Role of SIRT6 in Chromatin

Irene Santos Barriopedro

Doctorat en Biomedicina

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This thesis has been done under the supervision of **Dr Alejandro Vaquero García** from the Cancer and Epigenetics Biology Program (PEBC) in the Insitute d'Investigacions Biomèdiques de Bellvitge (IDIBELL).

Tutor: **Dr Esteban Ballestar Tarín**

Director:
Dr. Alejandro Vaquero García

Tutor:
Dr Esteban Ballestar Tarín

Student:
Irene Santos Barriopedro

A mi madre

Y a todos aquellos
que me han ayudado
y apoyado

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Abstract

Chromatin compaction is regulated by different factors, among them histone posttranslational modifications. There are different histone modifications, and among them, acetylation and methylation of lysine residues. Acetylation levels are regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), enzymes that catalyze addition and removal, respectively, of acetyl groups from histone lysine residues. Among the four classes of HDACs, the class III which correspond to the members of the Sir2 family or Sirtuins are quite unique. They participate in the response to a wide variety of stress stimuli through their requirement of NAD^+ as a cofactor in their enzymatic activity. Mammals harbor seven Sirtuins (SirT1-SirT7). Among them, SirT6 is a nuclear protein involved in both genomic stability and metabolic homeostasis. Interestingly, SirT6 regulates the majority of these processes through an effect on chromatin, based on deacetylation of a histone mark, H3K9ac. However, the mechanism involved has not been fully characterized. In this work, we aim to study the consequences of SirT6 function in chromatin organization. We show that SirT6 overexpression induces gene silencing, which fits with the role of SirT6 as a repressor, shown by previous reports. Our studies show that SirT6 interacts with proteins or multi-protein complexes also involved in gene silencing, such as components of NuRD complex, or the HMTs EZH2, Suv39h1. However, we have focused the project in understanding the functional relationship between SirT6 and an H3K9-specific methyltransferases that we have identified as Suv39h1 and G9a. Suv39h1 trimethylates H3K9 and is essential in the establishment and maintenance of pericentromeric and telomeric constitutive heterochromatin. Interestingly, Suv39h1 was previously found to interact with SirT1 in the context of constitutive and facultative heterochromatin formation. Our work shows that the functional relationship between Suv39h1 and SirT6 is quite different from the already described between SirT1 and Suv39h1. SirT6 mediates a non canonical monoubiquitination in Suv39h1 in three conserved cysteines of the pre-SET domain. We have also identified SKP2 as the E3 ubiquitin ligase responsible of this monoubiquitination. SKP2 has a well-known role in promoting poliubiquitination and degradation of proteins involved in G_1/S checkpoint such as p21 and p27 and its levels are regulated during cell cycle progression. Our data show that SKP2 levels are also regulated by SirT6 through deacetylation, which in turn induce a double phosphorylation that prevents its degradation. Suv39h1 monoubiquitination is induced by double thymidine block and nocodazole treatments that arrest cells in G_1/S phase and early mitosis, respectively. Furthermore, Suv39h1

monoubiquitination is induced by NF-kB pathway activation such as TNFα treatment, the overexpression of the NF-kB transcription factor RelA, or of the activator IKKα. Moreover, the promoter of the NF-kB global repressor, IkBα, is regulated by the interplay between Suv39h1, SKP2 and SirT6. Thus, upon activation of the pathway by TNFα treatment, SirT6 induces the Suv39h1 monoubiquitination through SKP2 activation and monoubiquitinated Suv39h1 is removed from the promoter allowing the transcription activation of IkBα by RelA. This novel model provides not only new insights in the regulation of NFkB pathway but also unveils new roles for SirT6, SKP2 and Suv39h1.

Resumen

La compactación de la cromatina es regulada por diferentes factores entre los cuales destacan las modificaciones post-traduccionales de las histonas. Hay diferentes modificaciones de histonas pero las mejores descritas son las desacetilationes y las metilaciones. Los niveles de acetilación están regulados por las histonas acetiltransferasas (HATs) y las histonas deacetilasas (HDACs), proteínas que añaden o quitan grupos acetil a las lisinas de las histonas, respectivamente. Hay cuatro clases de HDACs y los miembros de la familia Sir2 o de las sirtuínas constituyen la clase III de las HDACs. Participan en respuesta a muchas formas de estrés utilizando NAD^+ como cofactor para su actividad enzimática. En mamíferos hay 7 sirtuínas (SirT1-7). Entre ellas, está SirT6 que es una proteína nuclear involucrada tanto en la estabilidad genómica como en la homeostasis metabólica y regula la mayoría de los procesos gracias al efecto que produce en la cromatina basado en la desacetilación de H3K9 acetilado. Sin embargo, el mecanismo por el cual Sirt6 produce silenciamiento génico aún no ha sido caracterizado. En este proyecto hemos estudiado las consecuencias de la función de SirT6 en la organización de la cromatina. Mostramos que la sobreexpresión de SirT6 produce silenciamiento génico tal y como se había descrito previamente en artículos en los que se caracteriza SirT6 como represor. Nuestros estudios muestran que SirT6 interacciona con proteínas involucradas en silenciamiento génico como es el complejo NuRD y EZH2. Sin embargo, dirigimos la atención a la relación entre SirT6 y las metiltransferasas de H3K9 con las que interacciona que son G9a y Suv39h1. Suv39h1 trimetila H3K9 lo cual es esencial para la formación y mantenimiento de la heterocromatina constitutiva pericentromérica y telomérica. Además, previamente se había caracterizado la interacción entre Suv39h1 y SirT1 en el contexto de formación de la formación de heterocromatina constitutiva y facultativa. Aquí demostramos que la relación entre Suv39h1 y SirT6 es bastante diferente a la caracterizada entre Suv39h1 y SirT1. SirT6 media una monoubiquitinación no canónica en tres cisteínas conservadas del dominio pre-SET de Suv39h1. Entre las E3 ubiquitina ligasas que interaccionan con Suv39h1 y SirT6 como CHIP y CHFR, encontramos que la responsable de la monoubiquitinación de Suv39h1 es SKP2 que es una proteína oncogén involucrada en el ciclo celular y es muy conocida por poliubiquitinizar y promover la degradación de proteínas que participan en el checkpoint de G_1/S como p21 y p27. Los niveles de SKP2 fluctúan durante el ciclo. En este trabajo mostramos que los niveles de SKP2 además están regulados por

SirT6 la cual desacetila SKP2 e induce su fosforilación para evitar su degradación. La monoubiquitinación de Suv39h1 es inducida en las células bajo tratamiento de doble bloqueo y timidina, que paran las células en G_1/S y mitosis temprana, respectivamente. Además, la monoubiquitinación de Suv39h1 es inducida por la activación de la vía de NFkB a través de tratamiento con TNFα, la sobreexpresión de RelA, un factor de transcripción de NFkB o la sobreexpresión de IKKα, un componente de la vía NFkB. Además, la expresión del regulador negativo de la vía de NFkB, IkBα, está regulada por la interacción entre Suv39h1, SirT6 y SKP2 y bajo tratamiento con TNFα. SirT6 induce la monoubiquitinación de Suv39h1 a través de SKP2 y Suv39h1 monoubiquitinizado es desplazado del promotor permitiendo la activación de la transcripción de IkBα por RelA. Este modelo proporciona nuevas perspectivas de la regulación de la vía NFkB y nuevos papeles de Suv39h1, de SirT6 y de SKP2.

Abbreviations

Ac	Acetylation
APC/C	Anaphase-promoting complex /Cyclosome
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BER	Base excision repair
BSA	Bovine serum albumin
CBP	CREB binding protein
CDH1	Cadherin 1
CDC20	Cell division cycle
CDK	Cyclin-dependent kinase
CHD	Chromodomain helicase
CHFR	Checkpoint with Forkhead associated (FHA) and Ring finger domains
ChIP	Chromatin immunoprecipitation
CHIP	C-terminus of Hsc70-interacting protein
CLR4B	Culin 4B-Ring E3 ligase complex
CPS1	Carbamoyl phosphate synthetase 1
CPT	Camptothecin
CtIP	CtBP-interacting protein
Cul	Cullin
ΔChromo	Chromo domain deletion of Suv39h1
ΔN	N-terminal domain deletion of Suv39h1
ΔPRESET	Pre-SET domain deletion of Suv39h1
ΔSET	C-terminal domain deletion of Suv39h1
SETΔC	SET domain of Suv39h1
DAPI	4,6-diamino-2-phenylindole
DN	Double knockout
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA protein kinase catalytic subunit
DNMT	DNA methyltransferase
DSB	Double strand breaks
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic stem cells
EZH2	Enhancer of Zeste 2
FBS	Foetal Bovine Serum
FOXO	Forkhead box class O
G4BD	Gal 4 binding domain
G3BP	GTPase activating protein (SH3 domain) binding protein 1
GDH	Glutamate dehydrogenase
GFP	Green fluorescent protein
GLP	G9a-like protein
HA	Hemaglutinin A
HAT	Histone acetyltransferase
HDAC	Histone deacetylase

HDM	Histone demethylases
Hif1-α	Hypoxia inducible factor 1, alpha subunit
HMT	Histone methyltransferase
HP1	Heterochromatin Protein 1
HR	Homologous recombination
HSF	Heat shock factor
Hsp70	Heat shock 70KDa protein
HU	Hydroxyurea
IF	Immunofluorescence
IP	Immunoprecipitation
IR	Ionizing radiation
KO	Knockout
Ku70	70k autoantigen
LINE	Long interspersed nuclear domain
MBD	Methyl-CpG binding domain
me	methylation
MEF	Mouse embryonic fibroblast
NAD	Nicotinamide adenine dinucleotide
NBS1	Nigmejen breakage syndrome 1
NF-kB	Nuclear factor-kappa B
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
NuRD	Nuclear Remodeling complex
PARP	Poly(ADP-ribose) polymerase
PCH	Pericentric constitutive heterochromatin
PCR	Polymerase chain reaction
Plk1	Polo kinase 1
PMSF	Phenylmethylsulfonil fluoride
Pol-I	RNA polymerase I
Pol-II	RNA polymerase II
PRC	Polycomb repressive complex
PRMT	Protein arginine methyltransferase
RB	Retinoblastoma protein
RNA	Ribonucleic cid
RNF	Ring finger protein
ROS	Reactive oxygen species
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SAM	S-adenosyl-L-methyonine
SCF	Skp/cullin/F-box containing complex
SINE	Short interspersed nuclear domain
shRNA	Short hairping RNA
SirT	Silent mating type information regulation 2- homolog
SKP2	S-phase kinase-associated protein 2
Suv39h	Suppressor of variegation 3-9 homolog
SWI/SNF	Switch/Sucrose nonfermenting
TAD	Topological associated domain
TNF	Tumor necrosis factor
TSA	Trichostatin A

USP	Ubiquitin serine protease
UV	Ultraviolet
WB	Western Blot
WRN	Gene associated with Werner’s syndrome
WT	Wild type

INTRODUCTION

1. Chromatin organization

In eukaryotes, DNA is associated to specific proteins, histones, to form chromatin. The individual unit of chromatin is the nucleosome that is composed by two copies of core histone octamers (H2A, H2B, H3, H4) wrapped by 146 bp of DNA (*Figure 1A*). Histones are formed by the globular domain that allows histone-histone interactions, and N-terminal tails that are highly modified posttranslationally [1]. Chromatin is organized in the nucleus in a hierarchy of a successive order of compaction that range from the 11 mm fiber to the metaphase chromosome (*Figure 1B*).

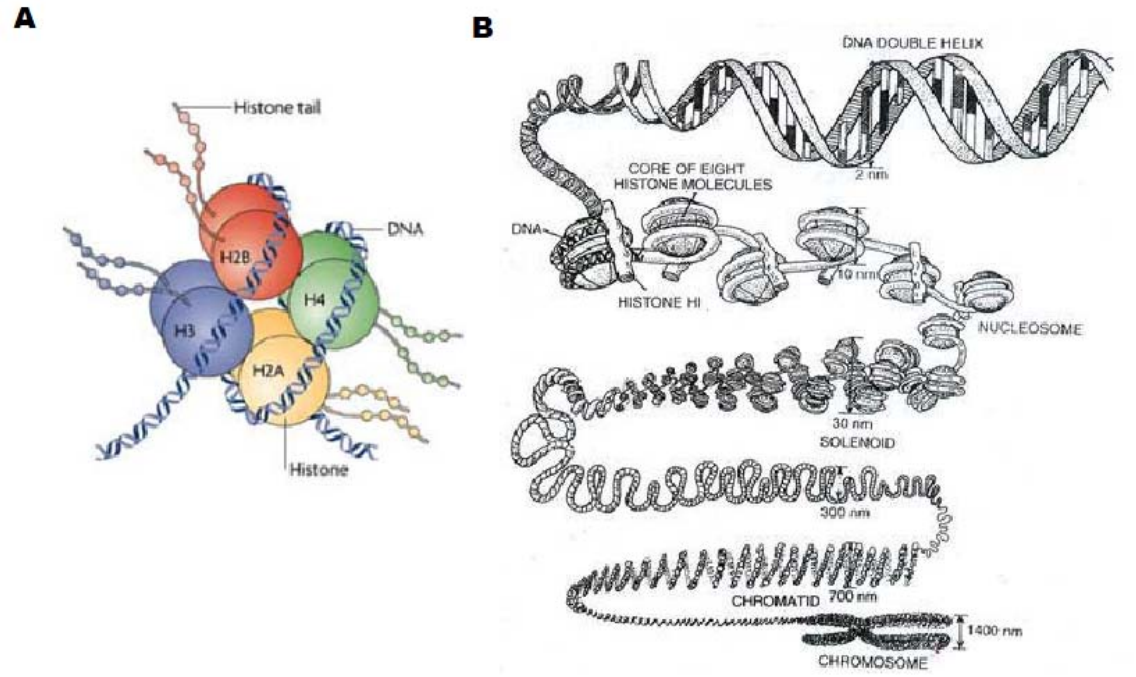

Figure 1. A. Scheme of the architecture of the nucleosome. **B.** Chromatin structure from DNA double helix to chromosome.

The level of chromatin compaction is important for the cell viability and is key for proper regulation of gene expression, DNA repair, DNA replication, genome stability and mitosis. Less compacted levels will allow the appropriate machinery to reach the DNA while higher levels of compaction would inhibit it. Thus, chromatin should be remodeled depending on specific cell conditions.

1.1. Regulation of chromatin functions

To allow an optimal modulation of cell physiology, an efficient management of the genetic information stored in DNA is required. For this purpose, two main regulatory mechanisms were developed during evolution.

1.1.1. ATP-remodeling activities

One of the factors responsible of the chromatin changes is the ATP-dependent chromatin remodeling that change nucleosomal organization allowing removal, deposition or redistribution of nucleosomes. Chromatin remodelers are required for multiple functions: deposition of nucleosomes after DNA replication, to hide or expose DNA sequence to DNA binding proteins by mobilizing nucleosomes, to provide rapid access to DNA after DNA damage by mobilizing or removing nucleosomes, to allow progression of RNA polymerase by removing nucleosomes (*Figure 2*) [2–4].

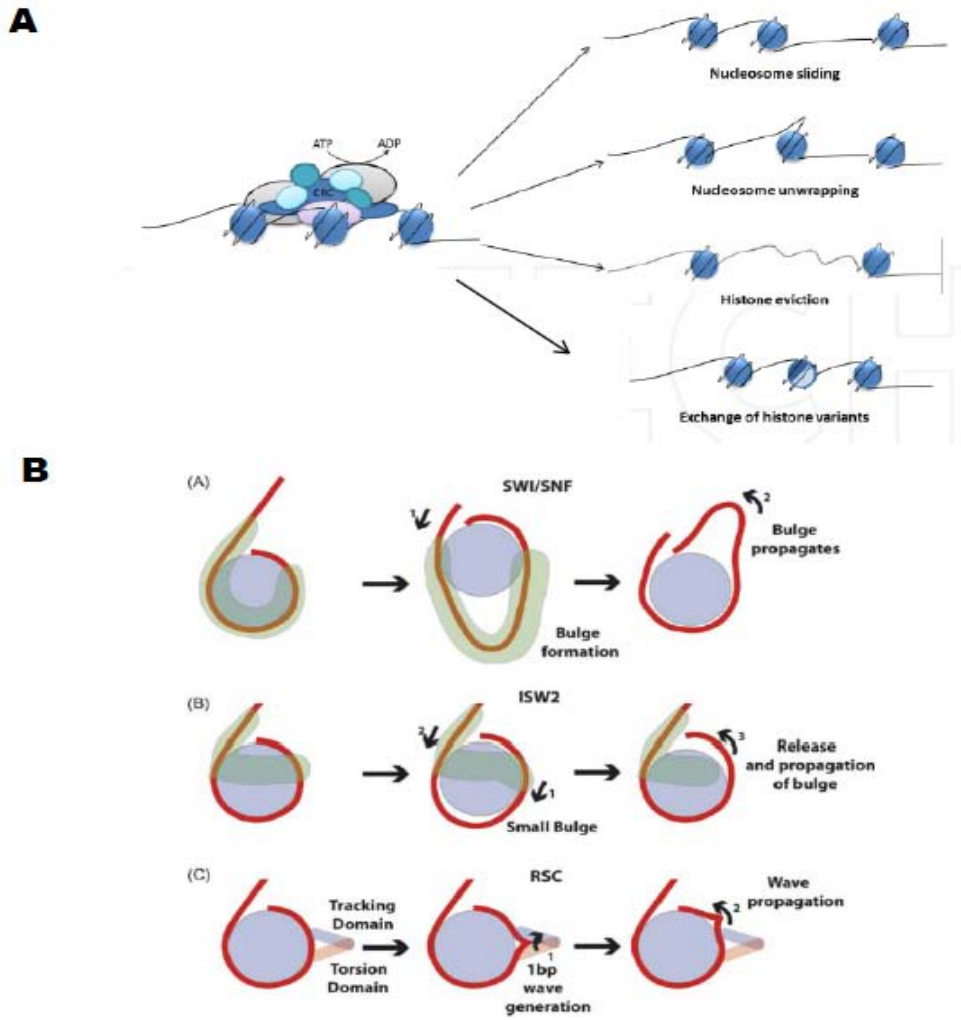

Figure 2. A. ATP-dependent remodeling chromatin functions [3]. **B.** Models of nucleosome remodeling mechanisms [4].

There are four families of ATP-remodeling chromatin enzymes: SWI/SNF, INO80, ISWI and CHD. Although they all have ATPase domain they are differentiated by their flanking domains. They are included in multisubunit complexes formed by other proteins that help to the function of the chromatin remodeler. SWI/SNF family (BAF and pBAF complexes) is involved mainly in ejection and sliding of nucleosomes. In general, ISWI family (NURF, CHRAC and ACF complexes) is important for transcription repression by spacing nucleosomes. CHD family (NuRD and CHD1 complexes) usually promotes transcription by ejecting nucleosomes but NuRD complex promotes gene silencing. INO80 family (INO80, SRCAP and TRRAP complexes) is required for DNA repair and transcription, the best described example of this family is the exchange of H2A-H2B dimer by H2AZ-H2B.

1.1.2. Posttranslational modifications of chromatin

The other key regulatory mechanism is the posttranslational modifications. They can target DNA or histones. In the first case involves cytosine methylation and is directly related to the inhibition of gene transcription. In the case of histones they take place mainly in N-terminal histone tails and involve a wide range of posttranslational modifications, the most relevant of which are acetylation, methylation, phosphorylation, ubiquitination, ADP-rybosilation and sumoylation (*Figure 3*) [5,6]. Depending on the type of modification and residue/s involved, the consequence of histone posttranslational modification may be different. Histone modifications or marks are dynamic and regulate chromatin function at two levels: first, they regulate chromatin architecture because they can destabilize nucleosomes, by interfering with histone-DNA, histone-histone and histone-chaperone interactions. Some examples are H3K56ac, H3R42me, H3K122ac, H3K64ac, H3T118phosphorylation, H1R54 citrullation, H3K79me, H4K91ac. However, little is known of most of them [7]. Second, the histone marks may be recognized by specific factors and therefore, act as docking sites for chromatin factors [8]. Several protein domains have been identified in this context. Each of them binds to particular modifications and residues. For instance, bromodomain can bind acetylated lysine residues in histones whereas chromodomain, PHD and Tudor domains bind to specific methylated lysine [1]. Therefore, different combination of histone modifications can determine different chromatin outputs because the different factors can recognize and bind to a particular combination or other factors that have been previously loaded to the chromatin [9].

Enzymes that perform these posttranslational modifications should be tightly regulated and bind to the specific sites of the chromatin. Most of them work in multisubunit complexes which can induce different outputs depending on the interacting partners of the complex. The different subunits recognizes different factors or histone modifications in order to target the chromatin site [8]. The multisubunit complexes can have multiple enzymatic activities. For instance, the conserved SAGA complex harbors both acetyl-transferase and an ubiquitin ligase activity.

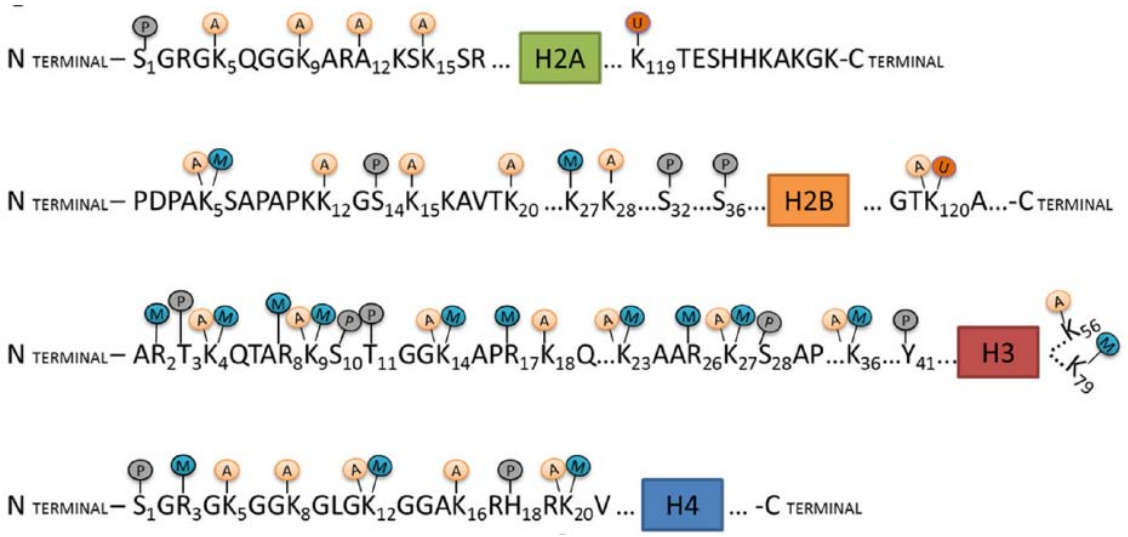

Figure 3. Major histone modifications [3].

Among these marks, from now on we will focus on acetylation and methylation marks.

2. Histone acetylation

Histone acetylation and methylation were the first histone modifications described by Vincent G Allfrey and collegues in 1964 [10]. Histone acetylation takes place in the ε-amino group of a lysine residue, is highly dynamic and it usually correlates with active chromatin. Initially, the theory was that the acetyl group would neutralize the positive charges of the histones so DNA would be found less tightly bound to the histones, in a more “open“ state. However, this theory has only been shown in vitro. In fact, one of the main reasons why chromatin is open and activate is the recognition of acetylated residues by the bromodomain of one or more subunits of chromatin remodeling

complexes and transcription factors [6]. Histone acetylation is related to a large number of functions and it interacts with a wide variety of proteins involved in different processes such as transcription, RNA splicing, DNA damage repair, cell cycle, nuclear transport and apoptosis [11].

One of the best studied acetylation marks is H4K16ac present in a 60% of H4 molecules and directly involved in chromatin structure regulation. H4K16ac prevents the formation of higher compacted structure, it correlates with gene activation and affects the binding to the DNA of chromatin associated proteins such as ISWI. H4K16ac is induced also upon DSB and it is necessary for DNA repair [12,13].

The proteins responsible for the dynamics of acetylation marks are the histone acetyltransferases (HAT) and the histone deacetylases (HDACs). They add or remove an acetyl group to or from the ε-amino group of a lysine that belongs to a histone or non-histone protein. HATs usually works in complexes and HDACs can also work either in complexes or binding different proteins.

2.1. HDACs

HDACs are classified in four classes of HDACs based in their homology to yeast HDACs: Class I (HDAC1, HDAC2, HDAC3, HDAC8), Class II (is subdivided: Class IIa: HDAC4, HDAC5, HDAC7, HDAC9, and Class IIb: HDAC6, HDAC10), Class III or sirtuins (SirT1-7) and Class IV (HDAC11). Class I shows homology to the yeast HDAC Rpd3p, Class II to the yeast Hda1 protein, Class III to the yeast Sir2 protein and Class IV shares sequence similarity to Class I and Class II (*Figure 4*).

Class I, II and IV require a zinc ion for their catalytic activity while class III or sirtuins require NAD^+. These enzymes are unable to bind directly to DNA so they should be recruited by other factors. They deacetylate different substrates, from histones to non-histone substrates. HDACs are involved in a wide variety of processes, such as transcription regulation, DNA repair or cell cycle regulation. Because of that, they are highly regulated also through posttranslational modifications, such as phosphorylations or acetylations. Interestingly, in the case of the majority of Class I members, HDAC1, HDAC2 and HDAC3, they are not fully active unless they are present in specific multi-protein complexes such as NuRD, Sin3, CoREST or NCoR/SMRT

	HDAC family member		Substrates	Binding Partners	Tissue Expression	mouse knock out phenotype
class I	HDAC1	N	p53, MyoD, E2F-1, Stat3, androgen	Sin3, Mi-2/NuRD, CoREST	ubiquitous	embryonic lethal day 9.5, p21 and p27 up-regulation, reduced overall HDAC activity
	HDAC2	N	Bcl-6, Stat3, glucocorticoid receptor, YY-1	Sin3, Mi-2/NuRD, CoREST	ubiquitous	viable until perinatal period, fatal mulitple cardiac defects, excessive hyperplasia of heart muscle, arrythmia
	HDAC3	N	GATA-1, RelA, Stat3, MEF2D, YY-1, SHP	N-CoR/SMRT	ubiquitous	embryonic lethal before day 9.5, defective cell cycle, DNA repair and apoptosis in embryonic fibroblasts. Conditional liver knock out results in hepatocyte hypertrophy and induction of metabolic genes
	HDAC8	N/C	nd	EST1B	ubiquitous	nd
class II A	HDAC4	N/C	GCMa, GATA-1, HP-1	ANKRA, RFXANK	heart, smooth muscle, brain	viable, premature and ectopic ossification, chondrocyte hypertrophy
	HDAC5	N/C	Smad7, HP-1, GCMa	REA, estrogen receptor	heart, smooth muscle, brain	myocardial hypertrophy, abnormal cardiac stress response
	HDAC7	N/C	FLAG1 and 2	HIF1a, Bcl-6,	heart, placenta, pancreas, smooth muscle	embryonic lethal, lack of endothelial cell-cell adhesion
	HDAC9	N/C	nd	FOX3P	smooth muscle, brain	viable at birth, spontaneous myocardial hypertrophy
class II B	HDAC6	C	a-Tubulin, HSP90, SHP, Smad7	nd	kidey, liver, heart, pancreas	viable, no significant defects, increase in global tubulin acetylation. MEFs fail to recover from oxidative stress
	HDAC10	C	HSP90?	nd	spleen, kidney, liver	nd
class IV	HDAC11	N/C	nd	HDAC6?	heart, smooth muscle, kidney, brain	nd

Abbreviations: MEFs, mouse embryonic fibroblasts; N, nuclear; C, cytoplsmic, N/C, nuclear and cytoplasmic; nd, no data

Figure 4. Summary of HDACs localization, non-histone substrates, binding partners, expression and mouse knock out phenotype.

In general, HDACs act as repressors of gene expression through histone deacetylation. However, they can also participate in activation given their capacity to deacetylate other non histone proteins [14,15].

3. Lysine histone methylation

Histone methylation is found in lysines, arginines and histidines. In lysines, methylation takes place in the ε-amino group (as acetylation), which could be mono-, di- or trimethylated. There are three types of arginine methylation: monomethylarginine, asymmetric dimethylarginine and symmetric dimethylarginine.

The histone methyltransferases (HMTs) are the enzymes that catalyze the methylation whereas the histone demethylases (HDMs) promote the removal of the methyl groups. Histone methyltransferases use S-adenosyl-L-methyonine (SAM) as a methyl donor in the reaction (*Figure 5*) [16,17]. Interestingly, HMTs not only target histones, but also other non-histone proteins. For instance, the HMT SETDB1 methylates ING2[18].

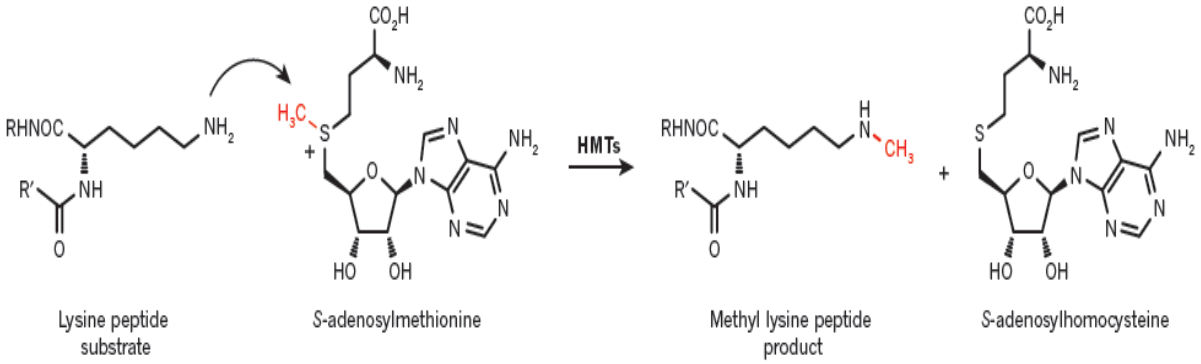

Figure 5. Mechanism of lysine methylation by HMTs. They need the cofactor S-adenosylmethyonine (SAM) as a methyl donor [17].

The histone methyltranferases are divided in three families: the SET-containing proteins (which include almost all lysines HMTs), Dot1 like proteins and the PRMTs. The first two encompasses the lysine HMTs, whereas the PRMTs include the arginine HMTs. In contrast, the HDMs are divided in two families: the amine-oxidases (LSD1 domain containing) and the Jumonji family.

There is a combinatorial code between methylation marks. For instance, H3K4me3 is an active mark while H3K9me and H3K27me3 are repressive marks for gene transcription. Upon an increase in H3K4me3, H3K9me and H3K27me3 are removed. Some specific marks such as H3K4me3 seem to be important for the binding of transcription factors or other proteins and, as a result, for initiation of transcription. In fact, the demethylase LSD1 removes monomethyl H3K4 and represses transcription [19]. Moreover, H3K4m3 inhibits the interaction between the H3K9 methyltransferases, with histone H3 abrogating their activity on H3K9 [18].

The di- and tri- methylation in H3K9 is a hallmark of compacted and silenced chromatin, or heterochromatin, conserved since early eukaryotes [20]. In mammals, the H3K9me2,3 methyltransferases are: Suv39h1, Suv39h2, G9a, GLP and SETDB1. Although they usually work independently, in some cases they seem to work together. For instance, in some cases, all four can form a multimeric complex that has been shown to regulate a subset of G9a target genes or pericentric major satellite repeats, considered a Suv39h1 target [21]. In this text, we will focus on G9a and Suv39h1.

3.1. G9a

G9a or EHMT2 is very similar to GLP or EHMT1: they share 45% of homology in their sequences. G9a has different domains: N-terminal region harboring the NLS sequence; ankyrin repeats-containing domain that mediate protein-protein interactions; and, the catalytic SET domain with a preSET and postSET domains [22].

G9a can mono- and dimethylate H3K9 and, in very specific conditions, it has been shown to target H3K27. G9a depletion triggers an accumulation of H3K9ac and H3K4me3 due to the absence of H3K9me [22,23]. G9a has a stronger specific activity in vitro than Suv39h1 [22,24]. In general, G9a is a H3K9 methyltransferase that targets euchromatic regions and induces repression in these active genes. However, G9a can induce activation in some specific genes but this effect is totally independent from its HMT activity, and it seems to depend on its interacting factors. G9a depletion causes an arrest in embryonic growth and death because some key genes are activated when they should be repressed [22,23]. In fact, G9a/GLP interacts with the polycomb repressive complex 2 (PRC2) complex that contains the H3K27me3-specific HMT EZH2, which is key for embryonic development. G9a activity is necessary for recruitment of PRC2 complex and interaction with a subset of genes involved in development that should remain silenced [25].

Either GLP deletion or G9a deletion in ES cells show the same phenotype: HP1 relocalization, activation of Mage-a gene expression and a notably decrease in mono- and dimethylation in H3K9 [22,23]. GLP and G9a forms heterodimers through their SET domain to bind euchromatin and they coordinate HP1 recruitment. In fact, G9a stability depends on GLP [26]. G9a can automethylate without affecting its catalytic activity. This automethylation creates a docking site for HP1, that may act as a binding platform for suppressor factors [27].

In euchromatin, like heterochromatin, both DNA and histone methylation are important for gene silencing. DNA methylation is mediated by Dnmts while histone H3K9 methylation in euchromatin is mainly based in G9a/GLP. In the establishment of silencing, histone methylation seem to be a preceding event before DNA methylation. In fact, depletion either G9a or GLP avoids Dnmt3 recruitment to the chromatin inducing gene derepression. However, the presence of both G9a and GLP even if involves catalytically inactive mutants does not avoid the recruitment of Dnmt3 and as a result the genes remain repressed. In contrast, Dnmts depletion does not induce per se derepression of these genes, since G9a/GLP are present and active [28]. G9a controls transcription of important genes such as p21. In a cycle-dependent manner

G9a binds to UHRF1 in the p21 promoter and they recruit DNMT1 and HDAC1 in order to repress p21 gene expression [29].

G9a is also necessary to maintain DNA stability during replication. DNMT1 binds to DNA in the replication fork through PCNA and recruits G9a to dimethylate H3K9 in order to maintain gene repression. After replication, Suv39h1 can bind H3K9me2 to trimethylate it in heterochromatin [30].

G9a and GLP are considered as oncogenes, and in fact, some cancers show overexpression of both enzymes. Interestingly, G9a and GLP KD cells are more prone to apoptosis [31].

3.2. Suv39h1

Suv39h1 and Suv39h2 share 55% of homology. Suv39h1 has different domains: N-terminal domain, chromo domain, preSET domain, SET domain and post SET domain. N-terminal domain is necessary for the interaction with DNA while the chromodomain is necessary for the proper localization of the enzyme. Suv39h1 uses monomethylated H3K9 as a substrate [32,33].

Depletion of Suv39h1 leads to a genomic stability and high lethality [34]. In contrast, transgenic mice overexpressing Suv39h1 show retarded growth, skeletal malformations and impaired erythroid differentiation [35].

3.2.1. Constitutive heterochromatin formation

Suv39h1 is the main regulator of constitutive heterochromatin in the cell, which encompasses pericentric and telomeric heterochromatin, and has a key structural role for the maintenance of genome integrity. The current model for heterochromatin formation and spreading predicts that upon arrival to chromatin, Suv39h1 methylates H3K9me3 in the neighboring nucleosomes, which creates docking sites for the protein HP1 that binds to the mark through its chromodomain. This, in turn, recruits Suv39h1 through its chromoshadow domain to the next nucleosome, spreading the mark and the structure. HP1 constitutes and essential and structural protein for an accurate heterochromatin formation and both interactions are required for HP1 recruitment [36–39].

Interestingly, Suv39h1 overexpression can form ectopic heterochromatin areas. In addition to its H3K9me3 HMT activity Suv39h1 also seems to play structural role in

heterochromatin structure as an important population of Suv39h1 molecules remains bound to the chromatin with a very low or no mobility in the pericentric heterochromatin structure. In fact, this seems to be part of a very dynamic process, since inhibition of deacetylases with Tricostatin A (TSA) induces a higher mobility of Suv39h1 present in heterochromatin [40,41].

Then, Suv4-20h is recruited by HP1 and Suv39h1 and catalyzes the deposition of H4K20me3, another histone mark for silencing and chromatin compaction (*Figure 6*) [42].

Other factors that are important for heterochromatin structure is that the general structure is hypoacetylated [39] and methylated by Dnmt3 in the DNA of the repeats [43].

Constitutive heterochromatin is usually enriched in repetitive sequences. H3K9me3 and H4K20me3 are very frequent marks found in repetitive sequences, including major and minor satellites, DNA transposons, retrotransposons, long interspersed nucleotide elements (LINEs) and short interspersed nucleotide elements (SINEs) and DNA methylation is not related to these patterns. In fact, Suv39h1 depletion induces an increase in the expression of these repetitive elements [44].

Regarding telomeric heterochromatin, both Suv39h1 and Suv39h2 are necessary for a correct telomere capping. Depletion of both enzymes induces an abnormal telomere length, a decrease in di-and tri- methylated H3K9 as well as less HP1 levels in the telomeres. Interestingly, Suv39h1 depletion is necessary to abolish critical shortening telomeres induced by myc overexpression in leukemias [45,46].

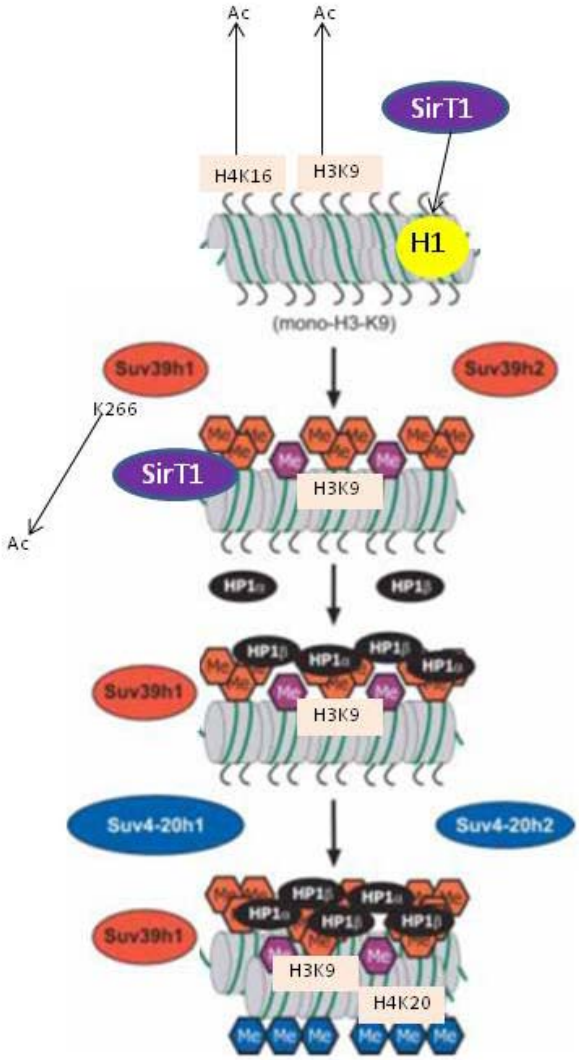

Figure 6. Summary of heterochromatin formation under stress. Suv39h1 is essential for heterochromatin [42].

3.2.2. Other mechanisms of gene silencing through Suv39h1 function

Suv39h1 also plays a role in facultative heterochromatin. Retinoblastoma (RB) protein, a repressor of gene transcription that represses E2F transcription factors, recruits Suv39h1 and HP1 to the promoters in order to silence target genes such as cyclin E and cyclin A (genes required for S-phase). Suv39h1 methylates H3K9me3 in these promoters inducing growth inhibition. Phosphorylation of RB catalysed by CyclinE/Cdk2 inhibits the interaction with Suv39h1 [47,48]. The second best example is the functional relationship between SirT1 and Suv39h1 in facultative heterochromatin

formation. SirT1 deacetylates H3K9ac and recruits Suv39h1 promoting H3K9me3 silencing chromatin. Furthermore, SirT1 deacetylates Suv39h1 in K266 enhancing its activity (*Figure 6*) [49,50]. An interesting example is the regulation of rDNA expression in the nucleolus. There, upon nutrient deprivation conditions, SirT1 forms the eNoSC complex together with Suv39h1 and the H3K9me2,3 binding protein nucleomethylin and represses ribosomal genes expression, inhibiting protein production [51].

For transcriptional repression, Suv39h1 has also been shown to associate with other proteins involved in silencing, specially, with HDAC1 and HDAC2. Suv39h1 interacts through its N-terminal domain with HDAC1 and HDAC2 in order to repress transcription and associates frequently to the called "core histone deacetylase complex" composed by HDAC1, HDAC2, RbAp48 and RbAp46 proteins [52]. There are numerous examples of cooperation between Suv39h1 and HDACs in the regulation of gene expression. Runx1 interacts with Suv39h1 and the repressor complex Sin3 (which contains HDAC1 and HDAC2) and HDAC3 to induce silencing [53] and Cabin-1 represses MEF2-target genes by interacting with Suv39h1 and the Sin3 complex and inhibits the association of MEF2 to the coactivator p300 [54]. Suv39h1 also plays a role in muscle differentiation because it seems to interact with the transcription factor MyoD in proliferating muscle cells in order to induce silencing of genes involved in muscle differentiation such as myogenin [55]. The transcription factor Sp1 which activates Cyclin B1 expression is methylated after hydrogen peroxide. This leads Sp1 to interact with HDAC1, HDAC2 and Suv39h1. This interaction inhibits in turn the capacity of Sp1 to activate Cyclin B1 expression and its downregulation induces cell cycle arrest in G_2 phase [56]. Another transcription factor, COUP-TF-interacting protein 2 (CTIP2), also interacts with HDAC1, HDAC2 and Suv39h1 in order to inhibit p21 expression. Suv39h1 recruitment is absolutely necessary for the control of p21 expression through CTIP2 [57].

In other cases, Suv39h1 has been functionally linked to other chromatin factors. For instance, Suv39h1 promotes cell migration, invasion and metastasis in breast cancer by repressing expression of E-cadherin, a protein that prevents the epithelial mesenchymal transition, a key process in development and cancer [58]. Snail binds to the E-cadherin promoter and recruits LSD1 to remove H3K4 methylation. Then, Snail recruits Suv39h1, which methylates H3K9me3 and induces gene silencing of E-cadherin. Suv39h1 also plays a role in adipogenesis. Ap-2α recruits Suv39h1 and G9a to the C/EBPα promoter, repressing its expression and inhibiting the pro-adipogenic activity of the transcription factor C/EBPα. Thus, G9a and Suv39h1 di- and tri-methylate, respectively, H3K9 [59]. Additionally, Suv39h1 and GLP form a complex with

MDM2 for binding p53 and the whole complex binds to p53 target promoters. GLP methylates p53 and inactivates it whereas Suv39h1 methylates and silences p53 target genes [60].

Suv39h1 also interacts with proteins involved in DNA methylation in order to induce gene silencing. Both Suv39h1 and HP1β interacts with the DNA methyltransferases Dnmt1 and Dnmt3A through their PHD-like motif. [43]. It has been reported that the complex Suv39h1-Dnmt3A-HP1α interacts to cullin4B-Ring E3 ligase complex (CRL4B) for repressing gene expression. CRL4B interacts to the promoter of tumor suppressor genes and recruits the complex [61]. Suv39h1 interacts with methyl-CpG binding domain 1 (MBD1) through its MBD domain. MBD1 recognizes methylated DNA and represses transcription by interacting with Suv39h1-HP1 complex. Then, HDAC 1 and 2 interacts indirectly to MBD1 through Suv39h1 and deacetylates histones promoting gene silencing. In this way, DNA methylation and silencing histone modifications are connected [62].

3.2.3. Cell cycle and Suv39h1

Suv39h1 is important for chromatin condensation during cell cycle progression. The levels of H3K9me3 in pericentric heterochromatin, mediated by Suv39h1 are essential for maintaining the pattern of other epigenetic marks such as H4K12ac and H3S10P. H3K9me3 avoids phosphorylation of H3S10 by the protein kinase Aurora and IKKα. Aurora A phosphorylates H3S10 in G2/M phase and this modification is necessary for chromatin condensation during mitosis [63]. Suv39h1 overexpression induces a redistribution of H3S10 and both Suv39h1 and HP1 all over the chromosome, a delay in G2/M phase and chromosome segregation defects.

In fact, Suv39h1 is dynamically distributed during cell cycle. Although it is dispensable for the enrichment in cohesions, it is found in centromeres from prometaphase until anaphase. From G_2 to metaphase, the levels of H3K9me3 in centromeres, Suv39h1 and SETDB1 increase while H3K9ac levels decrease to ensure chromosome condensation. In fact, Suv39h1 inhibition induces chromosome misalignments through an increase in Aurora B kinase activity which phosphorylates microtubule depolymerase (MCAK) interfering the interaction between kinetochore and microtubule (*Figure 7*). Methylation by Suv39h1 orchestrates kinetochore dynamics through feedback to polymerase regulation [32,64–68].

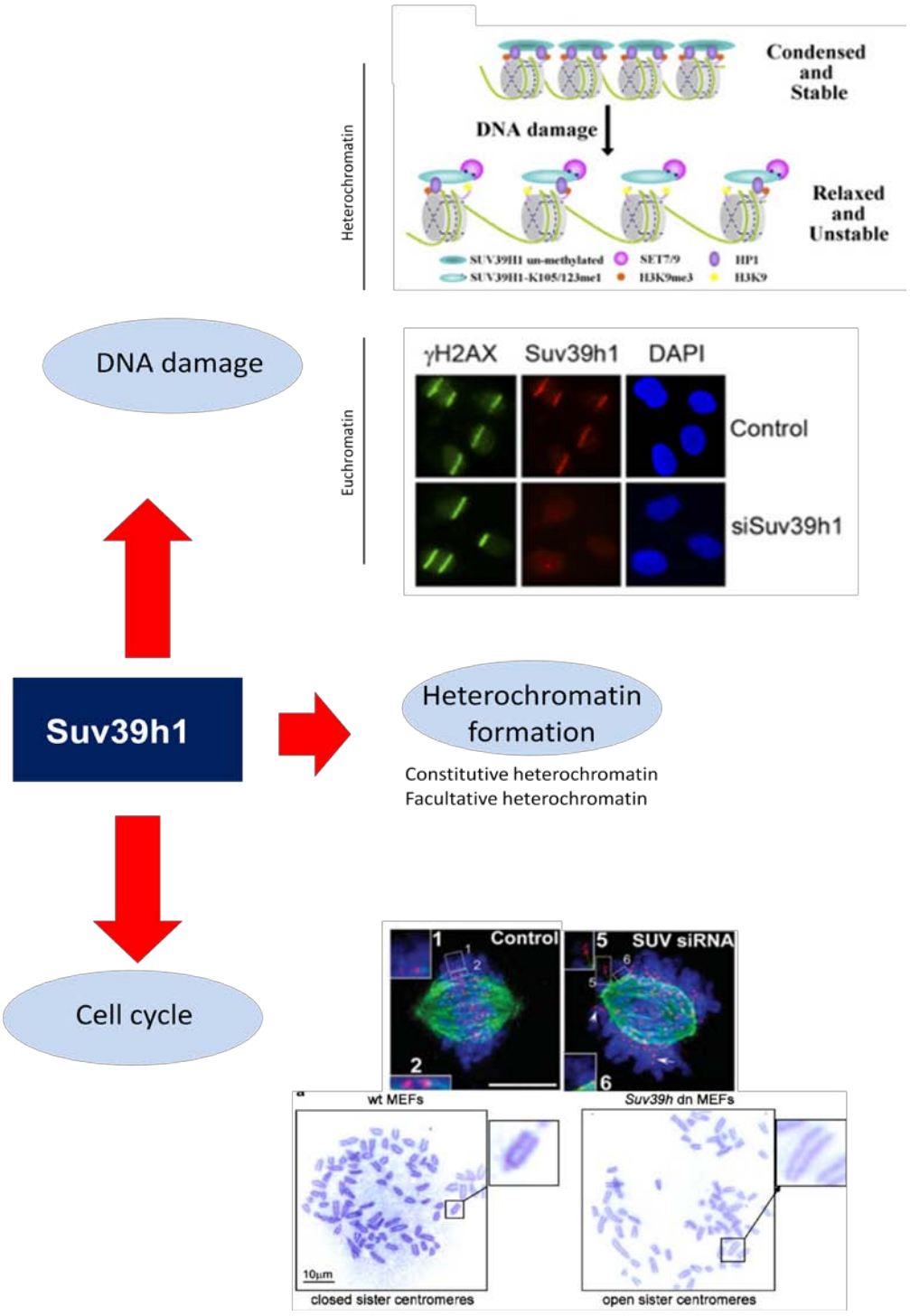

Figure 7. Scheme of Suv39h1 functions. Suv39h1 is involved in heterochromatin formation, cell cycle and DNA damage. **DNA damage**. Heterochromatin and euchromatin have different DNA damage signalization [69,70]. **Heterochromatin formation**: Suv39h1 is involved in both constitutive and facultative heterochromatin. **Cell cycle**. Defects in chromosome segregation. Red: centromeres, Green: microtubules, Blue: DAPI [68]. Cells arrested in prometaphase. Giemsa staining [66].

Suv39h1 is phosphorylated in G_1/S phase, and this phosphorylation is increased when Sbf1, a phosphatase inhibitor, is overexpressed. The phosphorylation inhibits the transcriptional repression mediated by Suv39h1 [71]. During S-phase CDK2 phosphorylates Suv39h1 in S391, which leads to a dissociation of Suv39h1 from the chromatin allowing a correct heterochromatin replication. This dissociation is accompanied by the recruitment of the demethylase JMJD2A in the heterochromatin foci. However, the complete absence of Suv39h1 induces a hypersensibility to the replication stress and impairs DNA replication [72].

After DNA replication, the HP1α-CAF1-SETDB1 complex interacts to pericentric heterochromatin in order to monomethylate H3K9 and provides the substrate for Suv39h1 that di- and trimethylates H3K9. Also, this complex provides HP1α, necessary for the heterochromatin formation. In this way, pericentric heterochromatin is restored again after replication [73].

3.2.4. DNA damage and Suv39h1

The H3K4 histone methyltransferase SET7/9 binds to the Suv39h1 chromodomain and methylates Suv39h1 in K105 and K123 upon DNA damage. Methylated Suv39h1 show a decreased activity but without alteration of neither the localization nor the protein stability. This drop in Suv39h1 activity induces a loss in H3K9me3 levels, which in turn associates with heterochromatin relaxation, satellite derepression and genomic instability (*Figure 7*) [69]. In constitutive heterochromatin to allow the full access to repairing machinery, demethylation of H3K9me3 and chromatin relaxation takes place. In fact, p53 regulates the heterochromatin repair because it represses Suv39h1 expression and upregulates the expression of the histone demethylase JMJD2b [74].

After DSB, the complex Suv39h1/kap1/HP1 rapidly goes to the foci and increases the H3K9me3 spreading along the DSB, which seems to help to stabilize damaged chromatin. Then, the HAT Tip60 is activated by binding H3K9me3, and in turn acetylates and activates ATM. As a negative feedback to facilitate a decondensed chromatin and avoid overactivation, activated ATM removes Suv39h1 activated complex. Supporting these evidences, ATM depletion triggers an increase in H3K9me3 at 53BP1 foci and the downregulation of Suv39h1 restores the ATM deficiency phenotype allowing an accurate repair. Pre-lamin A and progeronin interact with Suv39h1 and avoid their degradation leading to DNA repair defects [70,75].

4. Ubiquitination

Ubiquitin is a protein formed by 76 residues (MW 8KDa). Ubiquitin is convalently bound to proteins through the ε-amino group of the lysines. Ubiquitin is transferred to the substrate after a three-step reaction in which three different enzymes are involved: E1, E2 and E3. E1 is the activating enzyme and forms a thiol-ester bound to the ubiquitin using ATP; the E2 is the conjugating enzyme that receives the transference of the activated ubiquitin from E1; and E3 is the ubiquitin ligase that transfers ubiquitin from E2 to the substrate. Usually there is only a single E1, different E2 and many different E3 or E3 complexes that are characteristic for the recognition of the particular substrate, in fact, E3 ubiquitin ligase is very specific of the substrate. There are three main families of the E3 ubiquitin ligases: the HECT family, in which the ubiquitin forms a thiol esther intermediates with the E3; and the RING family and U-box family, in which the ubiquitin is directly transferred from the E2 to the substrate. Ubiquitination is important in order to develop some cellular functions such as cell cycle progression, repair, cellular differentiation and protein transport [76–78].

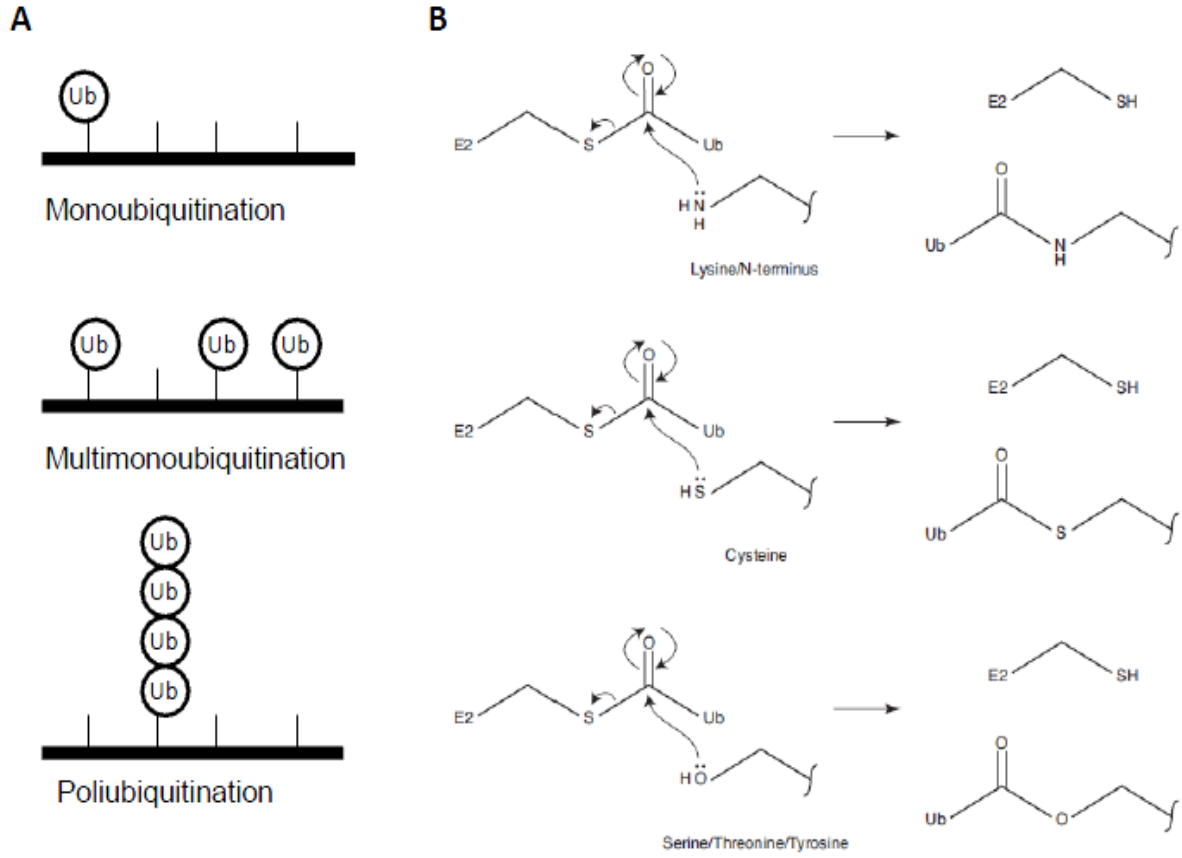

Figure 8. A. Different protein ubiquitination. **B.** Ubiquitin reaction of lysines, cysteines, serines, threonines and tyrosines [79].

Proteins can be monoubiquitinated or multimonoubiquitinated, when the protein has only one ubiquitin or when there is more than one residue with one ubiquitin, respectively; and, proteins can be polyubiquitinated when they have ubiquitin polymers (*Figure 8A*). Depending on the nature of the ubiquitination, it can lead to protein degradation or other functions [80]. Usually polyubiquitin is a target for proteasomal degradation, but, in fact ubiquitin can perform other functions, such as regulate protein subcellular localization, function or protein-protein interactions [81]. Monoubiquitination does not trigger proteasomal degradation. Another characteristic is that ubiquitination can be reverted by deubiquitinases. Moreover, it depends also in the place where ubiquitination takes place: it is different in nucleus and cytoplasm. Also different linkages of the ubiquitin to the substrate determine the ubiquitination function, as ubiquitin can bind to the target protein by: K6, K11, K27, K29, K33, K48, K63, Met1. Interestingly, all of them except K63 can target proteins for degradation [78,80]. It has been described domains that recognize specific ubiquitinations in certain proteins [82].

Recently, other alternatives to lysine ubiquitination have been described. Canonical ubiquitination is referred to the ubiquitination in lysine residues and noncanonical ubiquitylation refers to the one that occurs in non lysine residues (*Figure 8B*). Ubiquitination can be blocked by either removing lysines or adding postranslational modifications to this lysine such as acetyl or methyl groups [79].

Non canonical ubiquitination could take place in cysteines, threonines, serines and tyrosines. Cysteines link to ubiquitin through a thioester linkage. Until now there are very few examples of this ubiquitination due to the fact that this ubiquitination is weaker and is probably destroyed with reducing agents in the protein extraction process. So far, cysteine ubiquitination has been described in peroxisomal import factor, Pxp5, Pex20p and in immunosuppression induced by viral E3 ligases. Serines, threonines and tyrosines link to ubiquitin through hidroxyester linkages. In theory, the reason why these non canonical ubiquitinations occur is that they are more dynamic than lysine ubiquitination as they are not stable and could be an advantage depending on the function. Until now, it has not been reported any deubiquitinase of non canonical ubiquitination, maybe, because the ubiquitination can be reversed by other factors [79].

Next, it will take place an introduction about the E3 ubiquitin ligase CHIP (STUB1), CHFR and SKP2 and the importance of the ubiquitination in cell cycle progression because they are the aspects from the ubiquitination that the study has been interested on.

4.1. CHIP (STUB1)

The C-terminus of Hsc70-interacting protein (CHIP) or STUB1 is an ubiquitin ligase whose catalytic domain is a U-box [83]. CHIP is involved in cellular adaptation to stress. Its localization is ubiquitous but seems to be mainly present in the cytoplasm. Under stress, a lot of proteins are unfolded or not folded correctly, which requires their elimination from the cell [84]. CHIP participates in the quality control of the proteins by promoting degradation of these unfolded proteins in collaboration with the chaperones Hsc70 and Hsp90.[85]. For example, mutant p53 but not WT is degraded through the system of chaperon Hsc70 and Hsp90 and CHIP because mutant p53 remains unfolded [86]. CHIP function is regulated by Ataxin-3 and Ube2w. Ube2W is an E2 conjugating enzyme that monoubiquitinates CHIP. This monoubiquitination is recognized by the deubiquitinase Ataxin-3 which controls the ubiquitin chain length of the CHIP substrates and when it is critical induces the clearance of the substrate and deubiquitinates CHIP [87].

CHIP mRNA is increased after stress as a mechanism of adaptation [84]. Under heat shock, CHIP activates heat shock factor 1 (HSF1) which is the responsible of the activation of expression of genes involved in heat shock such as Hsp70 [88,89]. CHIP has preferences to ubiquitinate unfolded proteins but when there are not more unfolded proteins, it ubiquitinates Hsp70 in order to control protein levels of Hsp70 [83 90]. Under prolonged hypoxia, the system Hsp70-CHIP is also involved in degradation of Hif-1α, a transcription factor which controls glucose metabolism genes [91].

CHIP also regulates genome stability by regulating levels of proteins involved in base excision repair [92] and the telomerase reverse transcriptase [93] and it has a role in regulation of apoptosis by controlling tAIF, a protein that triggers apoptosis [94], and senescence by promoting degradation of oxidized proteins [95]. An interaction between CHIP and SirT6 has already been described in which CHIP stabilizes SirT6 levels. In fact, CHIP depletion leads to a premature senescence as SirT6 depletion does [96].

CHIP also acts as a tumor suppressor by controlling c-Myc levels [97] and NF-kB pathway that is tissue-dependent. In breast cancer cell NF-kB pathway is negatively regulated by CHIP through degradation of the TNF receptor (TRAF2), therefore, NF-kB transcription factors do not translocate to the nucleus and activate expression of genes such as urokinase plasminogen activator and matrix metalloproteinase 9 involved in breast cancer invasion [98]. In colorectal cancer, CHIP inhibits also NF-kB by inducing degradation of p65 NF-kB transcription factor [99]. However, CHIP can also activate the

pathway. For instance, in macrophages and dendritic cells, CHIP activates Toll-like receptor [100]. Also in T-cell CHIP-mediated ubiquitination of the NF-kB positive regulator CARMA1 is necessary for its activation instead of degradation [101].

4.2. CHFR

CHFR is involved mainly in cell cycle regulation and DNA damage. Accordingly, CHFR deficient mice develop spontaneous tumors and they have chromosomal instability [102]. CHFR has four different domains: N-terminal FHA domain, a RING domain, a Cys-rich domain and a C-terminal PBZ motif [103].

Levels of CHFR are regulated by both auto-ubiquitination, which can be reverted by USP7; and sumoylation induced by UBC9 and reverted by SENP2. Both of them induces CHFR degradation [104,105].

Regulation of mitotic stress and the regulation of progression to mitosis are two processes completely independently regulated by CHFR. CHFR induces degradation of Aurora A, a kinase required for cell cycle progression involved in tumorigenesis [102]. CHFR levels in cell cycle progression depend on its auto-ubiquitination. In response to mitotic stress, CHFR levels are increased and induces degradation of PARP1, an important regulator for cell cycle progression [106]; regulates p38 stress kinase [107,108], a protein involved antephase checkpoint; promotes degradation of polo-like kinase 1 (Plk1), that activates mitosis-promoting factor [109,110]; and regulates levels of TOPK kinase, necessary for the transition G_2/M [111], therefore CHFR induces cell cycle arrest at G_2/M by activation of mitotic checkpoints.

CHFR is involved in DNA damage repair. Upon DSB, CHFR is recruited to DNA damage sites and induces degradation of PARP1 [103]. Then, it works coordinately with RNF8 monoubiquitinating H2A and H2B that leads to downstream signal of DNA damage [112]. Other E3 ubiquitin ligases such as RNF20, RNF40 and RNF168 arrive to DNA damage foci [113,114].

CHFR is a tumor suppressor because of its relation with DNA damage response, regulates negatively NFkB pathway [115] and mainly because it is involved in early mitotic checkpoint. It is found downregulated in some cancers from lung, colon, esophageal, brain, bone, breast, gastric and hematopoietic origin. It is also absent in some cells lines such as HeLa, HCT116 and MCF7 and is mutated in U2OS, suggesting that CHFR depletion is an advantage to cultivate cells [102,116].

CHFR suppresses also metastasis. CHFR induces HDAC1 degradation upregulating the expression of p21 (important for the checkpoint in G_1/S phase) and metastasis suppressor factors such as KAI1 and E-cadherin. Furthermore, CHFR polyubiquitinates HLTF, a protein involved in chromatin remodeling which regulates expression of metastatic genes [116,117].

4.3. Cell cycle and ubiquitination

Ubiquitination plays an important role in cell cycle progression (*Figure 9*). Cell cycle is regulated mainly by phosphorylation by cyclin-dependent kinases (CDK). Since these CDKs are expressed in a constant way during cell cycle, there are other factors by which CDKs are regulated, for example, cyclins. The levels of these factors fluctuates during cell cycle because they are ubiquitinated and degradated [118].

The E3 ubiquitin ligases mainly involved in cell cycle control belong to the RING family and are anaphase-promoting complex/cyclosome (APC/C) and Skp/cullin/F-box-containing complex (SCF) [118].

APC/C is essential to separate sister chromatids in anaphase and exit from mitosis by polyubiquitinating securin (an inhibitor of separase) as well as to regulate late S phase replication by polyubiquitinating the replication factor CTD1 (a factor necessary to initiate replication). After polyubiquitination both factors are degraded by proteasome. APC/C has at least twelve subunits and it works together with the E2 ubiquitin ligases Ubch5 and Ubch10. The subunit Apc11 recruits Ubch5 [119]. APC/C can work with different cofactors, the best studied of which are Cdc20 (for the separation of chromatids) and Cdh1 (to exit from mitosis and star G1) [118]. APC/C should be inhibited in early mitosis to prevent premature chromatid segregation. Mitotic Checkpoint Complex inhibits APC/C-mediated securin degradation by interacting with the APC/C and changing the position of Cdc20 [120].

SCF complexes are composed by four core proteins: Skp1, Roc1(RBX), Cullin1 and F-Box E3-ubiquitin ligase protein [121]. Cullin1 (Cul1) works as the main scaffold protein of the complex. It interacts with SKP1 and Roc1. Roc1 recruits the E2 ubiquitin ligase and SKP1 binds to the F-box proteins through their F-box motif. Neither the F-box protein nor the E2 ubiquitin ligase interacts directly with Cul1 [122]. F-box proteins are divided in three classes depending on its substrate interaction domains: FBXW, FBXL

and FBXO [123]. The best characterized are β-TrCP1, FBW7 and SKP2 that participates in the cell cycle control.

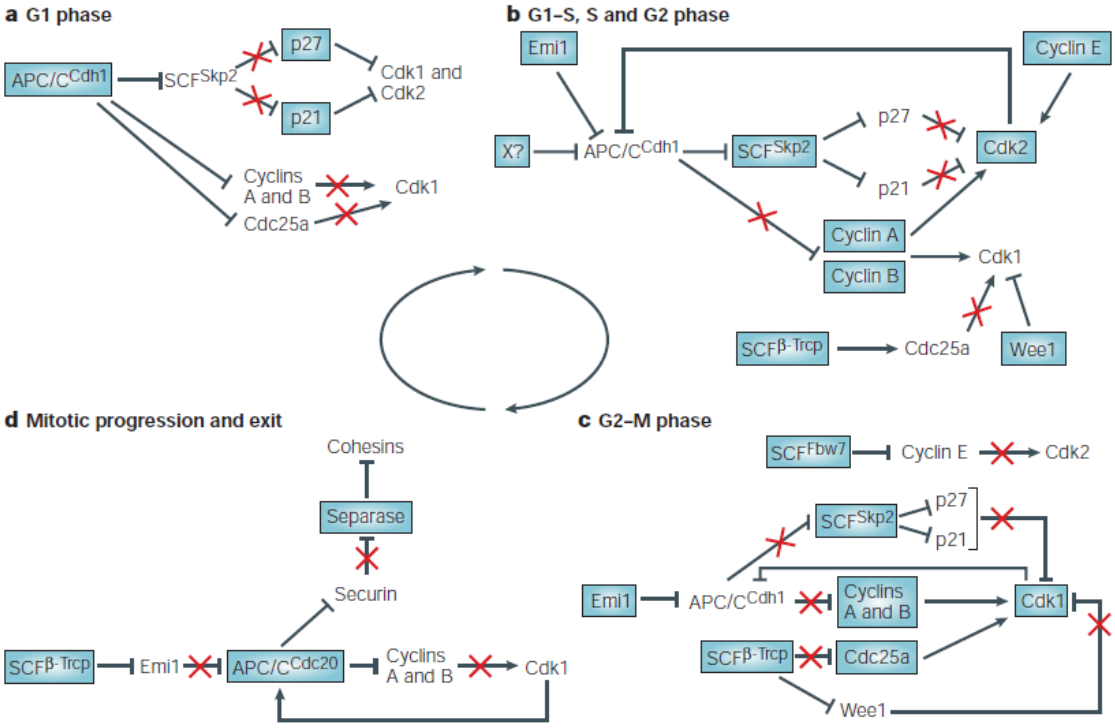

Figure 9. Cell cycle progression regulated by ubiquitination: APC/C complex and SCF complex have an important role in regulating levels of cyclines and other important factors for cell cycle progression [124].

Fbw7 induces poliubiquitination and degradation of cell cycle activators such as c-Jun, Notch1, cyclin E, c-myc. The substrates should be phosphorylated prior to the ubiquitination. At the early of mitosis, βTrcp induces degradation of Emi1, an APC/C inhibitor, and Wee1, an inhibitor of CDK activity. Both Fbw7 and βTrcp interact with the substrate through their WD40 domain [118].

4.4. SKP2

SKP2 is an F-box E3 ubiquitin that belongs to the FBXL class. It is localized mainly in the nucleus although it can be found in the cytoplasm. It has three domains: a D-box domain, an F-box motif and a LRR domain. D-box domain is localized at the N-terminal domain and is important for the Skp2 stability; F-box motif is localized around the mid-

protein and is the domain where Skp1 recruits SKP2 to the complex; and, LRR domain is the domain of recognition of the substrate [123].

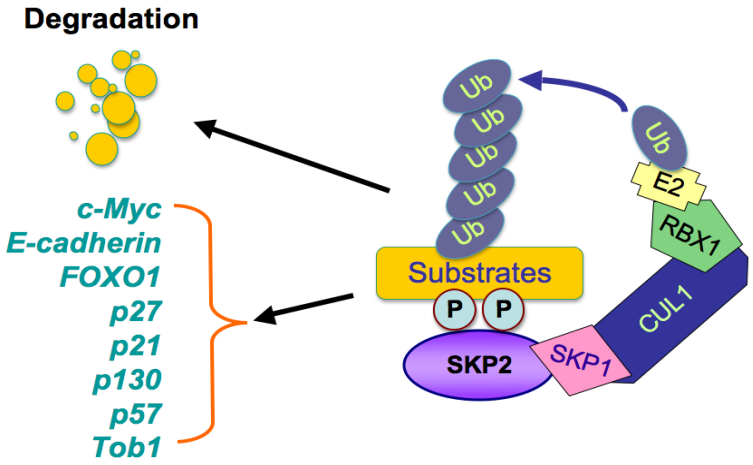

Figure 10. SCF^{skp2} complex regulates the degradation of important proteins such as c-myc and p21 among others [125].

The main role of Skp2 is to allow the cell cycle progression. Skp2 induces the progression of G_1/S phase through the degradation of some important proteins involved in the checkpoints such as p27, cyclinE, p21, p57 [125–128] (*Figure 10*). Skp2 KO cells have a delay in cell cycle progression and p27 and cyclin E levels are drastically increased [129]. p27 is the most important substrate of SKP2. However, p27 is also degraded during G_0/G_1 phase but in a Skp2-independent manner [130,131]. The substrates of Skp2 are usually phosphorylated previous to their ubiquitination. A previous phosphorylation in threonine 187 of p27 by CyclinE/CDK2 is essential for the 27 degradation by Skp2 [132]. Moreover, another factor is necessary for the degradation: Cks1 (CDK subunit 1) because it induces the recognition of the phosphorylated p27 by SKP2 [133].

p21 participates in cell proliferation, cell differentiation and apoptosis processes. p21 is regulated by different E3 ubiquitin ligases, among them, Skp2. p21 is phosphorylated by CyclinE/Cdk2. However, this phosphorylation is not required for its ubiquitination by Skp2 although it enhances the reaction [134].

SKP2 KO cells have also polyploidy and centrosome overduplication [129]. It is related with the fact that SKP2 controls re-replication: it degradates hOrc1 (human origin replication complex) and Cdt1 (a licensing factor for replication) that are necessary for replication. hOrc1 is the initiation complex for DNA replication and should be regulated

in order to avoid some extra replication. hOrc1 is phosphorylated by CyclinA/Cdk2 previous to its ubiquitination by Skp2 at S phase [135]. The degradation of Cdt1 is performed in the transition of G_1/S phase and also after UV DNA damage as a cell cycle checkpoint to inhibit the replication of DNA damaged [136]. Another example of the role of Skp2 in DNA damage response is the ubiquitination of NBS1. After irradiation CKII phosphorylates Skp2, which in turn ubiquitinates NBS1. NBS1 is a component of the MRN complex and its ubiquitination is required for the recruitment of ATM to DNA damaged foci and to start homologous recombination process. In this case, the ubiquitination does not lead to degradation and Skp2 is activated by stress instead of cell cycle progression [137]. Another example of alternative ubiquitination function is that Skp2 ubiquitinates Akt in order to activate it [138]

In general, Skp2 is considered an oncogene because it promotes cell proliferation. Skp2 is highly implicated in cancer as it controls cell cycle progression among other functions. It is found overexpressed in some cancers such as lymphomas, prostate cancer, melanoma, nasopharyngeal carcinoma, pancreatic cancer and breast carcinoma [125]. It has been described that Skp2 promotes the degradation of tumor suppressors such as p130, FOXO1, FOXO3 and Tob1. Interestingly, the ubiquitination of Foxo3, which suppresses estrogen-dependent breast cancer, requires a previous deacetylation by SirT1 or SirT2 [139]. Skp2 promotes the degradation of E-cadherin, therefore promoting cell migration [125]. Besides all of the different tumor suppressors degraded through polyubiquitination by Skp2, there are other mechanisms by which Skp2 promotes tumorigenesis. Skp2 inhibits p53-dependent apoptosis because it interacts to p300 inhibiting the interaction between p300 and p53 altering the transactivation capacity of p53 [140]. Although Skp2 ubiquitinates the oncogene myc for proteasomal degradation, Skp2 also activates a subset of myc target genes that are necessary to enter S phase [141]. In conclusion, Skp2 may be a potential therapeutic target for cancer.

The levels of Skp2, which has a very short half-life [123], fluctuate during cell cycle. Skp2 levels are increased at G_1/S phase by the APC/Cdh1 complex [131], although Skp1 and Cul1 levels do not change [123]. During cell cycle Skp2 levels are controlled. Thus, the E3 ubiquitin ligase Cdh1 binds to the D-box motif of Skp2 and induces its polyubiquitination triggering its degradation at early G_1 [123,142,143]. Interestingly some phosphorylation and acetylation of key residues present in the NLS region of Skp2 can inhibit its ubiquitination. For instance, Akt phosphorylates S72 and CKI phosphorylates S75 while p300 acetylates K68 and K71 [125,144–146]. SirT3 deacetylates Skp2 in the

acetylated residues catalyzed by p300, enhancing Skp2 degradation because it prevents the phosphorylation of S72 and S75 [125].

Another way of regulation of Skp2 levels is controlling its own expression. Transcription of Skp2 gene is positively regulated by myc, E2F1, NF-kB, SP1, CBF1, GABP and FoxM1. GABP binds to Skp2 promoter depending on the cell cycle. Until now, only a transcription repressor, Foxp3, has been described [123,147].

Skp2 expression is tightly regulated in cells even if they are in serum starvation conditions and totally independent from mitogen stimuli. E2F transcription factor induces SKP2 transcription, the Skp2 degradates p27 that binds to CyclinE/CDK2. CDK2 phosphorylates Rb that allows E2F release to regulate SKP2 expression [148]

Skp2 levels are also important for an optimal SCF complex formation. CAND1 binds Cul1 inhibiting the interaction between Skp1 and Cul1. In turn, Cul1 neddylation prevents CAND1 binding and, therefore, the stabilization of SCF complex. CyclinD1, a protein important for cell proliferation, induces this neddylation. Interestingly, phosphorylation of Skp2 in S72 of the NLS sequence by Akt facilitates the SCF complex assembly [123,149].

5. Sirtuins: Coordinators of the cellular stress response.

Sirtuins appeared first prokaryotes, where they seem to play a role in the regulation of cobalamin metabolism [150]. However, since the first stages of development of chromatin they seem to have adapted to perform a key role in the crosstalk between the environment and the genome. In eukaryotes, the number of Sirtuins in each organism has increased throughout evolution along with the complexity. Mammals harbor seven sirtuins which are classified in four classes based on phylogenetics: SirT1, SirT2 and SirT3 (class I), SirT4 (class II), SirT5 (class III), and SirT6 and SirT7 (class IV) [151].

Sirtuins are defined by a conserved core domain of approximately 250 residues, where the enzymatic activity of the protein resides. Additionally, the majority of them also contain non conserved N-terminal and C-terminal domains that may range from few residues to hundreds of them. The diversification of sirtuins during evolution is

reflected at many levels, including enzymatic activity, specificity of targets and subcellular localization (*Figure 11A*) [151].

A

Sirtuin	Localization	Main functions
SirT1	Nuclear	Metabolic regulation Cell survival in stress conditions. Chromatin organization. Gene silencing Cell differentation and development DNA damage repair
SirT2	Cytoplasmatic Nuclear	Cell cycle control
SirT3	Mitochondrial Nuclear	Metabolism
SirT4	Mitochondrial	Metabolism
SirT5	Mitochondrial	Metabolism
SirT6	Nuclear	Metabolism DNA damage repair Gene silencing
SirT7	Nuclear (nucleoli)	Activation of RNA-polymerase I

B

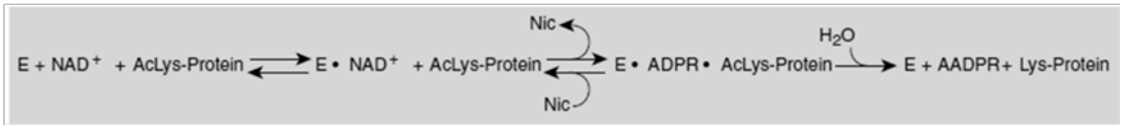

Figure 11. A. There are seven mammalian sirtuins with different localization and functions. **B.** Enzymatic reaction of deacetylation performed by Sirtuins. They need the cofactor NAD^+ in order to deacetylate the substrates [13].

Sirtuins catalytic mechanism is completely different from the rest of HDACs. They require NAD^+ as enzymatic cofactor (coenzyme) in order to catabolyze the deacetylation of a substrate. As a result, nicotinamide is removed from the NAD^+ molecule and the acetyl group of the substrate is transferred to the ADP-ribose (generated in the processing of the NAD molecule) producing O-acetyl-ADPribose. There is a release of the deacetylated substrate, nicotinamide and ADP-ribose (OAADPR) (*Figure 11B*). Because of that, nicotinamide is an important inhibitor of Sirtuins activity. Therefore, Sirtuins are sensitive to metabolic and energetic fluctuations that may alter the redox conditions reflected by changes in the NAD^+/NADH ratio. Interestingly, Sirtuins are also mono-ADP-rybosilases [50], because once they break the NAD^+ molecule they can just transfer the ADP ribose molecule to

another protein (or themselves). Because all of these reasons, they are major regulators of the response to a wide variety of stress conditions including genotoxic, oxidative and metabolic stress [152].

5.1. SirT1

SirT1 is by far the best described sirtuin. SirT1 is localized mainly in nucleus although it could be found in the cytoplasm for particular functions. It has four domains: N-terminal domain, allosteric site, catalytic core and C-terminal domain. SirT1 can deacetylate histones and non-histone proteins. In the case of histones, SirT1 deacetylates all four core histones and the linker H1 but shows a strong preference to the marks H4K16 and H3K9. In histone H1 it can deacetylate H1K26. SirT1 deacetylates non-histone substrates, such as many transcription factors among them, p53. SirT1 participates in a wide variety of functions, the most important of which are maintenance of genome stability, promoting survival in front of stress, and metabolic homeostasis regulation (*Figure 12*).

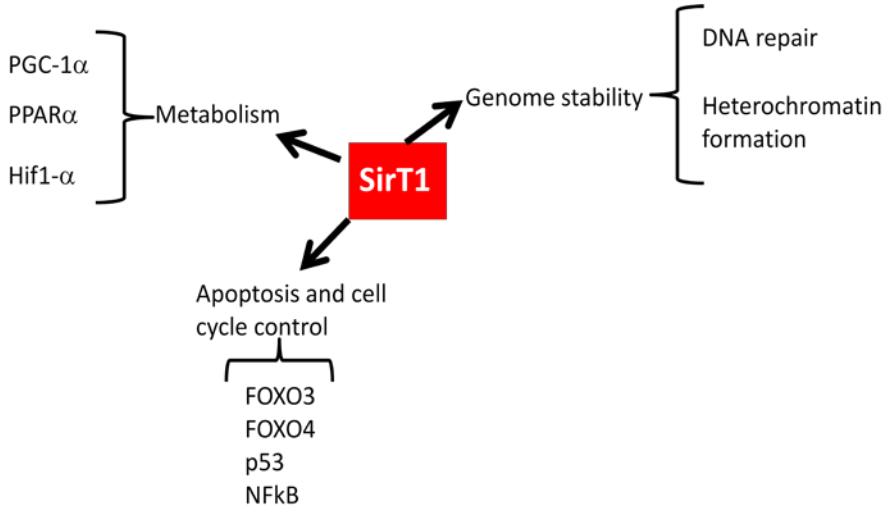

Figure 12. Brief summary about the main SirT1 functions in chromatin

In the case of genome stability, SirT1 participates in different processes to ensure a proper integrity of the genome such as constitutive and facultative heterochromatin regulation, DNA damage signaling and repair as well as cell cycle control [50].

SirT1 coordinates the facultative heterochromatin formation as a part of stress response. As mentioned before, SirT1 regulates the function of several master regulators of stress response such as FOXOs, p53, NFkB, etc. In this context, upon

stress and depending on the duration and time of the stimuli, SirT1 could move from simple gene silencing to induce facultative heterochromatin structures that can spam from one to many genes. For that purpose, SirT1 coordinates a sequence of events which include deacetylation of H4K16ac and H3K9ac, recruitment and deacetylation of histone H1 and the enrichment in the repressing marks such as H3K9me3 and H4K20me1 together with a decrease in the levels of the active mark H3K79me2 [153]. SirT1 can perform this function through a functional relationship with Suv39h1 described above (see section 3.2.2) [50].

SirT1 also regulates the role of Suv39h1 in constitutive heterochromatin under stress. SirT1 KO MEFs show a loss of H3K9me3 enrichment in the foci of pericentric constitutive heterochromatin (PCH) and also localization of HP1α in the same foci. Interestingly, SirT1 has also been detected with ChIP experiments in the PCH foci [154] but not in immunofluorescence experiments [49], suggesting that the levels of SirT1 in PCH are very low. Interestingly, SirT1 deacetylates K87 in Suv39h1 chromodomain and stabilizes the Suv39h1 levels by inhibiting polyubiquitination catalyzed by Mdm2. Thus, SirT1 ensures genomic stability by increasing Suv39h1 levels and increasing the turnover rate in heterochromatin foci under oxidative stress and caloric restriction [49,155].

Upon stress, such as caloric restriction or DNA damage, SirT1 activity is enhanced, in part by posttranslational modifications mediated by other pathways (such as AMP or JNK-1), also by changes in the NAD^+/NADH ratio, and also by direct increase in SirT1 transcription [156]. In many cases, this increase in transcription is mediated by stress-dependent transcription factors, such as NFkB or E2F [157]. Oxidative stress also affects to SirT1 due to an increase in reactive oxygen species (ROS). SirT1 can reduce ROS levels by specific antioxidant enzymes such as catalase or Superoxide dismutase [158]. SirT1 is negatively regulated by DBC1 that interacts with its catalytic domain [159,160].

SirT1 participates in DNA damage repair at different levels: signalization and regulation of DNA damage repair, BER and HR. For example, SirT1 deacetylates and activates NBS1, an important protein for signalization of DNA damage [161]. SirT1 also deacetylates Thymine DNA Glycosylase (TDG), a protein that participates in base excision repair (BER), and enhances its activity [162]. Furthermore, SirT1 promotes homologous recombination (HR) repair by deacetylating WRN protein [163]. In absence of DNA damage, SirT1 also prevents apoptosis and DNA damage repair by binding and deacetylating the HATs MOF and Tip60. Under DNA damage the binding is inhibited, allowing DNA repair [164]. SirT1 participates in telomere stability by inhibiting telomere

shortening and increasing homologous recombination in telomeres, arms and centromeres [165].

In the response to different forms of stress, SirT1 promotes the survival in front of these conditions in part by modulating the action of the key stress-response transcription factors, such as FOXOs, p53 or NFkB. SirT1 promotes silencing and facultative heterochromatin formation in a subset of genes targeted by these factors, enhancing their pro-survival side. In part this is achieved by direct deacetylation of the transcription factor and in part by direct silencing of the pro-apoptotic and pro-senescence genes targeted by them [152]. In the case of FOXO, for instance, SirT1 has been also shown to deacetylate, among other members, FOXO3 and FOXO4, which increases FOXO3 and FOXO4 dependent gene expression of some particular genes, attenuates apoptosis and potentiates FOXO induced cell cycle arrest. However, deacetylation of FOXO3 by SirT1 also induces ubiquitination by the E3 ubiquitin ligase SKP2 and degradation. Apparently, there are two mechanisms that balance the activity of FOXOs [139,166,167].

In the case of the proapoptotic transcription factor p53, SirT1 inhibits its activity by deacetylating K382 which prevents the nuclear localization of p53 and, therefore, avoids its arrival to the target promoters [152,168,169]. Under caloric restriction SirT1 also inhibits apoptosis by deacetylating the DNA repair factor Ku70, which in turn, inhibits its interaction with Bax and prevents apoptosis [170].

SirT1 has also an important role in metabolism in general, and also in particular in the tissues more sensitive to redox fluctuations, such as skeletal muscle and white adipose tissue [171]. SirT1 is involved in lipid metabolism repressing the transcription factor PPARα by binding NCoR and SMRT corepressors. It prevents fatty acid storage in white adipose tissue upon fasting [172,173]. SirT1 binds and deacetylates the transcription factor Hif1-α which prevents its interaction with p300 and inhibits its capacity to activate the expression of glycolytic genes. SirT1 also regulates glucose metabolism upon fasting conditions through activation of the master regulator PGC1α: activating gluconeogenic genes and suppressing glycolytic genes [174].

SirT1 has also a role regulating the important NF-kB pathway involved in cell cycle control, apoptosis, senescence, immunological response and inflammation. SirT1 inhibits the NF-kB transcription factor p65 (or RelA) through deacetylation of K310. Moreover, SirT1 enhances RelB gene transcription and the transcription of the negative regulator IkBα. In summary, SirT1 is negative regulator of NF-kB pathway (see section

7). Interestingly, NF-kB also inhibits indirectly SirT1function by activating miR-34a, a SirT1 inhibitor [175–177].

5.2. SirT2

SirT2, the only cytoplasmic Sirtuin, is also present in the nucleus in G_2/M phase, where it associates with chromatin. Despite its cytoplasmic localization, SirT2 regulates specifically global H4K16 acetylation levels during the cell cycle, as it seems to be the responsible for the dramatic drop in H4K16ac levels in G_2/M [178]. Interestingly, H4K16ac is a very unique mark, as is the only one that has been shown to directly regulate chromatin structure mentioned before [13]. SirT2 KO mice accumulate significant levels of genomic instability and chromosomal aberrations due to its important role in chromosome condensation. The H4K16ac deacetylation by SirT2 may be directly important for proper folding of the metaphasic chromosomes, but also because it regulates the deposition of H4K20me1, a mark keystone in cell cycle progression, mitosis and DNA replication. SirT2 regulates H4K20me1 deposition through H4K16 deacetylation but also through a functional interplay with the H4K20me1 HMT, PR-Set7 [179]. SirT2 deacetylates the H4K20 monomethylase PR-SET7, increases its levels and promotes its chromatin localization. PR-SET7 monomethylates H4K20 during G_2/M. Interestingly, both SirT2 and PR-SET7 seem to participate together in a not well-understood mitotic checkpoint. During mitosis, SirT2 also activates the APC/C complex, important for cell cycle regulation, by deacetylating its coactivators CDH1 and CDC20 [180].

SirT2 is phosphorylated by CDK1 in G_2/M phase increasing its levels until the end of mitosis, when the phosphatases CDC14A and CDC14B dephosphorylate SirT2 and decrease its levels [181,182]. In contrast, SirT2 phosphorylation at S331 residue by CyclinE/CDK2 and CDK5 in G_1/S phase inhibits its activity without affecting its levels [183].

SirT2 is also involved in the regulation of FOXO family transcription factors. Upon caloric restriction or oxidative stress, SirT2 is upregulated and deacetylates the transcription factor FOXO3 increasing its ability to bind to chromatin increasing the expression of its target genes, which include, for instance, scavengers or pro-apoptotic genes [184]. In adipocytes, SirT2 also regulates FOXO1 localization. It deacetylates FOXO1 preventing its phosphorylation, sequestering FOXO1 in the cytoplasm and

therefore avoiding activation of gene expression. In this way, SirT2 prevents adipocyte differentiation [185].

Furthermore, SirT2 have a role in inflammation by regulating negatively NFkB pathway by deacetylating p65 at K310 in the cytoplasm [186].

SirT2 is also involved in other functions, such as cell motility. SirT2 can deacetylate αtubulin together with HDAC6 modulating the dynamics of the cytoskeleton and regulating cell motility [183,187].

5.3. SirT3

SirT3 is mainly mitochondrial, where has been shown to be the major deacetylase activity. However, a small SirT3 population has been shown to localize to the nucleus in non-stressed conditions [188]. In fact, SirT3 also deacetylates H4K16ac and H3K9ac in the promoters of specific stress related genes [189]. However, upon DNA damage stress it translocates to the mitochondria where it is processed [188]. In the mitochondria SirT3 regulates key metabolic pathways such as respiratory chain, the tricarboxylic acid cycle, fatty acid β oxidation or ketogenesis by deacetylating specific enzymes [190] like acetyl Co-A synthetase 2 or glutamate dehydrogenase (GDH) [191].

5.4. SirT4

SirT4 and SirT5 are the two Sirtuins exclusively localized to mitochondria. So far, it has been described a mono-ADP-rybosil transferase activity for SirT4 but it has been found little or no deacetylase activity. SirT4 activity is very important for mitochondrial metabolism. SirT4 inhibits GDH by ADP ribosylation. GDH is necessary for insulin secretion, ATP production and inhibition of mitochondrial permeability. Following starvation SirT4 levels decreases in order to induce more ATP, but this is reversed under UVB stress. SirT4 also inhibits fatty acid oxidation [192–195].

5.5. SirT5

SirT5 is localized both in the mitochondrial matrix and the intermembrane space. SirT5 has a key role in the urea cycle. More than a deacetylase, it actually has a more general deacylase activity. It deacetylates cytochrome-c and also can deacetylate, demalonylase and desuccinylase carbamoyl phosphate synthetase 1 (CPS1).

Cytocrome C is involved in oxidative metabolism and apoptosis initiation and CPS1 is the first step enzyme of the urea cycle and catalyses ammonia [196–199].

5.6. SirT7

SirT7 is found in the nucleolus. It interacts with RNA polymerase I (Pol-I) and activates gene transcription in rDNA. SirT7 binds to UBF (a component of the Pol-I machinery) and to the B-WHICH chromatin remodeling complex, inducing chromatin relaxation and allowing the entry of the Pol-I machinery. In mitosis, SirT7 is inactivated by phosphorylation by CDK1-cyclin B although part of SirT7 remains bound to chromatin during all mitosis [200–202].

The H3K18 hypoacetylated state of a subset of genes in some tumors is regulated by SirT7 contributing to the maintenance of cancer phenotype and transformation. In fact, SirT7 is recruited to some of this genes by the transcription factor ELK4 that regulates genes involved in cellular transformation and cancer progression inhibiting their expression [203].

6. SirT6

SirT6 is, together with SirT1, present in the nucleoplasm and in non-nucleolar chromatin [204,205]. It is expressed in all tissues although is highly expressed in brain, heart, liver and muscle [204]. It is involved in important cellular functions such as DNA repair, telomere maintenance, glucose and lipid metabolism and inflammation [206] (*Figure 12*).

SirT6 has three domains: N-terminal domain, the conserved sirtuin domain and a C-terminal domain. The N-terminal domain is necessary for chromatin binding and catalytic activity together with the conserved domain. In contrast, the C-terminal domain is required for nuclear localization as it harbors the NLS sequence [207].

Together with SirT4, SirT6 is the only Sirtuin that has been shown to catalyze both ADP-rybosilase and deacetylase activity [204,208]. Moreover, SirT6 has been shown, like SirT5 to act also as a deacylase [209]. Probably, the most relevant substrates of SirT6 are the histone marks H3K9ac and H3K56ac. Interestingly, SirT6 is not very efficient deacetylase in vitro when using core histones as a substrate [208,210,211]. This is in part cause by the fact that SirT6 prefers nucleosomes as substrates instead of free core

histones [212]. In fact, in the regulation of fatty acid metabolism, SirT6 seems to have more preference to remove long-chain fatty acyl groups from lysines than acetyl groups [213]. Another observation is that SirT6 requires the binding of specific molecules, such as certain free fatty acids, to get fully activated [209]. SirT6 activity can be modulated by several factors such as nicotinamide as described below, 3-morpholinosydnonimine (SIN-1) that induces tyrosine nitration and decreases its catalytic activity [214] and different peptides and pseudopeptides [215]. There are some point mutations in SirT6 that also affects its activity: SirT6 S56Y lacks both deacetylase and mono-ADP-ribosyltransferase activity, SirT6 G60A lacks mono-ADP-ribosyltransferase activity and SirT6 R65A lacks deacetylase activity [216]. SirT6 H133Y inhibits catalytic activity, chromatin association and alters the binding of SirT6 to other proteins [207,217].

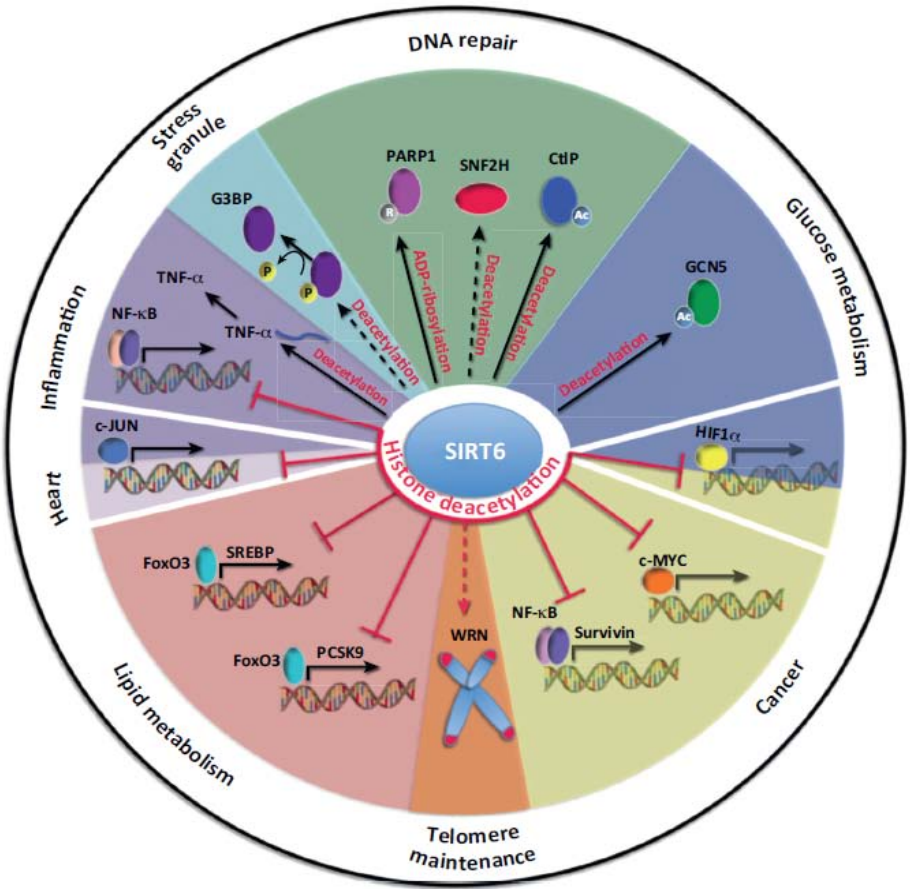

Figure 12. SirT6 has many substrate that deacetylates and ADP-ribosylates. Furthermore, their main functions are performed by its ability of silencing genes [206].

SirT6 levels are controlled by nutrient availability: nutrient deprivation increased SirT6 protein levels affecting the protein stabilization, particularly, in heart, brain and

kidney. Moreover, depletion of p53 decreases SirT6 protein levels in basal conditions [218]. Also, it has been suggested that, in some cell types, SirT6 levels fluctuates during cell cycle [219]. SirT6 levels are regulated through polyubiquitination by MDM2, the same E3 ubiquitin ligase that regulates p53. Interestingly, this ubiquitination requires a previous phosphorylation of SirT6 in S388 by AKT [220]. In contrast, USP10 deubiquitinates SirT6 preventing its proteasomal degradation [221]. SirT6 is also ubiquitinated in K170 by another the E3 ubiquitin ligase CHIP that stabilizes SirT6 protein levels [96].

SirT6 is phosphorylated in several residues at C-terminal domain but it is unknown the function of most of them. An exception is the phosphorylation in S338 which regulates SirT6 binding to other proteins [217].

SirT6 deficient mice develop a premature ageing phenotype and die at around four weeks of age. Two or three weeks after birth, they present a progeroid degenerative syndrome with several defects like lordokyphosis, lymphopenia, loss of subcutaneous fat, erosion of colonic epithelium among others. Therefore, SirT6, as SirT1 does, is implicated in lifespan [205]. In fact, it has been reported that SirT6 overexpression increases lifespan in male mice while in females lifespan is not affected [222]. In mice overexpressing SirT6, the lifespan increase is due to reduced levels of Insulin Growth Factor (IGF-1), a key factor in lifespan [223], in serum due to an upregulation of the IGF-binding protein 1 receptor [222].

Another factor of senescence that SirT6 regulates is the Long Interspersed element 1 (LINE L1), a group of repetitive elements present in many copies in the genome. LINE L1 expression is activated in old cells and is related to ageing. In young cells, SirT6 mono-ADP ribosylates KAP1, a key factor that interacts with HP1α and coordinates the assembly of HP1α-SETDB1-dependent corepressor complex in the LINE L1 promoters. ADP-ribosylation of KAP1 enhances its binding to HP1α. When cells get older or under stress, SirT6 leaves LINE L1 sequences and KAP1 and HP1α are unable to promote gene silencing, which in turn induces the overexpression of these repetitive elements [224].

As other sirtuins SirT6 also participates in stress response. G3BP1 promotes stress granule assembly and for the correct assembly is necessary SirT6 interaction to G3BP and SirT6 deacetylase activity is required [217,225].

6.1. Genome stability promoted by SirT6

SirT6 promotes genome stability by participating in DNA damage repair both in single and double strand breaks and in telomere maintenance.

6.1.1. DNA damage repair

SirT6 is involved in DNA damage repair at different levels: it is involved in signalization of DNA damage and DNA repair itself. Thus, SirT6 has been linked to base excision repair (BER) and double strand break (DSB) repair.

SirT6 deacetylates H3K56 and H3K9 in the DNA damage area for DNA repair signalization and recruitment of machinery repair [226]. It has been reported that deacetylation of H3K56 is necessary for genome stability because it is necessary for the early stages of DNA repair [227]. H3K56 in telomeres at S-phase is deacetylated by SirT6. However, the deacetylation at G_2/M is not performed by SirT6 [228,229].

SirT6 KO mouse embryonic fibroblasts (MEFs) and embryonic stem (ES) cells show higher sensitivity to ionizing radiation, peroxide and methylmethanesulfonate (MMS) [205].

Although factors that participates in BER are normally expressed in SirT6 KO cells and they do not interact with SirT6, overexpression of them can rescue the sensitivity [205]. The main reason for this effect is that SirT6 activates poly (adenosine diphosphote (ADP)-ribose) polymerase 1 (PARP1) by ADP-rybosilation. PARP1 is recruited to DNA damage sites and facilitates the recruitment of DNA damage repair factors involved in BER and DSB. In fact, SirT6 overexpression accelerates DNA damage repair and, in middle-aged and senescent cells, it rescues the homologous recombination efficiency shown by young cells through PARP1 activation [216,230].

For an accurate DNA damage repair in DSB it is necessary chromatin relaxation in order to facilitate accessibility of repair machinery. The factors responsible of the chromatin relaxation are chromatin remodelers. SirT6 is recruited to DSB foci by H3K9ac and probably by other unknown factors and deacetylates H3K9ac allowing access to DNA-PKcs and other repair machinery [231]. Then, SirT6 recruits the chromatin remodeler SNF2H to chromatin upon DNA damage through its terminal domain. This recruitment allows the formation of the foci enriched with 53BP1 and γH2AX necessary for DNA repair. Interestingly, SirT6 deacetylase activity is dispensable for the SNF2H recruitment [226]

SirT6 participates in both non-homologous end joining (NHEJ) and homologous recombination (HR) repair. SirT6 recruits DNA-PKcs, Ku80 and Ku70 to DSB, factors indispensable to achieve NHEJ [232]. Moreover, SirT6 activates and deacetylates CtIP, a protein involved in DNA end resection for homologous recombination. The deacetylation of CtIP is necessary for the recruitment of RPA to DNA. RPA induces coating of the single strand DNA in order to promote DNA resection [233].

6.1.2. *Telomere stability*

SirT6 KO cells harbors genome instability and many chromosome aberrations associated to telomeres [205]. In fact, SirT6 is necessary for telomere structure. SirT6 deacetylates both H3K9 and H3K56 in telomeres at S-phase. The H3K9 deacetylation allows the stable association of WRN to the telomeres in S-phase in order to protect them. Mutations in WRN induces Werner syndrome which is associated to a premature aging disorder. Supporting this relationship, like WRN-depleted cells, SirT6KO cells show chromosomal end-to-end fusions after DNA replication [208].

SirT6 is also important for maintaining telomere position effect: loss of SirT6 induces impairment in heterochromatin marks such as H3K9ac and H3K9me3 in telomeres. That leads to telomeres shortening and transcription of silencing genes due to telomere position which triggers degenerative syndromes and cancer [234].

6.2. NF-kB pathway regulation by SirT6

SirT6 shows a strong functional link with NF-kB. The model proposed to explain the negative regulatory role of SirT6 in Nf-kB pathway involves a binding of SirT6 to RelA and a subset of RelA-regulated promoters after NF-kB activation. Then, SirT6 represses gene expression of these genes by deacetylating H3K9ac destabilizing RelA interaction to the promoters [235]. In fact, SirT6 binds to different promoters depending on the time after NF-kB pathway activation. Depending on the dynamics of SirT6 occupancy in the promoters, genes can be classified in different subsets. Some of the gene promoters are occupied by SirT6 before NF-kB activation and after activation it leaves the promoters. Others are occupied by SirT6 only after NF-kB activation, and other gene promoters have a controversial behavior because SirT6 binding activates their expression [236]. Some of the genes that are regulated and silenced by SirT6 are involved in ageing linking SirT6KO phenotype with SirT6 function. Moreover,

senescence phenotype observed in SirT6 KO mice is similar to a NF-kB pathway hyperactivation and it can be rescued by an heterozygote RelA +/- [235,236]. However, SirT6 does not deacetylate directly RelA, and SirT6 overexpression does not affect NF-kB pathway: neither NF-kB translocation nor NF-kB target gene expression [210].

Another level of NF-kB regulation by SirT6 is the control of TNFα release (one of the activators of NF-kB pathway). SirT6 controls fatty acylation levels on TNFα by deacylating it which promotes its release [213]. Other regulatory mechanisms by SirT6 on NFkB pathway are very likely as SirT6 has been found to interact to NF-kB inhibitors: PIAS1 and MYBBP1A [237].

6.3. Metabolism and SirT6

SirT6 KO mice are hypoglycemic [205]. Hif1-α activates expression of glycolytic genes and SirT6 inhibits Hif1-α. SirT6 interacts with Hif1-α and deacetylates H3K9ac in the promoters of glycolytic genes silencing their expression. SirT6 KO cells show an increase in glycolysis and mitochondrial respiration and downregulation of Hif1-α rescue the phenotype of SirT6 KO cells. Moreover, SirT6 downregulates the expression of the glucose receptor GLUT-1 [238]. Consistently, SirT6 depletion activates AKT phosphorylation, which in turn increases insulin receptors and GLUT1 and GLUT4 levels in membrane inducing hypoglycemia [239].

Gluconeogenesis is also affected by SirT6. FOXO1 is a transcription factor that promotes expression of genes involved in gluconeogenesis such as G6PC and PCK1. FOXO1 is deacetylated by SirT6 and it translocates from nucleus to cytoplasm and, as a consequence, genes regulated by FOXO1 are not expressed. In fact, p53 regulates gluconeogenesis through activation of SirT6 expression [240].

Furthermore, SirT6 not only regulates glucose metabolism but also lipid homeostasis. SirT6 regulates expression of genes involved in lipid metabolism, and its overexpression avoids the accumulation of LDL-cholesterol, triglycerides and visceral fat [241].

6.4. Role of SirT6 in liver

The vast majority of our knowledge on the role of SirT6 in metabolism is based on studies in liver. In fact, these studies show that SirT6 is necessary for liver homeostasis. Induced SirT6 KO liver mouse shows fatty liver development due an increase in triglyceride levels. SirT6 depletion promotes an upregulation of genes involved in hepatic long-chain fatty acid uptake and a downregulation in genes involved in β-oxidation. Thus, fasting induces less accumulation of triglycerides preventing the formation of fatty liver [242]. Cholesterol synthesis is controlled also by SirT6. Both FOXO3 and SirT6 regulate cholesterol. They maintain low levels of the SREBP-2 expression, a factor involved in regulation of expression of genes involved in cholesterol metabolism; and Pcsk9 expression, a key factor for LDL-cholesterol synthesis. FOXO3 recruits SirT6 to the SREBP-2 or PcsK9 promoter and SirT6 silences the expression by deacetylating H3K9. In this way, FOXO3 and SirT6 prevent hypercholesterolemia [243,244]. In fact, rosiglitazone ameliorates hepatic steatosis (a disease of fatty liver) through SirT6 overexpression [245]. In liver, SirT1 activates SirT6 expression because it interacts with both FOXO3a and NRF1 forming a complex in the SirT6 promoter. SirT1 expression depends on nutrient availability in a way that with low nutrients SirT1 levels increases but NRF1 interacts to SirT6 promoter independently of nutrient levels. NRF1 interacts to SirT6 promoter independently of nutrient levels, but the presence of FOXO3a is regulated by SirT1. SirT1 gets activated under nutrient deficiency, and deacetylates FOXO3a, enhancing the binding of FOXO3a to NRF1 [243,244].

In the case of glucose homeostasis in liver in basal conditions, the interplay between SirT1 and SirT6 is completely opposed. In fact, while SirT1 activates hepatic gluconeogenesis, SirT6 inhibits it. In hepatocytes there is a balance between SirT1 and Sirt6 regulation [246]. PGC-1α factor enhances hepatic gluconeogenesis and its activity is regulated by acetylation. SirT1 activates PGC-1α by deacetylation and SirT6 activates the acetyltransferase GCN5 that inhibits PGC-1α.

SirT6 controls inflammation and liver cancer. SirT1 and SirT6 controls metabolic switch after inflammation inducing fatty oxidation and preventing glycolysis [247]. In fact, SirT6 KO mice develop chronic liver inflammation. Liver inflammation is caused by SirT6 deficiency in immune system and not hepatocytes. SirT6 binds to c-Jun in pro-inflammatory gene promoters and deacetylates H3K9 promoting gene silencing [248]. SirT6 also prevents liver cancer initiation by silencing the NF-kB target survivin. Survivin inhibits apoptosis. Liver cancer initiation is controlled by the c-Fos and c-Jun.

c-Fos promotes SirT6 gene expression (inhibits cancer initiation) and c-Jun promotes survivin gene expression (activates cancer initiation). This model is not valid for advanced stage in liver cancer [249].

6.5. Role of SirT6 in cardiovascular system

SirT6 function has also been studied in cardiovascular system. SirT6 prevents cardiac hypertrophy in two different ways: by inhibiting NF-kB target genes involved in cardiac hypertrophy and by binding to the transcription factor c-Jun which activates IGF. Interestingly, one of the main regulators of the cardiovascular homeostasis, the hormone angiotensin II (AngII) induces both cardiac hypertrophy and SirT6 expression, although the decrease in NAD^+ availability due to downregulation Nmat2 (a NAD^+ synthase) avoids SirT6 activation [250–252]. In case of damage in the cardiac tissue, , to ensure regeneration of the tissue by the cardiac fibroblasts, SirT6 prevents cardiac fibroblast differentiation into myofibroblast by regulating NF-kB pathway [253].

Also in endothelial cells SirT6 prevents DNA damage and senescence [254].

6.6. SirT6 as a tumor suppressor

The vast majority of evidences suggest that SirT6 has a tumor suppressor role: First, SirT6 is downregulated in different types of cancers. Second, it interacts with the tumor suppressor GCIP/CCNDBP1 in order to represses cyclinD1 and arrest cell proliferation[255]. Third, SirT6 acts as an antagonist of the oncogene c-myc [256]. Thus, SirT6 binds to c-myc and represses c-myc target genes, such as for instance, ribosome biogenesis-related genes, by deacetylating H3K56ac in their promoters. Supporting this role for SirT6, USP10 prevents tumors by stabilizing both SirT6 and p53 [221]. Fourth, SirT6 seems to have a role in the so-called Warburg effect, a metabolic switch shown by cancer cells in the process of transformation by which they switch from mitochondrial respiration to glycolisis, and therefore consume less O_2. Thus, loss of SirT6 associates with a marked increase in gycolysis and a decrease in respiration [256,257]. Fifth, SirT6 overexpression is toxic for cancer cells but not for normal cells. In cancer cells, SirT6 overexpression induces apoptosis through p53 and p73 by activating ATM pathway. This suggests that SirT6 could be a good candidate for cancer therapy in the near future [258]. Sixth, as it was described before, SirT6 levels are decreased due to a phosphorylation in S388 residue by AKT. In some breast cancer, AKT is hyperactivated so the treatment with trastuzumab (a drug that can block

specifically Her2 receptors) is not valid to stop cancer. However, the overexpression of the mutant SirT6-S388A confers sensitivity to trastuzumab, strongly suggesting that SirT6 is involved in the trastuzumab mechanism of action [220]. Seventh, in glioma, SirT6 downregulates gene expression of the RNA-binding protein PCBP2, which has been found overexpressed in this cancer [259]. Finally, SirT6 is downregulated in non-small cell lung cancer and its overexpression stops cell proliferation by downregulating TWIST, an oncogene of non-small cell lung cancer [260].

However, not all evidences point to this direction. SirT6 also may act as an oncogene in skin and pancreas. In skin, the expression of the proliferation promoter COX2 is upregulated by SirT6 through AMPK inhibition. In fact, SirT6 is overexpressed in some skin cancers, such as squamous cell carcinoma [261]. In pancreas, SirT6 promotes cell migration [262]. Moreover, SirT6 stimulates proinflamatory cytokines such as TNFα and IL8 without activating NF-kB pathway by regulating a second messenger in a Ca^{2+} response.

6.7. Future SirT6 perspectives

Many questions remain open. One of the most relevant is the fact that a wide variety of proteins have been found to interact with SirT6 but the functional consequences of these interactions have not been studied [225]. These interacting proteins can be classified in different groups according to their function: i) transcription regulation (proteins involved in DNA repair such as THRAP3, TRIM27 and proteins involved in NF-kB regulation such as NKFR, H2AFY and in RNA polymerase activity (COIL, NOLC1, XRN2); ii)chromatin organization (i. e. NUMA1, LMNA); iii)nuclear transport (i. e. TPR, Nup proteins) and in neurofilament networks (INA, NEFL); iv) mRNA processing (i. e. DDX41, RBM28, UTP18, DDX24) [217].

Future studies should clarify the real importance of each of them and help us define the full consequences of SirT6 function in cell physiology.

7. NF-kB

NF-kB pathway is necessary for the response to environmental changes and is involved in apoptosis, proliferation, differentiation and development [263]. NF-kB hyperactivation leads to an aging phenotype [264].

Five members form this transcription factor family: RelA(p65), RelB, c-Rel, NF-kB1 (p52) and NF-kB2 (p50) [265]. They form dimers or heterodimers, and they all have in common Rel homology domain (RHD) necessary for dimerization, interaction with the repressors IkBs and binding to DNA [266]. RelA, RelB and c-Rel have a TAD domain, necessary to induce gene transcription once they are in the gene promoter. However, the p50 and p52 do not harbor a TAD domain in their sequence so they are not able to induce transcription. In fact, when they form dimmers and bind the promoter they prevent the transcription of the gene [267]. Rel B is the only transcription factor that does not homodimerize, as only heterodimerizes with p52 and p50 and binds specifically to the IkB p100. In fact, it has been reported that RelB is the responsible of a constitutive activation of NF-kB in some tissues [267]. C-Rel homodimerizes and heterodimerizes with RelA and p50.

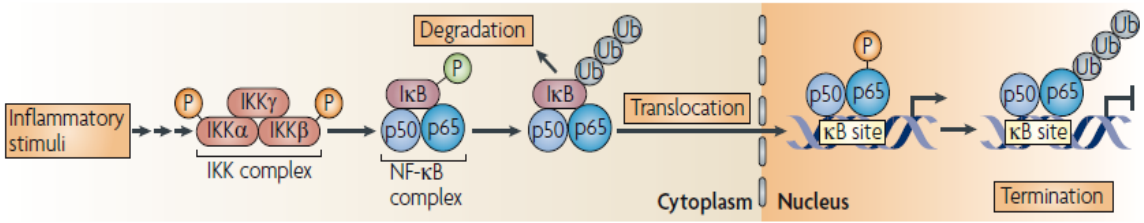

Figure 14. Activation of NF-kB canonical pathway [268].

In a resting condition, despite the NFkB NLS sequence, NFkB is found in the cytoplasm forming p65/p50 or p65/Rel-C dimers, bound to IkB repressors, which sequester these factors by masking their NLS. Proteasomal degradation of IkB proteins is required for the release of NF-kB degradation and translocation from the cytoplasm to the nucleus (*Figure 14*). The main responsible of this degradation are the IKK kinase complex, which phosphorylate IkB proteins allowing its recognition by the E3 ubiquitin ligases from the SCF complex. The recruitment of the E3 ubiquitin ligases is performed by ATM under TNFα conditions [269]. TNFα induces a phosphorylation of RelA at S276 by protein kinase A (PKAc) [269], which enhances the affinity of RelA for binding DNA and allows the interaction with the transcriptional coactivator CBP/p300 [267].

There are two kinds of NF-kB pathway: canonical and noncanonical. In the canonical both IKKα and IKKβ are activated and they form a complex with the regulator IKKγ (or NEMO) in order to phosphorylate IkB repressors. In contrast, in the noncanonical pathway only IKKα takes part of the pathway by phosphorylating p100, which in turn is processed to form p52 [266].

This pathway is triggered by different stresses. The canonical pathway activation involves signal Receptors such as Toll like receptors (TLRs), interleukin-1 receptor (IL-1R), tumor necrosis factor receptor (TNFR) and antigen receptors, as well as their corresponding signaling molecules l tumor necrosis factor α (TNFα), lipopolysaccharides (LPS) and interleukin-1 β (IL-1β) [265]. However, besides this cytokine-mediated signal, a wide variety of stimuli activate the pathway.

NF-kB pathway can be activated also by DSB. Thus, upon DNA damage, ATM phosphorylates IKKγ to activate the pathway [270]. Also, p65 has been involved in one of the two repair pathways of DSBs, HR, because stabilizes BRCA1 through interaction with the complex BRCA1 and CtIP, necessary for end resection. Then, the activation of NF-kB pathway accelerates the RPA foci formation necessary for the HR [271].

NF-kB response is stimulus and cell line-dependent [272]. However, one of the interesting aspect of the NF-kB pathway, is that the response is not only cell type-dependent, but also that in a single cell, the mechanisms exerted in the target genes are strikingly diverse [272]. Thus, after NF-kB entrance to the nucleus, genes regulated by NF-kB have not the same kinetics. Their promoters have different structure, and some of them need a chromatin remodeling before re-start their transcription [273].There is a classification of gene targets of NFkB due to their kinetics in which they are classified in two general types: First, “fast” or “early” genes, which refer to those genes with constitutive and immediate accessibility (CIA) such as IkBalpha or MnSOD. Second, “slow” or “late” genes, which refer to those with regulated and late accessibility (RLA) as Il6 [274]. Some of the genes are RelA dependent (the early ones) and some others are dependent on RelB. For example MnSOD, IkBalpha and GADD45B are RelA dependent [274–276]. Focusing in fast genes, there are many differences between them, as for example MnSOD is rapidly induced its gene expression and persists in time while IkBα is also rapidly induced but NFkB is removed from the promoter very fast to shut down the transcription. The fast transcriptional activation for a subset of early genes is due to a preloading of the transcriptional machinery such as Pol-II, TFIIB, CBP and p300 by the constitutive transcription factor Sp1 previous to the NF-kB activation and, then, when p65 arrives, the transcription takes place immediately, as for example, for A20 [277]. However, there are other early genes such as IL8 and Gro-β that require the phosphorylation of p65 at ser 276 in order to recruit the positive transcription elongation factor b (p-TEFb) that contains CDK9 and recruits Pol-II [269,278]. Another different characteristic between target genes is that NFkB binds to the DNA kB sites in the promoters and these kB sites are very divergent and depend on each

particular gene. The different kB sites make the different transcription regulation and NF-kB subunit binding [279]. The MAP kinase p38 affects to the H3S10 phosphorylation at IL6 and Il12p40 promoter but not at IkBα promoter and this phosphorylation produces a high affinity of p65 to these promoters [268]. For proper loading of p65 in some transcriptional active genes CDK6 binding to the promoter is required [280].

Some coactivators bind to p65 in order to enhance their transcription activity. For example, after TNFα treatment the HAT Tip60 binds to a subset of genes, acetylates H3 and H4 and p65 is recruited over there. Also, the HATs PCAF, CBP and p300 activates p65 transcription, but CBP HAT activity is not required for the activation [281–283]. Another example is the participation of the transcription coactivator E2F1 under LPS stimuli: E2F1 interacts to RelA to enhance some specific NF-kB gene target expression [284]. There are also some inhibitors such as HDAC1 and HDAC2. HDAC1 interacts to p65 and recruits HDAC2 and induces IL8 gene silencing [285].

7.1. IkB repressor proteins

There are nine IkB family members (IkBα, IkBβ, IkBε, IkBξ, BCL-3, IkB-NS, p100, p105, IkBη) and they have in common an ankyrin repeat domain and their function is inhibit the interaction between NF-kB and DNA [266]. They are negative regulators of NF-kB pathway. The best studied member, IkBα is synthesized very early and localizes to the cytoplasm where it stops the pathway. Although, IkBα is the classical IkB that sequesters the heterodimers p65/p50, in its absence IkBβ can also perform this function implying certain level of redundancy. BCL3 interacts in the nucleus with p50/p52 heterodimers in order to promoter gene expression. Interestingly, without BCL3, p50/p52 inhibits transcription. IkBε interacts with p65 homodimers and p65/c-Rel heterodimers, but its kinetics is much slower than IkBα [267].

IkBs are not only found in cytoplasm but also they could be found in the nucleus under special conditions. For example, some molecules of IkBα associates with HDACs in Notch promoters under basal conditions, and, in keratinocytes, IkBα recruits Polycomb repressor complex and is found in the HOX gene promoters [286,287].

7.2. IKK proteins

IKKα could be localized in cytoplasm and nucleus while IKKβ is restricted to cytoplasm. Apparently, IKKα is dispensable for IkBα phosphorylation while IKKβ is absolutely necessary. They do not only phosphorylates IkBα in order to release NF-kB transcription factors and activate the pathway but also they are involved in other processes [288]. IKKα and IKKβ also phosphorylate p65 subunit at S536 which alters the binding of transcriptional co-activators and co-repressors and has consequences in gene expression [289]. IKKα also phosphorylates other substrates that participate in chromatin regulation such as H3S10, SMRT or CBP. In general, phosphorylated H3S10 and CBP, together with the removal of the complex-SMRT-HDAC3, are involved in p65 activation [268,288,290,291]. IKKα also participates in the removal of p65 with the sumo E3 ligase PIAS1, but, the mechanism is not well understood [288].

IKKα can translocate to the nucleus in a NF-kB independent way and regulate cell cycle progression through E2F1 regulation, Notch dependent transcription or FOXA2 among other functions. It has also an important role in apoptosis by regulating p53 and p73 [288].

Interestingly, in keratinocytes, IKKα induces 14-3-3σ expression by preventing Suv39h1 and Dnmt3a interaction with its promoter without affecting H3S10 phosphorylated levels. 14-3-3σ is a G_2/M checkpoint induced after DNA damage in order to check DNA before entering in mitosis [292].

7.3. p65 posttranslational modifications

p65 is the target of several posttranslational modifications, such as phosphorylation, acetylation, methylation or ubiquitination (*Figure 15*) [293]. These marks are important to regulate NF-kB pathway. p65 is phosphorylated in several residues. Phosphorylation in S276 has a positive effect in transcription regulation because it enhances p65 binding to the coactivators p300, CBP and CDK9. Phosphorylation in S536 also enhances the binding to p300 and decreases the binding to the inhibitors SMRT and IkBα. Moreover, phosphorylation in S205, S281 and S311 induces NF-kB-dependent transcription. In contrast, phosphorylation of other residues has a negative effect on the pathway. For instance, phosphorylation in S468 negatively regulates NF-kB and also enhances binding of the COMMD1- containin E3 ligase complex for degradation. Also

the phosphorylation in T435 and T505 forms a docking site for the suppressor HDAC1 [294].

Acetylation in p65 takes place in K122, K123, K218, K221, K310, K314 and K315, all of them are catalyzed by CBP/p300, except K122 and K123 that are also acetylated by PCAF. p65 is acetylated and activated by CBP/p300 at lysine 310. At the same time, CBP/p300 acetylates K218 and K221 enhances DNA binding and impairs binding to IkBα [295]. However, p300 acetylates p65 at lysine 314 and 315 when it is bound to chromatin and after three hours of TNF treatment. The hyperactivation of genes when K314 and K315 were mutated suggests that these acetylations are repressive [294,296].

SirT1 has a negative role in NF-kB activation. After first gene activation by NF-kB pathway, SirT1 deacetylates both p300 and NF-kB at K310 in order to inactivate them. Also, SirT1 forms and stable structure with RelB in order to inhibit proinflammatory gene expression and favors IkBα expression [176]. PARP1 inhibits SirT1 activity by consuming NAD^+ allowing p65 acetylation. However, SirT2 is the most specific deacetylase at K310 of p65. SirT2 deacetylates p65 in the cytoplasm to avoid its tranlocation to the nucleus. According to this, depletion of SirT2 leads to a p65 hyperacetylation [186].

HDAC3 deacetylates p65 at K122, K123, K314 and K315 and activates it enhancing the NF-kB target gene transcription in response to IL-1 signal [297].

Acetylation and ubiquitination are two posttranslational modifications that are exclusive in most of the cases: when acetylation takes place, ubiquitination does not, and viceversa. In this sense, NF-kB signaling arrest is mediated also by polyubiquitination and degradation of p65 by binding to the COMMD1 complex which recruits an ubiquitin ligase complex composed by Elongins B/C, Cul2 and SOCS1[298]. For the interaction between p65 and COMMD1 complex is necessary the HAT GCN5, but its catalytic activity is dispensable. GCN5 interaction to p65 is enhanced by p65 S468 phosphorylation catalyzed by IKK complex [299]. Also, the E3 ubiquitin ligase PDLIM2 ubiquitinates p65 and induces its degradation [294]. The deubiquitinase USP7 removes ubiquitin from NF-kB and stabilizes it and regulates positively the transcription of NF-kB dependent genes [300].

p65 is also regulated by methylations in different residues. Monomethylation of K218 and K221 by the methyltransferase NSD1 enhances p65 DNA binding of a particular subset of genes. SET7/9 methylates K37 and also enhances p65 DNA binding, but the methylation of K314 and K315 by SET7/9 induces proteasomal degradation. K310

methylation by SETD6 induces NF-kB target genes silencing because it recruits GLP that is a repressor [301,302].

There is interplay between acetylation and methylation of p65. When K310 is acetylated, methylation of K314 and K315 by SET7/9 does not take place and, the opposite thing: when K310 is deacetylated by SirT1, K314 and K315 methylate inducing proteasomal degradation [303].

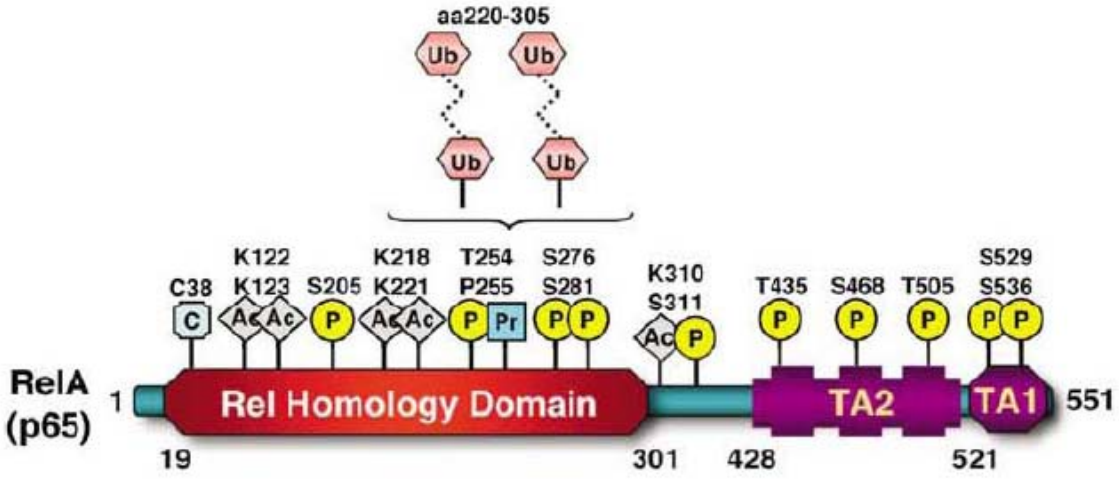

Figure 15. Scheme of RelA (p65) modifications [293].

7.4. NF-kB and cell cycle regulation

NF-kB plays an important role in cell cycle by regulating expression of genes involved in cell cycle progression such as CyclinE, CyclinD and p21.

Cyclin E is involved in G_1/S phase transition. NF-kB negatively regulates Cyclin E expression upon TNFα treatment because it recruits HDAC1 to the promoter. Indeed, it has been reported that p65 overexpression induces a G1 arrest [304]. In contrast, NF-kB regulates positively CyclinD1 expression. CyclinD1 is also involved in G_1/S phase and its expression is necessary for the phosphorylation of retinoblastoma [305]. NF-kB inhibition results in a deregulation in p53 and p21 checkpoint levels due to regulation of cyclins [306].

OBJECTIVES

SirT6 induces gene silencing in the context of stress response. The main objective of the project is to understand the mechanism through which SirT6 regulates chromatin functions under these conditions. We aim to fulfill this general objective by developing the following specific aims:

1.- The study of a possible link between SirT6 and histone methyltransferases. We aim to identify this/these activities and characterize their functional relationship with SirT6 and its role epigenetic silencing in the context of stress response.

2.- The identification of novel SirT6-binding partners that may collaborate with SirT6 to establish epigenetic repression of the target genes.

MATERIALS AND METHODS

1. Cell lines

For the experiments it has been used different cells lines: HEK293F, 293F T-REx, Platinum, HeLa, NIH3T3, H1299, HCT116, MCF7, U2OS, p19, MEF. All of them has been grown in DMEM (Gibco) medium supplemented with 10% serum bovine foetal (SBF), except MEFs that have been grown with 15% of SBF, at 37ºC in a 5% CO_2 incubator.

HEK 293F cells are inmortilized cell line from human kidney, its derivates are; 293F T-REx that is a stable cell line that express tetracycline repressor protein and should be grown with 5 μg/ml of blasticidine; and Platinum that are retroviral packaging cells and grow with 1 μg/ml of puromycine and 10 μg/ml of Blasticidine. HeLa cells are immortalized human cells from cervical cancer. NIH3T3 cells are immortalized mouse embryonic fibroblasts. H1299 is a human non-small cell lung carcinoma cell line. HCT116 is a human colon carcinoma cell line. MCF7 is a human breast cancer cell line. U2OS is a human osteosarcoma cell line.

MEFs (mouse embryonic fibroblast) can be primary or immortalized: primary MEFs should be considered cells that have less than three passes and immortalized are the ones with more than three passes and, most of times, they change their morphology or growth rate. We have used the W8 (Suv39h1 and 2 WT) and D5 (Suv39h1 and 2 KO) from Dr Jenuwein's lab and SirT6 WT and KO MEFs from Dr Bober's lab.

2. Cell Treatments

We treat cells with different reagents and also with radiation.

- Heat shock treatment. At 48 hours of transfection cells were incubated in an incubator with 5% CO_2 at 42 ºC during one hour and then cells were harvested.
- Different forms of stress. At 48 hours of transfection cells were treated with the following conditions before harvesting: 5mM of H_2O_2 (MERCK) for 2 hours, 2mM hidroxyurea (Sigma-Aldrich) for 4 hours , 1 μM campthotecin (Sigma-Aldrich) for 1 hour and 20ng/ml of TNFα (Peprotech) for the indicated times. Cells were irradiated 48 hours after transfection with 10 Gy and harvested at the indicated times.
- Cells were arrested at G_1/S phase with double thymidine block and nocodazole. For the double thymidine block they were plated for the treatment at 60% of confluency approximately, after 24 hours 4mM thymidine

from SIGMA was added to the media, then, 12 hours later, the media was removed and fresh media was added, 12 hours lates, 4mM thymidine was added and cells were harvested 12 hours later.

- Cells were arrested at mitosis with Nocodazole treatment. Cells were plated for the treatment and 24 hours later, 4mM of thymidine was added, then, 24 hours later, media was removed and fresh media was added, 3 hours later 250ng/ml nocodazole was added and cells were harvested 12 hours later.

3. Cell transfection

Plasmids were transfected with the polymer polyethylenimine (PEI), MW 25000, polysciense, Inc. ref 23966. The mix of DNA is done with DMEM, then, PEI is added (3 or 4 μl of 1mg/ml PEI for each DNA μg) and the mix is incubated during 5 minutes and then, added to the plates.

4. Formation and infection of retrovirus

The packing cells Platinum A were used to produce retrovirus. Transfect the cells with 2 μg of pVSVG plasmid and 8 μg of the retroviral vector, in our case the retroviral vector was with PMSCV, PMSCVmycSuv3h1 or PMSCVmycSuv39h1cysteine mutants. 24 hours after transfection collect the media that contains virus, add fresh media and collect the media 48 hours after transfection that contains virus. Media with virus can be stocked at -80ºC until utilization.

MEFs were infected with virus because transfection was inefficient for introducing a vector. For the infection, cells were plated in 6-well plate the previous day to the infection. The day of infection the media was change for the media retrovirus containing, 16 hours approximately after the infection, media were change for fresh media. 24 hours after infection cells were selected with puromycin (the resistance of the vector).

5. Preparation of cells for citometry analysis

For citometry analysis with propidium iodide approximately 1.5 x 10^6 cells are trypsinized and washed twice with PBS1X and 1% SBF. Them, they are fixed with 70% ethanol with PBS1X, 1%SBF that is added slowly while cells are being vortexed. Cells are incubated during at least 2h at -20ºC. Then, cells are washed twice with PBS1X, 1% SBF. They are resuspended in 400 μl of PBS1X, 1%SBF and 25 μl of propidium

iodide (1mg/ml) and 5 μl RNAse A (10mg/ml) are added to the cells. They are incubated at 37ºC during 45 minutes. Cells were analyzed in a Gallios Flow Cytometer.

6. Plasmids

SirT6 HA was subcloned in pcDNAT0 HA vector using the sequence from pcDEF FLAG SirT6 a gift from Dr Verdin, by the primers EcoRISirt6U and SirT6HA-NotI described in the table and the constructions SirT6 HA H133Y and G60A were made by directed mutagenesis and the primers are in the table.

SirT1HA was subcloned in pcDNA4T0 HA vector using the sequence from FLAG SirT1 vector [153] by the primers SirT1BamHIU and SirT1NotIL.

Myc Suv39h1 and the constructions ΔN, Δchromo and ΔSET were described [32]. SET construction was cloned in the same vector as Δchromo construction using the primers SuvmycSETNotIU and SuvmycSETXhoIL. ΔPRESET construction was made adding BamHI targets in the extremes of the PRESET sequence from the mycSuv39h1 vector by directed mutagenesis with the primers BamHIdeltaPRESET and BamHIdeltaPRESETfin, and then, removing this domain by cutting with BamHI. mycSETΔC constructions was made by adding a stop codon in the mycSET construction with the primers deltaCmycSuv39U. The retroviral vector was subcloned in the PMSCV puromycine resistant vector using the sequence from the mycSuv39h1 and the primers PMSCVmycSuv39h1F and PMSCVmycSuv39h1R. The mutant for three cysteines were made by directed mutagenesis with PRESETmutCAU and PRESETmutCAL primers described in the table and the mutant for eight cysteines was made with directed mutagenesis from the three cysteines mutant sequence with PRESETC818386AU and PRESETC818386AU primers to have mutated five cysteines and then with PRESETC9495AU and PRESETC9495AL primers described in the table to have the eight cysteines mutated.

MycG9a and mycGLP were cloned in pcDNA3.1myc by the primers mycG9aEcoRIU and mycG9aXhoIL for G9a; and mycGLPXbaIU and mycGLPXbaIL for GLP described in the table using the sequence of the vectors pRev G9a long E411 and pRev GLP E412 that were a kind gift from Dr Ait-Si-Ali. Myc SETDB1 vector was a kind gift from Dr Pfeifer.

FLAGSKP2 and FLAG SKP2 K68R/K71R was provided by Dr Wei and described in Inuzuka et al. 2012. FLAGSKP2 K68/71/73/77R and FLAG SKP2 S72D S75D were made by directed mutagenesis with the corresponding primers.

pcDNA3-Myc3-Cul1 was purchased in addgene, plasmid # 19896. Flag Cul2 was provided by Dr Hagen and it has been described in Cordero-Espinoza et al. 2013.

FLAGCHIP was cloned in PCMV4 FLAG using as a sequence a myc CHIP vector that was a kind gift from Dr Patterson using the primers CHIPFLAGEcoRIU and CHIPFLAGBamHIL. PCMV4 FLAG RNF181 was cloned from 293F cDNA with the listed primers. GFP RNF8 vector is a gift from Dr Lukas (Panum Instituttet, Denmark).

IKKα and IKKα DN were a kind gift from Dr Espinosa. IkBα HA was cloned in the pcDNA4T0HA vector by the primers IKbaBamHIU and IKbaNotIL, using the sequence of a vector pBabe-Puro-IKBalpha-wt purchased to addgene vector #15290.

GST fused N-terminal H3 tails vectors was provided by Dr Shinkai and they are described in Tachibana et al. 2001.

pLKO1 shSuv39h1 vector was provided by Dr D'Adda Di Fagagna and was described in Di Micco et al. 2011. pLKO1 shSirt6 was cloned using the primers described in the table whose sequence was taken from [235].

pcDNA4T0 G3BP1 HA, pcDNA4T0 USP10 HA and pcDNA4T0 UBAP2L HA were cloned from 293F cDNA with the listed primers.

FLAGCHD3 expressing vector is a gift from Dr Goodarzi (University of Calgary, Canada). mycHA MBD2 expressing vector is a gift from Dr Stunnenberg (Radboud University, Netherlands). FLAGMBD3 is a gift from Dr Da Costa (Cancer Research, London, UK). LSD1HA and FLAG MTA2 expressing vectors are a gift from Dr Reinberg (NYU, USA).

7. Protein extraction

There were used three different methods for protein extraction:

- *Modified Dignam Method (Dignam, 1983).* Cells are resuspended in Buffer A (10mM Tris pH7,8; 10mM KCl; 1,5mM $MgCl_2$) and incubated 10 minutes on ice. Then, extract is centrifuged a maximum speed 1 minute at 4ºC and the supernatant is collected (it will be the cytoplasmic fraction). The pellet is resuspended in Buffer C (10Mm Tris pH7,8; 0,42M NaCl; 1,5mM $MgCl_2$; 0,2mM EDTA; 25% glicerol) and incubated 20 minutes on ice, then, it is centrifuged at maximum speed 5 minute at 4ºC. The supernatant is collected and mixed with the other supernatant to have protein soluble extract.

- *Rippa extraction.* The pellet is resuspended in Rippa Buffer (50Mm Tris pH7,8; 150mM NaCl; 0,5% Deoxycholic acid; 0,1% SDS; 1% NP40) and incubated during 20 minutes on ice. It is centrifuged at maximum speed 5 minute at 4ºC and the supernatant is collected (whole cell extract). It is a more stringent method. In order to obtain the chromatin bound fraction of proteins Benzonase nuclease (SIGMA) incubation is used. We used 25U Benzonase in 500 μl Rippa Buffer / $5*10^6$ cells approximately and incubate with agitation over night at 4ºC. Next day, the extract is centrifuge 10 minutes, 7000g at 4ºC and the supernatant is collect, it is the chromatin bound fraction.
- *Acid extraction (Chromatin extraction).* The pellet is resuspended in lysis buffer (10mM HEPES ph7.4, 10mM KCl, 0.05% NP40) and incubated 20 minutes on ice. Then, it is centrifuged at maximum speed 10 minutes at 4ºC and the supernatant is collected (it is the cytoplasmic fraction). The pellet is resuspended with 0.2M HCl and incubated 20 minutes on ice. It is centrifuged at maximum speed 10 minutes at 4ºC and the supernatant is collected (there are the soluble acid proteins, the histones). The extract is neutralized with TrisHCl pH8.7.

For the protein extract treated with NaOH, the extract was performed with Dignam method and incubated with 60 mM NaOh one hour shaking at 32ºC. Then, it was loaded.

8. Core histone purification

Cells were lysed in RSB buffer (10 mM Tris pH 8.0, 10 mM NaCl, 3mM $MgCl_2$) and 1% NP40. The extract was digested with MNase and was run in a sacarose gradient. The fractions that contain nucleosomes were dialysed in 10 mM HEPES-KOH pH7.5, 1mM EDTA, 10mM KCl, 10% glycerol and 0.2mM PMSF. Then, the free core histones were purified with hydroxyapatite column that removes the DNA from the histones.

9. Recombinant protein purification with GST

The vectors are transformed in the BL21 *E.coli* competent bacteria. Four colonies are grown in four tubes with 5 ml LB over night. The content of the four tubes are transferred in an Erlenmeyer with 2L LB and they are grown until reaching an absorbance of 0.6. Then, 1mM IPTG is added to the culture and it is left growing during three hours for the induction of protein expression. Bacterial pellet is collected and resuspended in NETN buffer (20mM Tris pH7.8, 100mM NaCl, 1mM EDTA and 0.5% NP40) with 0.2% Sarcosyl, sonicated and centrifuged at 15000 rpm, 4ºC in a Sorval

centrifuge 15 minutes and the supernatant is collected. The pellet is resuspended again in NETN buffer with 2% sarcosyl and centrifuges at 15000 rpm, 4ºC in a Sorval centrifuge 15 minutes. Both supernatants are put together to constitute the protein extract.

Then, the extract is incubated with Glutathione Sepharose beads in columns and incubated in rotation 30 minutes at 4ºC. The beads are washed twice with NETN buffer 0.7M NaCl, twice with NETN bufferand and once with TST buffer (50 mM Tris pH 7.8, 150 mM NaCl and 0.1% Triton). The beads are eluted with 20 mM reduced glutathione in TST Buffer 5 mM DTT seven times approximately, in each elution the beads are vortexed and incubated with the reduced glutathione 2 minutes.

10. Western Blot

Samples were diluted in the denaturing Laemli buffer and then loaded in a sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE). Proteins were transferred from gel to a nitrocellulose membrane (GE Healthcare). Then, membrane was blocked with PBS1X, 0.1% Triton and 5% non-fat milk. Then, the incubation of the primary antibody was made with the indicated dilution of the antibody (antibody section) in 0.1%Tween, PBS1X one hour. The secondary antibody (Sigma) of mouse or rabbit was diluted 1:10000 in 0.1%Tween and PBS1X and membrane was incubated between 30 minutes and one hour. After that membrane was washed 4 times with 0.1% Tween PBS1X. The membrane was developed with luminol or HRP Enhanced Chemiluminiscence (ECL, GE Healthcare).

11. Silver staining and coloidal-coomassie staining

For Silver staining after the electrophoresis the gel is incubated one hour with agitation in a solution with 50% methanol and 10% acetic acid, then, 30 minutes in 30% of glutaraldehide at 25% for fixing, wash the gel with distillated water during at least two hours, changing often the water in order to get rid of the remaining of glutaraldehide. Meanwhile prepare the staining solution: make a mix (mix 1) of 10 ml water with 2 g of $AgNO_3$ and then, other mix (mix 2) with 0.74 ml 5M NaOH, 9.6 ml NH4OH and 45 ml water, add drop by drop mix 1 to mix 2 while mix 2 is agitating. Then, add staining solution to gel and leave it with shake during 30-45 minutes. Wash gel three times with distillated water during 10 minutes each time, and then, develop with a solution with 2.5ml citric acid at 1% and 0.25 formaldehide at 37% in one liter of distillated water. Once the gel is developed, stop the reaction with a solution with 50% methanol and 10% acetic acid.

For the colloidal-coomassie staining, the staining solution must be prepared at least one day before the staining and it is possible to store it but it is light sensitive. The staining solution is made preparing two mixes: mix 1: dissolve 340 g of ammonium sulfate in water (600mL) and acetic acid (10mL). mix 2: dissolve 2 g of coomassie (G250) in 680 ml methanol. Add mix 2 in mix 1 very slowly with agitation. Leave it over night with agitation. Adjust to 2 L with water. Put the gel directly with this colloidal-coomassie staining and leave it over night with agitation. For gel distaining, discart the staining solution and distain with distillated water.

12. RNA extraction, RT-PCR and Quantitative PCR

mRNA was extracted from cells using Trizol (Life Technologies) following the manufacturer's protocol. Retrotranscriptase PCR was performed with this mRNA using Transcriptor Reverse Transcriptase (ROCHE). Quantitave PCR was performed with Sybr green Master Mix of Applied Biosystems and the primers described in table II were used. The results were normalized using primers for EEF2, HPRT1 and NCL for human samples and EEF2, HPRT1 and RPL38 for mouse samples.

13. Protein immunoprecipitation

For immunoprecipitation experiments, cell extracts were incubated with either α-FLAG, α-HA resin (Sigma-Aldrich) or α-Myc tag antibody (Cell Signaling) crosslinked to proteinG-Agarose resin (MERCK), overnight. Beads were washed three times with BC100 buffer (10mM Tris pH 7.8, 0.5 mM EDTA, 0.1mM PMSF, 0.1 mM DTT, 10% glycerol, 100 mM KCl) and five times with BC500 buffer (500 mM KCl). Then, proteins were eluted with 0.2 M Glycine pH 2 or by incubation with the corresponding competing peptides for mass spectrometry analysis.

14. Chromatin immunoprecipitation (ChIP)

Cell crosslinking was perfomed with 1% of paraformaldehyde and the reaction was stopped with 125 mM Glycine. Then, cells were lysed with lysis buffer (50 mM Tris pH 7.8, 10 mM EDTA, 1% SDS) and chromatin was sonicated with a Bioruptor (Diagenode) until a range of 300bp-1000bp was reached. Samples were diluted at least six times by dilution buffer (1% Triton, 2 mM EDTA, 150 mM NaCl, 20 mM Tris pH7.8) and precleared with Protein-G Magnetic beads (MERCK) that were preincubated at least 6 hours in rotation with 5%BSA and 1mg/ml salmon sperm DNA. The precleared samples were incubated with the indicated antibodies at least 6 hours and incubated with Magnetic Protein G beads overnight. Wash the beads with TSE I

buffer (150 mM NaCl, 0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris pH 7.8), TSE II buffer (500 mM NaCl, 0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris pH 7.8), Buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH7.8) and PBS 1X. Beads were eluted with 0.1M $NaHCO_3$ and 1% SDS with agitation. The crosslinking was reverted at 65ºC overnight and DNA was purified. Quantitative Real Time was performed with hNFKBIA promoter primer (IkBαpromoter).

15. Immunofluorescence

Transfected cells are passed to coverslips in 6-well plates 24 hours after transfection. Then, at 48 hours of transfection the immunofluorescence protocol starts. Three fast washes with PBS 1X are made in the plates. Then, cells are fixed with 2% paraformaldehide diluted in PBS 1X during 10 minutes, wash fast with PBS 1X. Permeabilyze cells with 0.1% Triton in PBS1X. Wash three times with 0.1% Tween in PBS1X. Block the cells with 5% Bovine serum albumin (BSA) with PBS1X during at least 30 minutes. Incubate a drop of the mix of primary antibody in the indicated dilution with 5%BSA with the coverslip in a wet chamber at 37ºC during one hour. Put the coverslips again in the 6-well plate and wash the antibody three times with 0.1% Tween in PBS1X. Then, incubate the coverslips again in a wet chamber with a drop of the mix of the secondary antibody (1:1000 dilution) with 5% BSA PBS1X during 1 hour at room temperature. Put again the coverslips back to the 6-well plate and make three washes of 5 minutes with 0.1% Tween in PBS1X. Then, incubate them during 5 minutes in a wet chamber. Wash them again three times of 5 minutes in the 6-well plate. Mount the coverslips in the slips with mowiol.

The images were taken with a Zeiss LSM510 Metal confocal microscope. The primary antibodies used were myc antibody (Cell Signaling) dilution 1:150, HA antibody (Sigma H6908) dilution 1:150, H3K9me3 antibody dilution 1:150, G9a antibody dilution 1:150. The secondary antibodies were mouse Alexa Fluor 568 and rabbit Alexa Fluor 488, both of them from Molecular Probes.

16. In vitro deacetylation assay

For the in vitro deacetylation SirT1HA and SirT6HA purified proteins were used. Purified SirT1HA or SirT6HA were incubated with core histones in a buffer with 50mM Tris HCl pH8, 100mM NaCl, 2mM DTT and 0.5mM NAD^+ one hour at 37ºC. Then the reaction was loaded in a gel for TAU gel or for Western Blot.

17. In vitro methylation assay

Purified SirT6HA was incubated with 50 mM Tris pH 8.7, 5 mM $MgCl_2$, 4 mM DTT, ^3H labeled SAM and core histones or purified recombinant N-terminal histone tails at 30 degrees during one hour. Then, SDS sample buffer was added and samples were loaded in SDS-Page gel and transferred to a membrane. The autoradiography signal was enhanced by EN3HANCE spray (Perkin Elmer) and the loading was controlled by Coomassie Blue Staining.

18. Triton Acid Urea (TAU) gel

Resolving gel has the mix: 45% Urea, 12% Acrylamide, 0.08% Bis-acrilamide, 0.375% Triton X-100 and 25% Acetic acid. Stacking gel as the mix: 45% Urea, 20% Acrylamide: Bis-acrilamide (30:0.8), 0.37% Triton and 5% Acetic acid. Then, the gel should be run over night 200V from anode to catode in 5% Acetic acid with the Overlay Solution (2.5M Urea, 5% Acetic acid and 0.02% Pyronin Y), then, with the Scavenge Solution (2.5M Urea, 5% Acetic acid, 0.02% Pyronin Y and 2.5M Cystamine) and load the Solution of Protamine sulfate (2.5M Urea, 5% Acetic Acid, 0.02% Pyronin Y, 25 mg/ml Protamine Sulfate). Samples are diluted 1:2 in TAU Buffer (30%Urea, 10% Acetic Acid, 0.2% Pyronin Yand 5% Protamine Sulfate) and loaded in the gel.

19. Luciferase assay

Luciferase assay was performed with the kit Luciferase Assay System (Promega) according to the manufacturer's protocol. Luciferase has an emission wavelength of 482 nm and was measured by Junior LB 9509 Portable Tube Luminometer from Berthold Technologies.

20. Glycerol Gradient

Prepare two solutions with different glycerol percentage: 12.5% and 35% glycerol. In both solutions are included 20mM Tris pH7.8, glycerol in the indicated percentages, 25 μM of EDTA and 200mM KCl. Add the solutions in a gradient maker, each one in each compartment, then make the gradient in tubes suitable for ultracentrifuge. Leave the gradients at least 2 hours at 4ºC. Then, add the samples and centrifuge the mix at 35000 rpm at 4ºC during the time need it to separate the proteins (usually is from 16 to 24 hours) with maximal acceleration and not break option to stop the centrifuge. Collect fractions of 150 μl from the upper part (lighter fractions) to the bottom (heavier fractions).

21. Chromatography purification

Protein extract was purified by anion exchange column DEAE nitrocellulose DE52 (Whatman) and cation exchange column P11 (Whatman). Then, the extract was purified by Äkta protein purification system (GE Healthcare) using the columns of anion exchange column 5PW (GE Healthcare) and the gel filtration column Superose 6 (GE Healthcare). All of them were made by a gradient from BC100mM (already described) to BC1M (same buffer but KCl is 1M).

22. Antibodies

The antibodies used were α-HA (Sigma-Aldrich, H6908), α-FLAG (Sigma-Aldrich F1804), α-myc (Cell Signaling2276S), α-H3K9me3 (MILLIPORE 07-442 for Western Blot and immunofluorescence, ABCAM ab8898 for ChIP), α-H3K9ac (Cell Signaling, 9675S), α-actin (Sigma-Aldrich A1978), α-tubulin (Sigma-Aldrich T6199), α-NFkB p65 (Santa Cruz Biotechnology sc-372 X), α-SirT6 (ABCAM AB62739), α-H3 (Cell Signaling, 9715), α-GFP (MERCK, MAB2510), α-Gal4BD (MERCK, 06-262).

23. Primers

Table I. Primers used for cloning

EcoRISirt6U	CCGAATTCATGTCGGTGAATTACGCGGCGGGGCTG
SirT6HA-NotI	CCGCGGCCGCGCTGGGGACCGCCTTGGCCT
S6H133YL	CCACAAACATGTTCCCGTAGAGCTCTGC
S6H133YU	GCAGAGCTCTACGGGAACATGTTTGTGG
S6HAG60AU	AGCACTGCCTCTGCCATCCCCGACTTCAGG
S6HAG60AL	CCTGAAGTCGGGGATGGCAGAGGCAGTGCT
SirT1BamHIU	CCGGATCCATGGCGGACGAGGCGGCCCT
SirT1NotIL	CCGCGGCCGCTGATTTGTTTGATGGATAGT
mycG9aEcoRIU	AAGAATTCGCAAGCGGCGATGGCGGCGG
mycG9aXhoIL	GGCTCGAGCCTGTGTTGACAGGGGGCAGGGA
mycGLPXbaIU	CCTCTAGAATGGCCGCCGCCGATGCCGAGGCA
mycGLPXbaIL	GGTCTAGACGTAGGGGGTCGGCGGCAGCCGCGG
SuvmycSETNotIU	CGGCGGCCGCATCCGATATGACCTCTGCATCT
SuvmycSETXhoIL	GGCTCGAGCTAGAAGAGGTATTTGCGGCAGG
BamHIdeltaPRESETU	ATCACCCTCAACCAGGGATCCGTGGCTGTGAGTGCCAG
BamHIdeltaPRESETL	CTGGCACTCACAGCCACGGATCCCTGGTTGAGGGTGAT
BamHIdeltaPRESETfinU	GTAGTCCAGAAAGGCATCGGATCCGATCTCTGCATCTTC
BamHIdeltaPRESETfinL	GAAGATGCAGAGATCGGATCCGATGCCTTTCTGGACTAC
deltaCmycSuv39U	TACAACATGCAAGTGGACTGAGTGGACATGGAGAGTACC
deltaCmycSuv39L	GGTACTCTCCATGTCCACTCAGTCCACTTGCATGTTGTA
mycSuvS391AU	GCTGGGCTCCCCGGCGCCCCCAAGAAACGAGTCCGT
mycSuvS391AL	ACGGACTCGTTTCTTGGGGGCGCCGGGGAGCCCAGC
PRESETmutCAU	CAGCCCATCTACGAGGCCAACTCCCGCGCTTGCTGTGGCTATGACGC CCCAAACCGTGTAGTC
PRESETmutCAL	GACTACACGGTTTGGGGCGTCATAGCCACAGCAAGCGCGGGAGTTG GCCTCGTAGATGGGCTG

PRESETC818386AU	AACCAGGTAGCTGTTGGCGCTGAGGCCCAGGACGCTCTGTTGGCACCCACT
PRESETC818386AL	AGTGGGTGCCAACAGAGCGTCCTGGGCCTCAGCGCCAACAGCTACCTGGTT
PRESETC9495AU	GCACCCACTGGAGGCGCTGCCCCTGGAGCATCCCTGCAC
PRESETC9495AL	GTGCAGGGATGCTCCAGGGGCAGCGCCTCCAGTGGGTGC
mycSuv39h1K393RK394RU	GCTGGGCTCCCCGGCTCCCCCAGGAGACGAGTCCGTATTGAA
mycSuv39h1K393RK394RL	TTCAATACGGACTCGTCTCCTGGGGGAGCCGGGGAGCCCAGC
mycSuv39h1K401RU	GTCCGTATTGAATGCAGATGTGGGACAACGGCT
mycSuv39h1K401RL	AGCCGTTGTCCCACATCTGCATTCAATACGGAC
mycSuv39h1K409RU	ACAACGGCTTGCCGAAGATACCTCTTCTAGCCC
mycSuv39h1K409RL	GGGCTAGAAGAGGTATCTTCGGCAAGCCGTTGT
mycSETK263RK266RU	GTCCGCACCCTGGAGAGGATTCGCAGGAACAGCTTCGT
MYCSETK263RK266RL	ACGAAGCTGTTCCTGCGAATCCTCTCCAGGGTGCGGAC
SET K401U	CGTATTGAATGCAGGTGTGGGACTGAGTC
SET K401L	GACTCAGTCCCACACCTGCATTCAATACG
SET K409U	CTGAGTCCTGCCGCAGATACCTCTTCTAG
SET K409L	CTAGAAGAGGTATCTGCGGCAGGACTCAG
PMSCVmycSuv39h1F	CCCTCGAGGACCGGGGAAAGATGGGCGGACG
PMSCVmycSuv39h1R	CCGAATTCCTAGAAGAGGTATTTTCGGCAAGC
SKP2-S7275DF	CGGCTGAAGGACAAAGGGGACGACAAAGACTTTG
SKP2-S7275DR	CAAAGTCTTTGTCGTCCCCTTTGTCCTTCAGCCG
SKP2-K717377R-F	ACGGCTGAGAAGCAGAGGGAGTGACAGAGACTTTGTG
SKP2-K717377R-R	CACAAAGTCTCTGTCACTCCCTCTGCTTCTCAGCCGT
CHIPFLAGEcoRIU	CCGAATTCCATGAAGGGCAAGGAGGAGAAGGA
CHIPFLAGBamHIL	CCGGATCCGTAGTCCTCCACCCAGCCATTCTC
IKbaBamHIU	CCGGATCCATGTTCCAGGCGGCCGAGCGC
IKbaNotIL	GCGGCCGCTAACGTCAGACGCTGGCCTC
shSirT6KD2F	CCGGAAGAATGTGCCAAGTGTAAGACTCGAGTCTTACACTTGGCACATTCTTTTTTTG
shSirT6KD2R	AATTCAAAAAAAGAATGTGCCAAGTGTAAGACTCGAGTCTTACACTTGGCACATTCTT
RNF181EcoRIU	CCGAATTCCATGGCGTCCTATTTCGATGAACACG
RNF181BamHIL	CCGGATCCTCACGTGTACATGGCTCCATGG
G3BP1-EcoRI-U	CCGAATTCATGGTGATGGAGAAGCCTAGTCCCC
G3BP1NotIL	CCGCGGCCGCCTGCCGTGGCGCAAGCCCCCTTCCC
UBAP2L-EcoRI-U	TAGAATTCATGATGACATCGGTGGGCACTAACC
UBAP2LNotIL	CCGCGGCCGCGCTCTCAGCCGTCCAGAAATGCTTG
ups10NotIU	CCGCGGCGCATGGCCCTCCACAGCCCGCAG
UPS10NotIL	CCGCGGCCGCCAGCAGGTCCACTCGGCGGTAATAC

Table II. Primers used for Quantitative PCR.

hIkbalphaF	GATCCGCCAGGTGAAGGG
hIkbalphaR	GCAATTTCTGGCTGGTTGG
hA20-F	GTCCGGAAGCTTGTGGCGCT
hA20-R	CCAAGTCTGTGTCCTGAACGCCC
hIL8-F	GGCAGCCTTCCTGATTTCTG
hIL8-R	CTTGGCAAAACTGCACCTTCA
hGRO-1 F	AGGAAGCTCACTGGTGGCTG
hGRO-1 R	TAGGCACAATCCAGGTGGC
hIAP2F	ATGCTTTTGCTGTGATGGTG
hIAP2R	TGAACTTGACGGATGAACTCC
h14-3-3sigma-F	GTCTGATCCAGAAGGCCAAG
h14-3-3sigma-R	CTCCTCGTTGCTTTTCTGCT
hCyclinD1-F	AGCTCCTGTGCTGCGAAGTGGAAAC
hCyclinD1-R	AGTGTTCAATGAAATCGTGCGGGGT
hCyclinD2-F	GCGTGCAGAAGGACATCCAAC
hCyclinD2-R	GTTGTCGGTGTAAATGCACAGC
SUV39h1-fw	GTCATGGAGTACGTGGGAGAG
SUV39h1-rev	CCTGACGGTCGTAGATCTGG
RT.hHPRT1-F	TGACACTGGCAAAACAATGCA
RT.hHPRT1-R	GGTCCTTTTCACCAGCAAGCT
RT.hNCL-F	CTGCCTCAGAGGATGAG
RT.hNCL-R	TCTGTTTGGCCATTTCCTTC
hEEF2 F	TGGAGATCTGCCTGAAGGAC
hEEF2 R	GACTTGGAGAGGCAGAGAGCAC
IkbalphaU	AAATCTCCAGATGCTACCCGAGAG
IkbalphaL	ATAATGTCAGACGCTGGCCTCCAA
mIAP2F	GCTCAGAATCAAAGGCCAAG
mIAP2R	CACCAGGCTCCTACTGAAGC
mMnSODF	TTAACGCGCAGATCATGCA
mMnSODR	GGTGGCGTTGAGATTGTTCA
MajorsatU	GACGACTTGAAAAATGACGAAATC
MajorsatL	CATATTCCAGGTCCTTCAGTGTGC
MinorsatU	CATGGAAAATGATAAAAAC
MinorsatL	CATCTAATATGTTCTACAGTGTGG
L1_ORF1 - F	CACTCCCACCCCACCTAGT
L1_ORF1 - R	TAACTCTTTAGCAGTGCTCTCCTGT
RT.mHPRT1-1F	TCAGTCAACGGGGGACATAAA
RT.mHPRT1-1R	GGGGCTGTACTGCTTAACCAG
mEEF2 F	TGTCAGTCATCGCCCATGTG
mEEF2 R	CATCCTTGCGAGTGTCAGTGA
Mrpl38 f	AGGATGCCAAGTCTGTCAAGA
Mrpl38 r	TCCTTGTCTGTGATAACCAGG
hNFKBIApromoterF	GACGACCCCAATTCAAATCG
hNFKBIApromoterR	TCAGGCTCGGGGAATTTCC

RESULTS

In vitro deacetylase reaction of SirT6 and gene silencing induction

Previously to the start of this work, several articles had been published that reported that SirT6 promotes gene silencing by binding specific transcription factors [206]. Furthermore, in 2008, Dr Chua's laboratory showed that SirT6 has a specific H3K9ac deacetylation activity [208]. In order to understand better the role of SirT6 in gene silencing, we first aimed to confirm this H3K9-specific activity. For that purpose, we purified SirT6-HA by affinity chromatography using α-HA resin and we performed an *in vitro* deacetylation assay using the SirT6-HA elutions, hyperacetylated core histones purified from HeLa cells, and NAD^+ [50] *(Figure R1A).* In parallel, we performed the same assay using purified SirT1-HA as a control. The reaction was analyzed in a Western Blot using antibodies anti αH3 and αH3K9ac. Surprisingly, we only observed H3K9 deacetylation by SirT1, but not SirT6 *(Figure R1B).* To further confirm this result, we analyzed the same reaction in a Triton-Acetic Acid-Urea (TAU) gel, which can separate histones according to the charge of their N-terminal tails (histones are separated if they are acetylated or phosphorylated) *(Figure R1C).* Again, we observed a loss of the upper bands that correspond to acetylated histones in the SirT1 reaction whereas the same upper bands remain unaffected with SirT6. Therefore, in the case of SirT6, we were unable to detect the described H3K9 deacetylase activity for SirT6 with our conditions.

Next, we checked if the localization of SirT6 to gene promoters induces gene silencing as it was suggested [206]. For that, we have used and inducible system that had been previously used for SirT1 [153]. The system is based in the integration of two vectors in the Tetracyclin-inducible system cells T-REx (cells that express the tetracycline repressor): 1) a mammalian expression vector SirT6 fused to Gal4 binding domain (G4BD-SirT6) inducible by tetracycline (Tet-on promoter), and 2) a reporter vector containing the luciferase cDNA (pTK luc) under the control of five binding sites for Gal4BD *(Figure R2A).* In non-induced conditions, luciferase is expressed and its activity can be measured by absorbance at 482 nm. After tetracycline treatment during 24 h, G4BD fused protein is expressed, binds to the promoter of the luciferase gene and its effect on luciferase transcription can be monitored by measuring luciferase activity. Thus, our results with G4BD-SirT6 showed that arrival of SirT6 to the promoter correlated with five times less luciferase activity compared to the untreated control (*Figure R2B).*

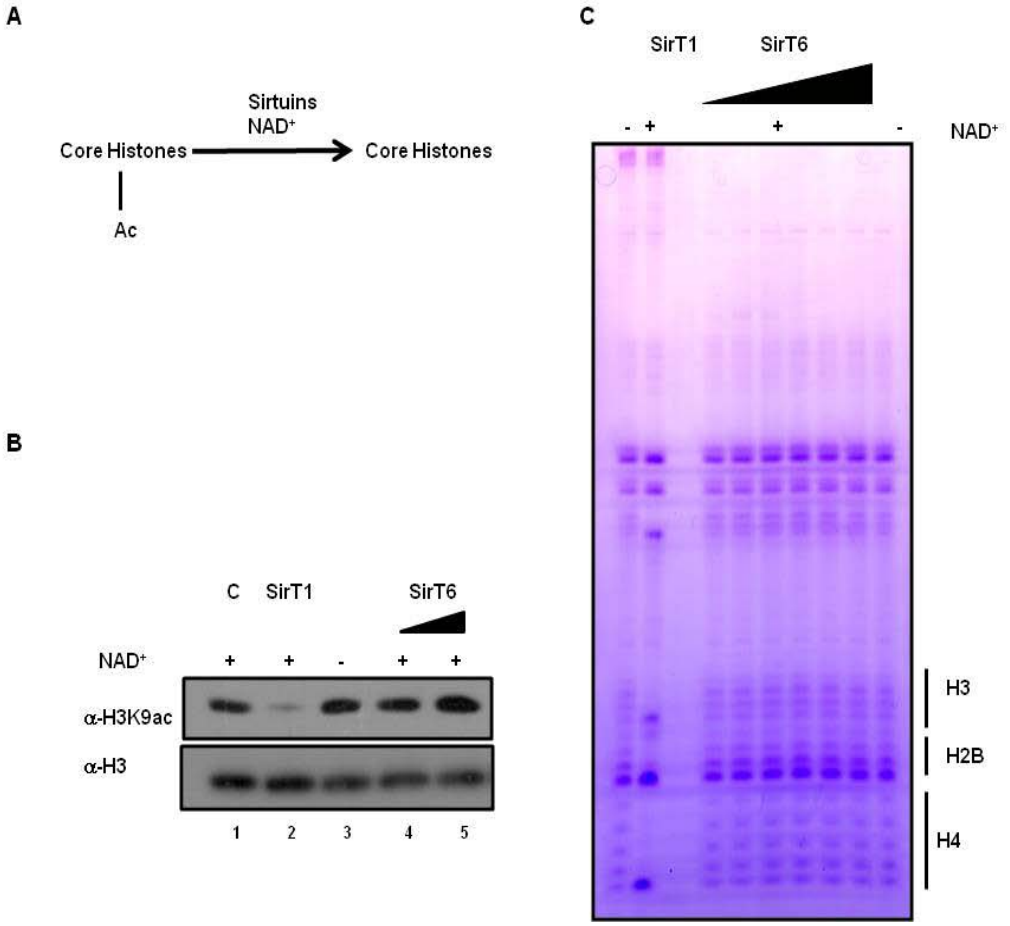

Figure R1. SirT6 in vitro deacetylation activity. A. Scheme of sirtuin deacetylation in vitro reaction. **B.** Western Blot αH3K9ac of deacetylation in vitro reaction with SirT1 or SirT6. **C.** TAU gel of deacetylation in vitro reaction with SirT1 or SirT6

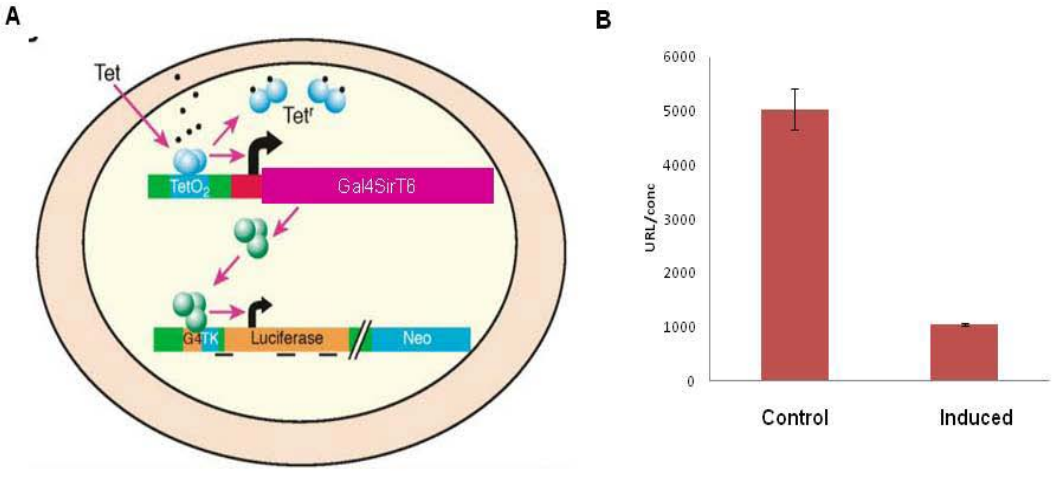

Figure R2. SirT6 promotes gene silencing. A. Scheme of system for detecting gene silencing upon binding of SirT6 to the promoter. **B.** Luciferase expression normalized by global protein levels.

In the establishment of gene silencing, HDAC activities do not work alone. In fact they cooperate with other activities in order to achieve this repressive state. In particular, HDACs show an intimate functional relationship with HMTs, a link that is key, not only for repression, but also to establish stable epigenetic silencing through facultative heterochromatin structures. In order to understand better the role of SirT6 in chromatin we followed two different complementary approaches: First, we addressed whether SirT6 fractionates with HMT activity/es and determined their identity. Second, we identified new interacting partners of SirT6.

1. Interaction of SirT6 with H3K9 methyltransferases

To address whether SirT6 interacts with HMT activities, we used an unbiased approach: We tested whether SirT6 purified from mammalian cells co-fractionates with an HMT activity. For that purpose, we purified SirT6-HA using α-HA resin. As a control, we used a similar purification but using cells that were not expressing SirT6HA. We then tested the elutions (C and SirT6-HA) in an *in vitro* histone methylation assay (see materials and methods) using purified core histones as the substrate and [H^3]-SAM as the methyl donor [16] *(Figure R3A).* Supporting our initial hypothesis, our results showed that SirT6 does co-fractionate with a HMT activity. Interestingly, this activity was specific for histone H3 as the band labeled with radioactivity corresponded to the histone H3 according to coomassie staining of the membrane *(Figure R3B).*

Next step was to identify the histone H3 residue methylated by SirT6-associated HMT activity. For that purpose, we performed a similar *in vitro* HMT assay, but instead of core histones as the substrate we used a set of recombinant proteins formed by GST fused to the N-terminal histone tail of histone H3 either WT (H3N) or containing specific mutations of K4, K9 or K27 to R, and different combinations of them [24], as indicated *(Figure R3C).* The methylation occurs in the H3 N-terminal histone tails that are WT, K4R (mutation in K4), K27R (mutation in K27) and K4RK27R (mutation in both K4 and K27), concluding that the methylation is produced only when lysine 9 is not mutated *(Figure R3D).* In conclusion, SirT6 co-fraccionates with an H3K9 histone methyltransferase activity.

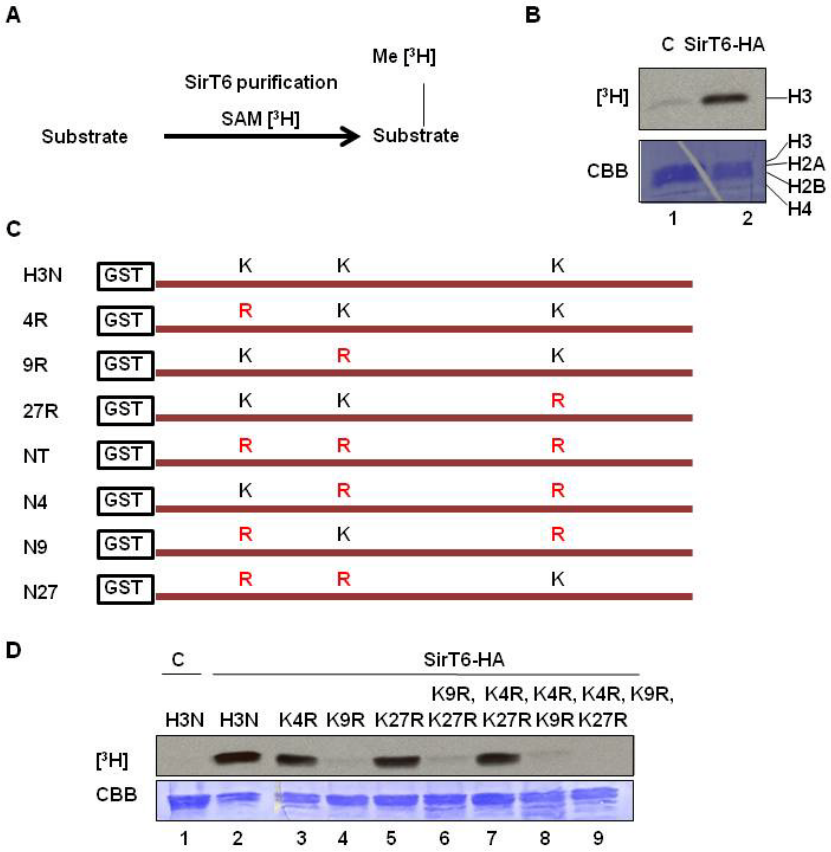

Figure R3. SirT6 co-fraccionates with methyltransferase activity. A. Scheme of the in vitro methylation reaction. **B.** Autoradiography and Coomassie blue staining membrane of the in vitro methylation reaction using core histones as substrate. **C.** Sequence of recombinant H3 N-terminal tails. **D.** Autoradiography and Coomassie blue staining of the in vitro methylation reaction using recombinant H3 N-terminal tails as substrate.

Our next step was to identifiy the HMT involved. In mammals there are four main H3K9 methyltransferases: Suv39h1, G9a, GLP, SETDB1 [21]. In order to identify the H3K9 methyltransferase that interacts to SirT6 and induces the methylation in the in vitro assays, cells were transfected with SirT6-HA together with each of the H3K9 methyltransferases and the interaction was tested by HA immunoprecipitation. We found that SirT6 interacts specifically with G9a and Suv39h1 (see lanes #9-12), but not with the rest of HMTs (see lanes #13-16) *(Figure R4A).* An immunoprecipitation was performed between SirT6, Suv39h1 and G9a in order to know whether both HMTs bind to the same domain of SirT6 and they could compete for interaction. Interestingly, as we described earlier, all the H3K9 methyltransferases can form a complex in in particular conditions [21]. After G9a immunoprecipitation both Suv39h1 and SirT6 interact with G9a even if the two of them are overexpressed *(Figure R4B)*, however,

SirT6 seems to precipitate less when Suv39h1 suggesting that SirT6 compete with Suv39h1 to bind G9a or Suv39h1 compete with G9a to bind SirT6.

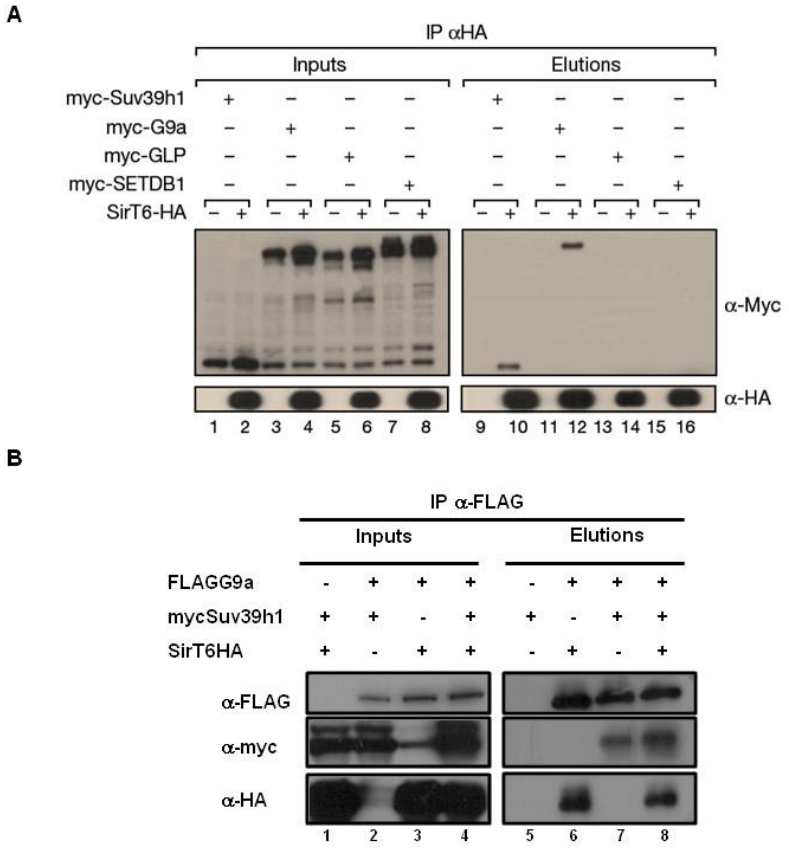

Figure R4. SirT6 interacts with Suv39h1 and G9a. A. HA immunoprecipitation from the 293F extracts of SirT6-HA and the H3K9 methyltransferases: myc-Suv39h1, myc-G9a, myc-GLP and myc-SETDB1. **B.** FLAG immunoprecipitation of 293F extracts of FLAG-G9a, myc-Suv39h1 and SirT6-HA.

In order to know whether SirT6 affects the global localization of G9a or Suv39h1 we performed immunofluorescence (IF) studies. In the case of Suv39h1, we do not have a good available IF antibody for endogenous Suv39h1, so we had to perform IF experiments in NIH3T3 cells overexpressed with mycSuv39h1 in the presence or absence of SirT6HA. Our results showed that overexpression of SirT6 does not affect the localization of mycSuv39h1, found mainly in constitutive heterochromatin as it has been described [64] *(Figure R5A).* In the case, of G9a, we could study endogenous G9a and therefore we tested its localization by IF in SirT6 WT and KO MEFs. Our studies could not detect any change in G9a localization upon SirT6 loss *(Figure R5B).*

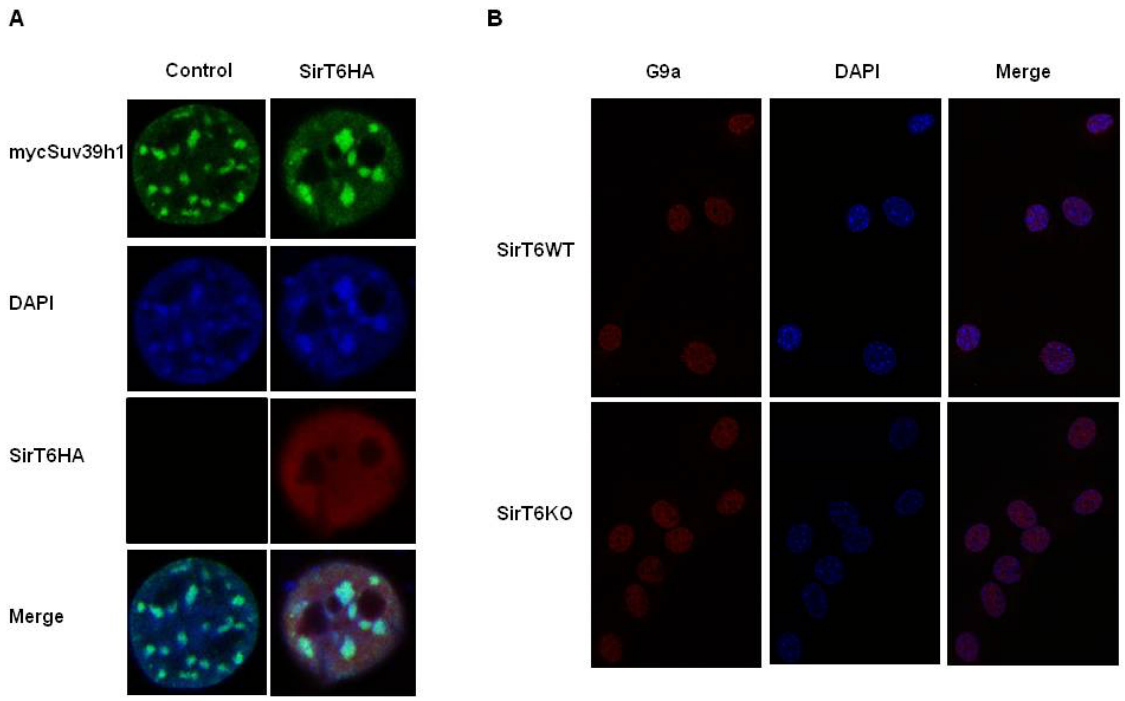

Figure R5. SirT6 does not affect neither to Suv39h1 nor to G9a subcellular localization. A. Immunofluorescence in NIH3T3 cells with overexpressed myc-Suv39h1 and with/without SirT6-HA. **B.** Immunofluorescence with αG9a and DAPI in MEFs SirT6WT and SirT6 KO.

1.1. Suv39h1 and SirT6

The finding that Suv39h1 interacts with SirT6 was a big surprise, given the close relationship between SirT1 and Suv39h1 in heterochromatin formation [49]. In order to understand better the differential relationship between Suv39h1 and SirT6 or SirT1, we first decided to determine whether the interaction between SirT6 and Suv39h1 is through SirT1. For that purpose, we performed an IP between SirT1 and SirT6. Our experiments confirmed that both Sirtuins do not interact and therefore the link between SirT6 and Suv39h1 is SirT1-independent *(Figure R6).*

We next aimed to determine whether the relationship between both Sirtuins and Suv39h1 is equivalent or involves a certain level of redundancy. Considering the role of SirT1 in the regulation Suv39h1-dependent constitutive heterochromatin structure, we next asked whether SirT6 relationship with Suv39h1 was also related to constitutive heterochromatin. For that purpose, we performed IF experiments with SirT1KO and SirT6KO MEFs (compared to their respective WT MEFs) to determine the levels of Suv39h1-dependent H3K9me3 in the constitutive heterochromatin foci. In contrast to

SirT1KO MEFs, that show a clear loss in Suv39h1-dependent H3K9me3 enrichment [49], SirT6KO MEFs did not show any defect in the levels of H3K9me3 in constitutive heterochromatin *(Figure R7).* Therefore, the relationship between SirT6 and Suv39h1 is not related to constitutive heterochromatin.

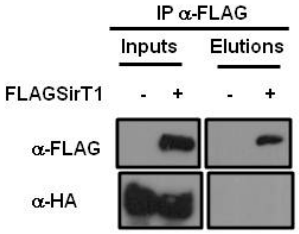

Figure R6. SirT1 and SirT6 do not interact. FLAG immunoprecipitation of extract from 293F cells transfected with FLAG-SirT1 and SirT6-HA.

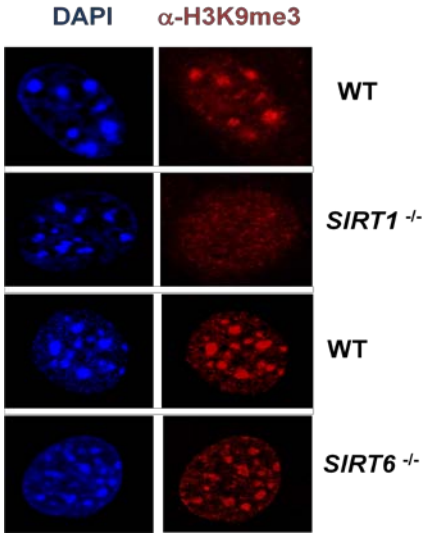

Figure R7. SirT6 depletion does not affect to H3K9me3 foci. Immunofluorescence of αH3K9me3 and DAPI in MEFs SirT1 WT, SirT1 KO, SirT6 WT and SirT6KO.

In order to understand the specific role of SirT6 in Suv39h1 function, we next determined the interacting domain of Suv39h1 with SirT6 compared to SirT1. For that purpose, we performed immunoprecipitation assays between SirT6-HA and different deletion mutants of Suv39h1 *(Figure R8A).* As a control, we also performed the same IPs with SirT1HA instead of SirT6-HA. Interestingly, the IPs showed that SirT6 interacts with all the mutants except the ΔSET mutant, which lacked the catalytic SET domain together with the very c-terminal short post-SET domain (*Figures R8A and R8B*). Supporting that SirT6 interacts through this extended SET domain (plus Post-SET

domain), a mutant only containing the extended SET domain was able to sustain interaction with SirT6 (*Figure R8C*). In contrast, as described previously[9] SirT1 interacts through the N-terminal region, including the N-terminal domain and the chromo domain *(Figure R8C).* We concluded that SirT6 interacts with the C-terminal domain of Suv39h1 while, in contrast, SirT1 does it through the N-terminal domain. In order to define better the Suv39h1 interaction domain to SirT6, we tested the ability of the strict SET domain of Suv39h1 to interact with SirT6. Interestingly, while the SET domain was able to bind to SirT6, loss of the post-SET domain decreased the binding of both proteins (*Figure R8C*). This suggested that both domains are required for the full binding ability of the extended SET domain construct.

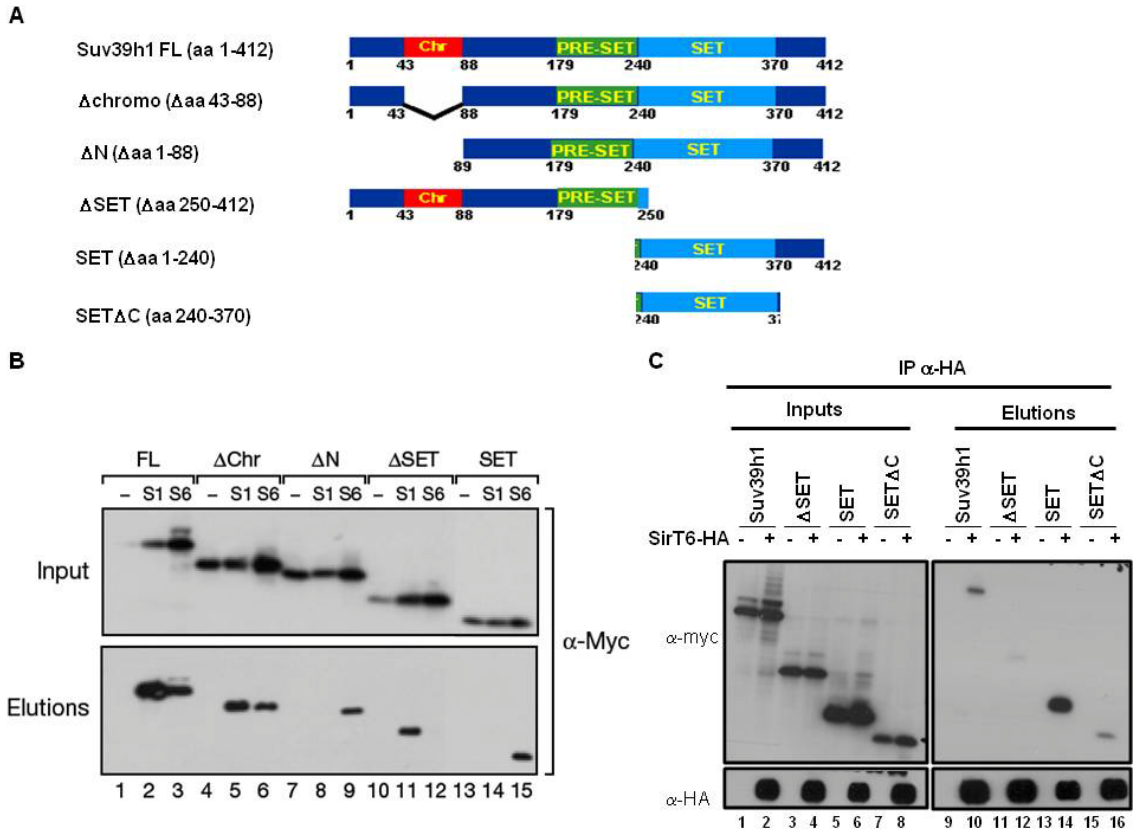

Figure R8. SirT6 interacts with Suv39h1 catalytic SET domain. A. Scheme of mycSuv39h1 deletion mutant constructions. **B.** HA immunoprecipitation from 293F extracts of SirT1HA and SirT6 and mycSuv39h1 constructions. **C.** HA immunoprecipitation from 293F extract of SirT6HA and mycSuv39h1 constructions.

Since SirT1 regulate Suv39h1 protein stability [155], we also studied whether SirT6 may be also involved in regulating Suv39h1 levels. A western blot of endogenous Suv39h1 was performed in WT and SirT6KO MEFs showed that Suv39h1 levels were

decreased in SirT6 KO MEFs *(Figure R9A)*. Consistently, SirT6 overexpression induced a twofold increase in the levels of myc-Suv39h1 while SirT1 overexpression increased the levels in more than threefold *(Figure R10 A, B)*. As in the case of SirT1, the SirT6-mediate increase in Suv39h1 levels was taking place at protein level and not at transcriptional level, since overexpression of SirT6 did not change the levels of either endogenous Suv39h1 mRNA or transfected myc-Suv39h1 mRNA measured by RT-PCR *(Figure R9B)*.

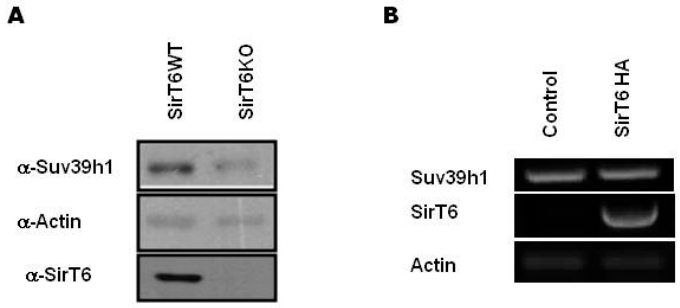

Figure R9. SirT6 stabilizes Suv39h1 protein levels. A. Western Blot of protein extracts from MEFs SirT6 WT and KO. **B.** RT-PCR from 293F cells with SirT6-HA and myc-Suv39h1 transfected.

Altogether, our evidences clearly demonstrate that, despite the effect of both sirtuins on Suv39h1 stability, the functional relationship between SirT1 and SirT6 with Suv39h1 is very different. Interestingly, a surprising observation supported even further specific relationship between SirT6 and Suv39h1. We observed that overexpression of SirT6 induces an 8-10 KDa postranslational modification in Suv39h1 *(Figure R10A).* We next aimed to identify the nature of this modification through mass spectrometry analysis. The results identified Suv39h1 and the 8.5kDa regulatory protein ubiquitin *(Figure R11A)* strongly suggesting that SirT6 induces monoubiquitination in Suv39h1

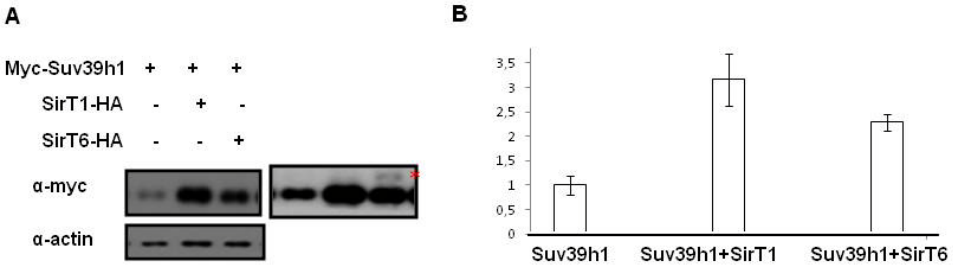

Figure R10. SirT6 overexpression upregulates Suv39h1 protein levels A. Western Blot of extracts from 293F cells tranfected with mycSuv-39h1, SirT1-HA and SirT6-HA. **B.** Quantification of myc-Suv39h1 levels with SirT1 and SirT6 overexpression.

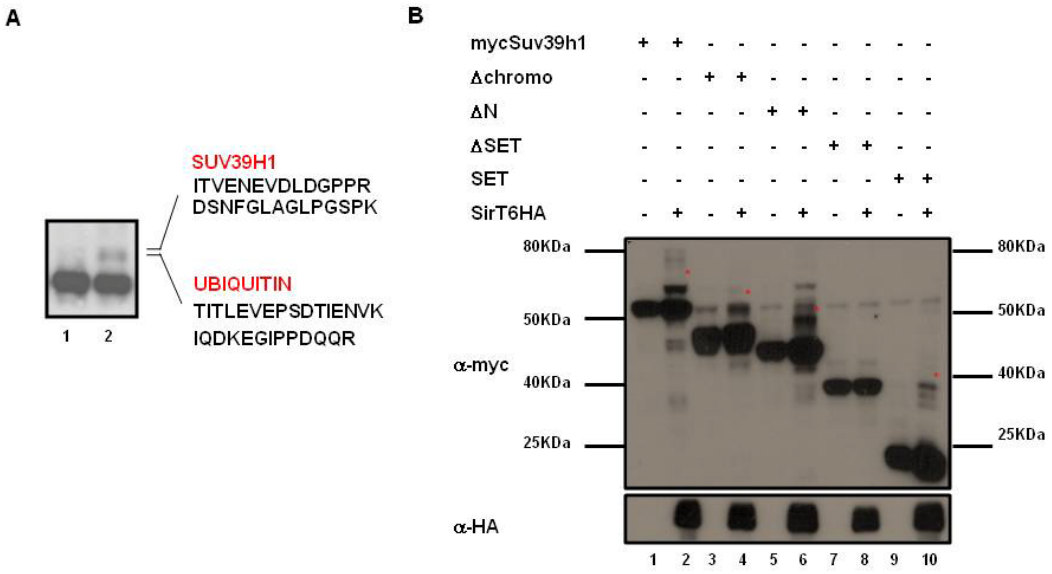

Figure R11. SirT6 induces a monoubiquitination. A. Western Blot of purified mycSuv39h1 from 293F cells with mycSuv39h1 and SirT6 and result from analysis of mass spectrometry of Suv39h1 upper band. **B.** Western Blot of protein extract from 293F cells with the indicated protein transfected.

1.1.1. **Characterization of Suv39h1 monoubiquitination**

First of all, we confirmed the SirT6 induction of the modification was a direct effect, mediated by the binding of SirT6 to Suv39h1. Then, we tested the ability of SirT6 to induce the modification in the Suv39h1 deletion mutants used before. As expected, loss of the SET domain correlated with a total loss of the monoubiquitination *(Figure R11B).*

We next aimed to characterize the residue/s monoubiquitinated. For that purpose, we first tested whether three lysine residues functionally linked to SirT1 could be involved in the monoubiquitination: a) The chromodomain residue K87, which is the main poly-ubiquitination site of Suv39h1 [155]; b) The residue K266, located in the catalytic SET domain and deacetylated by SirT1 [49]; c) The residue K263, just two aminoacids away from K266. The three lysine residues were mutated into alanines, a residue that could not be ubiquitinated *(Figure R12A).* Interestingly, none of these residues abrogated the modification by SirT6 suggesting that they were not involved in its deposition *(Figure R12B)*. As it has been observed that SET construction can have ubiquitination, we mutated every single lysine of the SET construction into arginines

and observed that even when every lysine was mutated, SirT6 overexpression was still able to induce ubiquitination suggesting that a different residue could be ubiquitinated *(Figure R12C).* We next tried to identify the identity of the residue/s involved by purifying mycSuv39h1 again after SirT6 overexpression but this time overexpressing Ubiquitin-HA to increase Suv39h1 ubiquitination and we analyzed again by mass spectrometry to identify the residue involved in this monoubiquitination *(Figure R13).*

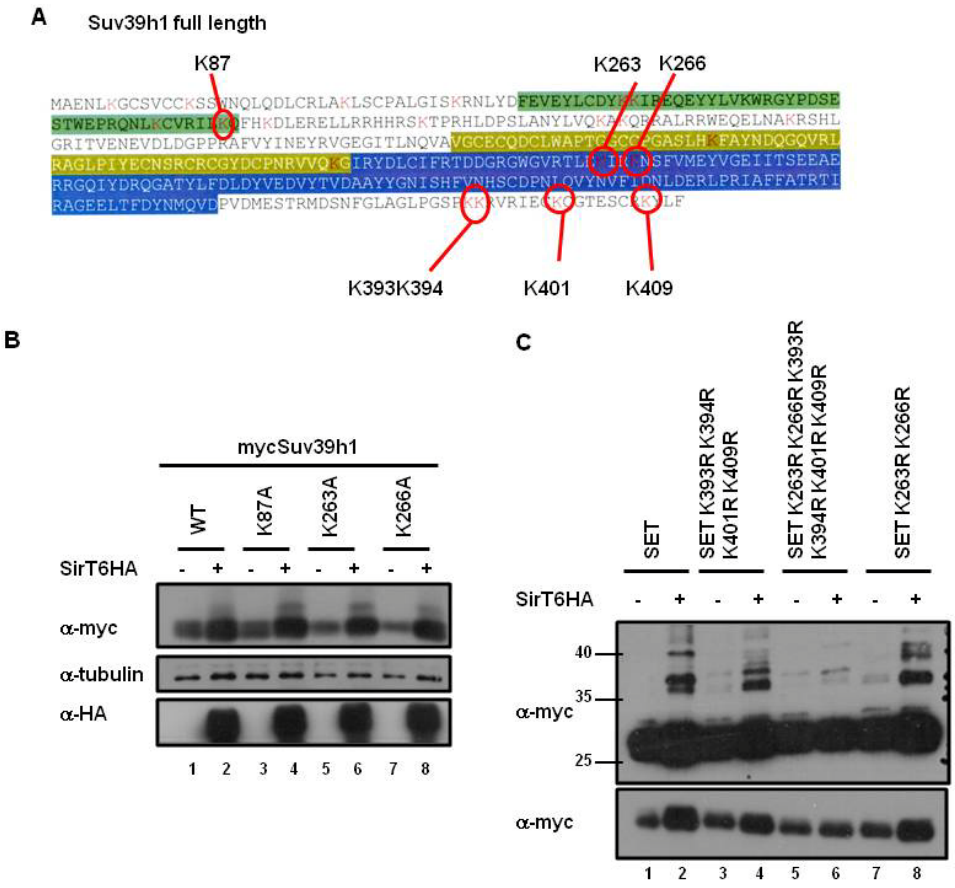

Figure R12. SirT6 does not induce monoubiquitination in any lysine. A. Suv39h1 full length aminoacidic sequence. Green: Chromo domain, Yellow: PRESET domain, Blue: SET domain. **B.** Western Blot of extract from 293F cells with Suv39h1 mutants transfected and SirT6-HA.

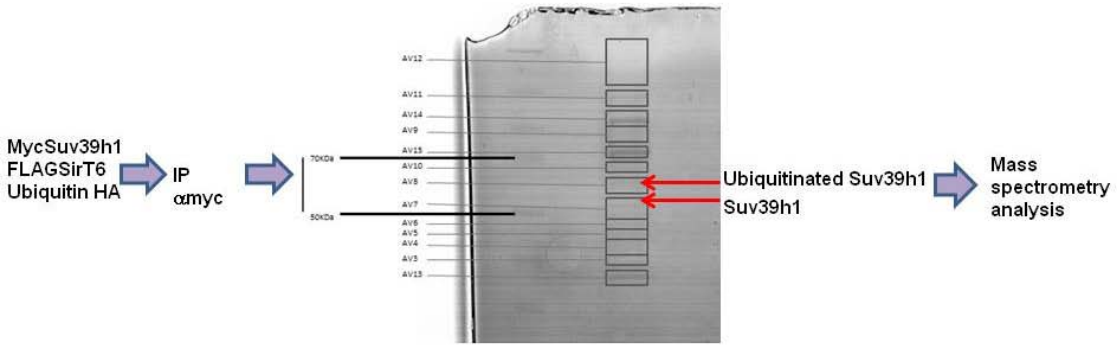

Figure R13. Suv39h1 monoubiquitination purification. Diagram of purification of Suv39h1 monoubiquitination for mass spectrometry analysis and coloidal staining of mycSuv39h1 purification.

Surprisingly, it was detected that the monoubiquitinated residue was not taking place in lysines, but in Cysteines and Serine. Exactly, we identified four Cysteines and a Serine residue (C49, C222, C226, C232 and S29) *(Figure R14A).* Of note, only one of these residues was monoubiquitinated in each Suv39h1 molecule, and therefore the "modified" Suv39h1 is a mix of Suv39h1 populations modified only in one of each of these residues. Given than the modified band is around 8-10 kDa bigger than regular Suv39h1, only one ubiquitin residue is attached to a single Suv39h1 molecule. Therefore, SirT6 induces monoubiquitination in at least four cysteines and a serine of Suv39h1. In fact, of the five identified residues, three cysteines (C222, C226 and C232) were localized to the pre-SET domain of Suv39h1, the other cysteine (C49) was present in the chromodomain of suv39h1, and the serine was localized in the HP1-binding N-terminal domain of Suv39h1. Strikingly, the three modified cysteine residues in the pre-SET domain actually correspond to three of the nine conserved cysteines that define the pre-SET domain *(Figure R14A).* We checked if Suv39h1 monoubiquitination induced by SirT6 is not taking place in lysines by inducing a mild alkaline hydrolysis with NaOH treatent. Amine bound that take place in lysine ubiquitination is not destroyed with this treatment, but it is destroyed the ester linkage that take place in non canonical ubiquitination [307]. We can observe that ubiquitin was removed with this treatment fitting with the spectrometry analysis result (*Figure 14B*).

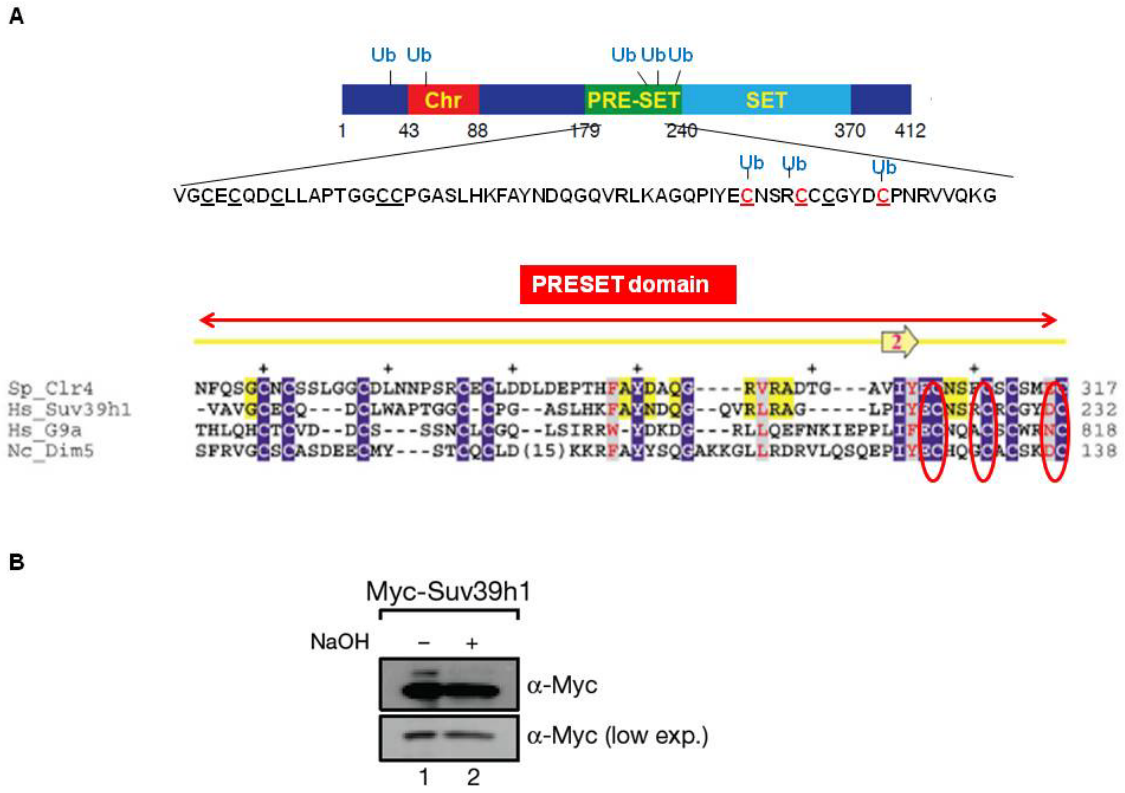

Figure R14. Suv39h1 is monoubiquitinated in cysteines. A. Scheme of ubiquitins found in the mass spectrometry analysis and scheme of the conserved cysteines in the pre-SET domain. **B.** Western Blot of extract from 293F cells transfected with myc-Suv39h1 and SirT6-HA treated with NaOH.

Given the fact that the majority of the monoubiquitinations take place in the pre-SET domain and that three of the nine conserved residues of the domain were modified, we decided to center our work on these modifications and leave the characterization of the others for future studies.

Overall, we hypothesize that a modification 8.5 kDa in a domain formed by around 70 residues (Pre-SET domain) within a 45 kDa protein (Suv39h1), would destroy the structure of the domain and alter the whole structure of the protein. In order to validate our hypothesis we performed a rescue experiment in Suv39 DN MEFs (lacking both Suv39h1 and Suv39h2) by re-introducing either wild type Myc-Suv39h1 or two cysteine mutants: A Suv39h1 mutant with the three modified Cysteines converted to Alanine (3C), or a mutant with eight of the nine conserved cysteines converted to Alanine *(Figure R15A).* We next performed IF experiments to determine the levels of H3K9me3 and Suv39h1 in constitutive heterochromatin foci *(Figure R15B).* As expected, Suv39DN MEFs showed a complete lack of H3K9me3 in constitutive heterochromatin foci, but re-expression of mycSuv39h1 restored completely these levels. In contrast,

neither 3C or 8C mutant were able to restore the H3K9me3 levels in constitutive heterochromatin foci. Moreover, both mutants were excluded from pericentric constitutive heterochromatin foci, suggesting that the pre-SET domain plays a role in the localization of Suv39h1 to chromatin. To further validate our hypothesis, we also tested whether the expression of the mutants correlated with an de-repression of the repetitive sequences found in pericentromeric constitutive heterochromatin [44]. Thus, next step was to analyze the expression levels of major satellites, minor satellites and LINE-L1s by qPCR. The analysis showed that only WT, but not mutant 3C could restore the repression of these sequences *(Figure R16A).* To confirm the drop in H3K9me3 levels in constitutive heterochromatin, we tested in the context of the same recovery experiment, whether the global levels of H3K9me3 were also affected. Thus, a Western Blot of H3K9me3 showed that H3K9me3 global levels were not rescued with the 3C and 8C Suv39h1 mutants *(Figure R16B).* An important question was whether in addition to an effect on localization, this loss of H3K9me3 could be attributed to a defect in HMT activity of the 3C and 8C mutants. However, an in vitro HMT assay of Suv39h1 WT, 3C or 8C showed that the mutation did not have any inhibitory effect on the HMT activity of Suv39h1. On the contrary, we actually detected an increase of the HMT activity in the mutants, probably due to the experimental conditions *(Figure R16C).*

Altogether these results strongly suggested that monoubiquitination of the pre-SET domain of Suv39h1 excludes the enzyme from chromatin. To test that, from a more biochemical point of view, we induced the modification by overexpressing SirT6 in mycSuv39h1-containing cells and then fractionated the cells according to the mild Dignam method. In this fractionation, we observed that the vast majority of modified Suv39h1 was present in the soluble nuclear extract. Moreover, when we solubilized the chromatin-bound proteins by washing the chromatin pellet with BC buffer containing increasing concentration of NaCl (100mM, 200mM, 500mM or 1M NaCl), we observed that the monoubiquitination was washed away almost completely by the 100mM wash (*Figure R17A*). We further confirmed this by extracting all soluble proteins with the stringent RIPA buffer and digesting the remaining insoluble pellet with the DNase benzonase. The RIPA-related insoluble pellet corresponds to the chromatin and the proteins tightly bound to it. When we tested the localization of the monoubiquitinated Suv39h1, we found it exclusively in the soluble fraction extracted by the RIPA buffer *(Figure R17B).* Overall, these evidences confirm that SirT6 induces Suv39h1 removal from chromatin through monoubiquitination.

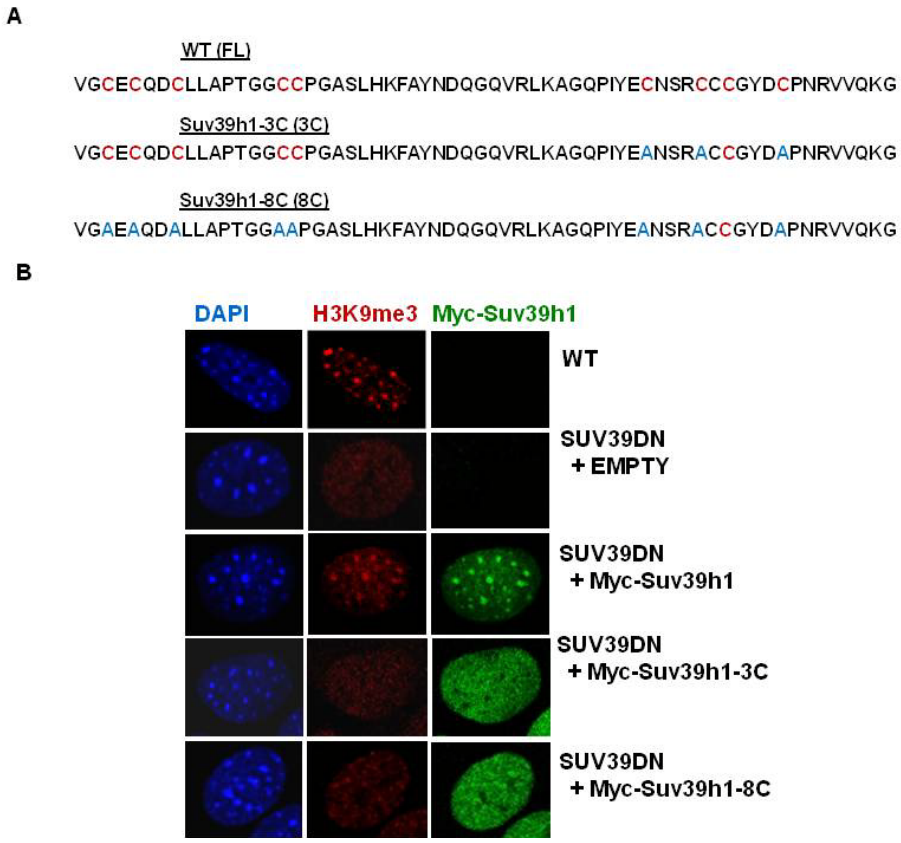

Figure R15. Suv39h1 mutant for cysteines does not recover H3K9me3 enriched heterochromatin foci. A. Scheme of Suv39h1 mutants for cysteines. **B.** Immunofluorescence of MEFs Suv39h1-2 WT and KD infected with Suv39h1WT and cysteine mutants.

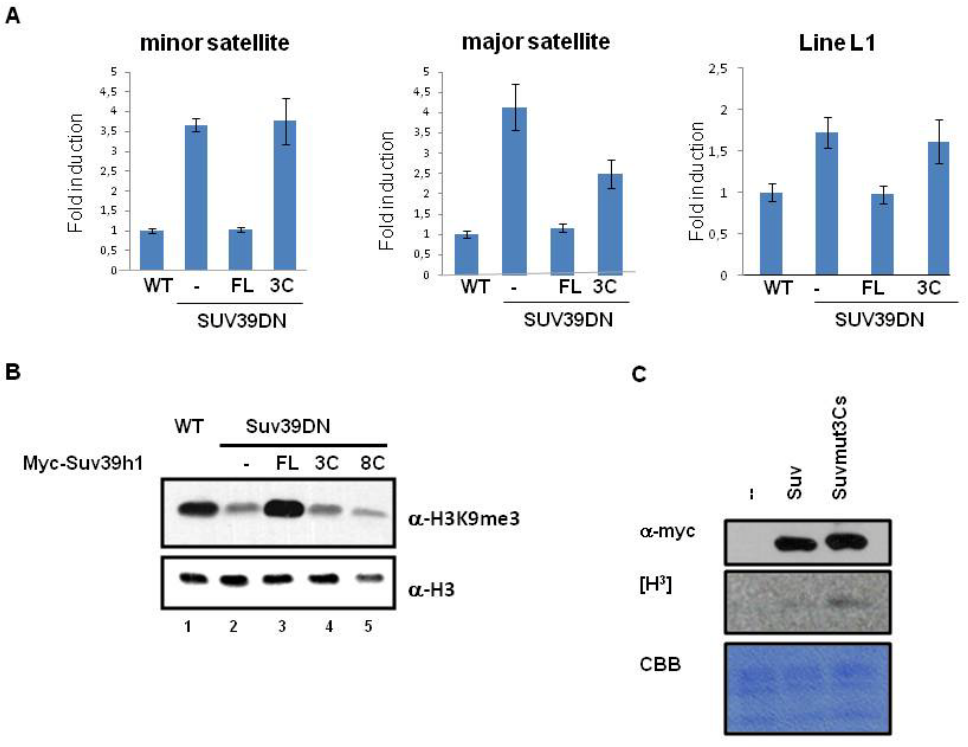

Figure R16. Suv39h1 mutant for cysteines allows the trancription of pericentric heterochromatin, does not recover H3K9me3 global levels but it has higher in vitro activity. A. qPCR from MEFs Suv39h1-2 WT and KO infected with Suv39h1WT and cysteine mutants. **B.** Western Blot of histone extract from MEFs Suv39h1-2 WT and KO infected with Suv39h1WT and cysteine mutants. **C.** Autoradiography, coomassie blue and Western Blot from an in vitro methylation reaction performed with core histones and purified Suv39h1WT and mutant for three cysteines.

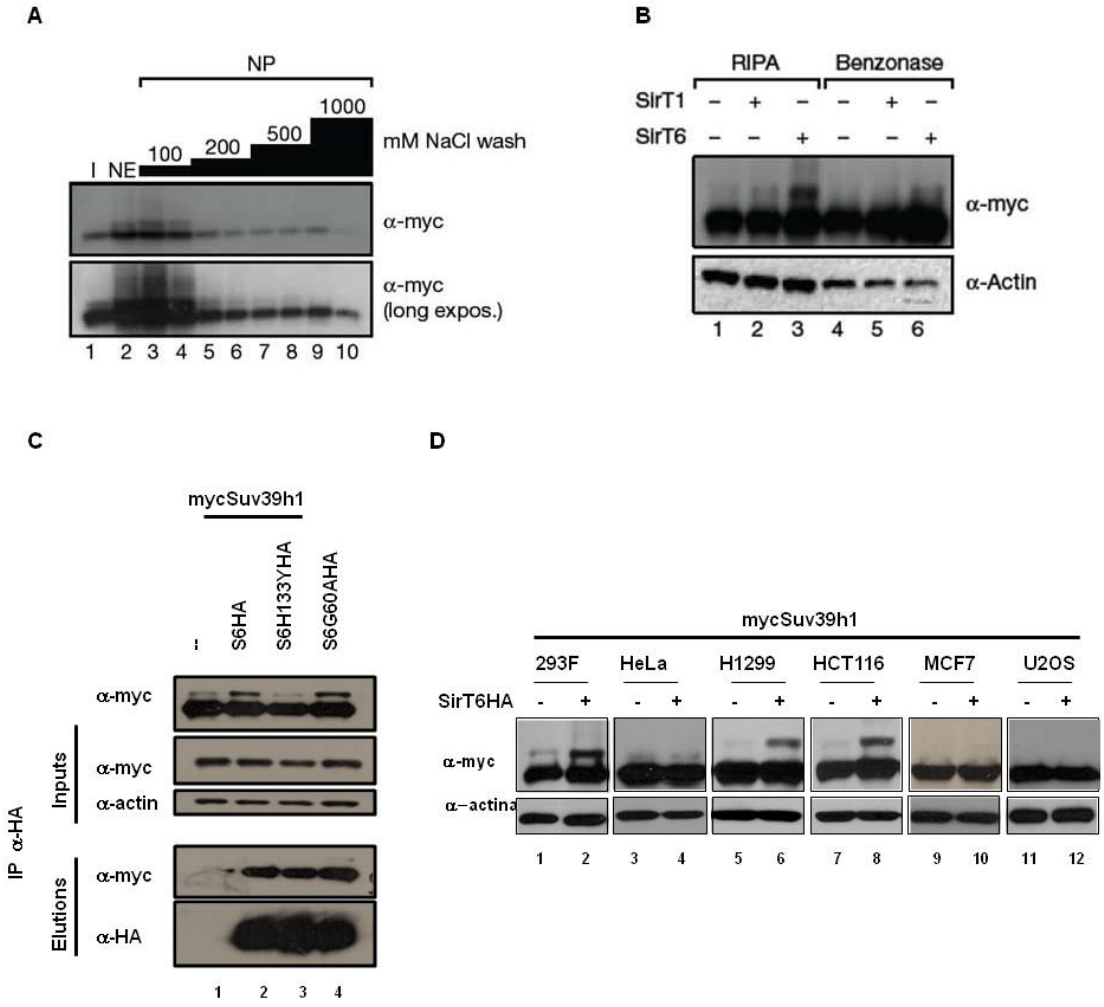

Figure R17. Other characteristics of the ubiquitinated band. A. Western Blot with myc-Suv39h1 co-transfected with SirT6HA. Protein extract was made with Dignam method for the two first lanes (Cytoplasmic and nuclear extract, NE) and nuclear pellet (NP) was washed sequencially with the indicated NaCl molarities. **B.**Western Blot of extract with Rippa buffer (soluble fraction) and benzonase (insoluble fraction) from 293F cells transfected with mycSuv39h1 with SirT1-HA or SirT6-HA. **C.** HA immunoprecipitation from 293F cells extracts transfected with myc-Suv39h1 and different SirT6-HA mutants. **D.** Western Blot of extracts from different cell lines: 293F, HeLa, H1299, HCT116, MCF7, U2OS transfected with myc-Suv39h1 with or without SirT6-HA.

SirT6 has both deacetylase and mono-ADP ribosylase activity as described in the introduction. It was described that SirT6 H133Y mutant eliminates both activities and SirT6 G60A mutant only inactivates the mono-ADP-ribosylase activity [216]. Interestingly, the overexpression of SirT6H133Y mutant did not induce the Suv39h1 monoubiquitination while SirT6G60A mutant did. Since both mutants interact to Suv39h1 *(Figure R17C),* this suggests that the deacetylase activity of SirT6 is required to induce the monoubiquitination of Suv39h1.

A curious feature about this modification is that it is cell type dependent. SirT6 overexpression induces the monoubiquitination in some specific cell lines such as 293F, H1299 and HCT166, while it does not in others such as HeLa, MCF7 or U2OS *(Figure 17D).*

1.1.1.1. Suv39h1 monoubiquitin and stress

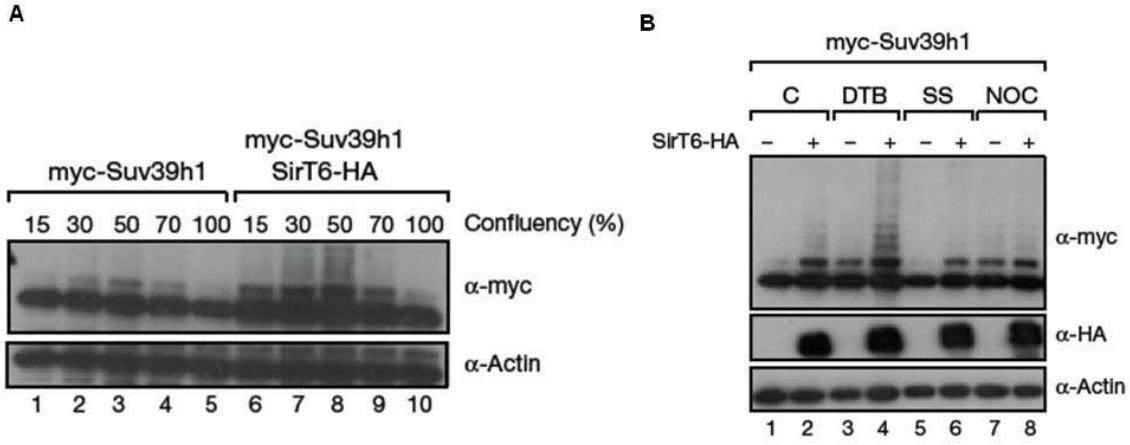

Figure R18. Suv39h1 monoubiquitination depends on cell cycle progression. A. Western Blot of extracts from 293F cells with different confluency transfected with mycSuv39h1 with or without SirT6. **B.** Western Blot of extracts from 293F cells with different treatment transfected with mycSuv39h1 with or without SirT6. C: Control, DTB: Double thymidine block, SS: Serum starvation, NOC: nocodazole.

We next tried to determine the physiological conditions under which SirT6 induces this modification in Suv39h1. An interesting clue came from proliferation studies performed upon overexpression of myc-Suv39h1 in absence or presence of SirT6. Both populations of cells proliferated similarly, but we realized that in the cells with SirT6 and Suv39h1 different states of confluency had a dramatic effect on the levels of monoubiquitination. In fact, the levels of monoubiquitination seemed to be high when cells were proliferating at their maximal rate, but decreased dramatically when it

reached confluency and proliferation stopped *(Figure R18A).* This suggested that the proliferative state of the cells affects the monoubiquitination and therefore that Suv39h1 monoubiquitination was probably related to the cell cycle progression. It is not technically possible to synchronize 293F cells so we arrested the cells in G_0 with serum starvation and in two key steps of the cell cycle, G_1/S and early mitosis, by treating the cells with double thymidine block (DTB) and Nocodazole (it induces mitotic stress), respectively. We observed that with double thymidine block and with nocodazole treatment the monoubiquitination increased significantly even without SirT6 overexpression. Interestingly, the highest levels of the modification where reached with the double thymidine block under SirT6 overexpression *(Figure R18B)*.

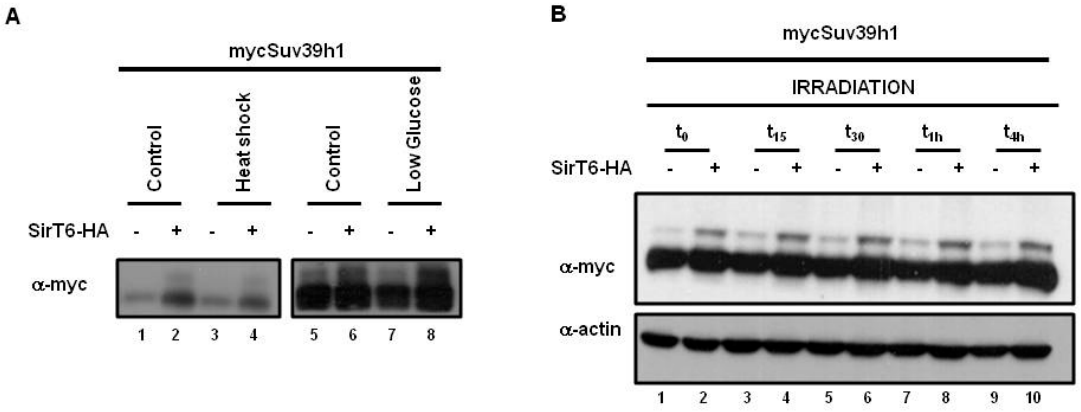

Figure R19. Suv39h1 monoubiquitination is not induced by neither heat shock nor low glucose nor irradiation. A. Western blot of extract from 293F cells with heat shock and low glucose treatment transfected with myc-Suv39h1 with or without SirT6-HA. **B.** Western Blot of extract from 293F cells irradiated with 10 Gy and harvested at different times after irradiation. Cells are transfected with myc-Suv39h1 with or without SirT6-HA.

As we described in the introduction section, Sirtuins are involved in coordinating the response to different types of stress conditions. Thus, we aimed to determine whether Suv39h1 monoubiquitination was induced under stress conditions. We transfected cells with myc-Suv39h1 and SirT6-HA and treated them with different types of stress related to SirT6 functions [206]. Treatment with heat shock, glucose restriction or DNA damage generated by IR under different recovering times, did not induce the monoubiquitination of Suv39h1 *(Figure R19)*. We have also treated the cells with peroxide (oxidative stress), hidroxyurea and camptothecin (replicative stress), but again, we did not observe any change in the modification *(Figure R20A).* However, when we treated the

cells with TNFα, a key mediator of inflammation and an inducer of NF-kB, we observed the same effect in monoubiquitination as we observed with double thymidine block and nocodazole: the monoubiquitination was induced even without the requirement of SirT6 overexpression *(Figure R20A).* Then, we transfected cells with mycSuv39h1 and SirT6-HA and performed a time course of TNFα incubation (from no treatment to 120 min of treatment) as indicated. The results showed that upon SirT6 overexpression, the levels of monoubiquitination reached a saturation level and the incubation with TNFα did not increase further the modification *(Figure R20B).* This was a clear indication that both SirT6 and TNFα were inducing the modification through the same pathway. In order to test that further, we generated three lines of shRNA containing empty vector(-), scrambled (Sc) or ShSirT6 (Sh6). After transfection, we selected the positive cells with puromycin and checked the levels of SirT6 in these cells. As expected, the levels of SirT6 were downregulated by more than 70% *(Figure R21A).* We then transfected these cells with mycSuv39h1 and tested for induction of Suv39h1 monoubiquitination under double thymidine block, nocodazole and TNFα treatment. The results clearly showed that downregulation of SirT6 decreased drastically Suv39h1 monoubiquitination in all cases, clearly indicating that their capacity to induce the modification involved SirT6 function (*Figure R21B).*

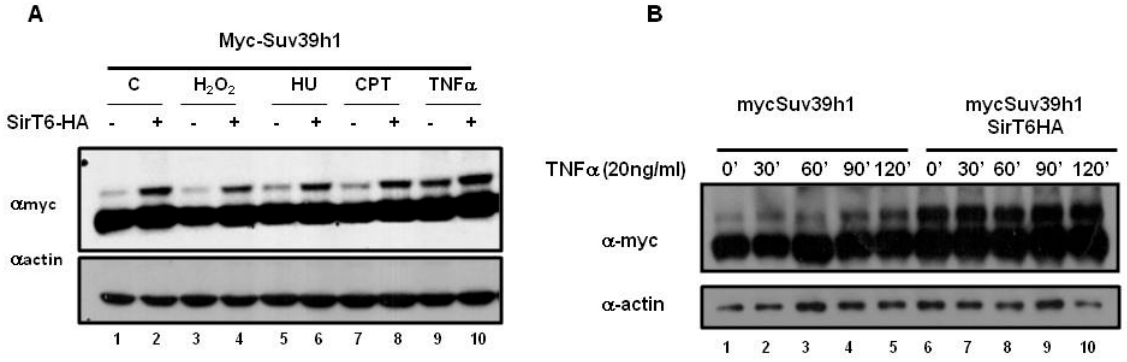

Figure R20. Suv39h1 monoubiquitination is induced by TNFα treatment. **A.** Western Blot of extract from treated 293F cells transfected with myc-Suv39h1 with or without SirT6-HA. C: control, HU: hidroxyurea, CPT: camptothecin. **B.** Western Blot of extract from 293F cells treated with TNFα and harvested in different times.

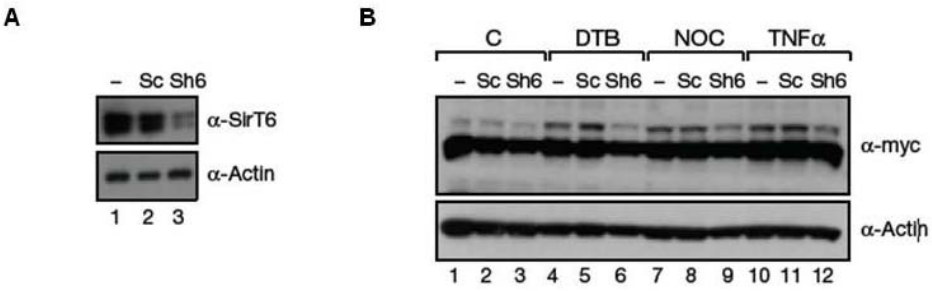

Figure R21. Suv39h1 monoubiquitination induction by double thymidine block, nocodazole and TNFα is through SirT6. Western Blot ofmextract from treated 293F cells with shRNA. - : Empty vector. Sc: shRNA Scramble, sh6: shRNA SirT6. C: control, DTB: Double thymidine block, Noc: Nocodazole.

1.1.1.2. E3 ubiquitin ligase

Ubiquitination of proteins involve three sequential reactions catalyzed by three different types of enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase [77]. The E3 ubiquitin ligase is the last enzyme in the ubiquitination process and the one that recognizes specifically the substrate. In order to characterize the monoubiquitination, it is crucial to identify the E3 ubiquitin ligase involved. SirT6 is not an E3 ubiquitin ligase, but it has been shown to interact with some E3 activities. Therefore, these SirT6 interacting partners with E3 ubiquitin ligase activity would be our best candidates.

Our first candidate was the ubiquitin ligase CHIP, because it was shown to interact with and monoubiquitinate SirT6, which results in its stabilization by preventing its degradation [96]. Immunoprecipitation experiments between CHIP and SirT6 confirmed the interaction as described. However, the same immunoprecipitation experiment but using the SirT6 catalytically-inactive point mutant H133Y mutant, completely abrogated the interaction *(Figure R22A)*. This result would fit with our previous experiment shown in figure R17C where we showed that SirT6 H133Y is not able to induce monoubiquitination of Suv39h1.

We also tested by immunoprecipitation experiments whether both CHIP and Suv39h1 interact directly. Interestingly, both factors did interact *(Figure R22B).* We next, determined the domain of interaction of Suv39h1 to CHIP by immunoprecipitation. As before, for that purpose we used a set of Suv39h1 deletion mutants. Our IP experiments between CHIP and these mutants showed that CHIP hardly interacts with the ΔSET construction, suggesting that, as SirT6, CHIP interacts with the SET domain of Suv39h1 *(Figures R22B and R27).* Interestingly, we were unable to detect CHIP-induced Suv39h1 monoubiquitination in the input (nuclear extracts) of these IPs.

However, we did detect the band in the elutions of the IPs in both full length Suv39h1 and SET constructions *(Figure R22B)*.

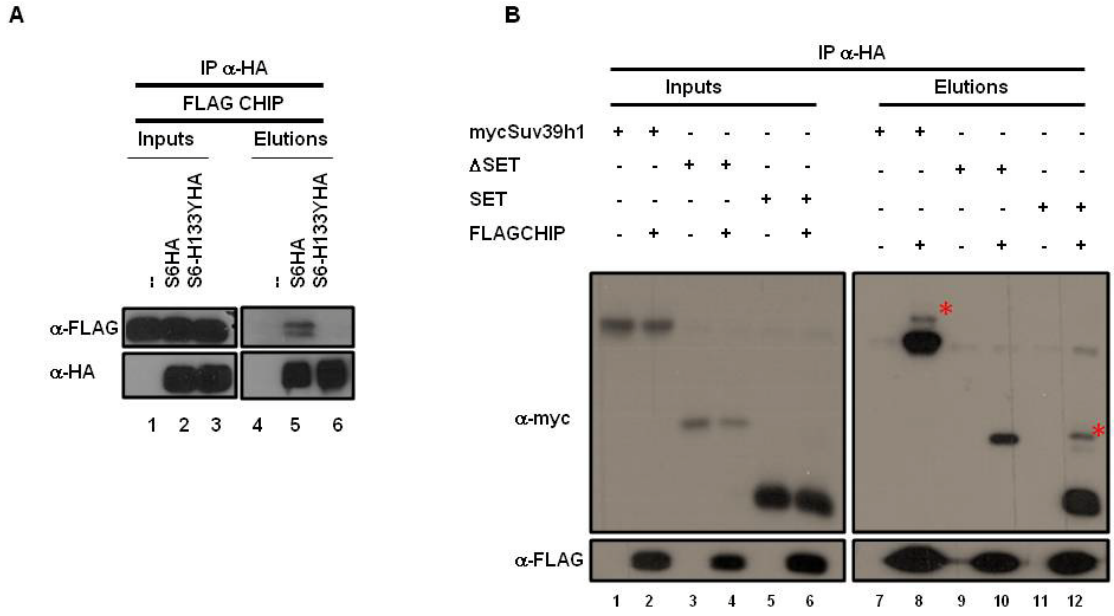

Figure R22. CHIP interacts with both Suv39h1 and SirT6. A. HA immunoprecipitation from 293F cells transfected with FLAG-CHIP and SirT6 WT or mutant. **B.** FLAG immunoprecipitation from 293F cells transfected with different myc-Suv39h1 constructions with or without FLAG-CHIP.

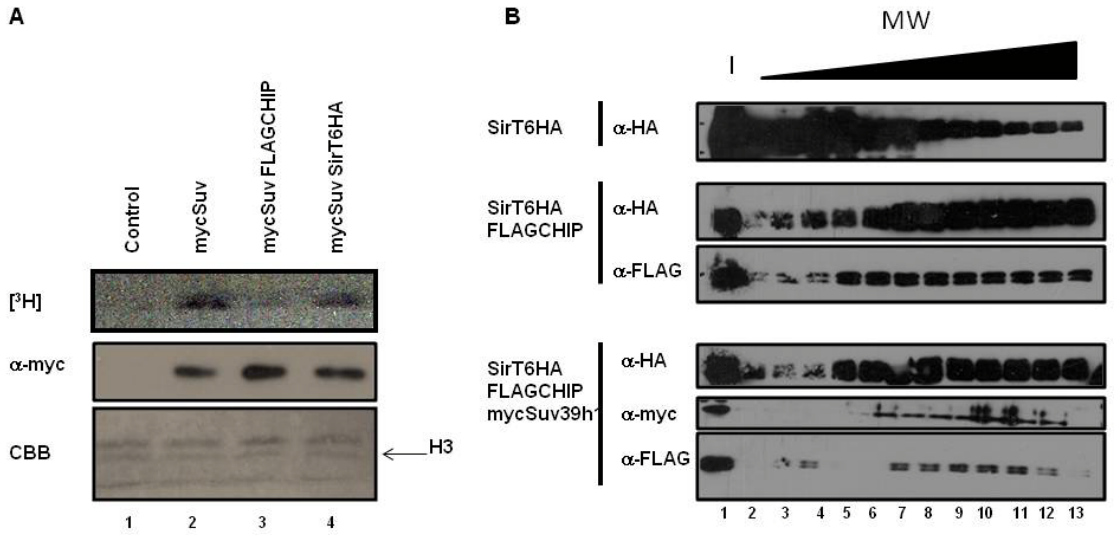

Figure R23. CHIP affects Suv39h1 activity and form a complex with Suv39h1 and SirT6. A. Autoradiography, Western Blot and coomassie blue staining of in vitro methylation reaction using as a substrate core histones and as enzyme purified mycSuv39h1 from cells transfected with mycSuv39h1, FLAGCHIP and SirT6HA. **B.** Western Blot of a glycerol gradient with protein extract of 293F cells transfected with SirT6HA, FLAGCHIP and mycSuv39h1. MW: molecular weight.

CHIP does not seem to be the activity we are looking for, but since both factors interact, CHIP may have a role in the regulation of Suv39h1. In order to determine whether CHIP had any effect on Suv39h1 activity, we purified Suv39h1 and performed an in vitro methylation assay. The result clearly showed that CHIP inhibits Suv39h1 activity, in contrast to SirT6, that did not produce any effect *(Figure R23A).* Our results suggest that CHIP interacts with Suv39h1 and SirT6 and therefore may form a stable complex with them. To test that, we transfected cells with mycSuv39h1, FLAG-CHIP and SirT6-HA and performed a glycerol gradient to determine whether they form a complex *(Figure R23B).* Glycerol gradient separates proteins according to the molecular weight of the complex. Of note that SirT6 transfected alone remains at the beginning of the gradient, which correspond to low molecular weight fractions that correspond to its monomeric form. When SirT6 and CHIP were transfected together, SirT6 migrates from the low molecular weight fractions to heavier fractions because of CHIP overexpression. Then, upon transfection of all three, SirT6, CHIP and Suv39h1 we found that all peaked in the same fractions. Interestingly, in these conditions, CHIP concentrated its fractionation pattern to these common fractions instead of spreading all over the gradient. Altogether these evidences suggested that they all three form a complex.

Future studies should determine all the components of this complex and study when, why and how is formed and what is its main function.

Another E3 candidate, CHFR, was chosen because its levels peak after nocodazole treatment, it has been showed to be involved in mitotic checkpoint, it interacts with NF-kB and it has been shown to be cell type specific, since it is not expressed in HeLa cells and it is mutated and non functional in U2OS cell. We first checked whether both SirT6 and Suv39h1 interact with CHFR. We transfected mycSuv39h1, SirT6-HA and FLAG-CHFR and performed an immunoprecipitation with FLAG antibody. Both mycSuv39h1 and SirT6-HA interacted with CHFR in 293F cells (*Figure R24*).

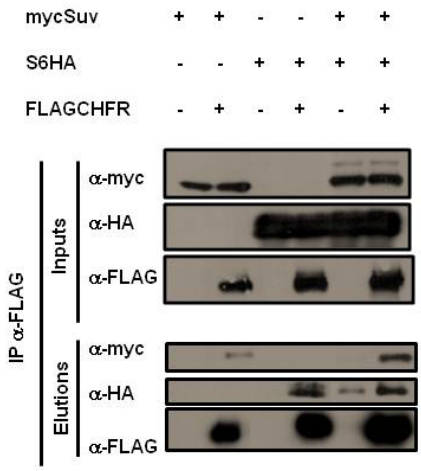

Figure R24. CHFR interacts with both SirT6 and Suv39h1. FLAG immunoprecipitation from 293F cells extracts transfected with myc-Suv39h1, SirT6-HA and FLAG-CHFR.

To check whether CHFR is the E3 we were looking for, we overexpressed myc-Suv39h1, G4BD-SirT6, FLAG-CHFR and HA-Ubiquitin in both 293F and HeLa cells *(Figure R25).* In 293F cells, CHFR not only did not induce the monoubiquitination of Suv39h1, but a titration of increasing levels of CHFR, induced a decrease in Suv39h1 monoubiquitination. However, upon SirT6 overexpression the same titration of CHFR did not change the levels of the modification. In contrast, in HeLa cells CHFR could induce Suv39h1 monoubiquitination induction. Interestingly, we observed a striking effect of SirT6 in CHFR levels: while in 293F cells, CHFR levels are increased by SirT6 overexpression, in HeLa cells, the effect is completely the opposite.

We also aimed to define the binding domain involved in Suv39h1. The first surprising thing that we observed is that in HeLa cells CHFR was able to induce the modification only in the full length of Suv39h1 *(Figure R26).* Moreover, the immunoprecipitation experiments determined that CHFR interacts with the N-terminal domain of Suv39h1 *(Figure R27).* In conclusion, as happened with CHIP, our results suggest that SirT6, Suv39h1 and CHFR are functionally involved.

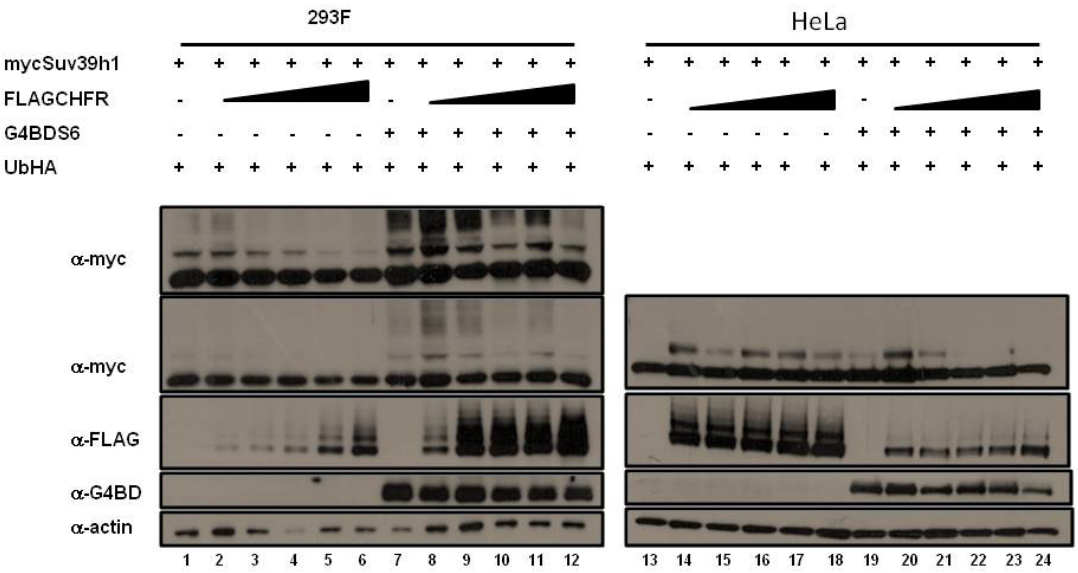

Figure R25. CHFR behaves differently in 293F and HeLa cells. Western Blot of extract from 293F and HeLa cells transfected with myc-Suv39h1, FLAG-CHFR, G4BD-SirT6 and Ubiquitin-HA.

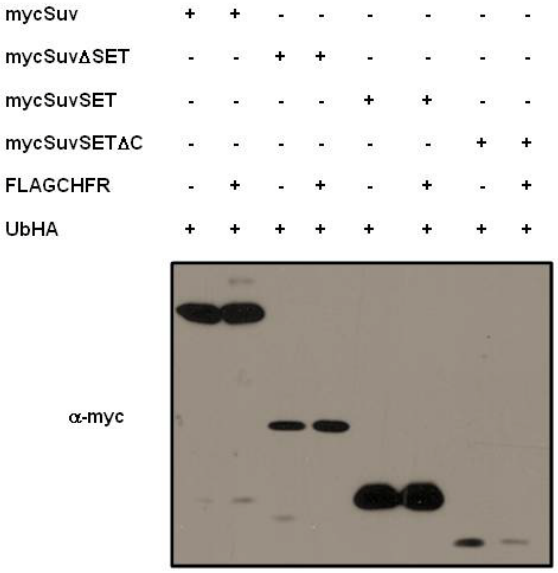

Figure R26. CHFR induces Suv39h1 monoubiquitination in full length and HeLa cells. Western Blot of extract from HeLa cells transfected with different Suv39h1 constructions, FLAG-CHFR and Ubiquitin-HA

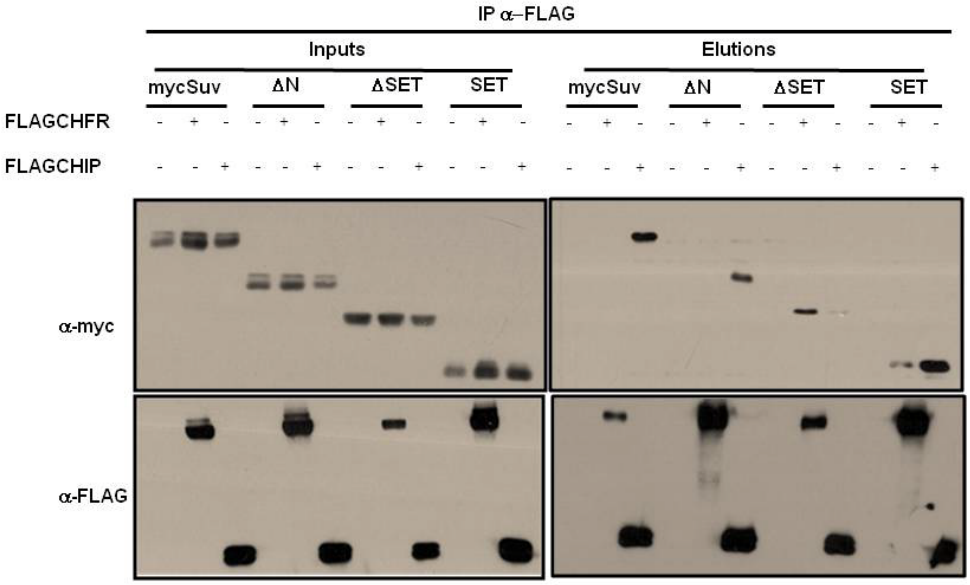

Figure R27. CHIP interacts with SET domain while CHFR interact with N-terminal domain. FLAG immunoprecipitation of protein extract from 293F cells transfected with different myc-Suv39h1 constructions, FLAG-CHFR and FLAG-CHIP.

We also had other candidates as RNF20-RNF40, RNF181 and RNF8. RNF20-RNF40 and RNF8 monoubiquitinates histones in a similar way to CHFR upon DNA damage stress [112]. However, RNF20-RNF40 overexpression does not induce the monoubiquitination in Suv39h1 *(Figure R28A)* and RNF8 does not even interact with Suv39h1 *(Figure R28C).* RNF181 was found in our search of interacting partners of Suv39h1 (data not shown) by mass spectrometry. However, RNF181 overexpression does not induce the Suv39h1 monoubiquitination *(Figure R28B).*

SKP2: the E3-ubiquitin ligase responsible of the Suv39h1 monoubiquitination

Finally, our last candidate was SKP2, an E3-ubiquitin ligase that was described to interact with SirT6. SKP2 belongs to the SCF complex and its levels peak at G_1-S phase [123]. To test the involvement of SKP2 in the modification, we overexpressed mycSuv39h1 with either SKP2, CHIP or Cul1 (the scaffold component of the SCF complex) to determine if any of them could induce the monoubiquitination in Suv39h1. Interestingly, both SKP2 and Cul1 induce it, while as we saw before CHIP did not *(Figure R29A)*. Furthermore, to determine that SKP2-induced monoubiquitination was taking place in the cysteines of the pre-SET domain, we overexpressed either SirT6-HA or FLAG-SKP2 in presence or absence of MycSuv39h1 WT, the mutant 3C or a Suv39h1 mutant lacking the PRE-Set domain (ΔPRESET). Strikingly, we found that both SirT6 and SKP2 induced the monoubiquitination in Suv39h1 WT but none of them

clearly induced the modification in the mutants 3C and ΔPRESET *(Figure R29B).* Moreover, induction of the modification by SKP2 is also cell-line dependent and the pattern was identical to SirT6 *(Figure R29C).*

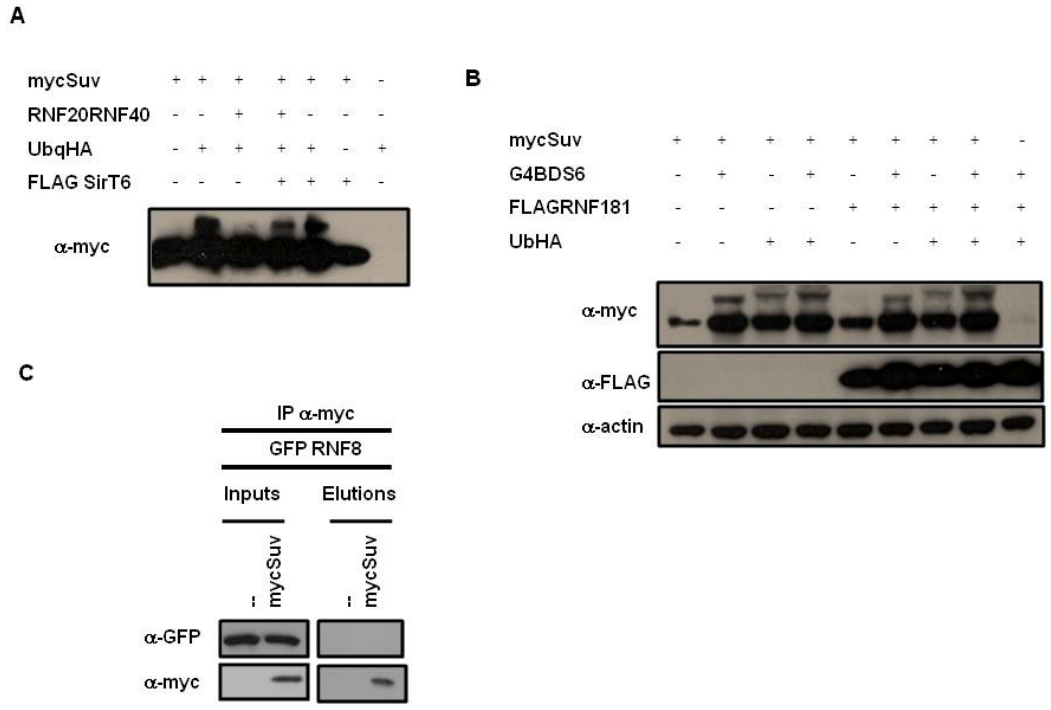

Figure R28. RNF20-RNF40, RNF8 and RNF181 do not induce Suv39h1 monoubiquitination. A. Western Blot of extract from 293F cells transfected with myc-Suv39h1, RNF20 and RNF40, Ubiquitin-HA and FLAG-SirT6. **B.** Western Blot of extract from 293F cells transfected with myc-Suv39h1, G4BD-SirT6, FLAG-RNF181 and Ubiquitin-HA. **C.** Myc immunoprecipitation of extract from 293F cells transfected with myc-Suv39h1 and GFP-RNF8.

To further confirm a direct involvement of SKP2 we next checked whether SKP2 interacts with Suv39h1. Immunoprecipitation experiments between SKP2 or Cul2 (another scaffold protein from another E3 ubiquitin ligase complex) and Suv39h1, showed that SKP2 interacts specifically with Suv39h1, but not Cul2 *(Figure R30A).* We then were aimed to define the interacting domain of Suv39h1 with SKP2. As before, we co-transfected SKP2 with the different Suv39h1 deletion mutants and we performed an immunoprecipitation. Interestingly, loss of the extended SET domain (ΔSET mutant) almost inhibited the interaction with SKP2, suggesting that is the main binding site of the enzyme is the SET domain. Supporting this observation, the extended SET domain alone was able to sustain interaction with SKP2 *(Figure R30B)*. Further supporting the involvement of SKP2 in SirT6-dependent monoubiquitination of Suv39h1, the overexpression of SKP2 induced the modification in all except the

ΔPRESET, which coincides with the pattern induced upon SirT6 overexpression *(Figure R11B).*

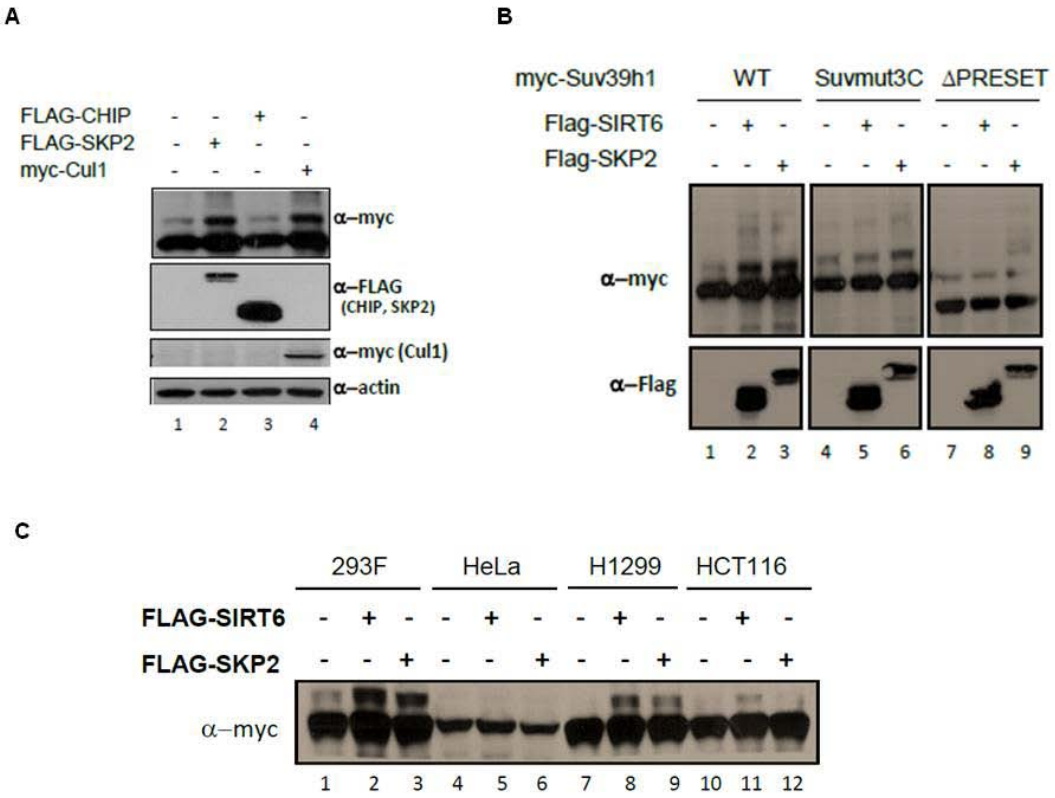

Figure R29. SKP2 induces ubiquitination in cysteines and it is cell line dependent. A. Western Blot of extract from 293F cells transfected with mycSuv39h1, FLAGCHIP, FLAGSKP2, mycCul1. **B.** Western Blot of extract from 293F cells transfected with different mycSuv39h1 constructions, FLAGSirT6 and FLAGSKP2. **C.** Western Blot ot extracts from different cell lines: 293F, HeLa, H1299, HCT116, MCF7, U2OS transfected with mycSuv39h1 and with or without SirT6 or SKP2

The interaction between SirT6 and SKP2 was also confirmed. Interestingly, SKP2 not only interacted with FL active SirT6, but also with the catalytic point mutant SirT6-H133Y *(Figure R30C)*. Therefore, the lack of ability of SirT6-H133Y to induce the monoubiquitination could not be attributed to a defect in interaction between both factors. We further confirmed that SKP2 is the responsible of the SirT6-dependent monoubiquitination of Suv39h1, we analyzed by mass spectrometry the monoubiquitination band induced by SKP2. In the analysis, we could confirm that at least one of the cysteines in the PRESET domain was monoubiquitinated.

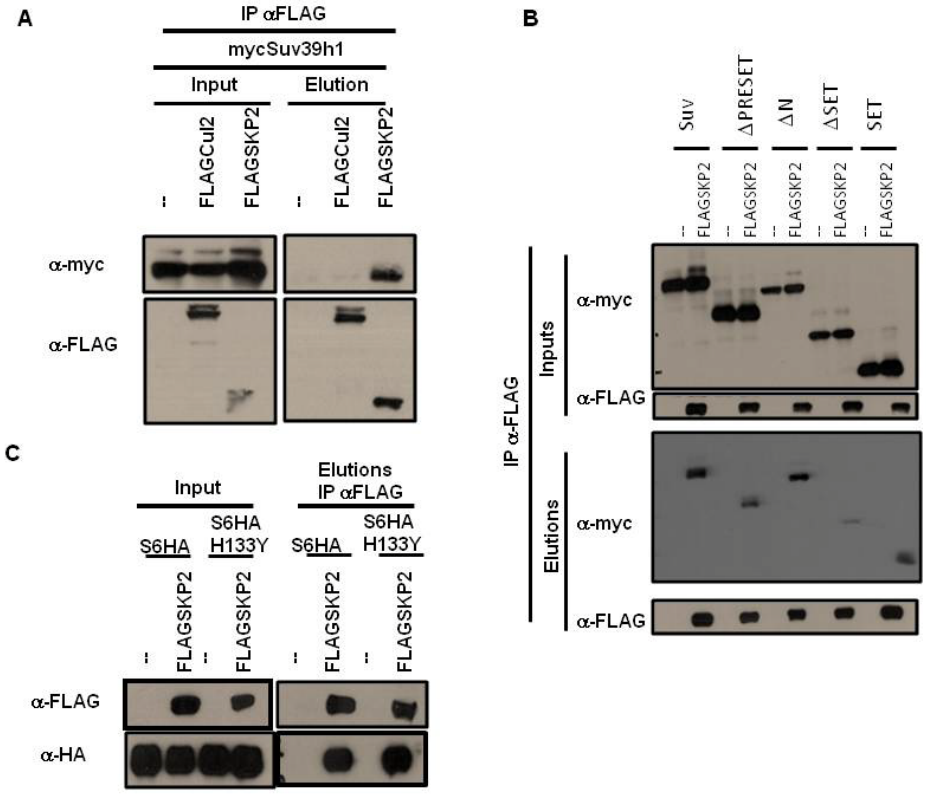

Figure R30. SKP2 interacts with both Suv39h1 and SirT6. A. FLAG immunoprecipitation of extracts from 293F cells transfected with myc-Suv39h1, FLAG-SKP2 and FLAG-Cul2 **B.** FLAG immunoprecipitation of extract from 293F cells transfected with different myc-Suv39h1 constructions and FLAG-SKP2. **C.** FLAG immunoprecipitation of extract from 293F cells transfected with FLAG-SKP2, SirT6-HA and SirT6-H133Y-HA.

A very important issue was related to the requirement of SirT6 activity to induce the modification. An important clue to help us understand this requirement came when we observed that SirT6 overexpression increases SKP2 protein levels in 293F cells but not in HeLa cells *(Figure R31A)*. Therefore, it is possible that the SirT6 activity is involved in the regulation of SKP2 levels. To test this, 293F cells where transfected with FLAG-SKP2 in absence or presence of SirT6-HA, and SKP2 was affinity purified with FLAG resin. The elutions were resolved in a SDS-PAGE and the SKP2 bands were analyzed by mass spectrometry. As a result, we found that SKP2 was acetylated in the aminoacids lysine 73 and lysine 77 in absence of SirT6 overexpression while SirT6 overexpression correlated with loss of these acetylation marks and instead two phosphorylations were detected in serines 72 and 75 *(Figure R31B).* Interestingly, this suggests that SirT6 deacetylates K73 and K77, which in turn allows phosphorylation of S72 and S75. In fact, all these modifications have been described before. All these residues are part of the NLS sequence. Acetylation/deacetylation of key lysines in this

sequence have been shown to regulate localization, protein levels and activity of SKP2 [144]. In particular, acetylation of K68 and K71 were reported to allow phosphorylation in S72 by Akt and S75 by CKI would inhibit SKP2 degradation by Cdh1 and facilitate SKP2 binding to its target [144]. This is in agreement with our observation that SirT6 regulates SKP2 protein levels but in this case deacetylation allows phosphorylation. Other studies have shown that mutation of SKP2 S72 and S75 to aspartic acid (D) mimics phosphorylation and increases SKP2 levels [145]. We then mutated SKP2 to S72D/S75D and overexpressed it with or without SirT6HA. Without SirT6, the levels of the mutant S72D S75D already increased sixfold compared to SKP2 WT and the ability of SirT6 to increase SKP2 WT levels was almost completely abrogated with the S72D/ S75D mutant *(Figure R31C).* We performed a similar experiment but mutating the lysines to arginines, which mimics deacetylated lysines. Since mutation of each of the residues K73R, K77R, or both together K73R/K77R did not have a clear effect on SKP2 levels (*Figure R31D*), we reasoned that probably the presence of any of the other two residues K68 and K71 could be enough to inhibit the phosphorylation of the serines. For that purpose, we mutated simultaneously all four lysines K68R/K71R/K73R/ K77R in SKP2 trying to mimic complete deacetylation in SKP2 and tested the ability of SirT6 to upregulate its levels. Consistent with our assumption, the quadruple mutant drastically reduced the effect of SirT6 *(Figure R31D).* Together, our data suggests that SirT6 binds, deacetylates SKP2, allowing phosphorylation of S72 and S75 and stabilizing SKP2 levels and very likely, activating also the enzyme.

Interestingly, SKP2 and the other SCF-related E3 activities usually require either a phosphorylation mark in the substrate or a phosphorylation in the same SKP2 molecule [123]. We considered the possibility that either the substrate (Suv39h1) or SKP2 may have to be phosphorylated. In fact, Suv39h1 has been reported to be phosphorylated at S391 residue by CDK2 in S phase [72], which induces a dissociation of Suv39h1 from the chromatin to allow replication. To test whether S391 of Suv39h1 was involved in the monoubiquitination, we mutated the residue to alanine mimicking a dephosphorylated serine. We transfected both Suv39h1WT and S391A, in presence or absence of SirT6. Arguing against a role of this residue in the modification, SirT6 did not show any alteration in its ability to induce the monoubiquitination *(Figure R32A)*. We analyzed the phosphorylation marks in the monoubiquitinated band of Suv39h1 by mass spectrometry, but the analysis only could identify the S391 phosphorylation. This suggests that either no phosphorylation in Suv39h1 is required for the modification, or if there is phosphorylation involved, it is very scarce. A more intensive mass spectrometry analysis should be performed in the future to confirm that.

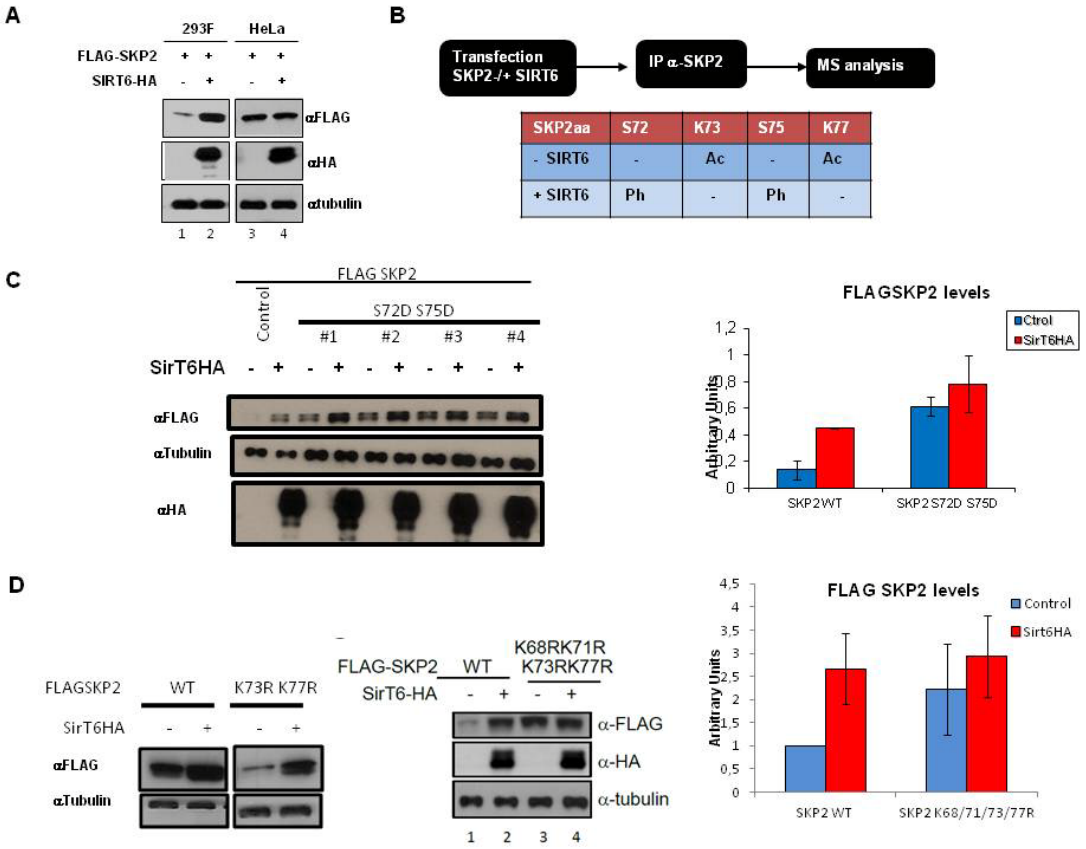

Figure R31. SirT6 regulates SKP2 levels. A. Western Blot of extract from 293F and HeLa cells. **B.** Diagram of purification of SKP2 for mass spectrometry analysis and results of the analysis. **C.** Western Blot of extract from 293F cells transfected with mutant for serins and WT FLAGSKP2 with or without SirT6HA and quantification of SKP2 levels. **D.** Western Blot of extract from 293F cells transfected with mutant for lysines and WT FLAGSKP2 with or without SirT6HA and quantification of SKP2 levels.

If a phosphorylation event in Suv39h1 was required for the modification, a good candidate to be the kinase involved would be IKKα, a component of the NF-kB pathway activator complex IKK. IKKα antagonize Suv39h1 H3K9 methyltransferase activity by phosphorylating H3S10 and by other unknown mechanisms [292]. Supporting a direct involvement of IKKα this process we observed by IP experiments that IKKα interacts with Suv39h1, SirT6 and SKP2. The interaction with all three factors was also sustained by IKKαDN (dominant negative), an IKKα catalytic inactive mutant. *(Figure 32B, C)*. Further studies should confirm this possible new role of IKKα.

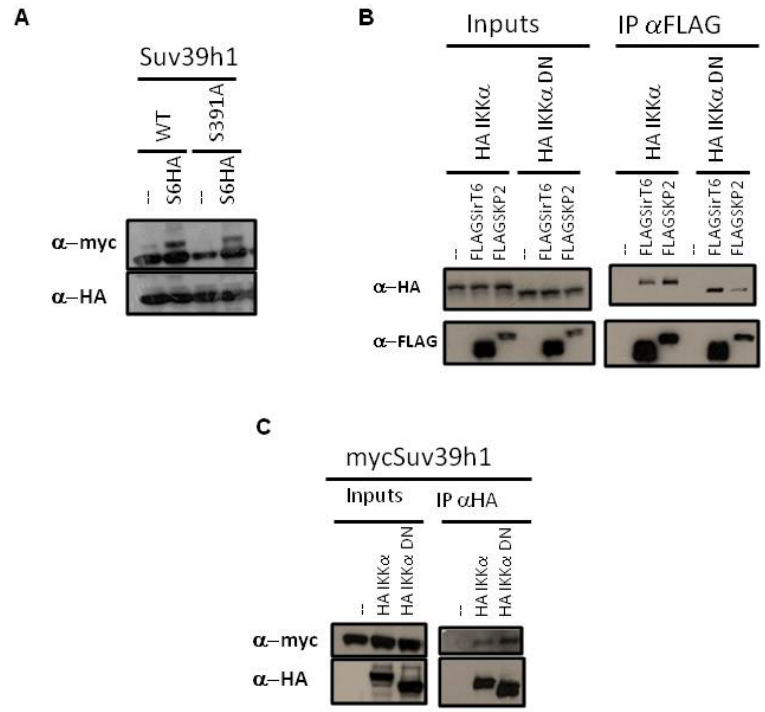

Figure R32. Myc-Suv39h1 S391 is not involved in the monoubiquitination and IKKα interacts with Suv39h1, SirT6 and SKP2. A. Western Blot of extract from 293F cells transfected with Wt or mutant mycSuv39h1 with or without SirT6HA. **B.** FLAG immunoprecipitation of extracts from 293F cells transfected with FLAGSirT6, FLAGSKP2, HA IKKα and HA IKKα DN. **C.** HA immunoprecipitation of extract from 293F cells transfected with mycSuv39h1, HA IKKα and HA IKKα DN.

1.1.1.3. Suv39h1 monoubiquitination and NF-kB pathway

We previously saw in figure R20 that Suv39h1 monoubiquitination was induced by NF-kB pathway activation. The inhibitor of the pathway IkBα sequesters NF-kB transcription factors in the cytoplasm impeding the translocation to the nucleus. Thus, supporting the involvement of the NF-kB pathway in the modification, IkBα overexpression inhibits the ability of both SirT6 and SKP2 to induce the Suv39h1 monoubiquitination *(Figure R33).* In contrast, activation of the NF-kB pathway requires phosphorylation and proteasomal degradation of IkBα, and in particular, both IKKα and IKKβ phosphorylate IkBα to promote its degradation and allow the release of NF-kB transcription factors. Moreover, IKKα also phosphorylates and activates p65 or RelA. Therefore, IKKα overexpression mimics activation of NF-kB pathway. In agreement with the previous results, IKKα overexpression increased significantly the levels of the monoubiquitinated Suv39h1 upon SirT6 or SKP2 overexpression *(Figure R33).*

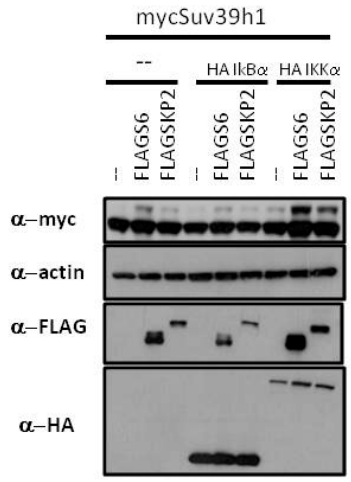

Figure R33. Suv39h1 monoubiquitination is regulated by NF-kB pathway. Western Blot of extract from 293F cells transfected with myc-Suv39h1, FLAG-SirT6, FLAG-SKP2, HA-IkBα and HA-IKKα.

SirT6 interacts with RelA or p65 (a kind of NF-kB transcription factor) after TNFα treatment to stop the transcription of RelA target genes by deacetylating H3K9 and destabilizing RelA from the promoter [235]. Based on all our previous experiments, we could hypothesize that RelA overexpression should also induce the monoubiquitination. To test this, cells were transfected with mycSuv39h1, RelA and SirT6 and also they were treated with TNFα *(Figure R34A).* We could observe that, as expected, RelA overexpression also induced the modification (input,lane 11). Given the described involvement of Suv39h1 in a couple of NF-kB genes [292,308], and the fact that both Suv39h1 and RelA interact with SirT6, RelA and Suv39h1 may also interact directly. To test this assumption, we performed immunoprecipitation experiments between RelA, Suv39h1 and SirT6 (*Figure R34A*). Interestingly, Suv39h1 not only did interact with RelA, also Suv39h1 overexpression inhibited the interaction between SirT6 and RelA. Therefore, it seems that both factors compete for the binding to RelA. We also tested whether also interact with SKP2. An immunoprecipitation assay confirmed that both factors also interact *(Figure R34B).*

We next checked whether SirT6 and Suv39h1 form a multi-protein complex under TNFα treatment. To study that, a glycerol gradient was performed with nuclear extracts from cells transfected with myc-Suv39h1 in the presence or absence of SirT6-HA and in normal conditions or under TNFα treatment. We could observe that under TNFα treatment, myc-Suv39h1 and SirT6-HA elutes at heavier fractions compared to non-induced conditions *(Figure R35A)*. The same protein extract was

immunoprecipitated with αmyc, resolved in an SDS-PAGE gel, and the differential protein bands were analyzed by mass spectrometry in order to determine the components of this complex formed by Suv39h1, SirT6 upon TNFα treatment *(Figure R35B).* A large amount of factors were identified. These proteins could belong to components of a complex or could just be interacting partners of SirT6 or Suv39h1 under the activation conditions.

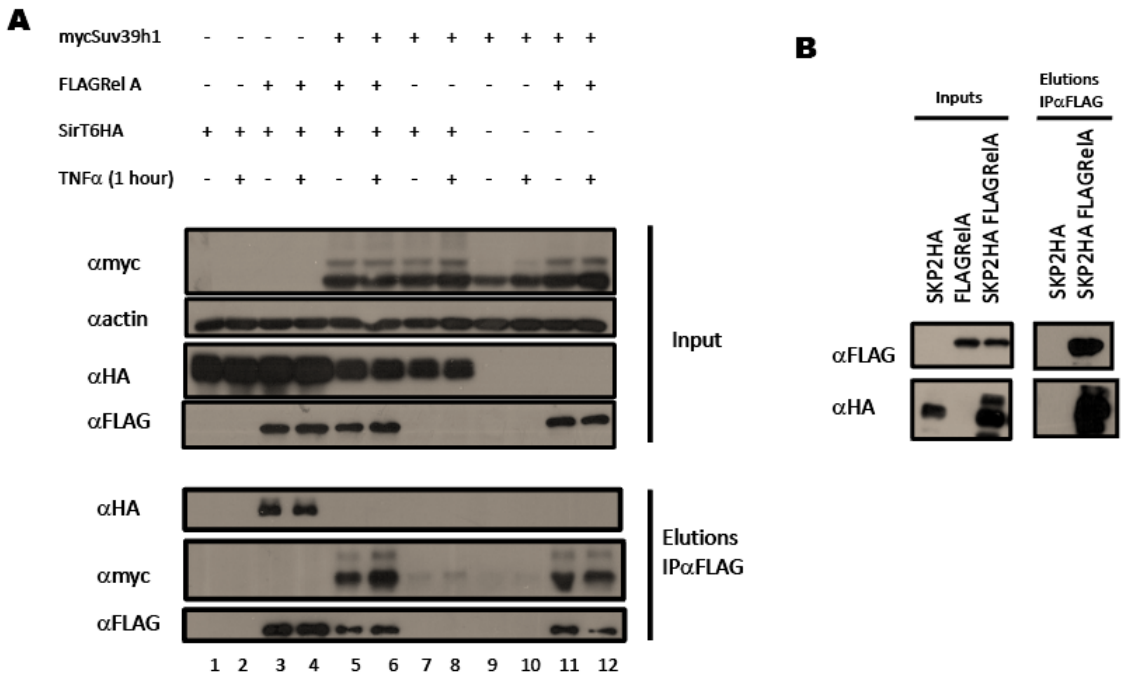

Figure R34. Suv39h1, SirT6 and SKP2 interact with RelA. A. FLAG immunoprecipitation of extract from 293F cells transfected with mycSuv39h1, FLAG-RelA and SirT6-HA and treated with TNFα. **B.** FLAG immunoprecipitation of extract from 293F cells transfected with SKP2-HA and FLAG-RelA

Our next step was to try to determine the functional implications of Suv39h1 monoubiquitination in cell physiology. Altogether, our evidences clearly suggest that the induction monoubiquitination, and therefore the inactivation of Suv39h1, is an event associated to NF-kB activation. Before understanding the modification, we first need to understand the contribution of Suv39h1 to the NF-kB pathway activation. For that purpose, we used shRNA cell lines of scrambled or of Suv39h1. We detected a drop in Suv39h1 activity in more than 60 %. We then performed a time course of TNFα treatment including control (no treatment), 30 minutes, 1 hour, 2 hours, 7 hours and 24 hours. We isolated RNA from these cells and measured by qPCR the mRNA levels of a group of NF-kB target genes under the TNFα treatment compared to the levels of non-treated cells *(Figure 36A)*. We observed that Il8, A20 and GRO1 mRNA levels peak at

two hours after TNFα treatment. Unexpectedly, the mRNA levels induction was much lower in shSuv39h1 cells. This suggests that the presence of Suv39h1 in the cell is required to allow the full activation of the NFkB pathway. That observation would argue against a general role of Suv39h1 as a repressor in the genes of the family and suggests that it may perform a very specific role. Strikingly, very few genes that we tested showed over-upregulation upon loss of Suv39h1. The most relevant of these few genes is the repressor IkBα, that peaks at one hour of TNFα treatment. Other target genes MCP1 and IAP2 peak after more than 24 hours of TNFα and show the same overexpression as IkBα upon Suv39h1 loss. Cyclin D1 and Cyclin D2 was also analyzed. Expression of these cyclins is downregulated after NFkB activation instead of being induced. In this case, Cyclins show a low decrease in their mRNA and loss of Suv39h1, only induce a slight upregulation at thirty minutes of TNFα treatment. Since SirT6 induced Suv39h1 monoubiquitination, in this case SirT6 show and antagonism with Suv39h1 activity. Thus, in cells containing shRNA against SirT6 (see figure R21A), IkBα expression showed a complete opposite pattern upon TNFα treatment. Thus, SirT6 loss associated to an attenuation of the signal *(Figure R36B).*

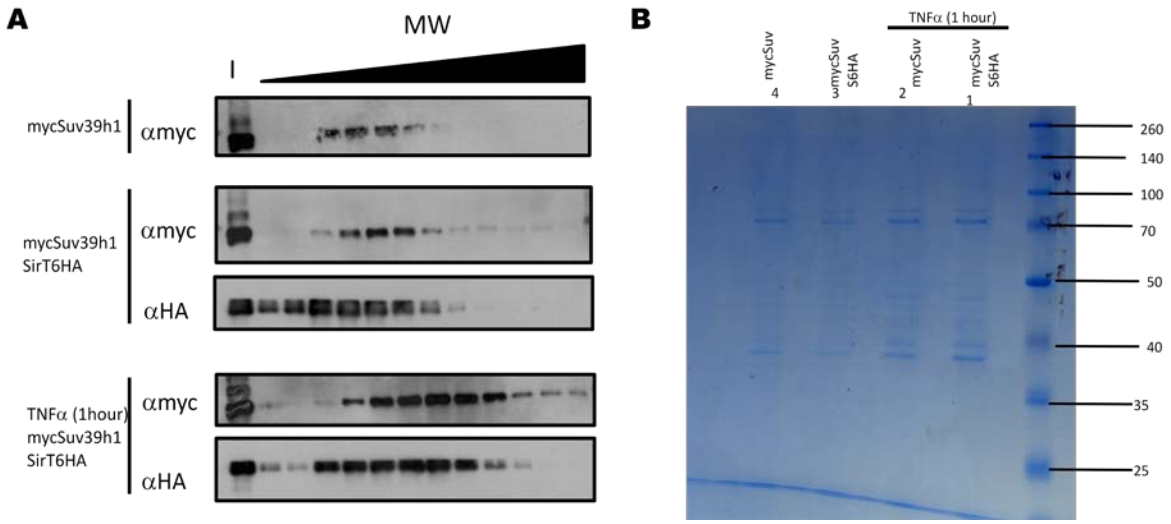

Figure R35. Suv39h1 and SirT6 form a complex under TNFα treatment. A. Western Blot of glycerol gradient with protein extract of 293F cells transfected with SirT6-HA and myc-Suv39h1 and treated with TNFα. MW: molecular weight. **B.** Coloidal-coomasie staining performed in acrilamyde gel of a myc immunoprecipitation from 293F cells transfected with myc-Suv39h1 and SirT6-HA and treated with TNFα.

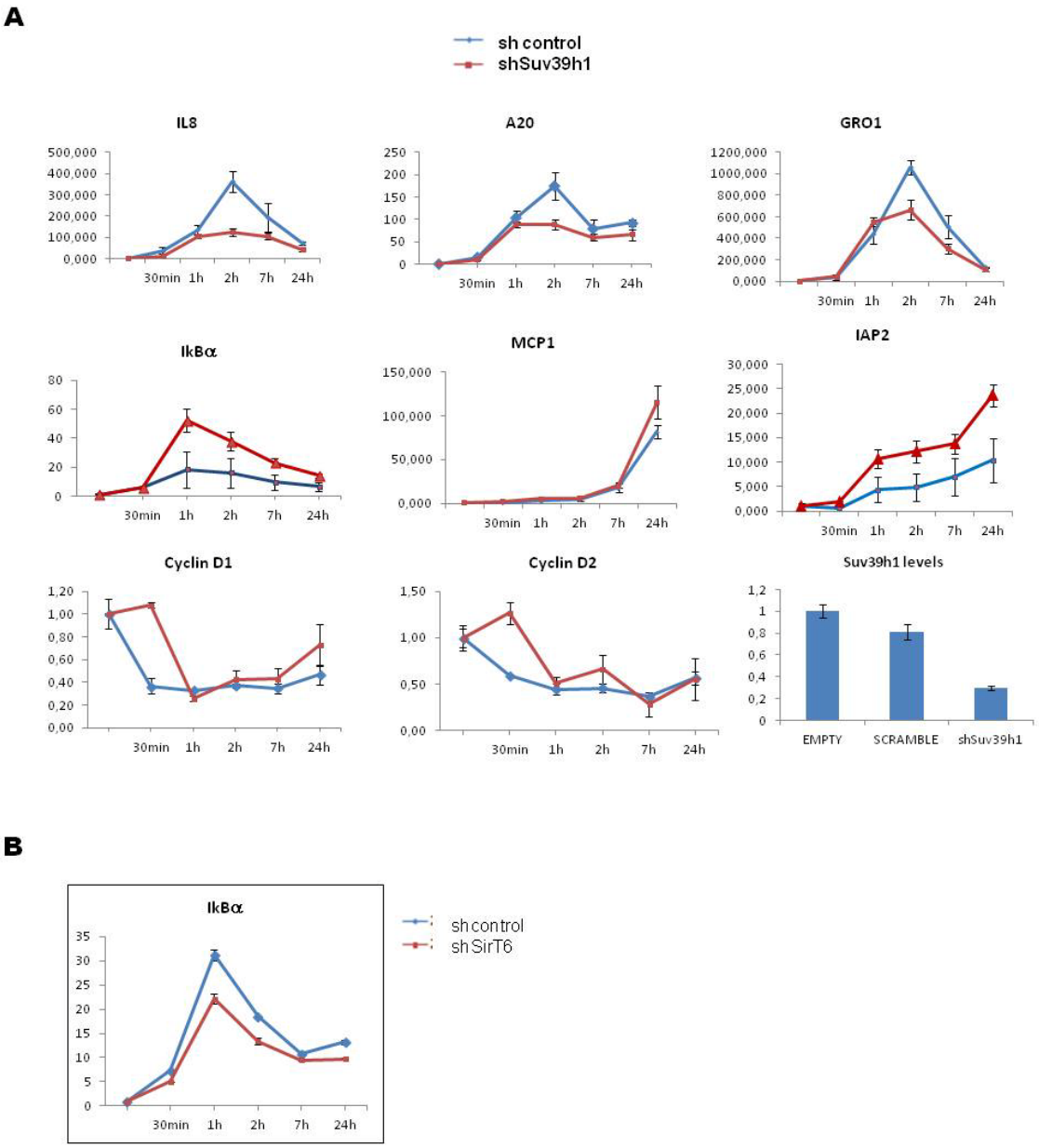

Figure R36. Suv39h1 donwregulation affects to NF-kB target genes expression in 293F cells. A. qPCR of the indicated genes of mRNA from 293F cells with shRNA Suv39h1 treated with TNFα and harvested at the indicated times. **B .** qPCR of IkBα of mRNA from 293F cells with shRNA SirT6 treated with TNFα and harvested at the indicated times.

Those results were obtained in 293F cells. However, NFkB pathway, as many other processes in cells, have different way of regulating genes in the different tissues and cell lines [272]. We also tested the expression pattern of the NFkB genes in MEFs cells. MEFs WT and KO for Suv39h1 were treated with TNFα and harvested at the same times that it has been done with the 293F cells. In this case IkBα expression was also upregulated during treatment with the absence of Suv39h1 and the target genes

MnSOD and IAP2 were less sensitive to the TNFα treatment as it happened more or less with Il8, A20 and GRO1 in 293F *(Figure R37A)*. However, in the case of SirT6 KO MEFs, IkBα behaved differently as in 293F cells. In this case, the absence of SirT6 induced an upregulation on IkBα expression *(Figure R37B)* as it has been described before for MEFs and HeLa cells [235].

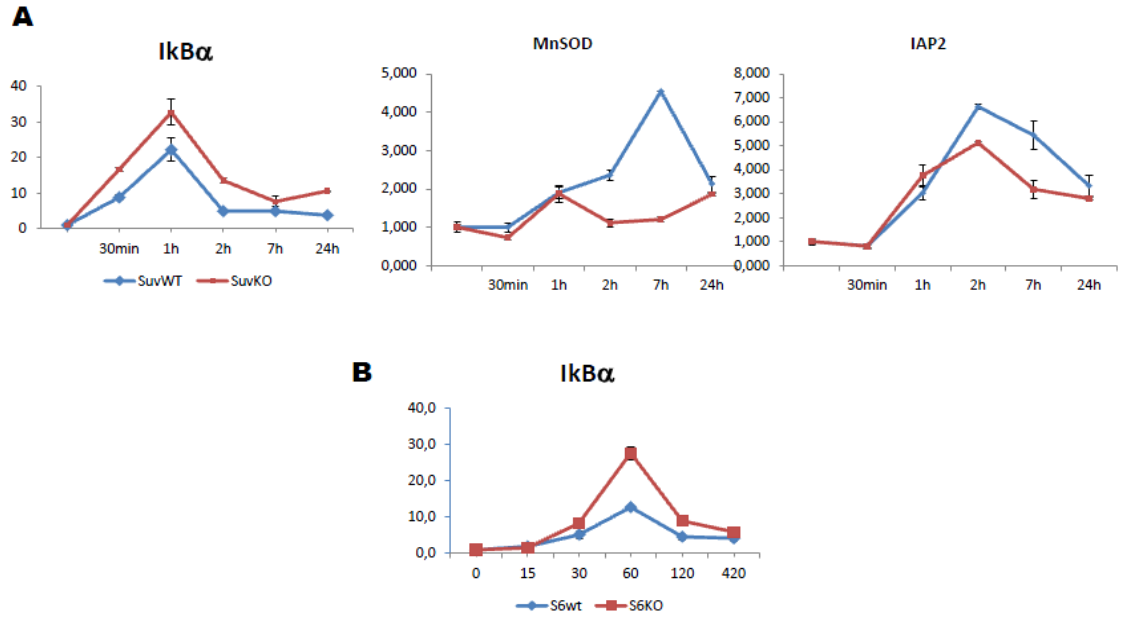

Figure R37. Suv39h1 downregulation affects to NF-kB target genes expression in MEF cells. A. qPCR of the indicated genes of mRNA from MEF cells Suv39h WT and KD treated with TNFα and harvested at the indicated times. **B.** qPCR of IkBα of mRNA from MEF cells SirT6 WT and KO treated with TNFα and harvested at the indicated times.

Therefore, in 293F SirT6 and Suv39h1 seems to have antagonistic effect on IkBα expression although both of them are known for the repressive function in gene expression. Gene expression is determined by the factors that bind to the promoter. Our next step was to study what happened in the IkBα promoter by using the chromatin immunoprecipitation technique (ChIP). A ChIP with the antibodies myc (myc-Suv39h1), SirT6, RelA, H3K9me3 and H3K9ac was performed in 293F cells that were treated with TNFα and harvested at one and two hours after treatment and the promoter amplified was from IkBα *(Figure R38A).* In absence of TNFα treatment, Suv39h1 is bound to the promoter, which correlates with high levels in H3K9me3 in the promoter and gene silencing. After one hour of treatment, Suv39h1 promoter occupancy decreases around six times to disappear completely after two hours of treatment. This decrease in Suv39h1 agrees with the observed decrease in H3K9me3. In contrast, SirT6

occupancy at the promoter is increased with TNFα treatment and also RelA. In fact, both RelA and SirT6 reach their occupancy peak at one hour of treatment, but after 2h RelA levels drop significantly whereas SirT6 remains at similar levels. ChIP performed with H3K9ac antibody reveals that levels after one hour of treatment increase that is probably due to a decrease in the H3K9me3.However, after two hours of treatment the levels decrease probably due to deacetylation by SirT6.

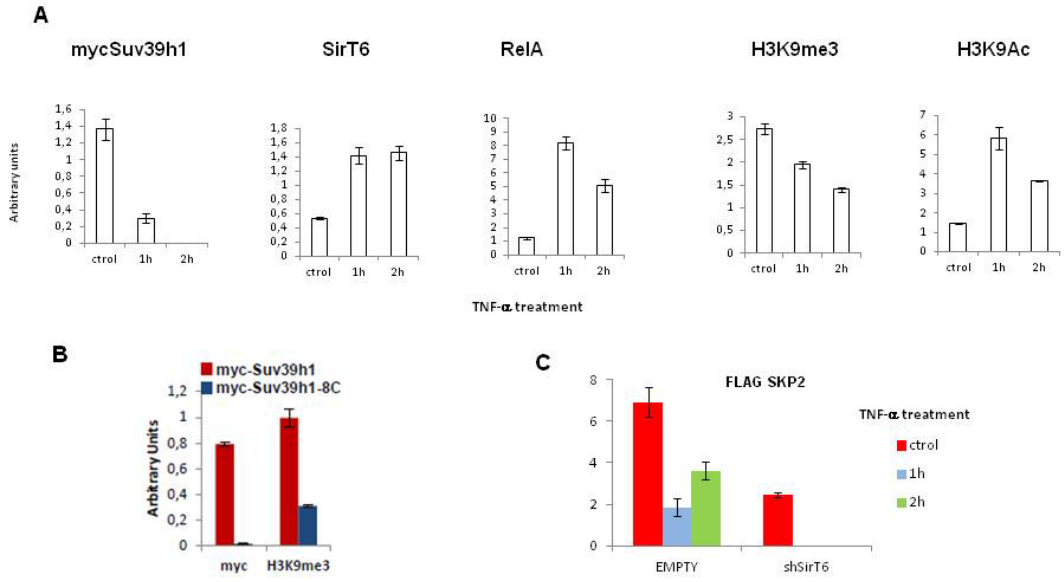

Figure R38. ChIP of IkBα promoter. A. ChIP αmyc, αSirT6, αRelA, αH3K9me3 and αH3K9ac of 293F cells treated with TNFα and harvested at the indicated times. **B.** ChIP αmyc of 293F cells. **C.** ChIP αFLAG of 293F cells with shRNA SirT6 and treated with TNFα and harvested at the indicated times.

Although we could not follow the ubiquitination of Suv39h1, we could try to understand what happened to this Suv39h1 dynamics in the IKBα promoter upon loss of pre-SET structure. For that purpose, we transfected either mycSuv39h1 WT or the 8C mutant described before. ChIP experiments showed that mutated Suv39h1 is not able to bind to the promoter and, as a consequence, H3K9me3 levels decrease in the promoter *(Figure R38B).* Therefore, Suv39h1 monoubiquitination, that destroys the structure, should affect the binding to IkBα promoter and, then, the H3K9me3 levels.

Considering our observations, we would expect that, SKP2 binds to the promoter before or around activation by TNFα. We then tested by ChIP whether that was the case and also whether its presence in the promoter depend on SirT6. Since no available antibody for SKP2 is recommended for ChIP, we perfomed ChIP with FLAG

antibody overexpressing FLAGSKP2 in cells containing shScrambled or shSIRT6 under TNFα treatment (*Figure 38C*). The first thing we observed is that SKP2 is already present in promoter before the induction, and 1h after activation completely disappears from the promoter. Strikingly, loss of SirT6 in the ShSirT6 cells correlates with a loss of SKP2. Therefore, SirT6 is required for the recruitment of SKP2 to the promoter previous to activation of the pathway.

2. Other SirT6 interacting proteins

2.1. SirT6 and EZH2

In the previous section, methylation assays was performed in extracts derived from 293F cells. These cells are well differentiated and derived from human embryonic kidney cells. In order to test whether SirT6 might interact with other methyltransferases in other physiological contexts, and in particular, in the process of differentiation, we performed HMT *in vitro* assays as before but this time SirT6 was purified from p19 cells, a type of pluripotent cells derived from a mouse embryonic teratocarcinoma *(Figure R39A).* SirT6 co-fraccionated with an H3 methyltransferase activity again. The identification of the specificity of the HMT using the GST fusions to the Wt and mutants of the N-terminal histone H3 tail rendered a very surprising result. While the activity was mainly H3K9 specific, mutation in K27 decrease the HMT activity. This suggested that in addition to a K9 specific activity, another H3K27 methylation activity was involved. The mammalian H3K27 methyltransferase, EZH2, belongs to the Polycomb Repressor Complex and is involved in the maintaining of the cell pluripotency by regulating transcription of some key genes [309]. Interestingly, supporting this finding, SirT6 and Ezh2 interacted in an immunoprecipitation experiment *(Figure R39B).*

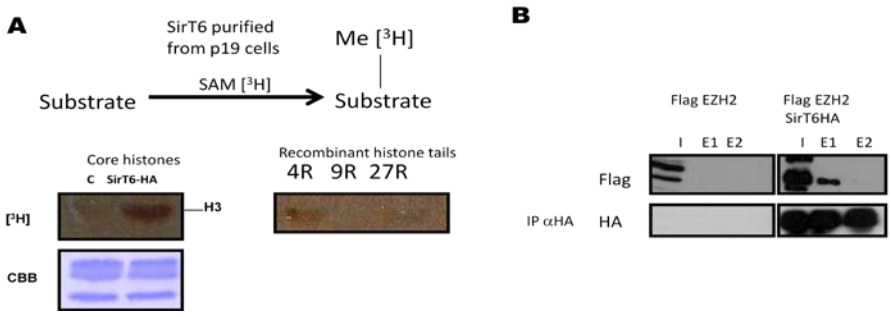

Figure R39. SirT6 interacts with EZH2. A. In vitro methylation assay performed with SirT6-HA purified from p19 cells. **B.** IP HA between FLAG-EZH2 and SirT6-HA in 293F cells.

2.2. Identification of SirT6 interacting proteins by purification

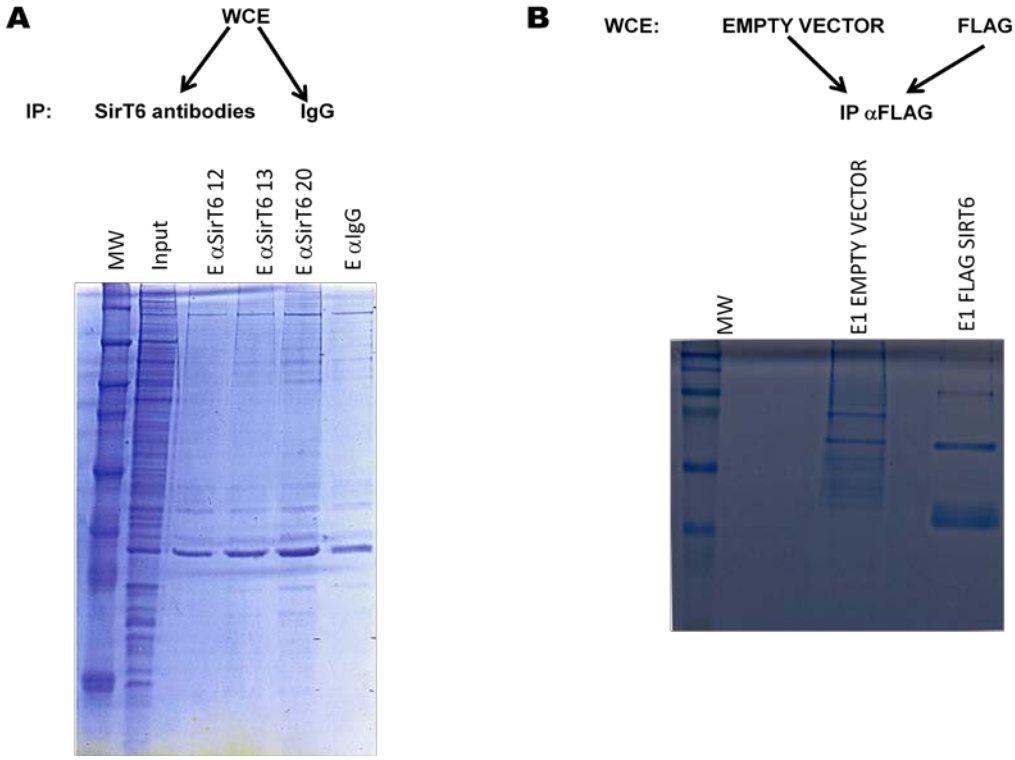

Figure R40. Identification of SirT6 interacting proteins by purification. A. Coloidal staining of the input and elutions of SirT6 endogenous purification from 293F cells whole cell extract. **B.** FLAG immunoprecipitation of extract from 293F cells transfected with empty vector and FLAG-SirT6. Coloidal staining of the elutions.

Aiming to identify SirT6 interaction-partners that could provide us with some clues about the role of SirT6 in chromatin regulation, we purified SirT6 and identified all proteins co-fractioning with SirT6 by using an affinity chromatography approach and analyzing the result by mass spectrometry. We followed the following approaches to identify these proteins:

- Immunoprecipitation of endogenous SirT6 *(Figure R40A)*. From a whole cell extract, immunoprecipitation with different SirT6 antibodies was performed. The, elutions were loaded in an acrilamide gel that was stained with colloidal coomasie. Differential bands comparing the immunoprecipitated SirT6 elutions with the control (immunoprecipitation with IgG antibody) were cut and analyzed by mass spectrometry.

- Immunoprecipitation of overexpressed FLAG-SirT6 *(Figure R40B).* 293F cells were transfected with empty vector and FLAG-SirT6 then the different cell extracts were immunoprecipitated with FLAG antibody and the elutions loaded in coomasie stained acrilamide gel. The differentiated bands between FLAG-SirT6 and empty vector elutions were cut and analyzed by mass spectrometry.
- Purification of SirT6-HA by different chromatography steps *(Figure R41A).* Protein extract from cells transfected with SirT6-HA was processed by several chromatography steps: anion exchange column (DE52), cation exchange column (P11), another anion exchange colum (DEAE 5PW), an immunoprecipitation with HA antibody and a gel filtration column (Superose 6). Then, the fractions from the superose 6 were loaded in a silver stain gel and there were made two pools: fractions 29-32 and fractions 47-50 *(Figure R41B).* These two pools were loaded in a gel that was stained with colloidal *(Figure R41C)*. Bands were cut and analyzed by mass spectrometry.

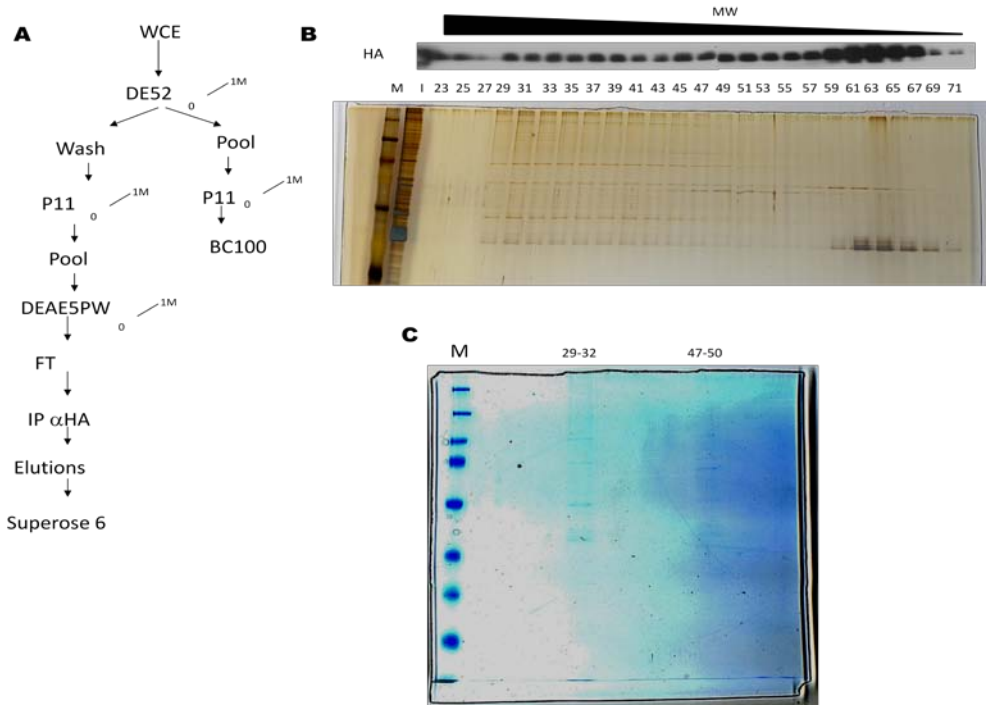

Figure R41. Identification of SirT6 interacting proteins by purification. A. Diagram of the purification steps made in a protein extract from 293F cells transfected with SirT6-HA. **B.** Western Blot of SirT6-HA and Silver staining from Superose 6 fractions. MW: molecular weight. **C.** Coloidal staining gel of the pool of indicated fractions loaded.

From the mass spectrometry analysis, many proteins were identified as potential SirT6 interactors. Overexpression and immunoprecipitations between some of the identified proteins and SirT6 were performed *(Figure R42).* We found that CAND1, PC2, USP10, G3BP1, MTA2, HDAC1, G3BP1 and UBAP2L interact with SirT6. USP10 and G3BP1 were found as SirT6 interacting proteins before, since they have been reported to form stress granules [221,310].

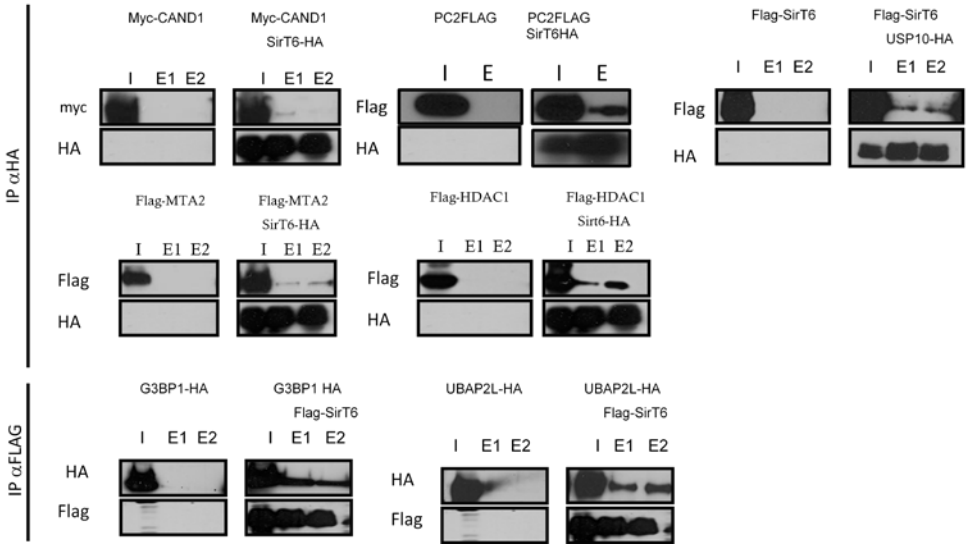

Figure R42. SirT6 interacts with CAND1, PC2, USP10, MTA2, HDAC1, G3BP1 and UBAP2L. FLAG or HA immunoprecipitation of extracts from 293F cells transfected with SirT6 and CAND1, PC2, USP10, MTA2, HDAC1, G3BP1 or UBAP2L.

MTA2 and HDAC1 are members of the Nucleosome Remodeling and Deacetylase (NuRD) complex. This complex is involved in gene repression and two HDACs belong to it: HDAC1 and HDAC2. Also there are other factors such as ATPase helicase and DNA binding factors that cooperate in heterochromatin formation [311]. We have verified the interaction between SirT6 and some of their components as MBD2, LSD1, RBBP4, MBD3 and CHD3 *(Figure R43A)* and we have found that some of them do not interact directly, such as for instance, MBD3, CHD3 and MBD2, maybe because SirT6 does not interact with them directly and the conditions are very stringent to see the interaction. We have also performed a glycerol gradient between SirT6 and some NuRD components: MBD2, RBBP4 and CHD4 and we have seen that their occupancy overlap only in a small range of fractions *(Figure R43B).* This is normal because SirT6 is also involved in other functions interacting with other proteins.

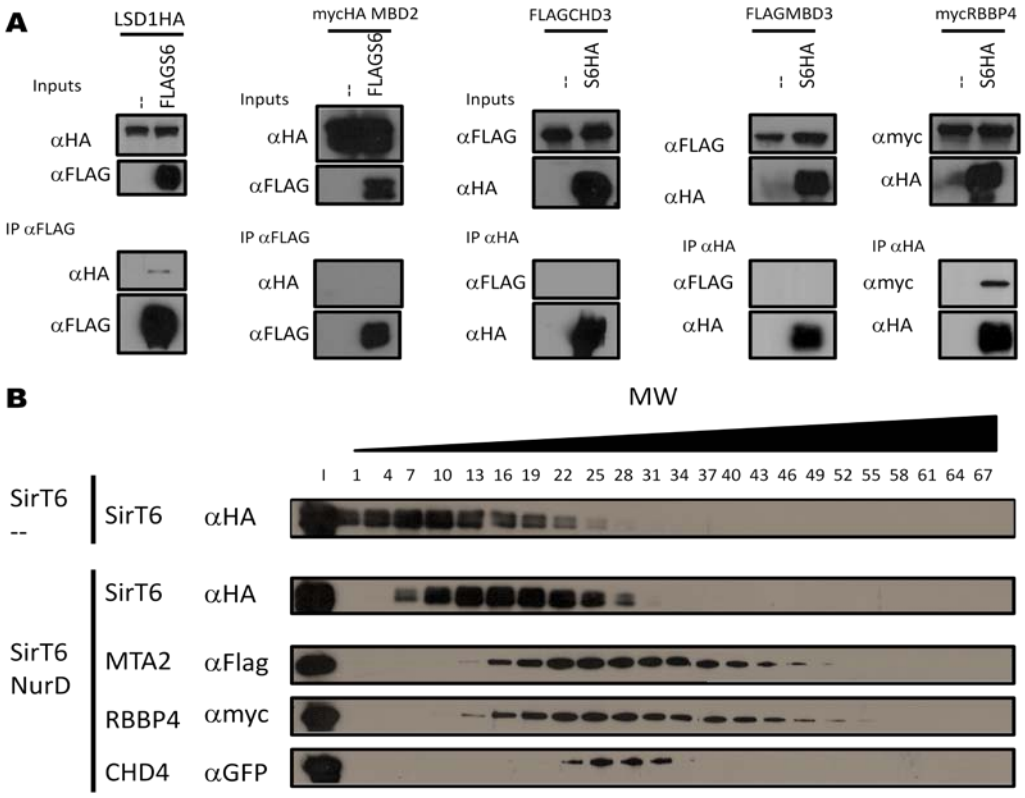

Figure R43. SirT6 interact with some NuRD complex components. A. FLAG or HA immunoprecipitation of extract from 293F cells transfected with FLAG-SirT6 or SirT6-HA and LSD1-HA, mycHA-MBD2, FLAG-CHD3, FLAG-MBD3 and myc-RBBP4. **B.** Western Blot of glycerol gradient of protein extract from 293F cells tranfected with SirT6-HA, FLAG-MTA2, myc-RBBP4 and GFP-CHD4.

DISCUSSION

In this work, we have aimed to understand the role of SirT6 in chromatin regulation, and in particular, the mechanisms through which regulate gene silencing in the context of stress response. Our studies have unveiled two types of novel modifications, cysteine and serine monoubiquitination, never described before in nuclear proteins or nuclear associated functions. Our work raises several interesting issues that we discuss in this section.

Role of SirT6 in chromatin silencing

The first studies focused on SirT6 catalytic activity, no deacetylation was detected and only an auto ADP-rybosilation activity was reported [204]. In 2008, the Chua lab reported that SirT6 deacetylates H3K9ac [206]. Since then, SirT6 deacetylation activity has been studied. The first surprising issue is that is not very efficient compared to for instance, SirT1 [210], it requires fatty acids to increase it activity [209] and targets histones organized in nucleosomes in contrast to the other Sirtuins that seem to prefer free core histones. Very recent studies have shown that SirT6 shows a stronger enzymatic activity as a deacetylase of long chain fatty acids rather than as a deacetylase of proteins like histones [212]. This is probably the main reason why in Figure R1 SirT6 does not deacetylate core histones *in vitro* comparing to SirT1, although others have reported *in vitro* deacetylation with core histones [235]. The differences between these experiments and ours might be due to the different conditions of cells when they were harvested or to technical differences in the in vitro deacetylase reaction assay, such as the level of enzyme. Another interesting possibility is that SirT6 requires other additional cofactors, or that only very specific stress conditions could increase specifically its K9-associated activity.

As it was mentioned before, SirT6 is associated to gene silencing but it has never been described before any other SirT6 interacting factor that cooperates in transcriptional repression. Here we show that SirT6 interacts with repressor factors among them HMTs, in particular, we describe the interaction between SirT6 and Suv39h1.

In this work we have been able to demonstrate a new mechanism to regulate NF-kB pathway. We have not only unveiled a novel SirT6-mediated mechanism, but a whole new modification in Suv39h1 with important implications in regulation of gene silencing and the response to stress. The mechanism involves a non canonical Suv39h1 ubiquitination induction through E3 ubiquitin ligase SKP2. Interestingly, our studies not

only report a new functional relationship between Suv39h1 and SirT6, but also describe a Sirtuin link to Suv39h1 that is completely different from the one described between Suv39h1 and SirT1 (Figure D1).

	SirT1	SirT6
Necessary for Suv39h1 localization in pericentric foci	Yes	No
Interacting Suv39h1 domain	N-terminal domain	SET domain
Suv39h1 protein levels	3 fold of increase	2 fold of increase
Monoubiquitination induction	No	Yes

Figure D1. Summary of relation between SirT1 and Suv39h1 and SirT6 and Suv39h1

The first issue is that SirT1 is absolutely necessary for pericentric constitutive heterochromatin formation through Suv39h1 recruitment while SirT6 is dispensable for Suv39h1 recruitment to the pericentric constitutive heterochromatin. This suggests that the role of SirT1 on Suv39h1 is actually wider that the one exerted by SirT6. This is supported by our studies with different types of stimuli, where we observed that only cell cycle arresting drugs and activation of NF-kB pathway promoted the modification of Suv39h1. Another important difference is that in our studies SirT6 promotes deacetylation of the NF-kB genes such as IkBα, but also seems to participate in the firing of the signaling in the gene, inducing desilencing of IkBα gene. Interestingly, since SirT1 has been involved in regulating RelA activity through deacetylation, it is not clear whether SirT1 may also interact with the Suv39h1 population present in the IkBα promoter. SirT6 has been shown to affect only the promoters. This is probably one of the most important issues regarding the differences between SirT1 and SirT6: *How do they both coordinate to regulate globally the Nf-kB pathway and in particular the IkBα expression*?

Another striking difference is that despite both proteins are relatively close in evolution, both SirT1 and SirT6 have developed the ability to bind to Suv39h1 through completely different domains. While SirT1 interacts with Suv39h1 through its N-terminal region, SirT6 does it through its c-terminal catalytic SET domain. This open the possibility that, although both factors cannot interact (Figure R6), they actually may bind simultaneously to the Suv39h1 molecule. Another interesting difference is the fact that SirT6 induces Suv39h1 stability but not at the same level as SirT1 does. The stability mediated by SirT1, which was described by our group in 2011, involves the inhibition of the polyubiquitination of the residue K87 in the chromodomain of Suv39h1 [155]. However, since SirT6 binds to the Suv39h1 protein through the C-terminal region, it is not likely that the mechanism may be the same. Future studies should address this point.

A final obvious difference is that SirT6 induces Suv39h1 monoubiquitination (see later). Altogether, all these evidences clearly demonstrate that SirT1 and SirT6 have different function relationship with Suv39h1. SirT1 interacts to Suv39h1 in order to form constitutive and facultative heterochromatin while SirT6 seems to be specifically restricted to NFkB specific genes.

Another important finding in our studies is that SirT6 also interacts with G9a protein. G9a is an H3K9me3 found exclusively in euchromatin, where it induces gene silencing interacting with HDACs. For example, it contributes to silence c-myc expression by interacting with PARP2 and HDAC5 and HDAC7 [312] and to silence p21 interacting with UHFR and HDAC1 [29]. Although we have concentrated our efforts on Suv39h1 and the monoubiquitination of cysteines in the pre-SET domain, Interestingly, G9a belongs to the same HMT superfamily, SUV39, as Suv39h1 and also contains a pre-SET domain. However, we have been unable to detect any monoubiquitination induced by SirT6. Whether it actually exists a G9a monoubiquitination in the pre-SET domain with specific stimuli or other proteins, it is completely unknown and will require more studies in the future. Interestingly, until our work, the only relation found between G9a and Sirtuins is that G9a can methylate SirT1 for an unknown purpose [313]. In our work we have only found the interaction between SirT6 and G9a. In fact, G9a is actually the best candidate to be the usual silencing partner of SirT6 in euchromatin. This actually would fit with the reported presence of G9a and GLP in the NF-kB pathway targets, although their relation with NF-kB pathway has been characterized for their binding to RelB [314–316]. In fact, activation of NF-kB pathway by TNFα, which induced Suv39h1 exit from the promoter only rendered loss of half of H3K9 methylation in the promoter,

which may indicate the presence of another HMT activity present in the promoter at all times (Figure R38A).

A new model for SirT6 and Suv39h1 regulation of NF-kB pathway

The NF-kB pathway is involved in a wide variety of key functions at cellular and organism level. For that reason, the activation of the pathway is very flexible and dynamic and involves many different factors, several different mechanisms. Not only that, the regulation of the transcription of NFkB target genes regulate completely different sets of genes, depending on the cell type or the stimuli [272]. In transcription regulation of NFkB target genes, different HDACs, HATs and chromatin remodeling complexes have been found to bind to the promoters. Interestingly, different genes seem to show different combinations of these chromatin-associated activities. Regarding to the regulation of NFkB pathway by Sirtuins, as we mentioned before, RelA can be deacetylated by SirT1 and SirT2 (in the nucleus and the cytoplasm, respectively) inhibiting RelA activity [176,186]. The general model of SirT6 regulation of the NF-kB pathway involves the interaction between SirT6 and RelA [235,236]. SirT6 inhibits RelA activity as a transcription factor by deacetylating H3K9 and destabilizing RelA from the promoter without deacetylation of RelA. This model has been described in HeLa and in MEFs cells and it is not as general as it seemed at the beginning *(Figure D2).* An interesting source of information reported is a ChIP-Seq experiment perfomed in similar conditions as our studies. The assay tried to determine in WT or SIRT6KO MEFs the chromatin localization of RelA and/or SirT6 under different times of TNFα treatment. The results showed that SirT6 distributes in the NF-kB target genes promoters dynamically upon TNFα treatment and in most of them colocalizes with RelA. Some genes were downregulated or upregulated in the absence of SirT6 and others behaved similar.

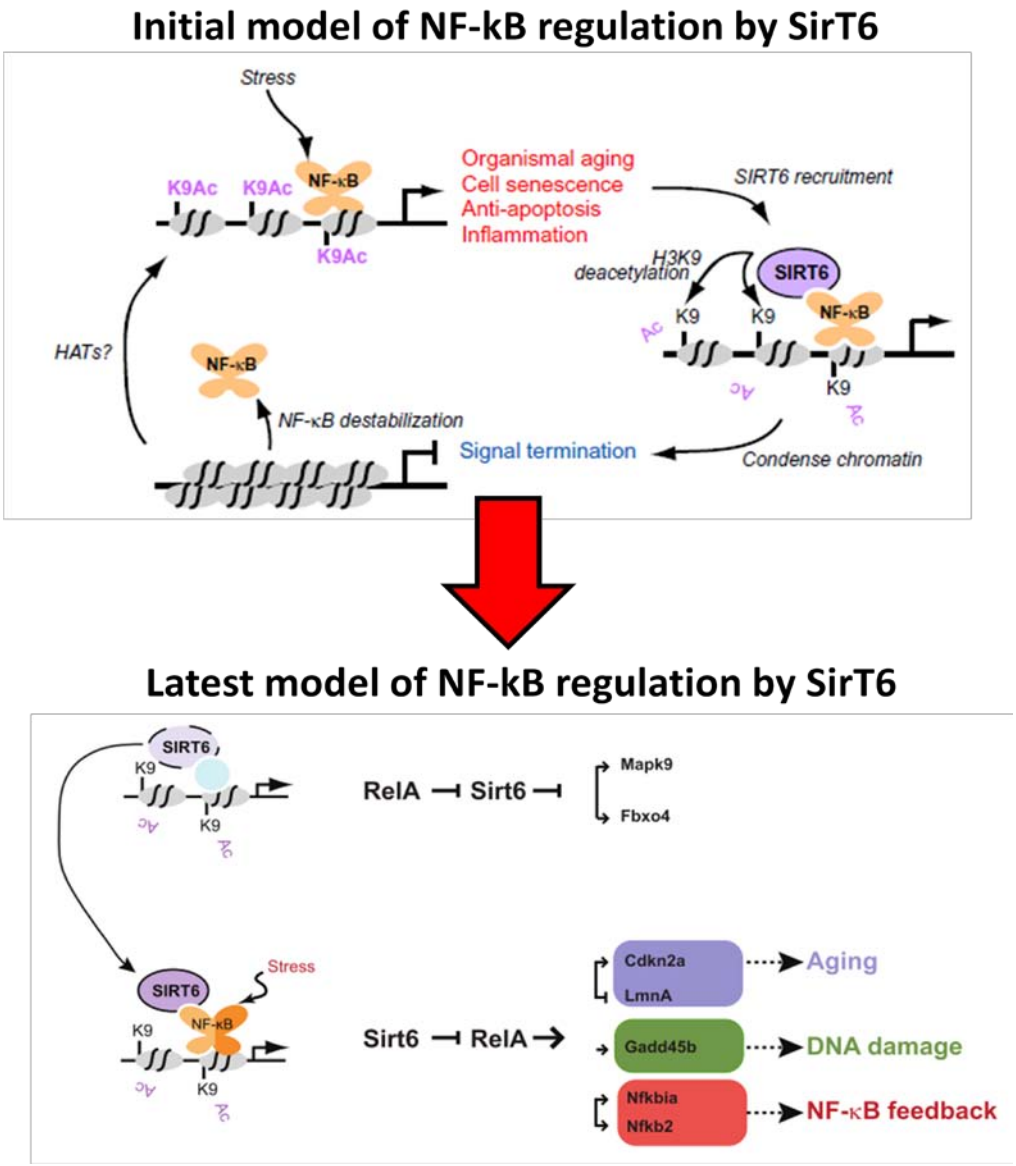

Figure D2. Models for NF-kB pathway regulation through SirT6 [235,236].

In our model, in basal conditions, in 293F cells IkBα promoter is bound by Suv39h1 to maintain high levels of H3K9me3 in order to avoid gene transcription. SirT6 is also binding to the promoter, where it maintains H3K9 deacetylated and recruits SKP2 to the same regions occupied by SirT6 and Suv39h1. Upon TNFα treatment, RelA translocates to the nucleus and binds to IkBα promoter, at the same time, SirT6 deacetylates SKP2 at residues K73 and K77 and induces phosphorylation in SKP2 at residues S72 and S75. These posttranslational modifications activate SKP2, which in turn monoubiquitinates Suv39h1. This is in turn followed by exit from Ub-Suv39h1 and SKP2 from the promoter and the arrival of more SirT6 molecules. RelA induces IkBα

expression until a certain point when SirT6 interferes with RelA activity and stops the IkBα expression through in part, H3K9 deacetylation (Figure D3).

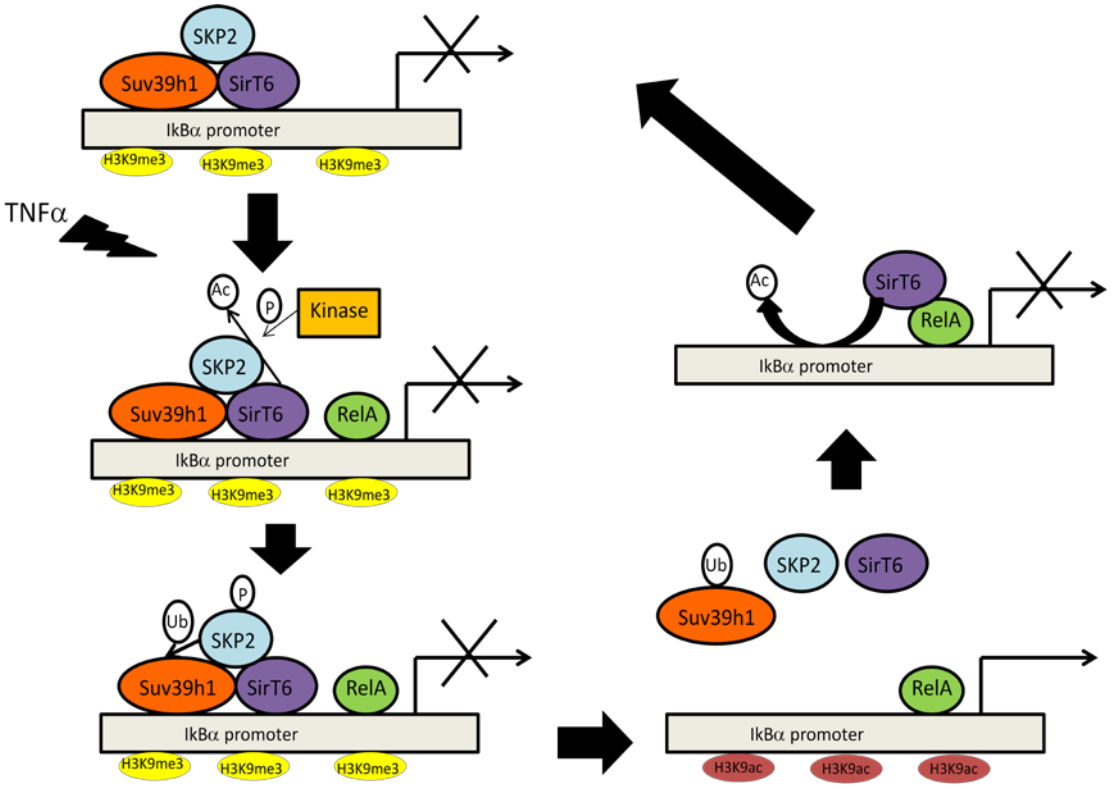

Figure D3. Our hypothetical model of IkBα promoter regulation in 293F cells upon TNFα stimulus

IkBα is the main negative regulator of NFkB pathway and cells should avoid a hyperactivation of NFkB pathway, then this specific regulation in some cell lines described with Suv39h1, SKP2 and SirT6 regulation is totally justified. It has already been described a regulation mechanism in IkBα promoter in 293F cells [275]. In the model propose there, IkBα promoter is regulated by coactivators and corepressors complexes. The coactivators involved were SRC1, SRC2 and SRC3 complexes that contain the HATs p300/CBP and PCAF: SRC1 would bind to the promoter at late stage of the TNFα treatment, SRC2 would activate at the beginning of the treatment and SRC3 would have an oscillatory behavior in the promoter. In contrast, the corepressors involved were SMRT and NCoR complexes where HDAC1 and HDAC3 would be necessary for silencing. The transcription of IkBα is determined by the balance between corepressors and coactivators as both groups are binding to the promoter. However, what triggers the initial activation process is not described. Our data suggest

that the interaction between SirT6, Suv39h1 and SKP2 is necessary for starting of IkBα expression after TNFα treatment. After this "permission" for transcription (the Suv39h1 removal from the promoter), the correpressors and coactivators would be maybe necessary to modulate the IkBα levels. Another possibility is that SirT6 presence before activation is not related to maintenance together with Suv39h1 of the gene silencing at this stage. In fact, interaction between Suv39h1 and these HDAC-containing complexes was previously reported [52].

Interestingly, in early transcribed NFkB target genes such as A20, transcriptional machinery is preloaded to the promoter without even TNFα treatment: TFIID, Pol-II, the coactivators CBP and p300 and other factors from the transcriptional machinery are found in promoter [277]. The Sp1 protein, that is constitutively expressed, helps to maintain all these proteins in the promoter in order to be ready once NF-kB will be activated. Transcriptional machinery is supposed to be preloaded in the IkBα promoter as it is an early gene. The persistent silencing of gene expression by Suv39h1 is necessary in order to not allow gene expression until TNFα treatment. The mechanism proposed by our work would allow a fast recruitment of Suv39h1 in the promoter, since Suv39h1 is only removed from the chromatin and not degraded.

Another interesting observation is the recruitment of SKP2 to the promoter. This is the first time that SKP2 is involved in regulation of gene expression. SKP2 binding to the IKBα promoter was analyzed by ChIP (Figure R38C). SKP2 left the promoter upon TNFα, a pattern that is similar to the Suv39h1 behavior. However, the decrease in SKP2 levels without SirT6 could be also due to a decrease in SKP2 global levels by lack of SirT6 that stabilizes SKP2 levels. In any case, SKP2 has an important role in NF-kB regulation at the IkBα promoter.

Our studies also suggest that there may be two types of SirT6 molecules. The molecules, limited in their levels, already present in the silenced promoter before the activation by TNFα, and the other population of SirT6 that arrives after activation. The first population would be the responsible to recruit SKP2 and maybe Suv39h1 to the promoter. A logical speculation, is that maybe what really leaves the IkBα promoter is a not an isolated Suv39h1-Ubiquitin molecule but a complex that may contain SirT6, SKP2 and Suv39h1-Ubiquitin. Then, a deubiquitinase could remove ubiquitin once the activation would be over to recycle the Suv39h1 molecule and maybe the complex. However, in any case, whether this is the case or not, and the enzyme/s that may be involved in this recycling, should be clarified in future studies.

To further study the mechanism, it would be useful design a Suv39h1 mutant that would prevent the monoubiquitination induced by SirT6-SKP2 without affecting to the structure or the activity. This would allow perform experiments with cells that only would harbor the mutant Suv39h1 and perform a xenograft to a mouse and study if there is a hyperactivation of NFkB pathway due to the lack of IkBα and if it produces a tumor as it has been reported when NFkB pathway is hyperactivated. This model shows a new vision of NFkB regulation.

Other mehyltranferases have been described to have a role in NF-kB pathway. They methylate RelA and affect DNA binding, RelA degradation and recruitment of other silencing factors [301,302]. For instance, SET7 also affects H3K4me1 at the promoter of particular NFkB target genes such as IL8 and MCP1 under specific conditions [317]. Moreover, H3K9 methyltransferases such as GLP and G9a have been involved in NFkB gene silencing [314–316]. Also, histone demethylases of H3K9, such as Aof1, have been described to bind to NFkB transcription factors in order to allow gene expression by removing H3K9 methylation mark [318]. Interestingly, Suv39h1 was already involved in NF-kB regulation. Thus, Suv39h1 was reported to be present in the RelA promoter, and leave the promoter upon hyperglycemic conditions to allow RelA expression [308]. .

In our studies, Suv39h1 downregulation induced an increase on IkBα transcription upon TNFα treatment that is in agreement with the described model (Figure R36). However, in other early genes (A20, GRO1 and IL8), Suv39h1 downregulation does not induce the expected increase in response to TNFα because the increase on IkBα levels decreases the NFkB transcription factors that are released upon TNFα treatment. Therefore, the effect on these genes, peaking their expression only one hour later than IkBα, may be explained by an attenuation of NF-kB activation due to a lower availability on nuclear NFkB transcription factors that activate their transcription (Figure D4). In late-activating genes MCP1 and IAP2, the effect of IkBα deregulation has already dissipated and their behavior is more complex, probably related with a requirement of chromatin remodeling to allow transcription and due by other regulatory signaling [274]. We hypothesize that SirT6 downregulation attenuates IkBα transcription upon TNFα treatment due to the impossibility of releasing Suv39h1 from IkBα promoter. In contrast, in MEF cells, SirT6 KO cells behave exactly as Suv39h1 KO cells. Given that we have not detected induction of Suv39h1 monoubiquitination in MEFs, the mechanism should be different. As it has been explained [272], transcription regulation of NFkB targets depends on cell type and stimulus.

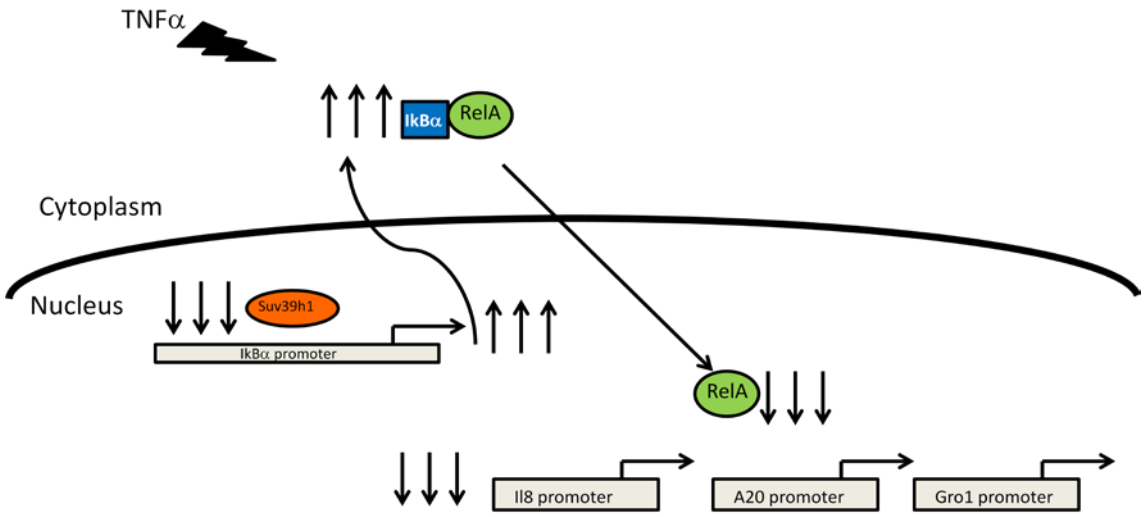

Figure D4. Model of NF-kB target gene expression in shSuv39h1 293F cells.

Monoubiquitination in a conserved domain of Suv39h1: pre-SET, SET and post-SET function

As we already explained, ubiquitination is very diverse. This correlates with a wide variety of functions associated to this modification [319]. Monoubiquitination is not as frequent as polyubiquitination. In most of the cases, monoubiquitination takes place does not lead to degradation such as for instance, the monoubiquitination in histone H2A and H2B is involved in silencing or in DNA damage signaling [112]. Moreover, monoubiquitination can be essential for controlling transcription. H2AK119 monoubiquitination and the H3K27 methylation mediated by PRC complex is necessary for polycomb epigenetic silencing [320]. Also H2AK119 monoubiquitination also has been found to directly inhibit RNA Pol-II elongation, acting coordinately with the transcription repressor NCoR/HDAC1/3 complex [321]. In contrast, H2BK123 monoubiquitination activates transcription, although this modification is also involved in transcription elongation [322].

Nevertheless, the non canonical ubiquitination, which involves non-lysine residues, are actually even less frequent and there are only very few cases described [79]. The main reason why it is difficult to describe is the unstable bound in non canonical ubiquitination that could be destroyed under some conditions of protein extraction. For example, Pex5p suffers non canonical ubiquitination in cysteine 11. Pex5p is a cytosolic receptor for peroxisome proteins and the ubiquitination is essential for the Pex5p shuttling between cytoplasm and nucleus [323]. Also the viral E3 ubiquitin ligase

mK3 can promote immunosuppression by ubiquitinating serine and threonine residues in the heavy chain of the major histocompatibility complex, which induces its degradation [307].

Interestingly, our findings describe a very special ubiquitination because it does not take place in one specific residue, it does in a whole a domain. This actually implies that the actual target of the modification is not a particular residue but the whole structure. In other words, the target is to destroy the structure of the pre-SET domain.

Of all SET domain-containing histone methyltransferases, the vast majority of HMTs found so far, can contain around the catalytic SET domain two conserved domains: pre-SET and post-SET domain. Of all the families in which these SET-containing HMTs are divided, only three contain pre-SETdomain and all of them except EZ family contain a post-SET domain. The best known of these families is the SUV39 family, which corresponds to Suv39h1, Suv39h2, and G9a, among them. The SUV39 family contain all three domains, Pre-SET, SET and Post-SET domains [324]. The crystal structure of the SUV39 HMTs have been solved: in the family, the PRE-SET domain show a very strong conservation in nine cysteines that are clustered in two groups of four and five [324–327]. The crystal structure of DIM-5 and Clr4 (orthologue of Suv39h1 in *S.pombe*) indicates that these nine cysteines coordinate three zinc ions that form an equilateral triangular cluster in the tridimensional space, each zinc ion is coordinated by four cysteines and there are some bridging cysteines that coordinate more than one ion (Figure D5). The Post-SET domain is a flexible domain that has also conserved cysteines, in particular, three, that coordinates one zinc ion. The homologies between cysteine structure found in the pre-SET and post-SET domain should be the main reason why we could still observe monoubiquitination induced by SirT6 overexpression in the extended SET construct (containing SET and post-SET domains) (Figure R11). We may then hypothesize that monoubiquitination may also involve the cysteines of the post-SET domain. Considering that this domain has been shown to be indispensable for the methyltransferase activity because it might bind to the methyl donor SAM, this hypothetical modification may be important to shut down the enzyme activity. Future studies should determine whether this hypothesis is real.

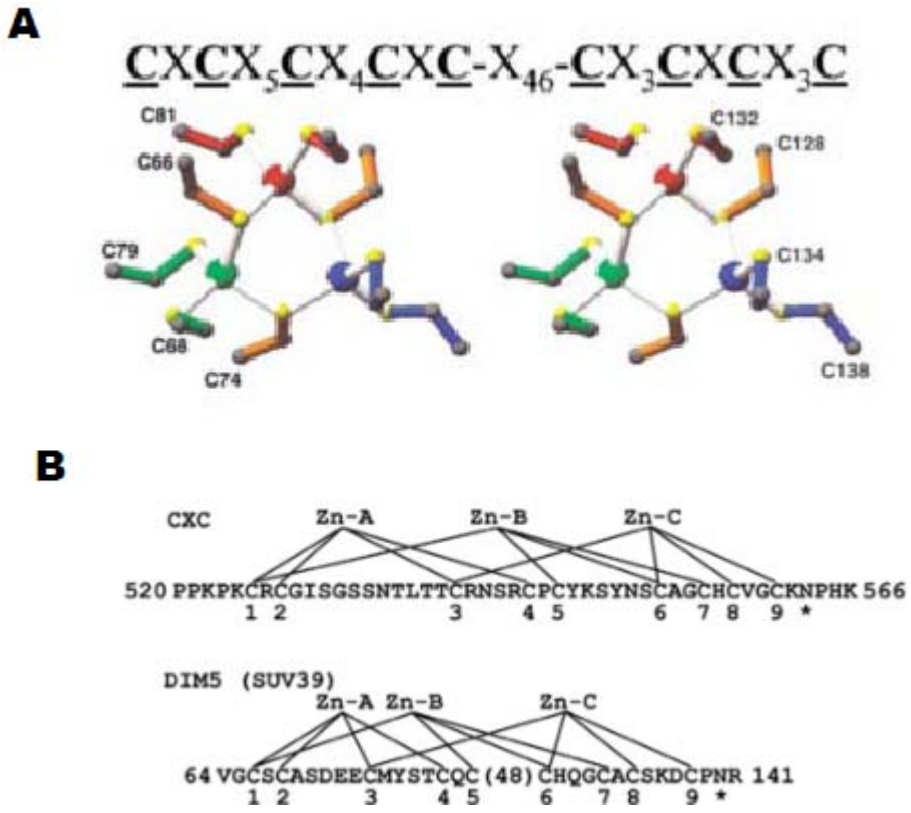

Figure D5. A. DIM-5 conserved pre-SET domain structure. Nine cysteines coordinate three zinc ions (represented in red, green and blue) [326]. **B.** Comparison between sequence of the MSL2 CXC domain and pre-SET domain of DIM5 and Suv39h1. They have nine conserved cysteines that coordinate three zinc ions [328].

For lysine ubiquitination, the ubiquitination machinery is promiscuous regarding to the substrate, and in general E3 ubiquitin ligases do not recognize a special sequence but a structure and accessibility to the residue and the aminoacids surrounding the lysine [329], it might happen something similar to cysteine ubiquitination. These experiments were done always by mutating the specific residue modified, which would force the enzyme to look for another acceptor of the ubiquitin covalent bond. However, in this case if the residue is intact, the enzyme tends to always modify the same lysine [330]. Therefore, our current case may be quite different as there may be not really a specific favorite cysteine residue. So far, we have detected only three cysteine events in the pre-SET domain, but we speculate that with enough analysis by mass spectrometry of the whole monoubiquitinated Suv39h1 population most of the cysteines, if not all, may be found to be modified.

As we explained before, the role of the SET domain has not been really characterized and it is not completely clear. The sequence and structure of the pre-

SET domain is strikingly similar to the CXC domain of the MSL2 of the Dosage Compensation Complex [328]. This CXC domain is required for binding to the X chromosome, which suggests a DNA binding role for the pre-SET domain. Our studies completely support this role for pre-SET domain since the destruction of the pre-SET domain structure leads to a loss of Suv39h1 binding to pericentric constitutive heterochromatin (Figure R15B). It is known that Suv39h1 binding to chromatin has a binding component independent on the histone mark H3K9me3 and the heterochromatin factor HP1, and is dependent on DNA. Based on this observation, our core hypothesis in this work is that monoubiquitination in the pre-SET domain is a mechanism aimed to remove Suv39h1 from direct contact to DNA.

It has been also observed that ΔSET construction has no monoubiquitination despite of the presence of the pre-SET domain (Figure R9A) but is important to remark that SirT6 and SKP2 bind to the SET domain, and therefore the ΔSET construction did not allow their binding.

Besides the cysteines identified in the pre-SET domain, we have also detected two other monoubiquitination events, one in serine (S29) and the other one in cysteine (C49), localized by mass spectrometry in the N-terminal and the chromo domain, respectively. Interestingly, the N-terminal region of Suv39h1 is the binding site for HP1, and the chromodomain binds to H3K9me3. Given the distance between both modifications, ubiquitination of any of these residues should alter drastically the structure and function of the whole N-terminal part of Suv39h1, which span the first 89 residues (N-terminal domain + chromodomain). We hypothesize that this modification in these residues would have the same effect as the modification of the pre-SET domain. Supporting this hypothesis, loss of these 89 residues in Suv39h1 were shown to completely abrogate the presence of Suv39h1 in mitotic chromosomes [35]. However, the fact that eliminating the pre-SET domain we almost eliminate the ability of SIRT6 and SKP2 to induce the modification suggests either that the pre-SET domain is the preference in this regulation over other domains of Suv39h1, or that there may be other E3-ubiquitin ligases that may be more specific of this E3 ubiquitin ligase. Another supporting evidence for the preference of SKP2 for the PRE-SET domain is the mass spectrometry analysis of the SKP2 where the induced modification found was only monoubiquitination in pre-SET but not in the N-terminal region of the protein (data not shown). Future studies should aim to characterize the ubiquitinations outside pre-SET domain in the context of Suv39h1 function.

A new role for SKP2

The E3 ubiquitin ligase SKP2 is considered an oncogene because it promotes cell cycle progression through degradation of key proteins involved in the cell cycle checkpoints. Among the proteins that SKP2 ubiquitinates are found the histone methyltransferase PR-SET7. PR-SET7 controls H4K20 methylation during cell cycle and its levels fluctuates since its activity can inhibit DNA replication [331]. The regulation of PRE-SET7 levels is performed by the participation of multiple ubiquitin ligases. SKP2 ubiquitinates and induces degradation of PR-SET7 in G_1/S and S phase [332]. However, our findings suggest for SKP2 another different way of regulation of HMTs in the context of the NFkB pathway. SKP2 is promoting a regulation through the non canonical ubiquitination of Suv39h1. In this case, by promoting a negative regulation of NFkB signaling, SKP2 seems to behave as a tumor suppressor since NFkB hyperactivation leads to inflammation and cancer [265,333].

This is not the first example where SKP2 and Sirtuins seem to work together to induce ubiquitination. SirT1 and SirT2 deacetylates K242, K259, K290 and K569 of FOXO3 and the deacetylation allows the FOXO3 ubiquitination at the same deacetylated residues by SKP2 [139]. However, in this case no direct interaction between SKP2 and these Sirtuins was detected.

Another interesting issue is the regulation of SKP2 levels by SirT6. SirT6 acts as a tumor suppressor in almost all the systems. Interestingly, SKP2 deacetylation in K73 and K77 by SirT6 induces SKP2 phosphorylation in S72 and S75 residues. Previously, K68 and K71 deacetylation of SKP2 by SirT3 induces degradation of SKP2 by ubiquitination by Cdh1 from the APC complex [146] and phosphorylation of S72 and S75 by Akt (S72) and CKI (S75) prevents degradation by Cdh1 [144]. It is particularly intriguing that both sirtuins behaves antagonistically in SKP2 regulation levels by inducing or repressing phosphorylation of the same residues. All of these residues are situated in the NLS sequence of SKP2 (Figure D6). Our results suggest that acetylation of K73 and K77 would have an opposite effect than K68 and K71 over SKP2 levels. While the deacetylation of the first two would induce phosphorylation of S72 and S75, the deacetylation of K68 and K71 would inhibit them. Interestingly, mutating only K73 and K77 do not inhibit significantly the effect of SirT6 on SKP2 (Figure R31D). However, loss of all four residues promotes upregulation of SKP2 and abrogates the ability of SirT6 to increase even further its levels (Figure R31D).

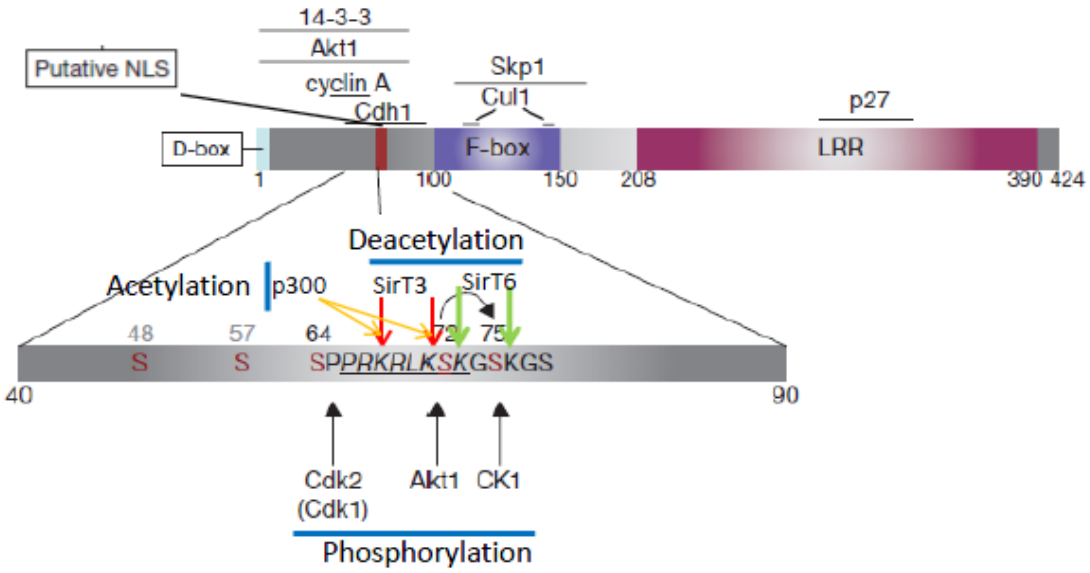

Figure D6. Summary of the main SKP2 posttranslational modifications in the NLS sequence [144].

Interestingly, one of the SirT6 interacting proteins found is CAND, a negative regulator of SCF complex. It binds to the unneddylated Cul1 preventing the binding of SKP1 and SKP2 to Cul1 [334]. SirT6 might interact with CAND1 and modify this protein preventing the interaction with the SCF complex in order to activate SKP2 activity and promote the Suv39h1 monoubiquitination. Alternatively, CAND1 may bind to the complex in order to finish the SKP2 activation.

Another question linked to the issue discussed above is that SirT6 has actually been shown to interact to Akt, the responsible for S72 phosphorylation, which open the possibility that other mechanism induced by SirT6 to stabilize SKP2 levels is by recruiting Akt to SKP2. Interestingly, two evidences argue against an involvement of Akt in our mechanism: First, Akt was also reported to activate RelA and therefore to induce NFkB target gene transcription [335]. Second, Akt can also phosphorylate SirT6 and induce its degradation[220], which we have not been detected. The best candidate to be the kinase responsible of this phosphorylation is IKKα given that is activated upon TNFα and interacts with SirT6, Suv39h1 and SKP2. The antagonistic relationship between IKKα and Suv39h1 has been described in keratinocyte differentiation through phosphorylation of H3S10, which inhibits Suv39h1 activity. However, whether IKKα antagonizes Suv39h1 function through other mechanism, including SKP2 activation, is not known. Another possibility is that IKKα could activate both proteins SKP2 and SirT6 at the same time (Figure D7). Recently, it has been characterized the phosphorylation

of S391 in Suv39h1 by CDK2 in S phase that also induces Suv39h1 removing from chromatin [72], but our data reveals that phosphorylation in this residue is not necessary for the ubiquitination by SKP2. However, it is possible an analogy between the phosphorylation in S391 and the ubiquitination in the pre-SET domain: both of them are found in the middle of the conserved structure of cysteines and zinc ions.

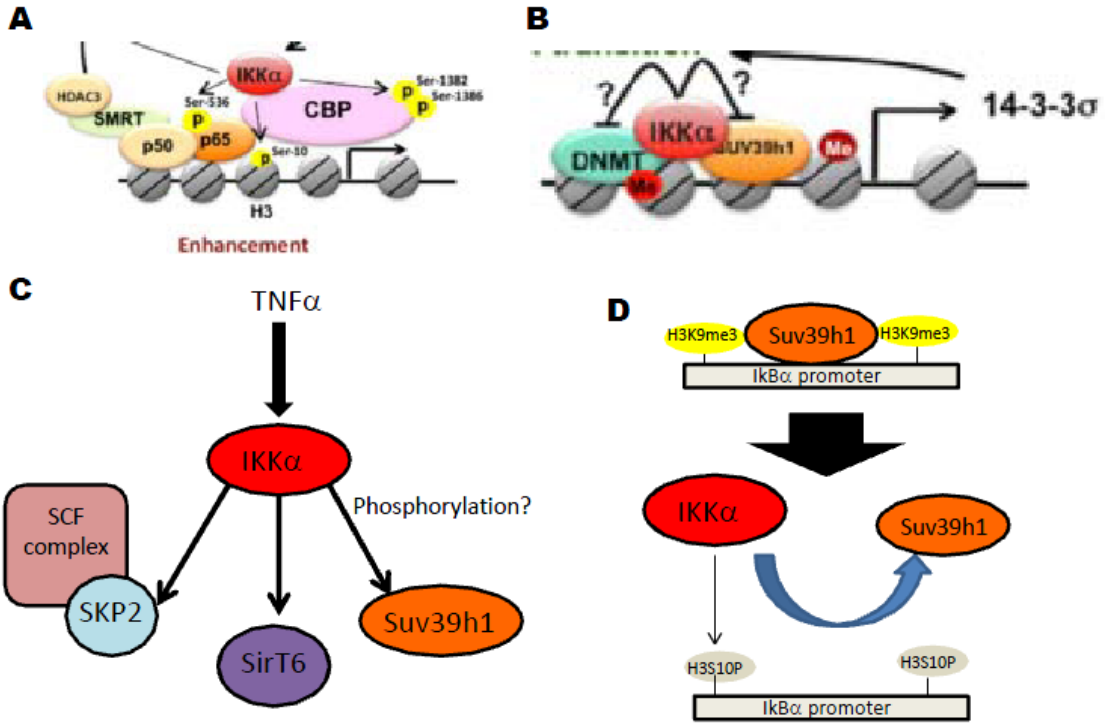

Figure D7. Role of IKKα in gene transcription. A. IKKα regulates some NFkB target genes transcription. **B.** In keratinocytes IKKα negatively regulates Suv39h1 and DNMT in the 14-3-3s. **C, D.** Hypothetical model of IKKα implication in our system.

A future interesting experiment would be to perform H3S10P ChIP in presence or absence of SirT6 in the IkBα promoter to confirm the link to SirT6 and whether this antagonism Suv39h1-IKKα is also valid in 293F cells, as it has been seen that in HeLa [291].

Suv39h1 monoubiquitination and cell cycle progression

This monoubiquitination is not only NFkB activation dependent, but also cell cycle dependent. An obvious explanation for this link with cell cycle is that it may be directly linked to SKP2 function, which has been shown to fluctuate during the cell cycle [131].

However, this cell cycle link to the modification may be also be directly linked to Nf-kB function as activation of the pathway regulates cell cycle progression through many key proteins including cyclins [304,305]. Nevertheless, nocodazole treatment, an anti-microtubule drug, activates NFkB pathway through IKKα and IKKβ [336]. This could suggest that Suv39h1 monoubiquitination induced by nocodazole treatment is due to the NFkB activation and the Suv39h1 monoubiquitination induced G_1/S phase arrest by double thymidine block could be attributed to the increase of SKP2 levels. Supporting the data, it has been reported a phosphorylation and, probably, a monoubiquitination (because of the weight of the band) takes place in Suv39h1 in the progression through G_1/S phase [71].

Another aspect that should be discussed is the fact that serum starvation and high confluency of cells should stop cells in G_o, however it should not induce the same effect because the high confluency avoids monoubiquitination induction (Figure R18A) and serum starvation does not have any effect on the modification (Figure R18B). The effects are not the same with both methods.

CHIP, SirT6 and Suv39h1 relationship

CHIP is an E3 ubiquitin ligase mainly involved in the degradation of unfolded proteins and the excess of proteins. However, CHIP regulates SirT6 levels by monoubiquitination, providing to SirT6 stabilization [96]. Our findings reveal that CHIP interaction with Suv39h1 is not involved in degradation as its levels are not altered with CHIP overexpression (Figure R22B). Moreover, we have shown that CHIP, SirT6 and Suv39h1 form a complex (Figure R23B). However, CHIP seems to have a negative effect on Suv39h1 activity (Figure R23A). Interestingly, CHIP regulates negatively gene transcription by ubiquitinating and promoting the degradation of transcription factors such as RelA [99] and Smad complexes [337], but it also promotes the degradation of transcriptional inhibitors such as CtBP2 inducing the activation of the transcription of its target genes [338]. Future studies should characterize this novel complex and determine its functional implications.

CHFR, SirT6 and Suv39h1 relationship

Both SirT6 and CHFR preserve genome stability. In DNA damage repair SirT6 function seems to be upstream of CHFR function. SirT6 activates PARP1 and CHFR

ubiquitinates and induces degradation of PARP1 once it has finished it action. We have seen in Figure R25, SirT6 regulates levels of CHFR and this regulation could be related to DNA damage repair or other contexts. However, it seems that interaction between CHFR and Suv39h1 could be indirect through SirT6 binding, because CHFR overexpression only induces Suv39h1 ubiquitination in HeLa cells and with overexpression of HA Ubiquitin, which means that we need to force the ubiquitination of the protein to detect it. In any case, CHFR has not been linked to neither histone methyltransferases nor with gene silencing, and therefore its relationship with Suv39h1 is completely unknown.

Furthermore, SirT6 may also regulate CHFR levels by induction of a Akt phosphorylation. Akt phosphorylates CHFR and induces its auto-ubiquitination for degradation, an event required for mitotic entry [109].

SirT6 interacts with other repressive proteins

We have also detected other interacting proteins that bind to SirT6. Interestingly, and supporting a role in silencing, many of them are actually repressive proteins. For example, SirT6 interacts with the methyltransferase EZH2, the responsible for H3K27me3 levels in the cell. As we mentioned previously, SirT1 is a component of the EZH2-containing PRC4 complex. Since SirT6 has been described as a H3K9 and H3K56 deacetylase but not H3K27, it is not really clear whether SirT6 would bind to the EZH2-related complexes through histone deacetylation or for modulating the activity of the complex to directly modify EZH2 or another component of the complex.

The interaction between SirT6 and NuRD components is difficult to explain because this complex is HDACs containing (in particular HDAC1 and HDAC2) and SirT6 could only add deacetylation to the histones in order to silence. However, it has also been described that NuRD complex has a role in DNA damage: it interacts at DNA damage foci and is recruited by PARP [339,340]. SirT6 could be interacting with this complex in this situation. Another possibility is that SirT6 modulates NuRD performance under stress. Future studies should address these issues.

CONCLUSIONS

1. SirT6 interacts with the H3K9 methyltransferases Suv39h1 and G9a.
2. SirT6 interacts with Suv39h1 in a different way than SirT1 interacts with Suv39h1. SirT6 is not involved in the regulation of Suv39h1-dependent constitutive heterochromatin.
3. SirT6 induces a non canonical monoubiquitination in well conserved cysteines in the Suv39h1 pre-SET domain.
4. Monoubiquitination of Suv39h1 abrogates its localization to tight chromatin.
5. Suv39h1 monoubiquitination is cell cycle dependent as it is induced after arresting cells in G_1/S phase and early mitosis with double thymidine block or nocodazole, respectively.
6. Suv39h1 monoubiquitination is activated byTNFα, a mediator of inflammation and an activator of the NFkB pathway.
7. Although both SirT6 and Suv39h1 interact with the E3 ubiquitin ligases CHIP and CHFR, SKP2 is the responsible of the Suv39h1 monoubiquitination induced by SirT6.
8. SKP2 levels are regulated by SirT6. SirT6 deacetylates and induces phosphorylation of SKP2.
9. Suv39h1 downregulation impairs NFkB target genes expression upon TNFα treatment.
10. SirT6 interacts to other proteins among them EZH2 and some NuRD components that are transcriptional repressors.

REFERENCES

1. Vaquero, A., Loyola, A. & Reinberg, D. The Constantly Changing Face of Chromatin. *Sci. Aging Knowl. Environ.* 1–16 (2003).

2. Clapier, C. R. & Cairns, B. R. The biology of chromatin remodeling complexes. *Annu. Rev. Biochem.* **78,** 273–304 (2009).

3. Xu, Y. Z., Kanagaratham, C. & Radzioch, D. Chromatin Remodelling During Host-Bacterial Pathogen Interaction. *Intech* (2013).

4. Gangaraju, V. K. & Bartholomew, B. Mechanisms of ATP dependent chromatin remodeling. *Mutat. Res.* **618,** 3–17 (2007).

5. Patel, D. J. & Wang, Z. Readout of epigenetic modifications. *Annu. Rev. Biochem.* **82,** 81–118 (2013).

6. Swygert, S. G. & Peterson, C. L. Chromatin dynamics : Interplay between remodeling enzymes and histone modifications. *Biochim. Biophys. Acta* **1839,** 728–736 (2014).

7. Tessarz, P. & Kouzarides, T. Histone core modifications regulating nucleosome structure and dynamics. *Nat. Rev. Mol. Cell Biol.* **15,** 703–708 (2014).

8. Lalonde, M.-E., Cheng, X. & Côté, J. Histone target selection within chromatin: an exemplary case of teamwork. *Genes Dev.* **28,** 1029–41 (2014).

9. Musselman, C. a, Lalonde, M.-E., Côté, J. & Kutateladze, T. G. Perceiving the epigenetic landscape through histone readers. *Nat. Struct. Mol. Biol.* **19,** 1218–27 (2012).

10. Allfrey, V. G., Faulkner, R. & Mirsky, A. E. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **315,** 786–794 (1964).

11. Choudhary, C. *et al.* Lysine Acetylation Targets Protein Complexes and Co-Regulates Major Cellular Functions. *Science (80-.).* **325,** 834–841 (2009).

12. Shogren-Knaak, M. *et al.* Histone H4-K16 Acetylation controls chromatin structure and protein interactions. *Science (80-.).* **311,** 844–848 (2006).

13. Vaquero, A., Sternglanz, R. & Reinberg, D. NAD+-dependent deacetylation of H4 lysine 16 by class III HDACs. *Oncogene* **26,** 5505–20 (2007).

14. Seto, E. & Yoshida, M. Erasers of histone acetylation: the histone deacetylase enzymes. *Cold Spring Harb. Perspect. Biol.* **6,** a018713 (2014).

15. Witt, O., Deubzer, H. E., Milde, T. & Oehme, I. HDAC family: What are the cancer relevant targets? *Cancer Lett.* **277,** 8–21 (2009).

16. Teperino, R., Schoonjans, K. & Auwerx, J. Histone methyl-transferases and demethylases ; can they link metabolism and transcription ? *Cell Metab.* **12,** 321–327 (2010).

17. Helin, K. & Dhanak, D. Chromatin proteins and modifications as drug targets. *Nature* **502,** 480–8 (2013).

18. Binda, O. *et al.* Trimethylation of histone H3 lysine 4 impairs methylation of histone H3 lysine 9: regulation of lysine methyltransferases by physical interaction with their substrates. *Epigenetics* **5,** 767–75 (2010).

19. Greer, E. L. & Shi, Y. Histone methylation: a dynamic mark in health, disease and inheritance. *Nat. Rev. Genet.* **13,** 343–357 (2014).

20. Krauss, V. Glimpses of evolution: heterochromatic histone H3K9 methyltransferases left its marks behind. *Genetica* **133,** 93–106 (2008).

21. Fritsch, L. *et al.* A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multimeric complex. *Mol. Cell* **37,** 46–56 (2010).

22. Shankar, S. R. *et al.* G9a, a multipotent regulator of gene expression. *Epigenetics* **8,** 16–22 (2013).

23. Tachibana, M. *et al.* G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev.* **16,** 1779–1791 (2002).

24. Tachibana, M., Sugimoto, K., Fukushima, T. & Shinkai, Y. Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. *J. Biol. Chem.* **276,** 25309–17 (2001).

25. Mozzetta, C. *et al.* The histone H3 lysine 9 methyltransferases G9a and GLP regulate polycomb repressive complex 2-mediated gene silencing. *Mol. Cell* **53,** 277–89 (2014).

26. Tachibana, M. *et al.* Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9. *Genes Dev.* **19,** 815–826 (2005).

27. Chin, H. G. *et al.* Automethylation of G9a and its implication in wider substrate specificity and HP1 binding. *Nucleic Acids Res.* **35,** 7313–23 (2007).

28. Tachibana, M., Matsumura, Y., Fukuda, M., Kimura, H. & Shinkai, Y. G9a/GLP complexes independently mediate H3K9 and DNA methylation to silence transcription. *EMBO J.* **27,** 2681–90 (2008).

29. Kim, J. K., Estève, P.-O., Jacobsen, S. E. & Pradhan, S. UHRF1 binds G9a and participates in p21 transcriptional regulation in mammalian cells. *Nucleic Acids Res.* **37,** 493–505 (2009).

30. Estève, P. *et al.* Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Genes Dev.* **20,** 3089–3103 (2006).

31. Huang, J. *et al.* G9a and Glp methylate lysine 373 in the tumor suppressor p53. *J. Biol. Chem.* **285,** 9636–41 (2010).

32. Melcher, M. *et al.* Structure-Function Analysis of SUV39H1 Reveals a Dominant Role in Heterochromatin Organization, Chromosome Segregation, and Mitotic Progression. *Mol. Cell. Biol.* **20,** 3728–3741 (2000).

33. Chin, H. G., Patnaik, D., Este, P., Jacobsen, S. E. & Pradhan, S. Catalytic Properties and Kinetic Mechanism of Human Recombinant Lys-9 Histone H3 Methyltransferase SUV39H1 : Participation of the Chromodomain in Enzymatic catalysis. *Biochemistry* **45,** 3272–3284 (2006).

34. Peters, A. H. F. M. *et al.* Loss of the Suv39h Histone Methyltransferases Impairs Mammalian Heterochromatin and Genome Stability. **107,** 323–337 (2001).

35. Melcher, M. *et al.* Structure-Function Analysis of SUV39H1 Reveals a Dominant Role in Heterochromatin Organization, Chromosome Segregation, and Mitotic Progression. *Mol. Cell. Biol.* **20,** 3728–3741 (2000).

36. Bannister, A. J. *et al.* Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410,** 2–6 (2001).

37. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410,** 116–120 (2001).

38. Yamamoto, K. & Sonoda, M. Self-interaction of heterochromatin protein 1 is required for direct binding to histone methyltransferase, SUV39H1. *Biochem. Biophys. Res. Commun.* **301,** 287–292 (2003).

39. Stewart, M. D., Li, J. & Wong, J. Relationship between Histone H3 Lysine 9 Methylation , Transcription Repression , and Heterochromatin Protein 1 Recruitment. *Mol. Cell. Biol.* **25,** 2525–2538 (2005).

40. Rea, S. *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406,** 593–599 (2000).

41. Krouwels, I. M. *et al.* A glue for heterochromatin maintenance: stable SUV39H1 binding to heterochromatin is reinforced by the SET domain. *J. Cell Biol.* **170,** 537–49 (2005).

42. Schotta, G. *et al.* A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev.* **18,** 1251–1262 (2004).

43. Fuks, F., Hurd, P. J., Deplus, R. & Kouzarides, T. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res.* **31,** 2305–2312 (2003).

44. Martens, J. H. a *et al.* The profile of repeat-associated histone lysine methylation states in the mouse epigenome. *EMBO J.* **24,** 800–12 (2005).

45. García-Cao, M., O'Sullivan, R., Peters, A. H. F. M., Jenuwein, T. & Blasco, M. A. Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. *Nat. Genet.* **36,** 94–9 (2004).

46. Vajen, B. *et al.* Histone Methyltransferase Suv39h1 Deficiency Prevents Myc - Induced Chromosomal Instability in Murine Myeloid Leukemias. *Genes, Chromosom. cancer* **430,** 423–430 (2013).

47. Nielsen, S. J. *et al.* Rb targets histone H3 methylation and HP1 to promoters. *Nature* **412,** 561–565 (2001).

48. Vandel, L. *et al.* Transcriptional Repression by the Retinoblastoma Protein through the Recruitment of a Histone Methyltransferase. *Mol. Cell. Biol.* **21,** 6484–6494 (2001).

49. Vaquero, A. *et al.* SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. *Nature* **450,** 440–4 (2007).

50. Vaquero, A. The conserved role of sirtuins in chromatin regulation. *Int. J. Dev. Biol.* **53,** 303–22 (2009).

51. Murayama, A. *et al.* Epigenetic control of rDNA loci in response to intracellular energy status. *Cell* **133,** 627–39 (2008).

52. Vaute, O., Nicolas, E., Vandel, L. & Trouche, D. Functional and physical interaction between the histone methyl transferase Suv39H1 and histone deacetylases. *Nucleic Acids Res.* **30,** 475–481 (2002).

53. Reed-Inderbitzin, E. *et al.* RUNX1 associates with histone deacetylases and SUV39H1 to repress transcription. *Oncogene* **25,** 5777–5786 (2006).

54. Jang, H., Choi, D.-E., Kim, H., Cho, E.-J. & Youn, H.-D. Cabin1 represses MEF2 transcriptional activity by association with a methyltransferase, SUV39H1. *J. Biol. Chem.* **282,** 11172–9 (2007).

55. Mal, A. K. Histone methyltransferase Suv39h1 represses MyoD-stimulated myogenic differentiation. *EMBO J.* **25,** 3323–34 (2006).

56. Chuang, J.-Y., Chang, W.-C. & Hung, J.-J. Hydrogen peroxide induces Sp1 methylation and thereby suppresses cyclin B1 via recruitment of Suv39H1 and HDAC1 in cancer cells. *Free Radic. Biol. Med.* **51,** 2309–18 (2011).

57. Cherrier, T. *et al.* p21 WAF1 gene promoter is epigenetically silenced by CTIP2 and Suv39h1. *Oncogene* **28,** 3380–3389 (2012).

58. Dong, C. *et al.* Interaction with Suv39h1 is Critical for Snail-mediated E-cadherin Repression in Breast Cancer. *Oncogene* **32,** 1351–1362 (2013).

59. Zhang, Z.-C. *et al.* Suv39h1 mediates AP-2α-dependent inhibition of C/EBPα expression during adipogenesis. *Mol. Cell. Biol.* **34,** 2330–8 (2014).

60. Chen, L. *et al.* MDM2 recruitment of lysine methyltransferases regulates p53 transcriptional output. *EMBO J.* **29,** 2538–52 (2010).

61. Yang, Y. *et al.* CRL4B promotes tumorigenesis by coordinating with SUV39H1/HP1/DNMT3A in DNA methylation-based epigenetic silencing. *Oncogene* **34,** 104–18 (2015).

62. Fujita, N. *et al.* Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. *J. Biol. Chem.* **278,** 24132–8 (2003).

63. Crosio, C. *et al.* Mitotic Phosphorylation of Histone H3: Spatio-Temporal Regulation by Mammalian Aurora Kinases. *Mol. Cell. Biol.* **22,** 874–885 (2002).

64. Aagaard, L., Schmid, M., Warburton, P. & Jenuwein, T. Mitotic phosphorylation of SUV39H1 , a novel component of active centromeres , coincides with transient accumulation at mammalian centromeres. *J. Cell Sci.* **829,** 817–829 (2000).

65. Czvitkovich, S. *et al.* Over-expression of the SUV39H1 histone methyltransferase induces altered proliferation and differentiation in transgenic mice. *Mech. Dev.* **107,** 141–153 (2001).

66. Koch, B., Kueng, S., Ruckenbauer, C., Wendt, K. S. & Peters, J.-M. The Suv39h-HP1 histone methylation pathway is dispensable for enrichment and protection of cohesin at centromeres in mammalian cells. *Chromosoma* **117,** 199–210 (2008).

67. Park, J.-A. *et al.* Deacetylation and methylation at histone H3 lysine 9 (H3K9) coordinate chromosome condensation during cell cycle progression. *Mol. Cells* **31,** 343–9 (2011).

68. Chu, L. *et al.* SUV39 H 1 orchestrates temporal dynamics of centromeric methylation essential for faithful chromosome segregation in mitosis. *J. Mol. Cell Biol.* **4,** 331–340 (2012).

69. Wang, D. *et al.* Methylation of SUV39H1 by SET7/9 results in heterochromatin relaxation and genome instability. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 5516–21 (2013).

70. Ayrapetov, M. K., Gursoy-Yuzugullu, O., Xu, C., Xu, Y. & Price, B. D. DNA double-strand breaks promote methylation of histone H3 on lysine 9 and transient formation of repressive chromatin. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 9169–74 (2014).

71. Firestein, R., Cui, X., Huie, P. & Cleary, M. L. Set Domain-Dependent Regulation of Transcriptional Silencing and Growth Control by SUV39H1, a Mammalian Ortholog of Drosophila Su(var)3-9. *Mol. Cell. Biol.* **20,** 4900–4909 (2000).

72. Park, S. H., Yu, S. E., Chai, Y. G. & Jang, Y. K. CDK2-dependent phosphorylation of Suv39H1 is involved in control of heterochromatin replication during cell cycle progression. *Nucleic Acids Res.* **42,** 6196–6207 (2014).

73. Loyola, A. *et al.* The HP1alpha-CAF1-SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin. *EMBO Rep.* **10,** 769–75 (2009).

74. Zheng, H., Chen, L., Pledger, W. J., Fang, J. & Chen, J. p53 promotes repair of heterochromatin DNA by regulating JMJD2b and Suv39h1 expression. *Oncogene* **33,** 734–744 (2014).

75. Liu, B. *et al.* Depleting the methyltransferase Suv39h1 improves DNA repair and extends lifespan in a progeria mouse model. *Nat. Commun.* **4,** 1868 (2013).

76. Pickart, C. M. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* **70,** 503–533 (2001).

77. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67,** 425–79 (1998).

78. Weissman, A. M. Themes and variations on ubiquitylation. *Nat. Rev.* **2,** (2001).

79. McDowell, G. S. & Philpott, A. Non-canonical ubiquitylation: mechanisms and consequences. *Int. J. Biochem. Cell Biol.* **45,** 1833–42 (2013).

80. Behrends, C. & Harper, J. W. Constructing and decoding unconventional ubiquitin chains. *Nat. Struct. Mol. Biol.* **18,** 520–8 (2011).

81. Schnell, J. D. & Hicke, L. Non-traditional functions of ubiquitin and ubiquitin-binding proteins. *J. Biol. Chem.* **278,** 35857–60 (2003).

82. Hurley, J. H., Lee, S. & Prag, G. Ubiquitin-binding domains. *Biochem. J.* **399,** 361–372 (2006).

83. Jiang, J. *et al.* CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. *J. Biol. Chem.* **276,** 42938–44 (2001).

84. Dikshit, P. & Jana, N. R. The co-chaperone CHIP is induced in various stresses and confers protection to cells. *Biochem. Biophys. Res. Commun.* **357,** 761–5 (2007).

85. Murata, S., Minami, Y., Minami, M., Chiba, T. & Tanaka, K. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep.* **2,** 1133–8 (2001).

86. Esser, C., Scheffner, M. & Höhfeld, J. The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation. *J. Biol. Chem.* **280,** 27443–8 (2005).

87. Scaglione, K. M. *et al.* Ube2w and Ataxin-3 coordinately regulate the ubiquitin ligase CHIP. *Mol. Cell* **43,** 599–612 (2011).

88. Kim, S.-A., Yoon, J.-H., Kim, D.-K., Kim, S.-G. & Ahn, S.-G. CHIP interacts with heat shock factor 1 during heat stress. *FEBS Lett.* **579,** 6559–63 (2005).

89. Dai, Q. *et al.* CHIP activates HSF1 and confers protection against apoptosis and cellular stress. *EMBO J.* **22,** 5446–5458 (2003).

90. Qian, S.-B., McDonough, H., Boellmann, F., Cyr, D. M. & Patterson, C. CHIP-mediated stress recovery by sequential ubiquitination of substrates and Hsp70. *Nature* **440,** 551–555 (2006).

91. Luo, W. *et al.* Hsp70 and CHIP selectively mediate ubiquitination and degradation of hypoxia-inducible factor (HIF)-1alpha but Not HIF-2alpha. *J. Biol. Chem.* **285,** 3651–63 (2010).

92. Parsons, J. L. *et al.* CHIP-mediated degradation and DNA damage-dependent stabilization regulate base excision repair proteins. *Mol. Cell* **29,** 477–87 (2008).

93. Lee, J. H., Khadka, P., Baek, S. H. & Chung, I. K. CHIP promotes human telomerase reverse transcriptase degradation and negatively regulates telomerase activity. *J. Biol. Chem.* **285,** 42033–45 (2010).

94. Oh, K. H. *et al.* Control of AIF-mediated cell death by antagonistic functions of CHIP ubiquitin E3 ligase and USP2 deubiquitinating enzyme. *Cell Death Differ.* **18,** 1326–36 (2011).

95. Sisoula, C. & Gonos, E. S. CHIP E3 ligase regulates mammalian senescence by modulating the levels of oxidized proteins. *Mech. Ageing Dev.* **132,** 269–72 (2011).

96. Ronnebaum, S. M., Wu, Y., McDonough, H. & Patterson, C. The Ubiquitin Ligase CHIP Prevents SirT6 Degradation through noncanonical ubiquitination. *Mol. Cell. Biol.* **33,** 4461–4472 (2013).

97. Paul, I., Ahmed, S. F., Bhowmik, A., Deb, S. & Ghosh, M. K. The ubiquitin ligase CHIP regulates c-Myc stability and transcriptional activity. *Oncogene* **32,** 1284–1295 (2012).

98. Jang, K. W. *et al.* Ubiquitin ligase CHIP induces TRAF2 proteasomal degradation and NF-κB inactivation to regulate breast cancer cell invasion. *J. Cell. Biochem.* **112,** 3612–20 (2011).

99. Wang, Y. *et al.* CHIP / Stub1 functions as a tumor suppressor and represses NF-κ B-mediated signaling in colorectal cancer. *Carcinogenesis* **35,** 983–991 (2013).

100. Yang, M. *et al.* E3 ubiquitin ligase CHIP facilitates Toll-like receptor signaling by recruiting and polyubiquitinating Src and atypical PKC{zeta}. *J. Exp. Med.* **208,** 2099–112 (2011).

101. Wang, S. *et al.* STUB1 is essential for T-cell activation by ubiquitinating CARMA1. *Eur. J. Immunol.* 1034–1041 (2013). doi:10.1002/eji.201242554

102. Yu, X. *et al.* Chfr is required for tumor suppression and Aurora A regulation. *Nat. Genet.* **37,** 401–6 (2005).

103. Liu, C., Wu, J., Paudyal, S. C., You, Z. & Yu, X. CHFR is important for the first wave of ubiquitination at DNA damage sites. *Nucleic Acids Res.* **41,** 1698–710 (2013).

104. Kwon, Y. E., Bae, S. J., Kim, M. & Seol, J. H. SUMOylation negatively regulates the stability of CHFR tumor suppressor. *Biochem. Biophys. Res. Commun.* **430,** 213–7 (2013).

105. Bae, S. J., Kwon, Y. E., Kim, M. & Seol, J. H. CHFR is negatively regulated by SUMOylation-mediated ubiquitylation. *Biochem. Biophys. Res. Commun.* **433,** 194–9 (2013).

106. Kashima, L. *et al.* CHFR protein regulates mitotic checkpoint by targeting PARP-1 protein for ubiquitination and degradation. *J. Biol. Chem.* **287,** 12975–84 (2012).

107. Matsusaka, T. & Pines, J. Chfr acts with the p38 stress kinases to block entry to mitosis in mammalian cells. *J. Cell Biol.* **166,** 507–16 (2004).

108. Scolnick, D. M. & Halazonetis, T. D. Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature* **406,** 430–435 (2000).

109. Kim, J.-S. *et al.* The auto-ubiquitylation of E3 ubiquitin-protein ligase Chfr at G2 phase is required for accumulation of polo-like kinase 1 and mitotic entry in mammalian cells. *J. Biol. Chem.* **286,** 30615–23 (2011).

110. Kang, D., Chen, J., Wong, J. & Fang, G. The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J. Cell Biol.* **156,** 249–59 (2002).

111. Shinde, S. R., Gangula, N. R., Kavela, S., Pandey, V. & Maddika, S. TOPK and PTEN participate in CHFR mediated mitotic checkpoint. *Cell. Signal.* **25,** 2511–7 (2013).

112. Wu, J. *et al.* Chfr and RNF8 synergistically regulate ATM activation. *Nat. Struct. Mol. Biol.* **18,** 761–768 (2012).

113. Luijsterburg, M. S. & van Attikum, H. Close encounters of the RNF8th kind: when chromatin meets DNA repair. *Curr. Opin. Cell Biol.* **24,** 439–47 (2012).

114. Huen, M. S. Y. *et al.* The E3 ubiquitin ligase RNF8 transduces the DNA damage signal via an ubiquitin-dependent signaling pathway. *Cell* **131,** 901–914 (2008).

115. Kashima, L. *et al.* CHFR, a potential tumor suppressor, downregulates interleukin-8 through the inhibition of NF-kappaB. *Oncogene* **28,** 2643–53 (2009).

116. Oh, Y. M. *et al.* Chfr is linked to tumour metastasis through the downregulation of HDAC1. *Nat. Cell Biol.* **11,** 295–302 (2009).

117. Kim, J. M. *et al.* CHFR functions as a ubiquitin ligase for HLTF to regulate its stability and functions. *Biochem. Biophys. Res. Commun.* **395,** 515–20 (2010).

118. Teixeira, L. K. & Reed, S. I. Ubiquitin ligases and cell cycle control. *Annu. Rev. Biochem.* **82,** 387–414 (2013).

119. Peters, J.-M. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat. Rev. Mol. Cell Biol.* **7,** 644–56 (2006).

120. Craney, A. & Rape, M. Dynamic regulation of ubiquitin-dependent cell cycle control. *Curr. Opin. Cell Biol.* **25,** 704–10 (2013).

121. Wang, Z. *et al.* Skp2 is a promising therapeutic target in breast cancer. *Front. Oncol.* **1,** 1–10 (2012).

122. Okumura, F., Matsuzaki, M., Nakatsukasa, K. & Kamura, T. The Role of Elongin BC-Containing Ubiquitin Ligases. *Front. Oncol.* **2,** 10 (2012).

123. Chan, C.-H., Lee, S.-W., Wang, J. & Lin, H.-K. Regulation of Skp2 expression and activity and its role in cancer progression. *ScientificWorldJournal.* **10,** 1001–15 (2010).

124. Cardozo, T. & Pagano, M. The SCF ubiquitin ligase: insights into a molecular machine. *Nat. Rev. Mol. Cell Biol.* **5,** 739–51 (2004).

125. Wang, Z., Inuzuka, H., Zhong, J., Liu, P. & Sarkar, F. H. Identification of acetylation-dependent regulatory mechanisms that govern the oncogenic functions of Skp2 ABSTRACT : **3,** 1294–1300 (2012).

126. Lonjedo, M. *et al.* The Rho family member RhoE interacts with Skp2 and is degraded at the proteasome during cell cycle progression. *J. Biol. Chem.* **288,** 30872–82 (2013).

127. Carrano, A. C., Eytan, E., Hershko, A. & Pagano, M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. **1,** 193–199 (1999).

128. Yeh, K. H. *et al.* The F-box protein SKP2 binds to the phosphorylated threonine 380 in cyclin E and regulates ubiquitin-dependent degradation of cyclin E. *Biochem. Biophys. Res. Commun.* **281,** 884–90 (2001).

129. Nakayama, K. *et al.* Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *EMBO J.* **19,** 2069–81 (2000).

130. Kamura, T. *et al.* Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase. *Nat. Cell Biol.* **6,** 1229–35 (2004).

131. Nakayama, K. I. & Nakayama, K. Regulation of the cell cycle by SCF-type ubiquitin ligases. *Semin. Cell Dev. Biol.* **16,** 323–33 (2005).

132. Montagnoli, a. *et al.* Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev.* **13,** 1181–1189 (1999).

133. Ganoth, D. *et al.* The cell-cycle regulatory protein Cks1 is required for SCF Skp2 -mediated ubiquitinylation of p27. **3,** (2001).

134. Bornstein, G. *et al.* Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. *J. Biol. Chem.* **278,** 25752–7 (2003).

135. Méndez, J. *et al.* Human Origin Recognition Complex Large Subunit Is Degraded by Ubiquitin-Mediated Proteolysis after Initiation of DNA Replication. *Mol. Cell* **9,** 481–491 (2002).

136. Kondo, T. *et al.* Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCFSkp2 complex. *J. Biol. Chem.* **279,** 27315–9 (2004).

137. Wu, J. *et al.* Skp2 E3 ligase integrates ATM activation and homologous recombination repair by ubiquitinating NBS1. **46,** 351–361 (2013).

138. Chan, C. *et al.* The SKP2-SCF E3 ligase regulates Akt ubiquitination, glycolysis, Herceptin sensitivity and tumorigenesis. **149,** 1098–1111 (2013).

139. Wang, F. *et al.* Deacetylation of FOXO3 by SIRT1 or SIRT2 leads to Skp2-mediated FOXO3 ubiquitination and degradation. *Oncogene* **31,** 1546–57 (2012).

140. Kitagawa, M., Lee, S. H. & McCormick, F. Skp2 suppresses p53-dependent apoptosis by inhibiting p300. *Mol. Cell* **29,** 217–31 (2008).

141. Kim, S. Y. *et al.* Skp2 Regulates Myc Protein Stability and Activity The State University of New York at Stony Brook. **11,** 1177–1188 (2003).

142. Bashir, T., Dorrello, N. V., Amador, V., Guardavaccaro, D. & Pagano, M. Control of the SCF SKP2-Cks1ubiquitin ligase by the APC / C Cdh1 ubiquitin ligase. *Nature* **428,** 2–5 (2004).

143. Wei, W., Ayad, N. G., Wan, Y. & Zhang, G. Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. **428,** 2–6 (2004).

144. Ecker, K. & Hengst, L. Skp2: caught in the Akt. *Nat. Cell Biol.* **11,** 377–379 (2009).

145. Gao, D. *et al.* Phosphorylation by Akt1 promotes cytoplasmic localization of Skp2 and impairs APCCdh1-mediated Skp2 destruction. *Nat. Cell Biol.* **11,** 397–408 (2009).

146. Inuzuka, H. *et al.* Acetylation-dependent regulation of Skp2 function. *Cell* **150,** 179–193 (2012).

147. Bretones, G. *et al.* SKP2 oncogene is a direct MYC target gene and MYC down-regulates p27(KIP1) through SKP2 in human leukemia cells. *J. Biol. Chem.* **286,** 9815–25 (2011).

148. Yung, Y., Walker, J. L., Roberts, J. M. & Assoian, R. K. A Skp2 autoinduction loop and restriction point control. *J. Cell Biol.* **178,** 741–7 (2007).

149. Jonason, J. H., Gavrilova, N., Wu, M., Zhang, H. & Sun, H. Regulation of SCFSKP2 Ubiquitin E3 Ligase Assembly and p27KIP1 Proteolysis by the PTEN Pathway and Cyclin D1. *Cell Cycle* **6,** 951–961 (2007).

150. Tsang, A. W. & Escalante-Semerena, J. C. CobB, a New Member of the SIR2 Family of Eucaryotic Regulatory Proteins, Is Required to Compensate for the Lack of Nicotinate Mononucleotide:5,6-Dimethylbenzimidazole Phosphoribosyltransferase Activity in cobT Mutants during Cobalamin Biosynthesis in Salm. *J. Biol. Chem.* **273,** 31788–31794 (1998).

151. Michan, S. & Sinclair, D. Sirtuins in mammals□: insights into their biological function. **404,** 1–13 (2009).

152. Bosch-Presegué, L. & Vaquero, A. The dual role of sirtuins in cancer. *Genes Cancer* **2,** 648–62 (2011).

153. Vaquero, A. *et al.* Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Mol. Cell* **16,** 93–105 (2004).

154. Oberdoerffer, P. *et al.* DNA damage-induced alterations in chromatin contribute to genomic integrity and age-related changes in gene expression. *Cell* **135,** 907–918 (2008).

155. Bosch-presegué, L. *et al.* Stabilization of Suv39H1 by SirT1 Is Part of Oxidative Stress Response and Ensures Genome Protection. *Mol. Cell* **42,** 210–223 (2011).

156. Lin, S., Defossez, P. & Guarente, L. Requirement of NAD and SIR2 for Life-Span Extension by Calorie Restriction in Saccharomyces cerevisiae. *Science (80-.).* **289,** 2126–2129 (2000).

157. Katto, J., Engel, N., Abbas, W., Herbein, G. & Mahlknecht, U. Transcription factor NF κ B regulates the expression of the histone deacetylase SIRT1. *Clin. Epigenetics* **5,** 1–9 (2013).

158. Salminen, A., Kaarniranta, K. & Kauppinen, A. Crosstalk between Oxidative Stress and SIRT1□: Impact on the Aging Process. *Int. J. Mol. Sci* **14,** 3834–3859 (2013).

159. Kim, J.-E., Chen, J. & Lou, Z. DBC1 is a negative regulator of SIRT1. *Nature* **451,** 583–6 (2008).

160. Zhao, W. *et al.* Negative regulation of the deacetylase SirT1 by DBC1. *Nature* **451,** 587–590 (2008).

161. Yuan, Z., Zhang, X., Sengupta, N., Lane, W. S. & Seto, E. SirT1 regulates the function of the Nijmegen breakage syndrome protein. *Mol. Cell* **27,** 149–162 (2007).

162. Madabushi, A., Hwang, B.-J., Jin, J. & Lu, A.-L. Histone deacetylase SIRT1 modulates and deacetylates DNA base excision repair enzyme thymine DNA glycosylase. *Biochem. J.* **456,** 89–98 (2013).

163. Uhl, M. *et al.* Role of SIRT1 in homologous recombination. *DNA Repair (Amst).* **9,** 383–93 (2010).

164. Peng, L. *et al.* SIRT1 negatively regulates the activities, functions, and protein levels of hMOF and TIP60. *Mol. Cell. Biol.* **32,** 2823–36 (2012).

165. Palacios, J. a *et al.* SIRT1 contributes to telomere maintenance and augments global homologous recombination. *J. Cell Biol.* **191,** 1299–313 (2010).

166. Giannakou, M. E. & Partridge, L. The interaction between FOXO and SirT1: tipping the balance towards survival. *Trends Cell Biol.* **14,** 408–412 (2004).

167. Brunet, A. *et al.* Stress-Dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase. *Science (80-.).* **303,** 2011–2016 (2004).

168. Luo, J. *et al.* Negative Control of p53 by Sir2a Promotes Cell Survival under Stress. *Cell* **107,** 137–148 (2001).

169. Vaziri, H. *et al.* hSIR2 SIRT1 Functions as an NAD-Dependent p53 Deacetylase. *Cell* **107,** 149–159 (2001).

170. Cohen, H. Y. *et al.* Calorie Restriction Promotes Mammalian Cell Survival by Inducing the SIRT1 Deacetylase. *Science (80-.).* **305,** 390–392 (2004).

171. Nogueiras, R. *et al.* Sirtuin 1 and Sirtuin 3: physiological modulators of metabolism. *Physiol Rev.* **92,** 1479–1514 (2012).

172. Picard, F. *et al.* SirT1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* **429,** (2004).

173. Rahman, S. & Islam, R. Mammalian Sirt1: insights on its biological functions. *Cell Commun. Signal.* **9,** 11 (2011).

174. Rodgers, J. T. *et al.* Nutrient control of glucose homeostasis through a complex of PGC-1 a and SIRT1. *Nature* **434,** 3–8 (2005).

175. Yeung, F. *et al.* Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J.* **23,** 2369–80 (2004).

176. Liu, T. F. & McCall, C. E. Deacetylation by SIRT1 Reprograms Inflammation and Cancer. *Genes Cancer* **4,** 135–47 (2013).

177. Kauppinen, A., Suuronen, T., Ojala, J., Kaarniranta, K. & Salminen, A. Antagonistic crosstalk between NF- κ B and SIRT1 in the regulation of in fl ammation and metabolic disorders. *Cell. Signal.* **25,** 1939–1948 (2013).

178. Vaquero, A. *et al.* SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev.* **20,** 1256–1261 (2006).

179. Serrano, L. *et al.* The tumor suppressor SirT2 regulates cell cycle progression and genome stability by modulating the mitotic deposition of H4K20 methylation. *Genes Dev.* **27,** 639–653 (2013).

180. Kim, H. *et al.* SirT2 maintains genome integrity and suppresses tumorigenes through regulating APC/C activity. *Cancer Cell* **20,** 487–499 (2011).

181. Dryden, S. C., Nahhas, F. a., Nowak, J. E., Goustin, a.-S. & Tainsky, M. a. Role for Human SIRT2 NAD-Dependent Deacetylase Activity in Control of Mitotic Exit in the Cell Cycle. *Mol. Cell. Biol.* **23,** 3173–3185 (2003).

182. North, B. J. & Verdin, E. Mitotic regulation of SIRT2 by cyclin-dependent kinase 1-dependent phosphorylation. *J. Biol. Chem.* **282,** 19546–55 (2007).

183. Pandithage, R. *et al.* The regulation of SIRT2 function by cyclin-dependent kinases affects cell motility. *J. Cell Biol.* **180,** 915–29 (2008).

184. Wang, F., Nguyen, M., Qin, F. X.-F. & Tong, Q. SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. *Aging Cell* **6,** 505–14 (2007).

185. Jing, E., Gesta, S. & Kahn, R. C. SirT2 regulates adipocyte differentiation involving FoxO1 acetylation/deacetylation. *Cell Metab.* **6,** 105–114 (2007).

186. Rothgiesser, K. M., Erener, S., Waibel, S., Lüscher, B. & Hottiger, M. O. SIRT2 regulates NF-κB dependent gene expression through deacetylation of p65 Lys310. *J. Cell Sci.* **123,** 4251–8 (2010).

187. North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M. & Verdin, E. The human Sir2 ortholog, SirT2, is an NAD+-dependent tubulin deacetylase. *Mol. Cell* **11,** 437–444 (2003).

188. Scher, M. B., Vaquero, A. & Reinberg, D. SirT3 is a nuclear NAD + -dependent histone deacetylase that translocates to the mitochondria upon cellular stress. *Genes Dev.* **21,** 920–928 (2007).

189. Iwahara, T., Bonasio, R., Narendra, V. & Reinberg, D. SIRT3 functions in the nucleus in the control of stress-related gene expression. *Mol. Cell. Biol.* **32,** 5022–34 (2012).

190. Giralt, A. & Villarroya, F. SIRT3, a pivotal actor in mitochondrial functions: metabolism, cell death and aging. *Biochem. J.* **444,** 1–10 (2012).

191. Schwer, B., Bunkenborg, J., Verdin, R. O., Andersen, J. S. & Verdin, E. Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 10224–9 (2006).

192. Haigis, M. C. *et al.* SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. *Cell* **126,** 941–54 (2006).

193. Nasrin, N. *et al.* SIRT4 regulates fatty acid oxidation and mitochondrial gene expression in liver and muscle cells. *J. Biol. Chem.* **285,** 31995–2002 (2010).

194. Dong, K., Pelle, E., Yarosh, D. B. & Pernodet, N. Sirtuin 4 identification in normal human epidermal keratinocytes and its relation to sirtuin 3 and energy metabolism under normal conditions and UVB-induced stress. *Exp. Dermatol.* **21,** 221–235 (2012).

195. Verma, M., Shulga, N. & Pastorino, J. G. Sirtuin-4 modulates sensitivity to induction of the mitochondrial permeability transition pore. *Biochem. Biophys. Acta* **1827,** 38–49 (2013).

196. Matsushita, N. *et al.* Distinct regulation of mitochondrial localization and stability of two human Sirt5 isoforms. *Genes Cells* **16,** 190–202 (2011).

197. Nakagawa, T. & Guarente, L. Urea cycle regulation by mitochondrial sirtuin , SIRT5. *Aging (Albany. NY).* **1,** 578–582 (2009).

198. Nakagawa, T., Lomb, D. J., Haigis, M. C. & Guarente, L. SirT5 deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. *Cell* **137,** 560–570 (2009).

199. Du, J. *et al.* SirT5 is an NAD-Dependent protein lysine demalonylase and desuccinylase. *Science (80-.).* **334,** 806–809 (2011).

200. Ford, E. *et al.* Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. *Genes Dev.* **20,** 1075–1080 (2006).

201. Grob, A. *et al.* Involvement of SIRT7 in resumption of rDNA transcription at the exit from mitosis. *J. Cell Sci.* **122,** 489–98 (2009).

202. Tsai, Y.-C., Greco, T. M., Boonmee, A., Miteva, Y. & Cristea, I. M. Functional Proteomics Establishes the Interaction of SIRT7 with Chromatin Remodeling Complexes and Expands Its Role in Regulation of RNA Polymerase I Transcription. *Mol. Cell. Proteomics* **11,** 60–76 (2012).

203. Barber, M. F. *et al.* SirT7 links H3K18 deacetylation to maintenance of oncogenic transformation. *Nature* **487,** 114–118 (2012).

204. Liszt, G., Ford, E., Kurtev, M. & Guarente, L. Mouse Sir2 Homolog SIRT6 Is a Nuclear ADP-ribosyltransferase *. **280,** 21313–21320 (2005).

205. Mostoslavsky, R. *et al.* Genomic Instability and Aging-like Phenotype in the Absence of Mammalian SIRT6. 315–329 (2006). doi:10.1016/j.cell.2005.11.044

206. Kugel, S. & Mostoslavsky, R. Chromatin and beyond □: the multitasking roles for SIRT6. *Trends Biochem. Sci.* **39,** 72–81 (2014).

207. Tennen, R. I., Berber, E. & Chua, K. F. Functional dissection of SIRT6 □: Identification of domains that regulate histone deacetylase activity and chromatin localization. *Mech. Ageing Dev.* **131,** 185–192 (2010).

208. Michishita, E. *et al.* SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. **452,** 492–497 (2008).

209. Feldman, J. L., Baeza, J. & Denu, J. M. Activation of the Protein Deacetylase SIRT6 by Long-chain Fatty Acids and Widespread Deacylation by Mammalian. **288,** 31350–31356 (2013).

210. Grimley, R. *et al.* Over expression of wild type or a catalytically dead mutant of Sirtuin 6 does not influence NFκB responses. *PLoS One* **7,** e39847 (2012).

211. Pan, P. W. *et al.* Structure and Biochemical Functions of SIRT6 * □. **286,** 14575–14587 (2011).

212. Gil, R. *et al.* SIRT6 exhibits nucleosome-dependent deacetylase activity. *Nucleic Acids Res.* **41,** 8537–8545 (2013).

213. Jiang, H. *et al.* SirT6 regulates TNF-a secretion through hydrolysis of long-chain fatty acyl lysine. *Nature* **496,** 110–113 (2013).

214. Hu, S. *et al.* Posttranslational modification of Sirt6 activity by peroxynitrite. *Free Radic. Biol. Med.* **79,** 176–185 (2015).

215. Kokkonen, P. *et al.* Peptides and Pseudopeptides as SIRT6 Deacetylation Inhibitors. *Med. chamistry Lett.* 4–9 (2012).

216. Mao, Z. *et al.* SIRT6 Promotes DNA Repair Under Stress by Activating PARP1. *Science (80-.).* **332,** 1443–1446 (2011).

217. Miteva, Y. V & Cristea, I. M. A Proteomic Perspective of Sirtuin 6 (SIRT6) Phosphorylation and Interactions and Their Dependence on Its Catalytic Activity * □. 168–183 (2013). doi:10.1074/mcp.M113.032847

218. Kanfi, Y. *et al.* Regulation of SIRT6 protein levels by nutrient availability. **582,** 543–548 (2008).

219. Ardestani, P. M. & Liang, F. Sub-cellular localization , expression and functions of Sirt6 during the cell cycle in HeLa cells. *Nucleus* **3,** 442–451 (2012).

220. Thirumurthi, U. *et al.* MDM2-mediated degradation of SIRT6 phosphorylated by AKT1 promotes tumorigenesis and trastuzumab resistance in breast cancer. *Sci. Signal.* **7,** 1–10 (2014).

221. Lin, Z. *et al.* USP10 Antagonizes c-Myc Transcriptional Activation through SIRT6 Stabilization to Suppress Tumor Formation. *CellReports* **5,** 1639–1649 (2013).

222. Kanfi, Y. *et al.* The sirtuin SIRT6 regulates lifespan in male mice. *Nature* **483,** 218–221 (2012).

223. Yuan, R. *et al.* Aging in inbred strains of mice: study design and interim report on median lifespans and circulating IGF1 levels. *Aging Cell* **8,** 277–87 (2009).

224. Van Meter, M. *et al.* SirT6 represses LINE1 retrotransposons by ribosylating KAP1 but this repression fails with stress and age. *Nat. Commun.* **5,** 1–10 (2014).

225. Simeoni, F. *et al.* Proteomic analysis of the SIRT6 interactome □: novel links to genome maintenance and cellular stress signaling. 1–6 (2013). doi:10.1038/srep03085

226. Toiber, D. *et al.* SirT6 recruits SNF2H to DNA break sites, preventing genomic instability through chromatin remodeling. *Mol. Cell* **51,** 1–15 (2013).

227. Battu, A., Ray, A. & Wani, A. a. ASF1A and ATM regulate H3K56-mediated cell-cycle checkpoint recovery in response to UV irradiation. *Nucleic Acids Res.* **39,** 7931–45 (2011).

228. Michishita, E. *et al.* Cell cycle-dependent deacetylation of telomeric histone H3 lysine K56 by human SIRT6. *Cell Cycle* **8,** 2664–2666 (2009).

229. Yang, B., Zwaans, B. M. M., Eckersdorff, M. & Lombard, D. B. The sirtuin SirT6 deacetylates H3K56ac in vivi to promote genomic stability. **8,** 2662–2663 (2009).

230. Mao, Z. *et al.* Sirtuin 6 (SIRT6) rescues the decline of homologous recombination repair during replicative senescence. **6,** (2012).

231. Lombard, B. Sirtuins at the breaking point : SIRT6 in DNA repair. **1,** 2–6 (2009).

232. Mccord, A. *et al.* SirT6 stabilizes DNA-dependent protein kinase at chromatin for DNA double-strand break repair. *Ageing* **1,** 109–121 (2009).

233. Kaidi, A., Weinert, B. T., Choudhary, C. & Jackson, S. P. Human SirT6 promotes DNA End Resection through CtIP deacetylation. **329,** 1348–1354 (2010).

234. Tennen, R. I., Bua, D. J., Wright, W. E. & Chua, K. F. SIRT6 is required for maintenance of telomere position effect in human cells. *Nat. Commun.* **2,** 433–437 (2011).

235. Kawahara, T. L. A. *et al.* SirT6 links histone H3 lysine 9 deacetylation to NF-kB-dependent gene expression and organismal lifespan. **136,** 62–74 (2009).

236. Kawahara, T. L. A. *et al.* Dynamic Chromatin Localization of Sirt6 Shapes Stress- and Aging-Related Transcriptional Networks. *PLoS Genet.* **7,** 1–12 (2011).

237. Polyakova, O. *et al.* Identification of novel interacting partners of Sirtuin6. *PLoS One* **7,** e51555 (2012).

238. Zhong, L. *et al.* The histone deacetylase SirT6 regulates glucose homeostasis via Hif1a. *Cell* **140,** 280–293 (2010).

239. Xiao, C. *et al.* SIRT6 Deficiency Results in Severe Hypoglycemia by Enhancing Both Basal and Insulin-stimulated Glucose Uptake. **285,** 36776–36784 (2010).

240. Zhang, P. *et al.* Tumor suppressor p53 cooperates with SIRT6 to regulate gluconeogenesis by promoting FoxO1 nuclear exclusion. **111,** (2014).

241. Kanfi, Y. *et al.* SirT6 protects against pathological damage caused by diet-induced obesity. **9,** 162–173 (2010).

242. Kim, H. *et al.* Hepatic-specific disruption of SirT6 in mice results in fatty liver formation due to enhanced glycolysis and triglyceride synthesis. *Cell Metab.* **12,** 224–236 (2010).

243. Tao, R., Xiong, X., Depinho, R. A., Deng, C. & Dong, X. C. Hepatic SREBP-2 and cholesterol biosynthesis are regulated by FoxO3 and Sirt6. *J. Lipid Res.* **54,** 2745–2753 (2013).

244. Tao, R., Xiong, X., Depinho, R. A., Deng, C. & Dong, X. C. FoxO3 Transcription Factor and Sirt6 Deacetylase Regulate Low Density Lipoprotein (LDL) - cholesterol Homeostasis via Control of the Proprotein Convertase Subtilisin / Kexin Type 9 (Pcsk9) Gene Expression *. *J. Biol. Chem.* **288,** 29252–29259 (2013).

245. Yang, S. J. *et al.* Activation of Peroxisome Proliferator-Activated Receptor Gamma by Rosiglitazone Increases Sirt6 Expression and Ameliorates Hepatic Steatosis in Rats. **6,** (2011).

246. Dominy, J. E. *et al.* The deacetylase SirT6 activates the acetyltransferase GCN5 and suppresses hepatic gluconeogenesis. *Mol. Cell* **48,** 1–14 (2012).

247. Liu, T. F., Vachharajani, V. T., Yoza, B. K. & Mccall, C. E. NAD+ -dependent Sirtuin 1 and 6 Proteins Coordinate a Switch from Glucose to Fatty Acid Oxidation during the Acute Inflammatory Response. **287,** 25758–25769 (2012).

248. Xiao, C. *et al.* Progression of Chronic Liver Inflammation and Fibrosis Driven by Activation of c-JUN Signaling in Sirt6 Mutant Mice * □. (2012). doi:10.1074/jbc.M112.415182

249. Min, L. *et al.* Liver cancer initiation is controlled by AP-1 through SIRT6-dependent inhibition of survivin. *Nat. Cell Biol.* **14,** 1203–1211 (2012).

250. Cai, Y. *et al.* Nmnat2 protects cardiomyocytes from hypertrophy via activation of SIRT6. *FEBS Lett.* **586,** 866–874 (2012).

251. Sundaresan, N. R. *et al.* The sirtuin SIRT6 blocks IGF-Akt signaling and development of cardiac hypertrophy by targeting c-Jun. *Nat. Med.* **18,** (2012).

252. Yu, S., Cai, Y., Ye, J., Pi, R. & Chen, S. Sirtuin 6 protects cardiomyocytes from hypertrophy in vitro via inhibition of NF- k B-dependent transcriptional activity. (2013). doi:10.1111/j.1476-5381.2012.01903.x

253. Tian, K. *et al.* Sirtuin-6 inhibits cardiac fibroblasts differentiation into myofibroblasts via inactivation of nuclear factor &kappaB signaling. *Transl. Res.* 1–13 (2014). doi:10.1016/j.trsl.2014.08.008

254. Cardus, A., Uryga, A. K., Walters, G. & Erusalimsky, J. D. SIRT6 protects human endothelial cells from DNA damage , telomere dysfunction and senescence. *Cardiovasc. Res.* 571–579 (2012). doi:10.1093/cvr/cvs352

255. Ma, W. *et al.* GCIP / CCNDBP1 , a Helix – Loop – Helix Protein , Suppresses Tumorigenesis. **1386,** 1376–1386 (2007).

256. Sebastián, C. *et al.* The histone deacetylase SirT6 is a tumor suppressor that controls cancer metabolism. **151,** 1185–1199 (2012).

257. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg Effect: the metabolic requirements of cell proliferation. *Science (80-.).* **324,** 1029–1033 (2009).

258. Meter, M. Van, Mao, Z., Gorbunova, V. & Seluanov, A. SIRT6 overexpression induces massive apoptosis in cancer cells but not in normal cells. **10,** 3153–3158 (2011).

259. Chen, X. *et al.* The histone deacetylase SIRT6 suppresses the expression of the RNA-binding protein PCBP2 in glioma. *Biochem. Biophys. Res. Commun.* **446,** 364–369 (2014).

260. Han, Z., Liu, L., Liu, Y. & Li, S. Sirtuin SIRT6 suppresses cell proliferation through inhibition of Twist1 expression in non-small cell lung cancer. **7,** 4774–4781 (2014).

261. Ming, M., Han, W., Zhao, B., Sundaresan, N. R. & Deng, C. SIRT6 Promotes COX-2 Expression and Acts as an Oncogene in Skin Cancer. 5925–5934 (2014). doi:10.1158/0008-5472.CAN-14-1308

262. Bauer, I. *et al.* The NAD -dependent Histone Deacetylase SIRT6 Promotes Cytokine Production and Migration in Pancreatic Cancer Cells by Regulating Ca 2 Responses *. **288,** 40924–40937 (2012).

263. Oeckinghaus, A. & Ghosh, S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb. Perspect. Biol.* **1,** a000034 (2009).

264. Salminen, A. & Kaarniranta, K. NF-kappaB signaling in the aging process. *J. Clin. Immunol.* **29,** 397–405 (2009).

265. Hoesel, B. & Schmid, J. a. The complexity of NF-κB signaling in inflammation and cancer. *Mol. Cancer* **12,** 86 (2013).

266. Ghosh, S. & Hayden, M. S. Celebrating 25 years of NF-kB research. *Immunol. Rev.* **246,** 5–13 (2012).

267. Hayden, M. S. & Ghosh, S. Signaling to NF- B. 2195–2224 (2004). doi:10.1101/gad.1228704.bone

268. Ghosh, S. & Hayden, M. S. New regulators of NF-kB in inflammation. *Nat. Rev.* **8,** (2008).

269. Fang, L. *et al.* ATM regulates NF-κB-dependent immediate-early genes via RelA Ser 276 phosphorylation coupled to CDK9 promoter recruitment. *Nucleic Acids Res.* **42,** 8416–32 (2014).

270. Wu, Z.-H. & Miyamoto, S. Induction of a pro-apoptotic ATM-NF-kappaB pathway and its repression by ATR in response to replication stress. *EMBO J.* **27,** 1963–73 (2008).

271. Volcic, M. *et al.* NF-κB regulates DNA double-strand break repair in conjunction with BRCA1-CtIP complexes. *Nucleic Acids Res.* **40,** 181–95 (2012).

272. Sen, R. & Smale, S. T. Selectivity of the NF- k B Response. 1–15 (2009).

273. Natoli, G. Control of NF-kappaB-dependent transcriptional responses by chromatin organization. *Cold Spring Harb. Perspect. Biol.* **1,** a000224 (2009).

274. Saccani, S., Pantano, S. & Natoli, G. Two waves of nuclear factor kappaB recruitment to target promoters. *J. Exp. Med.* **193,** 1351–9 (2001).

275. Gao, Z. *et al.* Coactivators and corepressors of NF-kappaB in IkappaB alpha gene promoter. *J. Biol. Chem.* **280,** 21091–8 (2005).

276. Liebert, M. A. *et al.* Regulation of the gadd45 b Promoter by NF- k B. **21,** 491–503 (2002).

277. Ainbinder, E. *et al.* Mechanism of Rapid Transcriptional Induction of Tumor Necrosis Factor Alpha-Responsive Genes by NF- B. *Mol. Cell. Biol.* **22,** 6354–6362 (2002).

278. Nowak, D. E. *et al.* RelA Ser276 phosphorylation is required for activation of a subset of NF-kappaB-dependent genes by recruiting cyclin-dependent kinase 9/cyclin T1 complexes. *Mol. Cell. Biol.* **28,** 3623–38 (2008).

279. Wang, V. Y.-F. *et al.* The transcriptional specificity of NF-κB dimers is coded within the κB DNA response elements. *Cell Rep.* **2,** 824–39 (2012).

280. Handschick, K. *et al.* Cyclin-dependent kinase 6 is a chromatin-bound cofactor for NF-κB-dependent gene expression. *Mol. Cell* **53,** 193–208 (2014).

281. Kim, J.-W. *et al.* New molecular bridge between RelA/p65 and NF-κB target genes via histone acetyltransferase TIP60 cofactor. *J. Biol. Chem.* **287,** 7780–91 (2012).

282. Gerritsen, M. E. *et al.* CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2927–2932 (1997).

283. Sheppard, K. *et al.* Transcriptional Activation by NF-kB Requires Multiple Coactivators. *Mol. Cell. Biol.* **19,** 6367–6378 (1999).

284. Lim, C.-A. *et al.* Genome-wide mapping of RELA(p65) binding identifies E2F1 as a transcriptional activator recruited by NF-kappaB upon TLR4 activation. *Mol. Cell* **27,** 622–35 (2007).

285. Ashburner, B. P., Westerheide, S. D. & Baldwin, a S. The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Mol. Cell. Biol.* **21,** 7065–77 (2001).

286. Espinosa, L., Bigas, A. & Mulero, M. C. Alternative nuclear functions for NF-kB family members. *Am. J. Cancer Res.* **1,** 446–459 (2011).

287. Mulero, M. C. *et al.* Chromatin-bound Ikbalpha regulates a subset of Polycomb target genes in differentiation and cancer. *Cancer Cell* **24,** 151–166 (2013).

288. Huang, W.-C. & Hung, M.-C. Beyond NF-κB activation: nuclear functions of IκB kinase α. *J. Biomed. Sci.* **20,** 3 (2013).

289. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T. & Toriumi, W. IkB Kinases Phosphorylate NF-kB p65 Subunit on Serine 536 in the Transactivation Domain. *J. Biol. Chem.* **274,** 30353–30356 (1999).

290. Anest, V. *et al.* A nucleosomal function for IkB kinase-alpha in NF-kB-dependent gene expression. *Nature* **423,** 660–663 (2003).

291. Yamamoto, Y., Verma, U. N., Prajapati, S., Kwak, Y.-T. & Gaynor, R. B. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature* **423,** 655–9 (2003).

292. Zhu, F. *et al.* IKKalpha shields 14-3-3sigma, a G(2)/M cell cycle checkpoint gene, from hypermethylation, preventing its silencing. *Mol. Cell* **27,** 214–27 (2007).

293. Perkins, N. D. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* **25,** 6717–30 (2006).

294. Huang, B., Yang, X., Lamb, A. & Chen, L.-F. Posttranslational modifications of NF-kB: another layer of regulation for NF-kB signaling pathway. *Cell signal* **22,** 90–109 (2011).

295. Kauppinen, T. M., Gan, L. & Swanson, R. A. Poly(ADP-ribose) polymerase-1-induced NAD+ depletion promotes nuclear factor-kB transcriptional activity by preventing p65 de-acetylation. *Biochi* **1833,** 1985–1991 (2013).

296. Rothgiesser, K. M., Fey, M. & Hottiger, M. O. Acetylation of p65 at lysine 314 is important for late NF-kappaB-dependent gene expression. *BMC Genomics* **11,** 22 (2010).

297. Ziesché, E. *et al.* The coactivator role of histone deacetylase 3 in IL-1-signaling involves deacetylation of p65 NF-κB. *Nucleic Acids Res.* **41,** 90–109 (2013).

298. Maine, G. N. & Burstein, E. COMMD Proteins and the Control of the NF κ B Pathway ND SC ND ES RIB. 672–676 (2007).

299. Mao, X. *et al.* GCN5 is a required cofactor for a ubiquitin ligase that targets NF-kB/RelA. *Genes Dev.* **23,** 849–861 (2009).

300. Colleran, A. *et al.* Deubiquitination of NF-κB by Ubiquitin-Specific Protease-7 promotes transcription. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 618–23 (2013).

301. Wei, H. *et al.* PRMT5 dimethylates R30 of the p65 subunit to activate NF-κB. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 13516–21 (2013).

302. Lu, T. *et al.* Role of lysine methylation of NF-κB in differential gene regulation. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 13510–5 (2013).

303. Yang, X.-D., Tajkhorshid, E. & Chen, L.-F. Functional interplay between acetylation and methylation of the RelA subunit of NF-kappaB. *Mol. Cell. Biol.* **30,** 2170–80 (2010).

304. Janbandhu, V. C., Singh, A. K., Mukherji, A. & Kumar, V. p65 Negatively regulates transcription of the cyclin E gene. *J. Biol. Chem.* **285,** 17453–64 (2010).

305. Hinz, M. *et al.* NF-kB Function in Growth Control□: Regulation of Cyclin D1 Expression and G0/G1-to-S-Phase Transition. *Mol. Cell. Biol.* **19,** 2690–2698 (1999).

306. Kaltschmidt, B., Kaltschmidt, C., Hehner, S. P., Dro, W. & Schmitz, M. L. Repression of NF- k B impairs HeLa cell proliferation by functional interference with cell cycle checkpoint regulators. *Oncogene* **18,** 3213–3225 (1999).

307. Wang, X. *et al.* Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *J. Cell Biol.* **177,** 613–24 (2007).

308. Brasacchio, D. *et al.* Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that coexist on the lysine tail. *Diabetes* **58,** 1229–36 (2009).

309. Cao, R. & Zhang, Y. The functions of E (Z)/ EZH2-mediated methylation of lysine 27 in histone H3. *Curr. Opin. Genet. Dev.* **14,** 155–164 (2004).

310. Jedrusik-bode, M. *et al.* The sirtuin SIRT6 regulates stress granule formation in C . elegans and mammals. *J. Cell Sci.* **9,** 5166–5177 (2011).

311. Basta, J. & Rauchman, M. The nucleosome remodeling and deacetylase complex in development and disease. *Transl. Res.* **165,** 36–47 (2014).

312. Liang, Y.-C., Hsu, C.-Y., Yao, Y.-L. & Yang, W.-M. PARP-2 regulates cell cycle-related genes through histone deacetylation and methylation independently of poly(ADP-ribosyl)ation. *Biochem. Biophys. Res. Commun.* **431,** 58–64 (2013).

313. Moore, K. E. *et al.* A general molecular affinity strategy for global detection and proteomic analysis of lysine methylation. *Mol. Cell* **50,** 444–456 (2013).

314. Ea, C.-K., Hao, S., Yeo, K. S. & Baltimore, D. EHMT1 protein binds to nuclear factor-κB p50 and represses gene expression. *J. Biol. Chem.* **287,** 31207–17 (2012).

315. Chen, X., El Gazzar, M., Yoza, B. K. & McCall, C. E. The NF-kappaB factor RelB and histone H3 lysine methyltransferase G9a directly interact to generate epigenetic silencing in endotoxin tolerance. *J. Biol. Chem.* **284,** 27857–65 (2009).

316. Levy, D. *et al.* SETD6 lysine methylation of RelA couples GLP activity at chromatin to tonic repression of NF-κ B signaling. *Nat. Immunol.* **12,** 29–36 (2011).

317. Keating, S. & El-osta, A. Transcriptional regulation by the Set7 lysine methyltransferase. *Epigenetics* **8,** 361–372 (2013).

318. Van Essen, D., Zhu, Y. & Saccani, S. A feed-forward circuit controlling inducible NF-κB target gene activation by promoter histone demethylation. *Mol. Cell* **39,** 750–60 (2010).

319. Sadowski, M., Suryadinata, R., Tan, A. R., Roesley, S. N. A. & Sarcevic, B. Protein monoubiquitination and polyubiquitination generate structural diversity to control distinct biological processes. *IUBMB Life* **64,** 136–42 (2012).

320. Hu, H. *et al.* CRL4B catalyzes H2AK119 monoubiquitination and coordinates with PRC2 to promote tumorigenesis. *Cancer Cell* **22,** 781–95 (2012).

321. Zhou, W. *et al.* Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation. *Mol. Cell* **29,** 69–80 (2008).

322. Minsky, N. *et al.* Monoubiquitinated H2B is associated with the transcribed region of highly expressed genes in human cells. *Nat. Cell Biol.* **10,** 483–8 (2008).

323. Okumoto, K. *et al.* Cysteine ubiquitination of PTS1 receptor Pex5p regulates Pex5p recycling. *Traffic* **12,** 1067–83 (2011).

324. Dillon, S. C., Zhang, X., Trievel, R. C. & Cheng, X. The SET-domain protein superfamily: protein lysine methyltransferases. *Genome Biol.* **6,** 227 (2005).

325. Min, J., Zhang, X., Cheng, X., Grewal, S. I. S. & Xu, R.-M. Structure of the SET domain histone lysine methyltransferase Clr4. *Nat. Struct. Biol.* **9,** 828–32 (2002).

326. Zhang, X. *et al.* Structure of the Neurospora SET domain protein DIM-5, a histone H3 Lysine methyltransferase. *Cell* **111,** 117–127 (2002).

327. Wilson, J. R. *et al.* Crystal Structure and Functional Analysis of the Histone Methyltransferase SET7 / 9. *Cell* **111,** 105–115 (2002).

328. Zheng, S., Wang, J., Feng, Y., Wang, J. & Ye, K. Solution structure of MSL2 CXC domain reveals an unusual Zn3Cys9 cluster and similarity to pre-SET domains of histone lysine methyltransferases. *PLoS One* **7,** e45437 (2012).

329. Danielsen, J. M. R. *et al.* Mass spectrometric analysis of lysine ubiquitylation reveals promiscuity at site level. *Mol. Cell. Proteomics* **10,** M110.003590 (2011).

330. De Juan-Sanz, J., Núñez, E., López-Corcuera, B. & Aragón, C. Constitutive endocytosis and turnover of the neuronal glycine transporter GlyT2 is dependent on ubiquitination of a C-terminal lysine cluster. *PLoS One* **8,** e58863 (2013).

331. Yin, Y., Yu, V. C., Zhu, G. & Chang, D. C. SET8 plays a role in controlling G 1 /S transition by blocking lysine acetylation in histone through binding to H4 N-terminal tail. *Cell Cycle* **7,** 1423–1432 (2008).

332. Wu, S. & Rice, J. C. A new regulator of the cell cycle. The PR-Set7 histone methyltransferase. *Cell Cycle* **10,** 68–72 (2011).

333. Rayet, B. & Gélinas, C. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* **18,** 6938–47 (1999).

334. Zheng, J. *et al.* CAND1 Binds to Unneddylated CUL1 and Regulates the Formation of SCF Ubiquitin E3 Ligase Complex. *Mol. Cell* **10,** 1519–1526 (2002).

335. Madrid, L. V. *et al.* Akt Suppresses Apoptosis by Stimulating the Transactivation Potential of the RelA/p65 Subunit of NF-kappa B. *Mol. Cell. Biol.* **20,** 1626–1638 (2000).

336. Mistry, P., Deacon, K., Mistry, S., Blank, J. & Patel, R. NF-kappaB promotes survival during mitotic cell cycle arrest. *J. Biol. Chem.* **279,** 1482–90 (2004).

337. Li, L. *et al.* CHIP Mediates Degradation of Smad Proteins and Potentially Regulates Smad-Induced Transcription. *Mol. Cell. Biol.* **24,** 856–864 (2004).

338. Lee, J. S. & Yoo, S. J. C-terminus of Hsc70-interacting protein regulates C-terminal binding protein 2 and the expression of its target genes. *Biochem. Biophys. Res. Commun.* **432,** 418–24 (2013).

339. Smeenk, G. *et al.* The NuRD chromatin-remodeling complex regulates signaling and repair of DNA damage. *J. Cell Biol.* **190,** 741–9 (2010).

340. Chou, D. M. *et al.* A chromatin localization screen reveals poly (ADP ribose)-regulated recruitment of the repressive polycomb and NuRD complexes to sites of DNA damage. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 18475–80 (2010).