidences suggest that upon stressful conditions, SIRT2 strongly binds to PR-Set7, promoting an enrichment of both proteins in insoluble chromatin, which may correlate with the blockage of mitotic progression and an accumulation of H4K20me1. According to this mechanism, our data demonstrated how SIRT2 loss causes deficiency in H4K20me1 levels

during mitosis (Figure R25) and defects in G_2/M arrest upon stress conditions (Figure R27). Our FACs experiments showed how SIRT2 deficiency allowed the entrance into mitosis under genotoxic stress, and other authors also demonstrated how following SIRT2 knockdown, the percentage of cells escaping the G_2/M checkpoint and entering mitosis after IR was significantly increased (North et al., 2014). Nonetheless, we have not determined if increased levels of H4K20me1 are required for mitotic arrest or are the consequence of the arrest; therefore future studies will have to investigate further the function of H4K20me1 levels at the G_2/M border. At this point, it is important to consider that depletion of PR-Set7 would not be the best way to study this possibility because of its role in genome stability maintenance throughout the rest of the cell cycle (Houston et al., 2008; Tardat et al., 2007; Jorgensen et al., 2007).

Nevertheless, what we have been able to corroborate is the important role of the regulation of H4K16Ac levels in the control of mitotic entry at G_2/M. According to our results, MOF depletion in mice cells, and the subsequent loss of H4K16Ac, leads to cell cycle arrest at G_2/M (Figure R24); which has already been observed by previous studies (Smith et al., 2005; Taipale et al., 2005). In understanding this information, the additional SIRT2 depletion in MOF knockout cells abrogated the G_2 blockage by MOF deficiency (Figure R29), indicating that the accurate regulation of H4K16Ac at the entrance of mitosis by SIRT2 and MOF is essential for the control of cell cycle progression. Therefore, our findings suggest a clear involvement of SIRT2 in the G_2/M checkpoint. Although the specific role of PR-Set7 and H4K20me1 deposition in this mechanism still has to be clarified, it is possible that a proper H4K20me1 deposition may be required for mitotic entrance (see Figure D3).

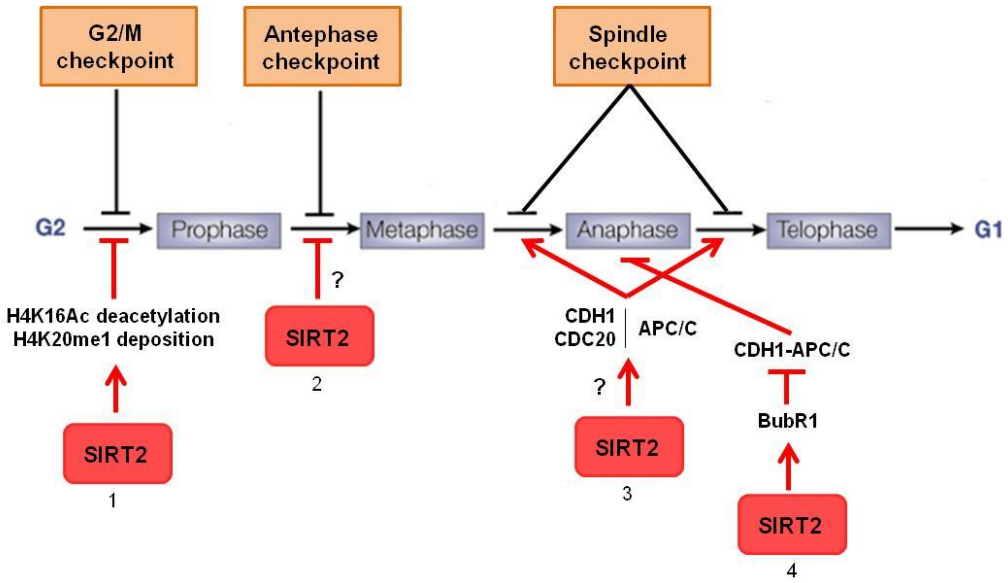


Figure D3. Role of SIRT2 in the mitotic checkpoints. (1) SIRT2 may participate in the G_2/M checkpoint as a key component in the regulation of chromatin structure by deacetylating H4K16Ac and modulating H4K20me1 deposition. (2) SIRT2 has been described to participate in the Antephase checkpoint (Inoue et al, 2007), but its role on this checkpoint is still unknown. (3) SIRT2 seems to positively regulate APC/C activation by favoring CDH1 and CDC20 binding, but it does not correlate with SIRT2 cell cycle distribution. (4) SIRT2 may participate in cell cycle arrest at spindle checkpoint by favoring BubR1 activity (North et al, 2014).

Additionally, SIRT2 and PR-Set7 may also influence the regulation of mitotic progression or exit throughout an additional mitotic checkpoint. Indeed, in our experiments upon stressful conditions, not only were SIRT2 knockout cells capable of entering mitosis, but also G_2-M sorted SIRT2 knockout cells were able to progress and exit mitosis with a high percentage of apoptotic cells (Figure R27.C). The regulation of the cell cycle is an important mechanism to ensure genome and cellular integrity, and thus, it must be accurately controlled by several checkpoints at the different cell cycle stages. Among these cell cycle phases, mitosis is precisely regulated throughout all its duration, keeping cells from proliferating uncontrollably. For that reason, at least three checkpoints have been described to govern mitotic progression: G_2/M, antephase and spindle checkpoints. Therefore, the behavior observed upon SIRT2 deficiency in stress conditions could be explained by the involvement of SIRT2 in the regulation of few of these mechanisms during mitosis (apart from the G_2/M checkpoint): First, SIRT2 could be acting in another mitotic checkpoint between G_2/M and metaphase. In that case, SIRT2 absence, would allow progression throughout early mitosis with no restrictions reaching the spindle checkpoint, where the cells would get blocked. Due to the increased DNA damage accumulated in previous phases (including G_2-M first steps), the spindle checkpoint would either promote the apoptotic pathway or suffer mitotic slippage, letting some cells escape from mitosis (Mikhail V. Blagosklonny, 2007). This possibility would explain the increased apoptotic levels upon SIRT2 deficiency, as well as the higher percentage of cells progressing after mitosis in comparison with wild-type MEFs (Figure R27.C), but will not support the non affected length of G_2-M phase in the SIRT2 KO cells (Figure R30.A). Accordingly, previous findings related SIRT2 with the antephase checkpoint (Inoue et al, 2007) as a response to chronic stress produced by microtubule inhibitors (see Figure D3).

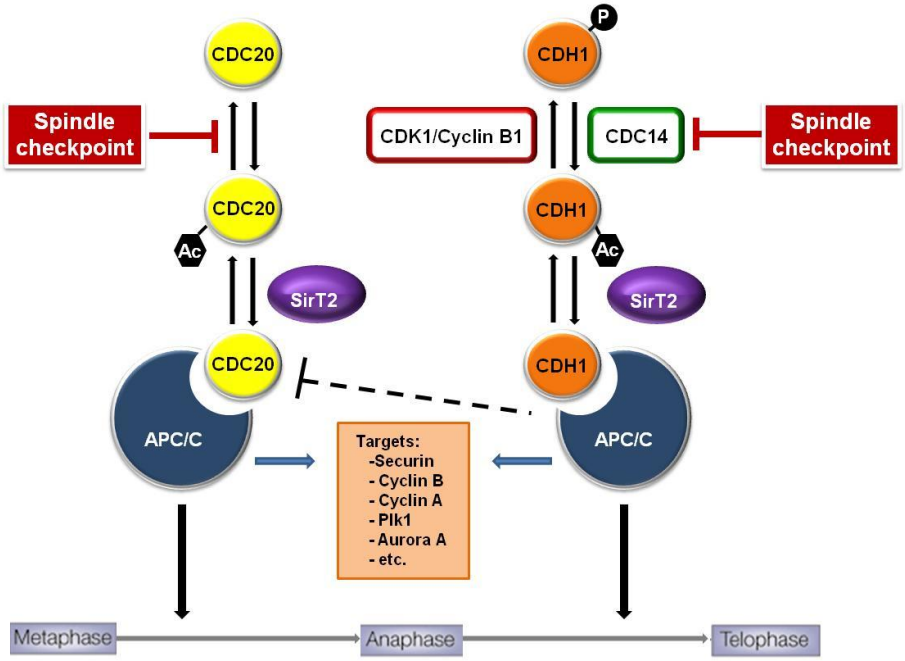


Figure D4. Role of SIRT2 in positively regulating the APC/C activation. SIRT2 deacetylase CDH1 and CDC20 favoring their binding to the APC/C complex. The binding of CDH1 and CDC20 activates APC/C ubiquitination capacity and promote the degradation of several targets in order to exit mitosis. Both CDH1 and CDC20 are accurately regulated in order to ensure that the cell do not progress throughout mitosis under unfavorable conditions.

However, this mitotic checkpoint has been described as a preventing mitotic entry checkpoint that reduces chromatin condensation until the proper conditions allow mitotic progression. Accordingly, the described SIRT2 and PR-Set7 mechanism upon stress conditions may not be implicated in this checkpoint because the interplay between these two enzymes promotes increasing H4K20me1 levels, which would in turn favor chromatin compaction. Therefore, if SIRT2 is really implicated in this checkpoint, it will have to be addressed in future, but it does not seem to be related with H4K20me1.

Second, SIRT2 could be part of the spindle checkpoint as it has been described before for different groups (Kim et al, 2011; North et al, 2014). However SIRT2 role inside this control step seems to be under debate, because whereas Dr. Deng's lab described SIRT2 in promoting APC/C complex by CDH1 and CDC20 (Figure D4) (Kim et al., 2011), Dr. Sinclair's lab has recently demonstrated the role of SIRT2 in inhibiting BubR1 degradation (North et al., 2014) (Figure D5), which would in turn block anaphase release.

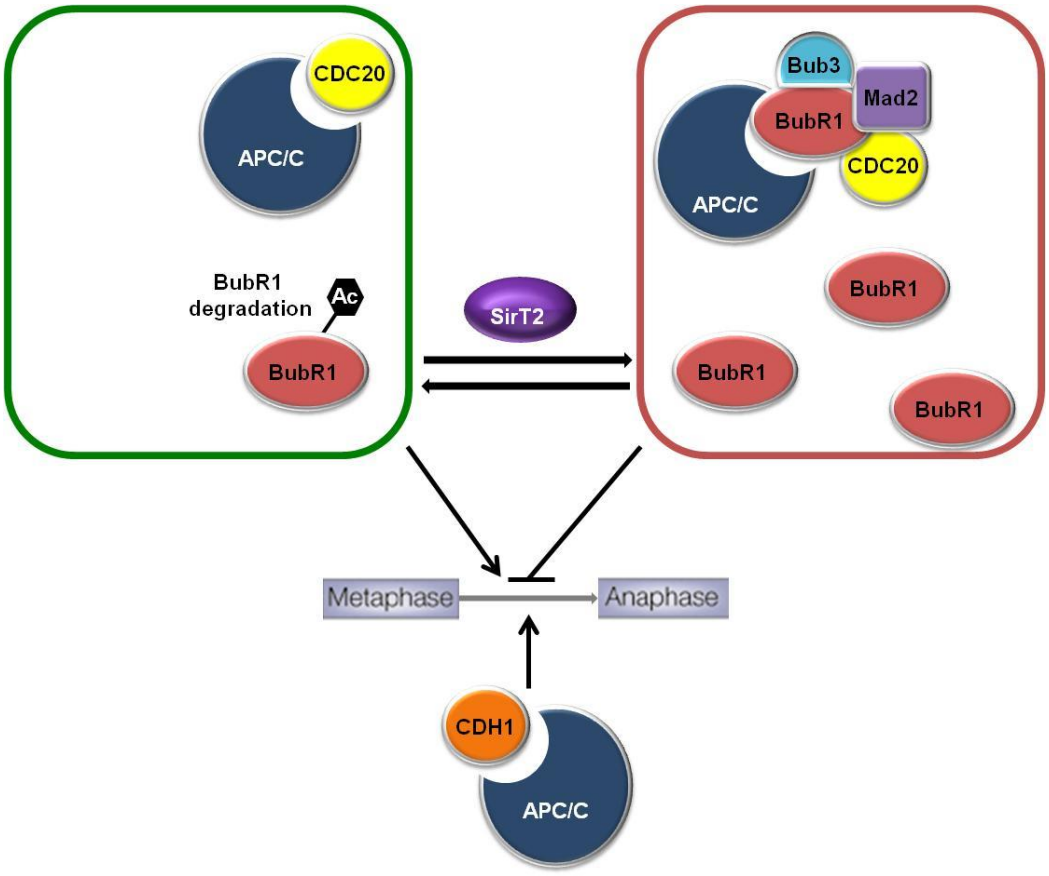


Figure D5. Role of SIRT2 in partially regulating the spindle checkpoint. Under normal conditions, the APC/C complex binds to CDC20 and CDH1 to promote mitotic exit. However, when the conditions are unfavorable the spindle checkpoint machinery blocks the APC/C complex activation. One of these mechanism may be due to the deacetylation of BubR1 by SIRT2 in order to inhibit its protein degradation. Thus, BubR1 is able to bind to and sequestrate CDC20 inhibiting APC/C^{CDC20} complex activation and favoring mitotic arrest. On the other hand, the APC/C^{CDH1} complex is not regulated by SIRT2.

Our evidence suggests that SIRT2 could not be implicated in upstream regulation of APC/C^{CDH1} complex, as Kim et al 2011 suggested, because of its role in PR-Set7 protein degradation. According to our results, SIRT2 loss does not influence total levels of PR-Set7 protein, and the main pathway implicated in the ubiquitin-dependent degradation of this enzyme is the APC/C^{CDH1} complex at mitotic exit (Wu et al., 2010); therefore, this pathway could not be governed SIRT2. Moreover, the cell cycle distribution of SIRT2 KO cells does not show any influence in G2-M phases length (Figure R30), and in case that SIRT2 would favor APC/C activation, its loss would have delayed mitotic exit. In addition, the role of SIRT2 as a tumor suppressor corroborated by our study and previously described by Kim et al (2011), drastically rejects the idea of SIRT2 as a promoter of the spindle checkpoint release; whereas the SIRT2 relationship with BubR1, part of the spindle checkpoint machinery, does support this idea. BubR1 is known to play two very different but complementary roles in promoting spindle checkpoint, the first to promote the checkpoint complex assembly (composed of three components, namely BubR1, Bub3 and Mad2, and the APC/C co-activator Cdc20). The second is to block substrate recruitment to APC/C^{CDC20} (Han et al., 2013; Lara-Gonzalez et al., 2011). Therefore, BubR1 functions inhibiting the APC/C^{CDC20} complex activation, and not the APC/C^{CDH1} complex. This data may tip the balance toward the possible function of SIRT2 in promoting the spindle checkpoint by deacetylating and inhibiting BubR1 degradation, and not by regulating the whole APC/C complex. Altogether this indicates that SIRT2 could participate in the spindle checkpoint regulation, but PR-Set7 involvement would have to be determined in the future (see Figure D3 and D5). In this case, SIRT2 would only participate in the inhibition of APC/C^{CDC20}, but not APC/C^{CDH1}, therefore SIRT2 loss would not completely suppress this checkpoint. This possibility would also explain the elevated levels of cells leaving mitosis upon DNA damage, and the subsequent increase of apoptotic events (Figure R27.C). The cells that were able to progress to G_1-phase, due to the mitotic slippage favored by the defect in APC/C^{CDH1} pathway, suffered apoptosis as a result of the high levels of accumulated DNA damage.

In addition, some of the observations in SIRT2 knockout cells by Dr. Deng's lab could be attributed to alternative mechanisms that do not include the regulation of APC/C^{CDH1} complex, and actually, some of these mechanisms support the role of SIRT2 in regulating PR-Set7 and H4K20me1 deposition. For instance, Kim et al (2011) detected centrosome amplification upon SIRT2 absence, and they justified this observation because of higher levels of Aurora A, caused by inactivation of APC/C^{CDH1}. However, other pathways could lead to centrosome amplification. On one hand, this aberration could be due to downregulation of H4K20me1, as it has been observed under PR-Set7 loss (Oda et al 2009) and according to its correlation with delay in S-phase due to DNA replication problems; indeed, centrosome duplication is under cell cycle regulation control, which also controls DNA replication and thereby coordinates the two events (Fukasawa, 2005). On the other hand, different hypotheses support the role of spindle checkpoint arrest in centrosome amplification. One example is the Cdk2-dependent reduplication of centrioles by the inactivation of the APC/C upon prolonged

arrest (Ma et al., 2009); and other example describes how inefficient inhibition of Separase during a mitotic delay or arrest might result in premature centriole disengagement followed by formation of multipolar spindles (Maiato & Logarinho, 2014). This last explanation could also correlate with the possible involvement of SIRT2 in control BubR1 levels (North et al, 2014), and subsequently, APC/C^{CDC20} inhibition. Therefore, under SIRT2 deficiency, APC/C^{CDC20}-dependent Securing degradation could not be efficiently inhibited, and consequently Separase levels would increase, leading to centrosome amplification. Another observation made by Kim et al, includes a delay in APC/C substrates degradation (Aurora A, Aurora B, Cyclin B1, PLK1, Securin and Cyclin A2) after mitotic release. The degradation of these proteins would be delayed in case of arrest at the spindle checkpoint, as they claimed. However, whereas they justify this arrest because of SIRT2 role in promoting APC/C activation; this process could also be happening if the genome stability accumulated in previous phases, due to SIRT2 loss, caused cell cycle arrest at mitotic exit and the cells suffer mitotic slippage.

Therefore, all the above data indicates a clear role of SIRT2 (and the regulation of H4K16Ac levels) in favoring G_2/M checkpoint, possibly associated to PR-Set7 modulation. In addition, SIRT2 could be involved in another/other mitotic checkpoints in order to control mitotic progression, which could be independent of PR-Set7.

4. SIRT2 is a tumor suppressor.

Our data described above supports the hypothesis of SIRT2 as a tumor suppressor, previously formulated by Dr. Deng's lab in 2011, and suggested by Dr. Vaquero's lab (Bosch-Presege & Vaquero, 2011). Our DMBA-TPA assay corroborated that SIRT2 knockout mice are more prone to develop tumors (Figure R37-40), correlating with increased genome instability and the role of SIRT2 in control cell cycle progression. Contrary to the spontaneous tumor development described by Kim et al (2011), we needed to perform this tumorigenesis assay in order to confirm SIRT2 as a tumor suppressor. As we already mentioned, it should be due to the mice strain. Our C57-BL6 mice are known to be more resistant to tumorigenesis than the mice used by the other lab, which consist of a quimera (Swiss/C57-BL6) (DiGiovanni et al., 1993).

In addition, several of the characteristics described upon SIRT2 depletion in our study, together with previous findings, are associated with tumorigenesis. That is the case of centrosome amplification, aneuploidies, heterochromatin formation and re-replication.

Higher number of centrosomes have been found in absence of SIRT2 (Kim et al., 2011), and centrosome amplification has been observed in most, both solid and hematological, cancer entities and by now is viewed as a "hallmark" of cancer cells (Anderhub, Kramer & Maier, 2012). As SIRT2 knockout cells, cancer cells display elevated centrosome numbers and are often aneuploid (Anderhub et al., 2012). In addition, proper higher-order three-dimensional organization of chromatin is crucial for normal cell function and maintenance of genomic

stability, influencing gene expression, DNA replication and repair. Thus global dysregulation of heterochromatin might also increase cancer susceptibility. In agreement with this hypothesis, a strongly reduced level of H4K20me3 was found to be a hallmark of many human cancers (Fraga et al., 2005; Tryndyak, Kovalchuk & Pogribny, 2006; Van Den Broeck et al., 2008), and reduced levels of HP1α have been found in highly invasive and metastatic breast cancer cell lines (Kirschmann et al., 2000). It is not clear if these findings represent cause or consequence of tumorigenesis; however, several lines of evidence suggest that impairment of heterochromatin can increase cancer susceptibility (Hahn M et al, 2010). Our results show how SIRT2 loss not only decreases H4K20me levels, but also affects heterochomatin structure according to the reduced H3K9me3 levels. Finally, the re-replication events observed in our SIRT2 knockout MEFs have also been associated with tumorigenesis. Additional rounds of replication of genomic DNA, or even sections of it, within a given cell cycle would result in gene amplification, polyploidy and other kinds of genome instability, which is a hallmark of tumorigenesis (Albertson, 2006; Cook, 2009; Levine, 2007; Schimke et al., 1986).

Our study focuses on the role of SIRT2 as a tumor suppressor in chromatin and cell cycle regulation but this sirtuin also regulates cell death in response to certain conditions of DNA damage-induced stress (Li et al., 2011; Matsushita et al., 2005). In fact, SIRT2 is also involved in autophagy inhibition throughout FoxO1 deacetylation, which in turn has been associated with tumor development (Levine, 2007; Zhao et al., 2010).

This altogether justify the downregulation of SIRT2 in gliomas, gastric carcinomas and melanomas (Hiratsuka M et al, 2003; Inoue T et al, 2007). Notwithstanding, our mice were not able to develop spontaneous tumors, but the classical skin tumorigenesis assay demonstrated how the absence of SIRT2 was associated with tumor progression and bad prognosis. These results may indicate that SIRT2 is not essential for blocking tumor initiation, but it may function as a tumor progression inhibitor.

5. SIRT1 contributes to the accurate regulation of PR-Set7 and Suv4-20h2.

Contrary to SIRT2's positive effect on PR-Set7, the role of SIRT1 with respect to this histone methyltransferase seems to be related to the balance of its expression.

As it has been pointed out from the introduction, the levels of PR-Set7 must be regulated throughout the cell cycle in order to preserve genome integrity and ensure a proper cell cycle progression (Oda et al., 2009; Tardat et al., 2007; Tardat et al., 2010; Jorgensen et al., 2007). PR-Set7 levels peak during mitosis favoring chromatin compaction, chromosome structure and precise chromosome segregation; accordingly, it is during mitosis when SIRT2 is promoting H4K20me1 deposition. However, PR-Set levels need to be downregulated during the rest of cell cycle to inhibit its activity beyond mitosis (Tardat et al., 2010; Abbas et al., 2010; Oda et al., 2010; Jørgensen et al., 2011). Defects on this KMT leads to several genome instability

associated phenotypes (Oda et al., 2009; Tardat et al., 2007; Jorgensen et al., 2007), and the absence of its degradation during S-phase also affect genome integrity (Tardat et al., 2010). For that reason, several proteosome pathways coordinately work to ensure that PR-Set7 protein levels follow the correct pattern (Tardat et al., 2010; Abbas et al., 2010; Oda et al., 2010; Jørgensen et al., 2011). So far, all the information published about the regulation of PR-Set7 levels is based on ubiquitin-dependent mechanisms that control protein degradation; notwithstanding, our results demonstrated for the first time the possible regulation of not only PR-Set7, but also Suv4-20h2, by mRNA degradation. In addition, it is also the first time that SIRT1 is involved in a mechanism involved in controlling mRNA stability.

As our results have determined, the negative effect of SIRT1 in the mRNA stability of both KMTs appears to be restricted to a specific cell cycle stage (Figure R40). In the case of PR-Set7 this regulation seems to be happening in S-phase; whereas for Suv4-20h2 the degradation is linked to G_1-phase. The association of SIRT1-dependent PR-Set7 mRNA degradation with S-phase could be the main reason for the inability to detect the effect of SIRT1 and Actinomycin D. The cells used for these experiments were asynchronous, which indicates that the main population was in G_1-phase, and the rest was divided between S-phase and G_2-M-phases. Thereby, the amount of cells in S-phase was not sufficient enough to detect a quantifiable effect.

Both histone modifiers, PR-Set7 and Suv4-20h2, have been widely related with S-phase progression, as it has been disscused before. PR-Set7 creates the H4K20me1 sites during G_2-M (Oda et al., 2009; Rice et al., 2002) that are used by Suv4-20h2 to trimethylate the residue beyond mitosis; and H4K20me3 is in turn used as a recruitment site for the pre-RCs during late G_1 and S-phase (Beck et al., 2013). The accurate control of DNA replication is essential to maintain genome integrity, as it has also been pointed out before; and SIRT1 has been previously related with this process. According to other studies, SIRT1 may participate in the control not only of DNA replication, but also of histone replication (He et al., 2011). So far, the role of SIRT1 in DNA replication is mainly based in the regulation of Mcm10, a protein involved in the pre-initiation complexes, essential for DNA replication initiation (Remus & Diffley, 2009). The loss of SIRT1 was related with increased levels of this protein in the chromatin-bound fraction, and SIRT1 overexpression led to decrease of Mcm10 levels (Fatoba et al., 2013); indicating a negative effect of SIRT1 in the replication process. This mechanism would correlate with the negative effect of SIRT1 in PR-Set7 and Suv4-20h2 mRNA stability during G_1 and S-phase, when the DNA replication machinery is being orchestrated. Then, SIRT1 may be involved in the accurate control of the DNA replication process. Accordingly, contrary with what happened after PR-Set7 depletion, SIRT1 loss do not alter inter-origins distances but it tend to accelerate fork velocity (Fatoba et al., 2013); whereas PR-Set7 downregulation was shown to decrease both, fork velocity and the density of active replication forks (Tardat el at., 2007).

In order to establish an appropriate explanation of SIRT1 role in DNA replication, it would be also important to consider the SIRT1 activity during each cell cycle stage due to NAD+/NADH ratios. This ratio is known to be high during early G1, and continually decreases

until arriving at G_1/S border and during S-phase, and increase at late S-phase (Yu et al., 2009). Accordingly, Mcm10 degradation and the inhibition of its binding to the chromatin would be happening during early G_1-phase and late S-phase. Additionally, if the regulation of PR-Set7 and Suv4-20h2 mRNA is dependent on SIRT1 activity, the reduction of Suv4-20h2 mRNA levels might happen during early G_1, and PR-Set7 mRNA levels negative regulation would be likely to happen during late S-phase. Altogether this indicates that SIRT1 could be involved in the accurate regulation of the DNA replication timing, which starts at late G_1 and proceeds until late S-phase. Therefore, SIRT1 activity seems to be essential to regulate the on/off of the DNA replication process through controlling different mechanisms that include PR-Set7 and Suv4-20h2 expression. Our new findings may consist of an additional mechanism to inhibit re-replication and regulate S-phase progression. However, the mechanism by which SIRT1 favors both PR-Set7 and Suv4-20h2 mRNA degradation by lysosomes still have to be determined.

Chromosome	start	end	width	names	peak	Start position	End position	Inside Feature	Distance to Feature	Shortest Distance	From Overlapping Or Nearest	symbol
19	3767533	3768363	831	04876 ENSMUSG00000045098	4876	3767421	3818302	inside	112	112	NearestStart	**Suv420h1**
19	3768636	3769043	408	04877 ENSMUSG00000045098	4877	3767421	3818302	inside	1215	1215	NearestStart	**Suv420h1**
7	4691339	4692032	694	08569 ENSMUSG00000059851	8569	4691717	4699116	overlapStart	-378	315	NearestStart	**Suv420h2**
5	124895163	124895640	478	07826 ENSMUSG00000049327	7826	124889939	124912316	inside	5224	5224	NearestStart	**PR-Set7**
5	124889631	124890087	457	07825 ENSMUSG00000049327	7825	124889939	124912316	overlapStart	-308	148	NearestStart	**PR-Set7**

Table D2. SIRT1 bind to PR-Set7 and Suv4-20h genes (*GSE17067)*. The "symbol" column refers to the name of the gene and the "Inside feature" described the position of SIRT1 along the gene length.

In addition to the mRNA regulation, SIRT1 seems to be modulating PR-Set7 and Suv4-20h enzymes by extra mechanisms. On one hand, our results also suggest a possible role in modulating PR-Set7, Suv4-20h1.1 and Suv4-20h1 enzymes through a direct binding to the proteins (Figure R36). As we already know, this interaction is not the responsible for the protein degradation, but could influence other functions such as the enzymatic activity or gene targeting. In any case, it would be important to distinguish the cell cycle stage during the study, because, as we have already demonstrated, SIRT1 absence did not affect H4K20me1 levels in mitosis, but…what about S-phase? It will be important to find out. And on the other hand, previous studies, using ChIP-seq technique in mice, have found SIRT1 bound to PR-Set7 and Suv4-20h1-2 genes (Table D2, *GSE17067)(Macisaac & Fraenkel, 2010)*. This extra information may indicate that maybe SIRT1 is regulating PR-Set7 and Suv4-20h by three complementary mechanisms: expression, mRNA stability and enzymatic function.

Finally, according to the control of PR-Set7 and Suv4-20h expression by SIRT1, together with the role of SIRT2 in the regulation of PR-Set7 and the subsequent H4K20me1-3

deposition, the sirtuins seem to be clearly involved in the global control of H4K20me1-3 levels throughout the cell cycle. Notwithstanding, the role of SIRT1 in the regulation of this histone mark deposition may be involved somehow in the embrionary development, due to the importance of both proteins in this process. Accordingly, not only a great percentage of the SIRT1 knockout mice died at early postnatal stages, but also the PR-Set7 knockout mouse embryos are not viable. A deeper analysis of H4K20me1-3 deposition during embrionary development in SIRT1 KO mice would be important to characterize this possibility.

6. Interplay between H4K16Ac and H4K20me1.

Finally, one of the most interesting points of our study relies on the antagonism between H4K16Ac and H4K20me1. Despite the clear role of H4K16Ac in relaxing the chromatin, favoring transcription, DNA replication or repair mechanisms (Shogren-Knaak et al., 2006; Robinson et al., 2008; Taylor et al., 2013); and the function of H4K20me1 in favoring chromatin compaction, silencing and heterochromatin formation (Oda et al., 2009; Rice et al., 2002); the interplay between both histone marks is still under debate.

The possible antagonism between these two histone marks was pointed out in 2002 when Nishioka et al (2002) found contrasted levels of H4K16Ac and H4K20me1 in the hyperactivated X-male chromosome of Drosophila. Their publication demonstrated how H4K16Ac was enriched in X-male chromosome, meanwhile H4K20me1 was hardly detectable. Accordingly, their enzymatic assays also supported the role of each of these histone marks in inhibiting the deposition of the other. However, those original histone methyltransferase assays used peptides as substrates, and a PR-Set7 mutant capable of methylating non-nucleosome substrates, to demonstrate their theory (Nichioka et al., 2002). Here we have been able to demonstrate the H4K16Ac-H4K20me1 antagonism *in vitro*, using recombinant nucleosomes and wild-type PR-Set7, and also *in vivo*, by analyzing H4K20me1 levels in absence of SIRT2. Our *in vitro* assays demonstrated how H4K16ac interferes in PR-Set7 activity; and our *in vivo* experiments support the antagonism of these histone marks during mitosis, when the absence of SIRT2 increases H4K16Ac levels and subsequently decreases H4K20me1 levels.

However, in other studies this is not the case. According to other publications, H4K20me1 is also found in promoters of transcribed regions coinciding with H4K16Ac (Vakoc et al., 2006; Barski et al., 2007; Talasz et al., 2005; Beisel et al., 2002). Indeed, not only is the loss of H4K20me3 considered to be a hallmark in cancer, but the loss of both H4K16Ac and H4K20me3 is a hallmark in cancer cells (Fraga et al., 2005). And accordingly with this coexistence, H4K16Ac levels peak during S-phase (Vaquero et al, 2006), correlating with DNA-replication (Eaton et al., 2011; Rampakakis et al., 2009); and H4K20me is an essential histone mark for replication origins. Therefore, do these histone marks coexist or repel one another? According to our results, the presence of H4K16Ac seems to inhibit H4K20me1 deposition, but maybe, H4K20me1 would not be enough to block H4K16Ac deposition. In fact, the enzymatic

activity of PR-Set7 is highly dependent on the substrate. This KMT needs the nucleosome conformation to perform its activity, being incapable of methylating core histones (Nishioka et al., 2002). Likely, it is possible that PR-Set7 may bind to a platform of nucleosomes instead of single nucleosomes or histone tails, indicating that PR-Set7 may need H4K16 to be deacetylated in order to get a closer conformation between the nucleosomes. Meanwhile, acetylation of H4K16 could be less dependent on substrate or favored by other histone modifiers or remodelers. For example, the yeast Esa1 HAT fails to acetylate nucleosomal histones, but its parent multisubunit NuA4 complex does acetylate nucleosomes (Ep1/Yng2/Esa1) (Boudreault et al., 2003). Therefore, it would be important to address if MOF would be able to acetylate nuclesomes, alone or as part of the MSL complexes. Determining the precise conditions for this antagonism should be an important research focus of future epigenetic studies.

Conclusions

Conclusions

1. SIRT2 is the main histone deacetylase of H4K16Ac during mitosis *in vivo*.

2. SIRT2 regulates H4K20me1 deposition by two complementary functions: the deacetylation of H4K16Ac and the modulation of PR-Set7 during mitosis.

3. SIRT2 binds and deacetylases PR-Set7 in K90 in order to favor its spreading throughout the chromatin, affecting H4K20me1 deposition.

4. The loss of SIRT2 affects H4K20me2-3 levels due to H4K20me1 alteration, causing several phenotypes associated to the loss of Suv4-20h1-2.

5. SIRT2 deficiency alters H4K20me1 deposition and G2-M progression upon oxidative stress. These findings suggest the involvement of both, SIRT2 and PR-Set7, in a mitotic checkpoint.

6. SIRT2 and the regulation of H4K16Ac levels are involved in the G2/M checkpoint. The specific role of PR-Set7 in this checkpoint will have to be addressed in the future.

7. SIRT2 and its role in modulating H4K16Ac and H4K20me1-3 levels severely affect genome integrity increasing DNA damage levels and promoting chromosome aberrations.

8. The DMBA-TPA classical tumorigenesis assay demonstrated that SIRT2 knockout mice are more prone to develop tumors. This finding suggests the role of SIRT2 as a tumor suppressor.

9. SIRT1 and SIRT2 seem to participate in the regulation of PR-Set7 during different cell cycle stages.

10. SIRT1 may contribute to regulate H4K20me1-3 levels by the control of PR-Set7 and Suv4-20h enzymes outside mitosis.

11. H4K20me1 levels seem to be negatively influenced by the presence of H4K16Ac. Our results support the hypothesized antagonism between both histone marks.

Annex

Most of the work presented in this thesis was published in this article:

The tumor suppressor SirT2 regulates cell cycle progression and genome stability by modulating the mitotic deposition of H4K20 methylation. Lourdes Serrano[1*], Paloma Martínez-Redondo[2*], Anna Marazuela-Duque[2], Berta N. Vazquez[1], Scott J. Dooley[1], Philipp Voigt[3] , David B. Beck[3], Noriko Kane-Goldsmith[1], Qiang Tong[4], Rosa M. Rabanal[5], Dolors Fondevila[5], Purificación Muñoz[6], Marcus Krüger[7], Jay A. Tischfield[1] & Alejandro Vaquero[2]. 2013. *Genes & Development.*

[1] Department of Genetics and the Human Genetics Institute of New Jersey, Rutgers University, 145 Bevier Road, Piscataway, New Jersey 08854, USA

[2] Chromatin Biology Laboratory, Cancer Epigenetics and Biology Program (PEBC), **Bellvitge Biomedical Research Institute** (IDIBELL), Av. Gran Via de l'Hospitalet, 199-203, 08908 L'Hospitalet de Llobregat, Barcelona, Spain

[3] Department of Biochemistry, New York University School of Medicine, New York, 10016, USA

[4] Children's Nutrition Research Center, Baylor College of Medicine, 1100 Bates Street, Houston, TX 77030, USA

[5] Centre de Biotecnologia Animal i Teràpia Gènica (CBATEG), Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra (Cerdanyola del Vallès), Spain

[6] Ageing and Cancer Laboratory, Cancer Epigenetics and Biology Program (PEBC**), Bellvitge Biomedical Research Institute** (IDIBELL), Av. Gran Via de l'Hospitalet, 199-203, 08908 L'Hospitalet de Llobregat, Barcelona, Spain

[7] Max Planck Institute for Heart and Lung Research, Department of Cardia development and Remodeling, D-61231 Bad Nauheim, Germany

[*] These authors contributed equally to the work

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