Sirt7 acts as a novel regulator of chromatin dynamics through modulation of the Sirt1/Suv39H1 axis: Possible role in cancer

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Contents

Zusammenfassung

Sirtuine bilden eine Familie von Histon- und Protein-Deacetylasen, die in eine Vielzahl physiologischer Prozesse wie Zellwachstum, Metabolismus, Genom-Stabilität und zelluläre Stressantwort regulatorisch involviert sind. Interessanterweise wurde kürzlich entdeckt, dass sich die Sirtuine auch gegenseitig regulieren können. Sirt7 blockiert die katalytische Aktivität von Sirt1 dadurch, dass es die Sirt1 Autodeacetylierung am Lysin 230 verhindert. Im Fettgewebe unterbindet Sirt7 somit die negative Rolle von Sirt1 in der Adipozyten-Differenzierung und resultiert in einer Akkumulierung von weißem Fettgewebe.

In der vorliegenden Arbeit zeige ich, dass eine regulatorische Interaktion zwischen Sirt1 und Sirt7 eine entscheidende Rolle bei der Etablierung des konstitutiven Heterochromatins spielt. Ich konnte experimentell belegen, dass Sirt7 durch die Inhibierung von Sirt1 zu einer weiteren Inhibierung eines Sirt1-Targetproteins, Suv39H1 führt. Suv39H1, eine Histon-Methyltransferase, katalysiert die Trimethylierung des Histon H3 (H3K9me3) und ist somit maßgeblich für die Bildung des Heterochromatins verantwortlich. Unter genotoxischer Stresseinwirkung ist wiederum die Entfernung von Suv39H1 vom Heterochromatin um eine Chromatinrelaxierung und DNA-Reparatur zu ermöglichen essentiell. Suv39H1 wird durch Sirt1 deacetyliert und aktiviert. Da Sirt7 die katalytische Aktivität von Sirt1 hemmt, verhindert Sirt7 gleichzeitig die Aktivierung von Suv39H1. In Sirt7 defizienten Zellen liegt Sirt1 autodeacetyliert und hyperaktiviert vor und kann somit Suv39H1 stark aktivieren. In Abwesenheit von Sirt7 kommt es demnach zu einer gesteigerten H3K9me3 Deposition und einer übermäßigen Heterochromatin-Bildung.

Ich habe weiterhin eine entscheidende Rolle der hier entdeckten negativen Regulierung von Sirt1 und Suv39H1 durch Sirt7 in der zellulären Stressantwort dokumentiert. Unter Einwirkung von genotoxischem Stress ist Sirt7 notwendig um Suv39H1 zu reduzieren und eine Lockerung der Chromatinstruktur zu bewirken damit die DNA-Reparatur stattfinden kann.

Im letzten Abschnitt der Arbeit konnte ich zusätzlich zeigen, dass Sirt7 die Rekrutierung von Suv39H1 zu euchromatischen Genloci blockieren kann. Meine Resultate deuten darauf hin, dass diese Funktion von Sirt7 für die Regulierung der essenziellen Stressantworten wie Apoptose und Proliferation wichtig ist.

Sirtuine können eine duale Rolle entweder als Tumorsuppressoren oder als Onkogene in der Tumorgenese ausüben. Die genauen molekularen Mechanismen, die den beiden widersprüchlichen Sirtuin-Funktionen zugrunde liegen, sind noch unzureichend untersucht. In meiner Arbeit konnte ich eine solche duale Sirt7-Funktion aufdecken. Sirt7 kann eine onkogene Wirkung entfalten, indem es die Menge von Suv39H1 am konstitutiven Heterochromatin reduziert und dadurch zu Heterochromatin-Verlust und Genom-Instabilität beiträgt. Desweiteren kann Sirt7 zumindest teilweise durch Suv39H1-Regulierung die Zellproliferation fördern. In der Tat ist die Expression von Sirt7 in vielen Tumoren erhöht. Unter Stresseinwirkung dagegen kann Sirt7 eine tumorsuppressive Wirkung entfalten: durch Beförderung der Chromatinrelaxierung wird DNA-Reparatur ermöglicht. Zur vollständigen

Entschlüsselung der Rolle der Sirt7/Sirt1/Suv39H1-Achse in der Cancerogenese sind weitere experimentelle Arbeiten notwendig. Meine Arbeit hat jedoch deutlich gezeigt, dass eine Manipulierung dieser Regulationsachse eine geeignete Alternative darstellt um neue, innovative Ansätze für die Krebstherapie zu entwickeln.

Abstract

Sirtuins build a family of NAD^+-dependent histone and protein deacetylases that are involved in a variety of cellular processes including proliferation, metabolism, genomic stability and stress response among others. Recently, it was discovered that one member of the sirtuin family, Sirt7, can regulate the activity of another sirtuin, Sirt1. Sirt7 inhibits Sirt1 catalytic activity by preventing its autodeacetylation at lysine 230. Through this mechanism, Sirt7 represses Sirt1-mediated inhibition of adipocyte differentiation and promotes accumulation of white adipose tissue.

In this work, I unravel another layer of complexity in the crosstalk between Sirt7 and Sirt1. I demonstrate that Sirt7-mediated inhibition of Sirt1 plays a crucial role in the regulation of the catalytic activity of a prominent Sirt1 target: the methyltransferase, Suv39H1. Suv39H1 is an important histone modifier, which plays decisive role in the formation of constitutive heterochromatin mainly by catalyzing deposition of the histone mark H3K9me3. On the other hand, dissociation of Suv39H1 from constitutive heterochromatin is a critical event that ensures heterochromatin relaxation following genotoxic stress to implement DNA repair. Sirt1 promotes Suv39H1 activation through direct deacetylation. Here I demonstrate that Sirt7 indirectly inhibits Suv39H1 by restraining Sirt1 catalytic activity. In absence of Sirt7, Sirt1 is autodeacetylated and hyperactive and thus binds Suv39H1 with high efficiency. As a result, overactive Suv39H1 effectuates H3K9me3 deposition and leads to aberrant formation of constitutive heterochromatin in Sirt7 deficient cells.

I further provide evidence that Sirt7-mediated inhibition of Sirt1/Suv39H1 axis is a crucial mechanism that establishes heterochromatin relaxation in response to stress. Cells lacking Sirt7 fail to reduce Suv39H1 recruitment to constitutive heterochromatin resulting in blunted heterochromatin relaxation following DNA damage, indicating an essential role of Sirt7 in DNA repair mechanism.

Finally, I demonstrate that the Sirt7/Suv39H1 axis is involved in regulation of gene expression following genotoxic stress. My results suggest that Sirt7 controls the recruitment of Suv39H1 to euchromatin loci and ensures essential cellular responses to stress such as evasion from apoptosis and cell survival.

Sirtuins possess a dual role in cancerogenesis based on their ability to act both as tumor suppressors mainly by facilitating DNA repair, or as oncogenes by stimulating cancer growth through different mechanisms. However, the exact mechanism underlying this duality still remains poorly characterized. My work reveals such a dual action of Sirt7. Since Sirt7 is upregulated in different types of cancer it might promote cancer progression by reducing Suv39H1 recruitment to the constitutive heterochromatin resulting in heterochromatin loss and genomic instability. Further, Sirt7 increases cell survival following DNA damage at least in part through Suv39H1. On the other hand, I show that Sirt7 possess potential tumor suppressor function by enforcing heterochromatin relaxation to allow DNA repair under stress. Although further work is required to better characterize the role of the

Sirt7/Sirt1/Suv39H1 axis in cancer, my work suggests that the manipulation of this pathway may represent a novel alternative for the development of innovative anti-tumor therapies.

Chapter 1: Introduction

1.1 Discovery and the main characteristics of sirtuins

The first sirtuin was discovered in Saccharomyces cerevisiae in 1979, referred to as Sir2 [1-4]. In years to follow, sirtuin homologues were discovered in all living forms ranging from bacteria, plants to mammals, evidencing a conserved and primordial character of the sirtuin protein family.

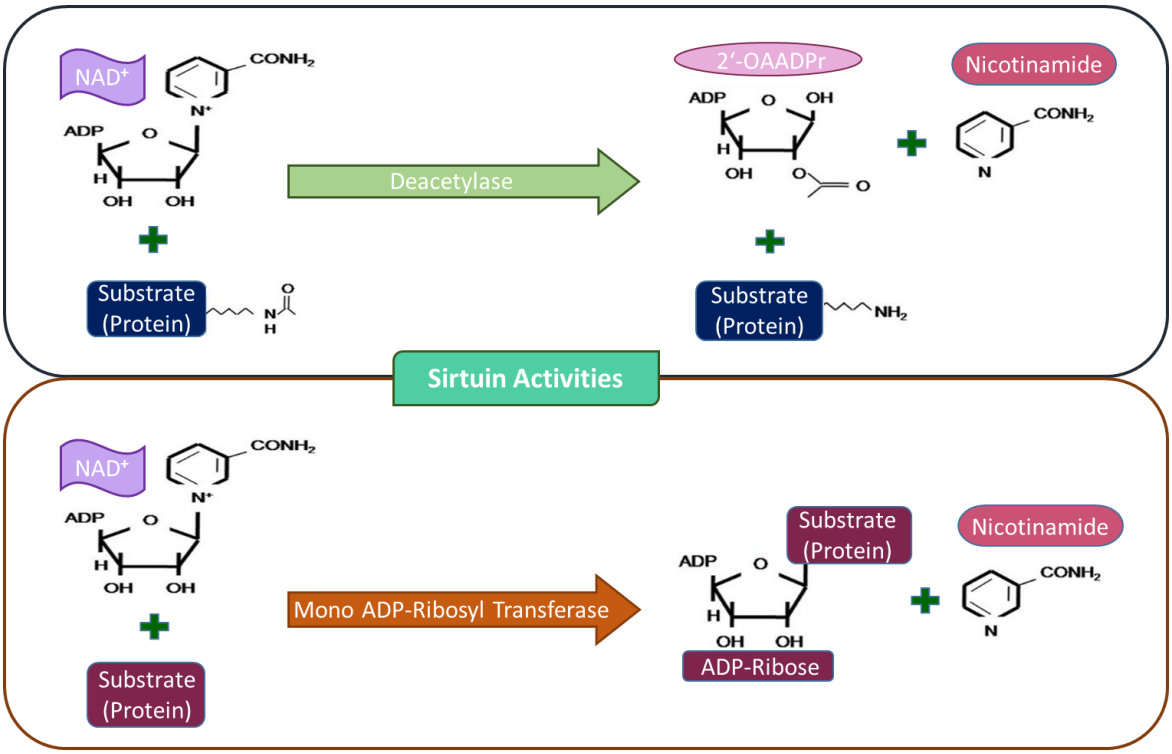


Fig.1.1. Enzymatic activity of sirtuins. Sirtuins possess either deacetylase or mono-ADP ribosyl transferase activity. In both reactions NAD^+ is used as a co-substrate. In the deacetylation reaction, an acetyl moiety is transferred from acetylated substrate to ADP-ribose molecule of NAD^+ releasing O-acetyl ribose and nicotinamide. In ADP-ribosylation, ADP-ribose is transferred to the substrate protein (Figure adapted from Kitada et al [5]).

Sirtuins encode class III histone deacetylases (HDACs), which belong to a separate category based on the distinct mechanism of deacetylation compared to other HDACs. While HDAC families I, II and IV employ zinc for the catalytic activity, sirtuins require NAD^+ as coenzyme. Although sirtuins possess a zinc binding domain, it is mainly responsible for the structural stability. The sirtuin molecule consists of a conserved catalytic core domain that is approximately 250 amino acid long and of distinct N-terminal and C-terminal domains, which specify their subcellular localization and binding partners. The catalytic domain contains three conserved motifs namely, a NAD binding domain, a reverse Rossman fold domain and a zinc-binding domain [6-8].

Two major catalytic activities are known for sirtuins: deacetylation and mono-ADP-ribosylation (mADPRT). In both reactions, NAD^+ is required as a co-substrate that binds to the enzyme, leading to the formation of an intermediate complex upon releasing nicotinamide. In case of deacetylation, the sirtuin enzyme transfers the acetyl group from the substrate to ADP-ribose molecule releasing O-acetyl-ADP-ribose (OAADPr) and nicotinamide. In case of mADPRT, sirtuin transfers ADP-ribose to the substrate completing the reaction as illustrated in **Fig.1.1** [9].

Although these are the two most observed and characterized catalytic activities of sirtuin family members, in recent years several other functions have also come to light. For example, Sir2A in Plasmodium falciparum has NAD^+-dependent glycohydrolase activity [10]. Mammalian Sirt5 performs demalonylation [11-14], desuccinylation [11-13] and deglutarylation [15]. Another mammalian sirtuin, Sirt7 has been shown to possess desuccinylation activity [16]. Recently, it has been documented that Sirt1, Sirt2, Sirt3, Sirt6 and Sirt7 also orchestrate debutyrylase activity toward butyrylated nucleosomes [17]. These findings suggest that there could be more unidentified enzymatic functions of sirtuins.

1.2 Mammalian sirtuins

Mammalian sirtuins constitute a family of seven members (Sirt1-7). All sirtuins possess a conserved core catalytic domain of approximately 275 amino acids, whereas N-terminal and C-terminal domains are unique and specify their sub-cellular localization and the binding partners. Sirt1, Sirt6 and Sirt7 are mainly present in the nucleus with Sirt7 predominantly located in the nucleolus and Sirt1 also shuttling to cytoplasm [18, 19]. Sirt2 is primarily localized in cytoplasm [20]. Sirt3, Sirt4 and Sirt5 are located in mitochondria [21, 22], with full length Sirt3 also present in nucleus [23]. In terms of activity, Sirt1-Sirt3 and Sirt5-Sirt7 show deacetylase activity [24-29]. Sirt2, Sirt3, Sirt6 and Sirt7 have both deacetylase and mono-ADP ribosyl transferase activity [21, 30]. Sirt4 shows only mono-ADP ribosyl transferase activity (**Fig.1.2**). Recent studies have shown that Sirt5 can also perform demalonylation [11-14], desuccinylation [11-13] and deglutarylation [15] and Sirt7 possesses desuccinylation activity [16].

In mammals, sirtuins play a major role in a multitude of cellular processes such as proliferation, differentiation, aging, metabolism, cell cycle and genome maintenance, among others. In the last decade, sirtuins have emerged as major players in stress responses. Almost all of the mammalian sirtuin family members have been implicated as leading regulators of stress response such as metabolic, oxidative and genotoxic stress. Sirtuins regulate these processes either by direct deacetylation of different protein targets or by modulating chromatin dynamics [31].

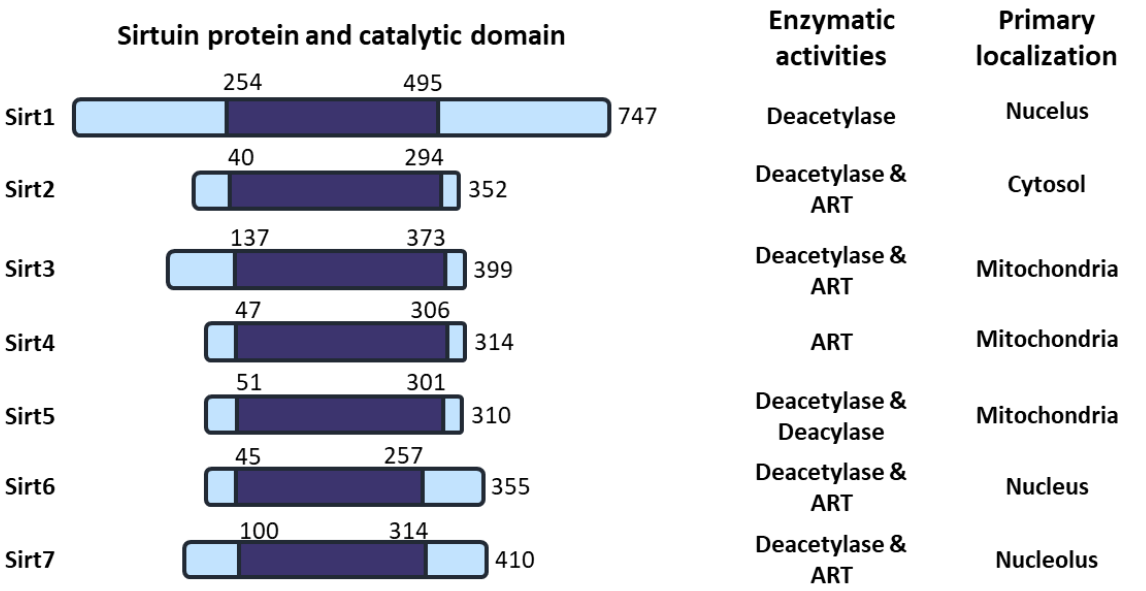


Fig.1.2. Mammalian sirtuins localization and function. Protein organization of seven sirtuin family members with their respective enzymatic activity and sub-cellular localization is outlined in the figure. (Figure adapted from Roth et al [32]).

Key focus of this dissertation is to understand the interplay between two sirtuin family members, Sirt1 and Sirt7, in the regulation of chromatin dynamics under physiological conditions and during stress response.

1.3 Mammalian sirtuins regulate chromatin structure and genomic stability

In eukaryotes genomic DNA is packaged as chromatin, in which the DNA is wrapped around histones that are kept at variable densities. Chromatin exists in two principle states, euchromatin and heterochromatin, as shown in **Fig.1.3**. Euchromatin is described as open form of chromatin that can be actively transcribed. It is characterized by high levels of histone acetylation and low levels of methylation. In contrast, heterochromatin is a more compact form of chromatin that is normally not transcribed [33-35]. Heterochromatin is characterized by high degree of histone methylation and low degree of acetylation. Broadly, heterochromatin can be further sub-divided in two categories, facultative and constitutive heterochromatin. Facultative heterochromatin includes heterochromatin regions that can be transcribed under certain physiological conditions, e.g. differentiation, cell cycle or specific developmental stages [36], while constitutive heterochromatin contains regions that are maintained in a very tight conformation and are generally not transcribed [36-42].

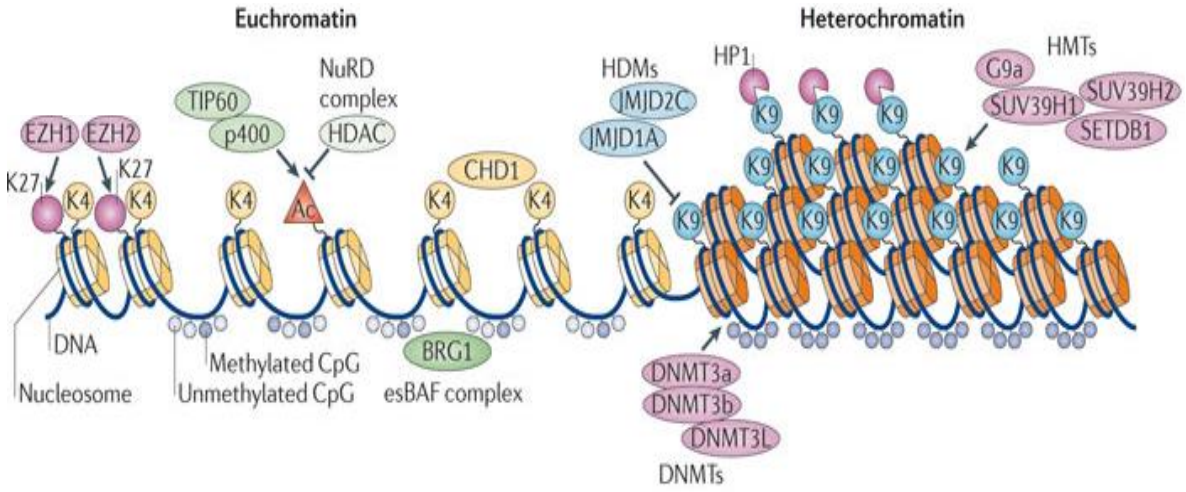


Fig.1.3. Euchromatin and heterochromatin. Chromatin exists mostly in two states, the relaxed euchromatin and the compact heterochromatin. Several histone modifiers involved in the chromatin dynamics are indicated. (Figure adapted from Gasper-Maia et al [43]).

Constitutive heterochromatin contains highly repetitive sequences that are over-represented in specific heterochromatin regions such as telomeres, centromeres and pericentomeres [36, 42, 44]. If these repeats are in relaxed state, they are prone to homologous recombination which will lead to chromosomal rearrangements and aneuploidy. Therefore, it is imperative for the cell to maintain the constitutive heterochromatin in tight conformation to preserve genomic stability [45, 46].

Besides the general acceptance of these two principle types, modern day characterization recognizes several intermediate stages. Heterochromatin can shuttle between different sub-types ranging from more open to compact chromatin by modification of histone residues. Enzymes performing these modifications are termed as histone modifiers. Histone acetyl transferases (HATS), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs) are among some of the major modifiers of histones. Generally, histone acetylation at lysine residues promotes a more open form of chromatin while methylation leads to formation of compact chromatin. There are several other histone modifications that regulate the chromatin dynamics, such as phosphorylation, ubiquitination, sumoylation, poly-ADP ribosylation, carbonylation, deamination, butyrylation, biotynilation and others, as well as their reverse processes. Dynamic and flexible nature of chromatin allows for selective expression or repression of certain genes.

Different types of histone modifications controlled by sirtuins and their role in chromatin regulation will be discussed in the following sections.

1.3.1 Regulation via direct deacetylation of histones

Regulation through H3K9Ac deacetylation. Sirtuins have emerged as key modifiers of chromatin either by directly deacetylating histones or by controlling the activity of other

histone modifiers, mainly through direct deacetylation. One of the most prominent targets of sirtuins is histone H3 acetylated at lysine 9 (H3K9Ac) [22]. H3K9Ac is deacetylated by four mammalian sirtuins, Sirt1-Sirt3 and Sirt6.

Sirt1 coordinates the formation of constitutive and facultative heterochromatin by deacetylating H3K9Ac [47, 48]. Deacetylation of this residue is prerequisite for the deposition of another histone mark, the methyl group, forming H3K9me2/me3, which is a hallmark for higher order chromatin compaction. Sirt2 deacetylates H3K9Ac but to a much lesser extent. Sirt3 is primarily a mitochondrial sirtuin but a small pool of a full length Sirt3 is also found in the nucleus. It has a strong deacetylase activity for H3K9Ac when artificially recruited to a reporter gene, which leads to the repression of the target gene [23]. However, no physiological function of Sirt3-dependent H3K9Ac deacetylation has been demonstrated. In contrast, deacetylation of H3K9Ac by Sirt6 is important for maintaining telomere structure and DNA double strand breaks (DSBs) repair [49-51]. Consistently, loss of Sirt6 has been associated with increase in H3K9Ac levels at telomeres which results in telomeric defects, premature senescence and end-to-end chromosomal fusions [52-55].

Regulation via H4K16Ac deacetylation. Another major histone residue targeted by mammalian sirtuins is histone H4 acetylated at lysine 16 (H4K16Ac) [22]. This residue is recognized and deacetylated by three sirtuins Sirt1-Sirt3 [47, 56]. Deacetylation at H4K16Ac by Sirt1 coordinates the formation of constitutive and facultative heterochromatin similarly to H3K9Ac deacetylation [47, 48]. H4K16Ac deacetylation by Sirt2 plays a major role in cell cycle control. Sirt2 is primarily a cytoplasmic sirtuin but during G2/M phase of the cell cycle it shuttles to the nucleus and globally deacetylates H4K16Ac [57]. Deacetylation of H4K16Ac during G2/M phase leads to deposition of another histone mark H4K20me1. These two modifications are necessary for chromatin condensation prior to mitosis entry. The deacetylation by Sirt2 is not only necessary for cell cycle progression but also for development, DNA repair signaling and genomic stability. Lastly, Sirt3 has strong deacetylase activity for H4K16Ac when artificially recruited to a reporter gene, similarly to what is observed for H3K9Ac [23].

Regulation via H3K56Ac deacetylation. H3K56Ac is deacetylated by four sirtuin family members, i.e. Sirt1-3 and Sirt6 [58-62]. H3K56 is localized at the entry and exit points of nucleosomes were it is deacetylated by sirtuins upon genotoxic stress to help chromatin remodeling for the DNA damage response. H3K56Ac deacetylation by Sirt6 is mainly restricted to the telomeres where it regulates the dynamic changes of telomeric chromatin throughout the cell cycle [61]. H3K56Ac deacetylation by Sirt6 is important for maintenance of telomere stability and prevention of telomere dysfunction and end-to-end chromosomal fusion [52, 61, 62].

Regulation via H3K18Ac deacetylation. H3K18Ac is a relatively new histone mark that has come to focus in recent years. Sirt2, Sirt6 and Sirt7 deacetylate H3K18Ac to control different cellular functions. Sirt2 deacetylates H3K18Ac leading to transcriptional repression [63]. Sirt6-dependent deacetylation of H3K18Ac is important for pericentric heterochromatin

condensation, which ensures genomic stability and protects against cellular senescence and mitotic defects [64]. Sirt7 deacetylates H3K18Ac at promoters of several tumor suppressor genes through its binding with the transcription factor ETS-like transcription factor 4 (ELK4). Deacetylation of H3K18Ac by Sirt7 at these promoters is important for the maintenance of critical features of cancer cells, e.g. escape from contact inhibition and insurance of anchorage-independent growth. Sirt7 is highly expressed in several tumors and consequently Sirt7 depletion from human cancer xenograft results in decreased tumorigenicity [26].

Regulation by modification of core histones. Core histone H2A variant H2A.Z is a deacetylation target of Sirt1. Sirt1 deacetylates H2A.Z, resulting in its ubiquitination at residue K115 and K121 leading to proteasome-mediated degradation and promoting transcriptional silencing of oncogenes expression in prostate cancer [65, 66].

Regulation via modifications of linker histone H1. Sirtuins not only deacetylate core histones but they can also regulate linker histone H1. Deacetylation of H1 at its N-terminal domain at K26 by Sirt1 leads to the formation of more compact chromatin structure [47]. Sirt1 deacetylates H1K26Ac, which enables its methylation by EZH2 to form H1K26me2. H1K26me2 is then recognized by heterochromatin protein 1 (HP1) that contributes to facultative heterochromatin formation. This favors specific regulation of gene expression during cell differentiation and transformation [31, 67]. Sirt1 also localizes at chromatin from pro-metaphase to telophase during mitosis, where it leads to global chromosomal compaction by histone deacetylation and promotes loading of linker histone H1, thus contributing to maintenance of stability and integrity of the chromosomes [68].

1.3.2 Regulation via control of chromatin modifiers

As mentioned previously, sirtuins not only mediate chromatin formation by direct deacetylation of histone marks but also by modulating the enzymatic activity of several other histone modifiers (**Fig.1.4**).

Suppressor of variegation 3-9 homolog 1 (Suv39H1) is the first methyltransferase identified, which principally catalyzes the deposition of H3K9me3 at pericentric and telomeric constitutive heterochromatin. Loss of Suv39H1 and its variant Suv39H2 leads to a complete eradication of H3K9me3 at pericentric heterochromatin and reduced H3K9me3 at telomeres [69-71]. Loss of H3K9me3 is also coupled with the loss of HP1, which further leads to heterochromatin relaxation. As a result, mice lacking both of these methyltransferases have compromised chromatin segregation, damaged DNA and delayed G2/M phase. Interestingly, Sirt1 knock out (KO) mouse embryonic fibroblasts (MEFs) show decreased H3K9me3 at constitutive heterochromatin, which is accompanied by the mislocalization of HP1. This observation led to the discovery of functional link between Sirt1 and Suv39H1. Sirt1 modulates the activity of Suv39H1 through a range of sequential events that results in an increased activity of Suv39H1. Sirt1 interacts with, deacetylates and recruits Suv39H1 to the chromatin [72]. Sirt1 binding to Suv39H1 stimulates Suv39H1 activity through three different mechanisms: (I) Sirt1 deacetylates Suv39H1 at its catalytic SET domain, at residue K266,

thereby activating it, (II) Sirt1 binding induces a conformational change in Suv39H1 further increasing its activity [48], and (III) Sirt1 also inhibits poly-ubiquitination of Suv39H1 at K87 and therefore its degradation through E3 ubiquitin ligase MDM2 [72]. Additionally, Sirt1 also deacetylates H3K9Ac to free the site for methylation by Suv39H1. Overall, through regulation of Suv39H1, Sirt1 promotes spreading of H3K9me3 establishing formation of heterochromatin [48]. This deposition of H3K9me3 is important for conservation of pericentric heterochromatin and rDNA repeats [72-75].

DNA methyltransferase 1 (Dnmt1) is another methyltransferase regulated by sirtuins. Sirt1 directly deacetylates Dnmt1 resulting in its increased activity at a number of genomic loci, such as ribosomal DNA (rDNA) and some tumor suppressor genes [76-78]. Dnmt1 is important for Sirt1 recruitment at rRNA genes which is essential to sustain deacetylated state of H3K9Ac. Together they keep the rRNA loci in methylated and deacetylated state preserving nucleolar integrity [77]. Sirt7 is another sirtuin located at rDNA locus and it helps to maintain genomic stability by recruiting Dnmt1 and Sirt1 to the rRNA genes [79]. Sirt7 also leads to heterochromatin silencing at rDNA genes by associating with SNF2H, a component of the nucleolar silencing complex (NoRC) [80].

Enhancer of zeste homolog 2 (Ezh2) is a methyltransferase that co-localizes with Sirt1. Ezh2 is a part of polycomb repressive complex 2 (PRC2), which is important for healthy embryonic development through epigenetic control [81]. There is no evidence suggesting that Sirt1 deacetylates Ezh2. However, deacetylation of H1K26 by Sirt1 could facilitate the successive methylation of same residue by Ezh2. In addition, loss of Sirt1 leads to an increased stability of Ezh2 protein levels [82]. Furthermore, HP1 recognizes H1K26me2 residue and hence contributes to the formation of facultative heterochromatin [67].

PR/SET domain containing protein 7 (PR-SET7) is the methyltransferase responsible for H4K20 mono-methylation. Sirt2 mediates the methylation of H4K20me1 indirectly by de-acetylating H4K16Ac which facilitates H4K20me1 formation by PR-SET7. Additionally, Sirt2 directly deacetylates PR-SET7, increasing its catalytic activity and stimulating its recruitment to the chromatin. During G2/M phase of cell cycle, there is an increased binding of Sirt2 and PR-SET7 that consequently results in H4K20me1. Accumulation of H4K20me1 is also important for subsequent deposition of H4K20 di/tri-methylation at the same residue which is important for proper cell cycle control and maintenance of genomic stability [83].

Additional methyltransferases known to be regulated by Sirt1 include disruptor of telomeric silencing-1 (Dot1), mixed-lineage leukemia 1 (MLL1) and histone demethylase/lysine demethylase 1A (Kdm1A/LSD1). Sirt1 positively regulates the methyltransferase responsible for H3K79 methylation, Dot1, leading to repression of its target genes [84]. Moreover, Sirt1 can also directly deacetylate the methyltransferase MLL1, modulating the cyclic expression of circadian clock genes [85]. Finally, Sirt1 physically interacts with LSD1 promoting the suppression of genes of the notch signaling pathway preventing notch-dependent tumorigenesis and developmental abnormalities in humans [86-88].

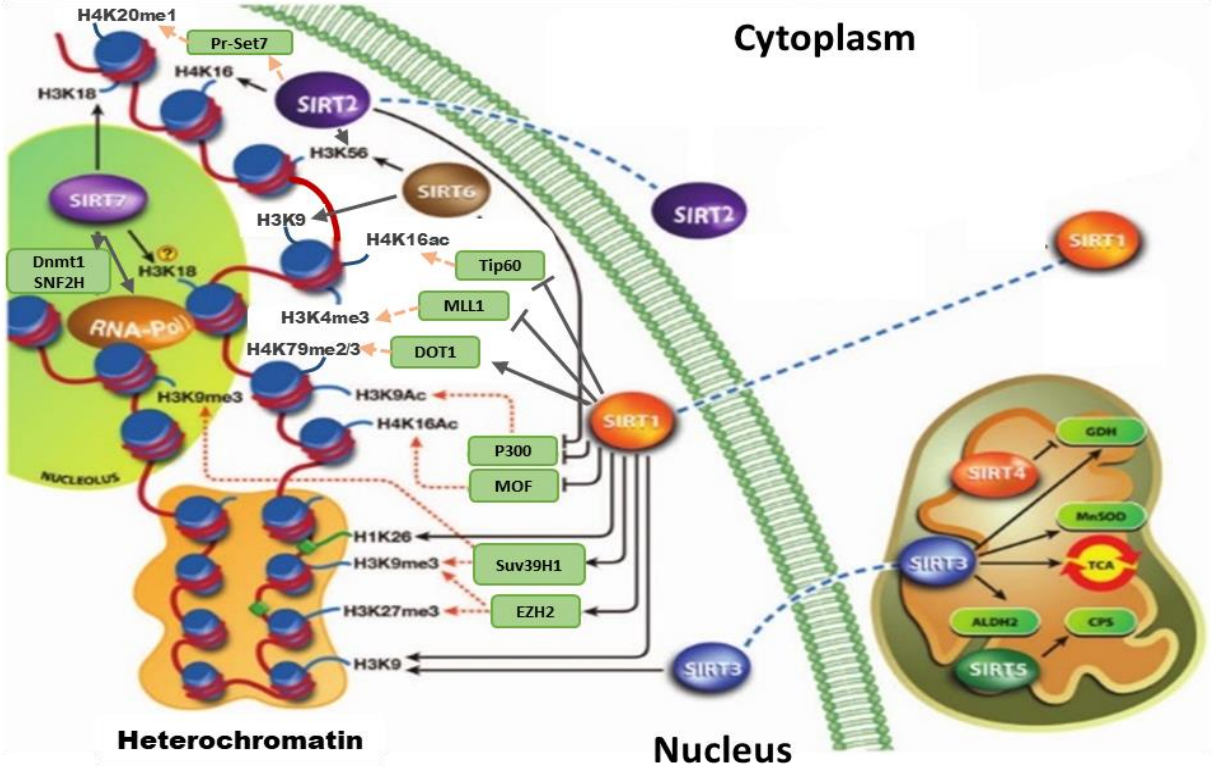


Fig.1.4. Sirtuins in regulation of chromatin structure. The picture depicts crucial sirtuin targets and the regulation of histone modifications either directly or through histone modifiers. (Figure adapted from Martinez-Redondo et al. [22]).

Remarkably, sirtuins not only modulate methyltransferases to form facultative and constitutive heterochromatin but they can also regulate their own antagonist histone acetyl-transferases (HATs), for instance, p300. p300 is a major HAT which acts as a transcriptional coactivator for a wide range of pathways regulated by p53, NFκB, PARP1 and fork head box class O (FOXO) factors through deacetylation of H3K9Ac, H3K27Ac, H3K36Ac and H3K56Ac and is required for cellular processes like survival, growth and differentiation [59, 89-94]. Therefore, tight control of p300 is a necessity for maintenance of chromatin structure and for activation/repression of several essential cellular pathways. Sirt1 and Sirt2 have been shown to deacetylate p300 and inhibit its enzymatic activity [95, 96].

In addition to p300, sirtuins also deacetylate several members of the MYST family of HATs. Sirt1 regulates males absent on the first (MOF) and Tat-interactive protein (TIP60). MOF is the main HAT in mammals which catalyzes H4K16Ac and hence stimulates transcriptional activation. Sirt1 deacetylation of MOF reduces its enzymatic activity and protein levels through ubiquitin-dependent degradation [22, 97-99]. In contrast to this role, deacetylation of MOF by Sirt1 can also increase the binding of MOF to some specific chromatin loci and hence stimulate the expression of a subset of target genes probably in a cell specific manner [99]. TIP60 regulation by Sirt1 is especially important in DNA damage response and will be discussed in detail in the next chapter.

1.4 Mammalian sirtuins in stress response

The critical dependence of the sirtuin catalytic activity on the levels of NAD^+ makes sirtuins the perfect candidates for detection of changes in energy homeostasis and metabolism [67, 100-103]. Indeed, sirtuins play major roles in the maintenance of genomic stability, cell cycle control, apoptosis and other essential cellular processes responsible for preservation of the cellular integrity under different stress conditions [57, 72, 83, 104-106]. Furthermore, sirtuins promote cellular survival in response to various stressors by preventing apoptosis [21, 28, 107, 108]. The capacity of sirtuins to promptly respond to different kinds of stress stimuli is achieved by different mechanisms. First of all, exposure to various kind of stressors might lead to the rapid induction of expression of sirtuins´ genes [109]. On the other hand, accumulation of different post-translational modifications (PTMs) such as phosphorylation, methylation, ubiquitination, etc, might modulate sirtuin enzymatic activity, their capacity to bind to specific targets and/or their recruitment to particular promoters of target genes [109-113].

Sirtuins facilitate the cellular adaptation to the adverse external stimuli by controlling different molecular pathways. On one hand, sirtuins primarily deacetylate different transcription factors, enzymes and other molecules that are involved in cellular apoptosis, inflammation, DNA repair etc. Interestingly, several of these targets are shared between different members of mammalian sirtuins, for instance, p53, NFκB, HIF1α, FOXOs, HSF1, E2F1 and MnSOD, among others [21, 114].

Additionally, sirtuins ensure the metabolic adaptation to stress mainly by deacetylating specific factors involved in the maintenance of mitochondrial function. For example, Sirt3 prevents the accumulation of reactive oxygen species (ROS) by deacetylating and activating the mitochondrial enzyme manganese superoxide dismutase (MnSOD) and components of electron transport chain [115, 116]. In addition, in response to oxidative stress and calorie restriction, Sirt3 deacetylates isocitrate dehydrogenase 2 (IDH2), leading to detoxification of ROS. As a consequence, ablation of Sirt3 results in altered energetic and metabolic homeostasis which can promote damage permissive and tumorigenic environment [117-119].

Similarly to Sirt3, Sirt5 is also responsible for protection against oxidative damage by reducing the levels of cellular ROS through desuccinylation of isocitrate dehydrogenase (IDH) and deglutarylation of glucose-6-phosphate dehydrogenase (GPDH) [120]. Altered activities of these targets have been implicated in cancer cell metabolic dysregulation [13].

1.4.1 Sirtuins control the cellular stress response through epigenetic regulation

In response to different stress stimuli, sirtuins control different molecular pathways through epigenetic regulation of gene expression [39, 121, 122]. One example is represented by Sirt3. In response to genotoxic or oxidative stress, full length Sirt3, which is localized in nucleus, undergoes rapid degradation by ubiquitin-proteasome pathway. This process leads to de-repression of nuclear encoded mitochondrial and stress related genes through increase in acetylation of H3K9Ac and H4K16Ac at their promoters [23, 123]. In addition, Sirt6 participates in the cellular stress response by inhibiting the expression of gene involved in inflammation

and metabolic response by deacetylating H3K9Ac at the promoters of NFκB and HIF1α target genes [124-126].

Besides the epigenetic regulation of gene expression, sirtuins also play a pivotal role in the control of constitutive and facultative heterochromatin dynamics following stress. For instance, Sirt1 helps to restore homeostasis during nutrient and energy scarce conditions by promoting rDNA silencing through its association with the energy dependent nucleolar silencing complex (eNoSc). Sirt1 is a part of eNoSC together with Suv39H1 and nucleomethylin (NML). It is hypothesized that NML tethers the complex to rDNA and facilitates the spreading of heterochromatin and rDNA silencing through Sirt1-mediated deacetylation of histones and Suv39H1-dependent deposition of H3K9me3. Hence, through silencing of rDNA locus, energy balance is restored, accompanied by augmented genome stability via suppression of recombination between highly repetitive rDNA repeats [73, 100, 127, 128].

As mentioned before, constitutive heterochromatin is mostly located in the pericentromeric regions and telomeres. Sirt1 regulates constitutive heterochromatin in response to different stressors mainly by its functional link to Suv39H1. An upregulation of Sirt1 expression is observed in response to oxidative and metabolic stress, which leads to an increase in Suv39H1 expression and stability along with its heightened localization on pericentromeric heterochromatin. This sustains genomic integrity of the cells in response to stress [72]. HP1 also works in coordination with Suv39H1 for the maintenance of heterochromatin structure under normal conditions and in response to stress [129].

However, relaxation of constitutive heterochromatin especially in response to genotoxic stress plays also a critical role in ensuring genomic stability, mainly by facilitating the recruitment of DNA repair factors. In this work, I demonstrate that Sirt7 is a key factor that controls this process, indicating that sirtuins might build a complex network that finely controls constitutive heterochromatin dynamic following stress.

1.4.2 Regulation of DNA repair mechanisms

In human body, a single cell can undergo up to 1 million DNA mutations per day. Permanent uncorrected errors that are sustained after DNA replication and proof reading are estimated to range from $1x10^{-4}$ to $1x10^{-6}$ mutations per gamete for a given gene. A rate of $1x10^{-6}$ amounts to one mutation in a specific gene locus per one million gametes [130]. Furthermore, this rate does not take into account any kind of external/internal stressor or DNA damaging agent affecting a cell. Therefore, it becomes imperative that an organism ensures its genomic integrity in order to propagate genetic information to the next generation. Cells are exposed to various kinds of stressors on a daily basis. The genomic stability in cells is ensured by the activation of different mechanisms of DNA repair. Different pathways are activated in response to various kinds of DNA damage to promote the repair of single strand DNA breaks (SSBs) or double strand DNA breaks (DSBs). SSBs are mostly repaired by nucleotide excision

repair (NER) or by base excision repair (BER). DSBs are primarily repaired by non-homologous end joining repair (NHEJ) or by homologous recombination (HR).

Interestingly, sirtuins have been implicated in the activation of DNA damage response (DDR) pathways of both SSBs and DSBs by controlling chromatin remodeling and activation of critical factors of the DNA repair machinery. Among all the sirtuin family members, Sirt1 and Sirt6 are known to be most active in DNA repair. Recently, some studies revealed that Sirt7 also acts as a major player in some of these mechanisms [131-133].

Sirt1 is involved in SSBs repair through NER by modulating two key components of the pathway, i.e. xeroderma pigmentosum complementation group A (XPA) and xeroderma pigmentosum complementation group C (XPC). Sirt1 interacts with and deacetylates XPA that leads to an increased binding of XPA to RPA32 and promotes efficient DNA repair by NER [134]. Inhibition of Sirt1 decreases the transcription of XPC, which subsequently impairs NER pathway [135]. In addition, Sirt1 is involved in DSB repair through several factors. Foremost, Sirt1 is localized to DSBs in an ATM-dependent manner mainly through phosphorylation of H2AX, which is a primary target of ATM [136]. Sirt1 also interacts with and deacetylates NBS1, a subunit of the MRE11-Rad51-NBS (MRN) DSB DNA repair complex. NBS1 deacetylation by Sirt1 leads to its phosphorylation and activation [137-141]. Phosphorylated NBS1 then initiates the DNA repair pathway and delays cell cycle progression to allow time for damage repair [141]. Sirt1 is also important for the formation of γ-H2AX, Rad51, NBS1 and BRCA1 foci upon γ-irradiation during early phases of DSB which is important for efficient DDR [142]. Additionally, Sirt1 is involved in DSB repair through modulation of NHEJ repair pathway by controlling the function of Ku70. Ku70 is primarily located in the nucleus where it associates with the pro-apoptotic factor Bax. In response to stress, Ku70 is acetylated inducing a conformational change in Bax, which results in its subsequent localization to mitochondria to promote apoptosis. However, deacetylation of Ku70 by Sirt1 prevents the conformational change in Bax, thus suppressing the induction of apoptosis and allowing the cells to repair damaged lesions [109, 143, 144]. Sirt1 also deacetylates two members of MYST family of acetyltransferases, MOF and TIP60. Both are important in cell growth and arrest, apoptosis and DNA repair. Under normal conditions, Sirt1-dependent deacetylation of MOF and TIP60 leads to an inhibition of their activity and promotes their ubiquitination-mediated degradation. In response to stress, binding of Sirt1 to these two acetyl transferases decreases, leading to their stabilization and activation of the DNA damage response [145-150]. Furthermore, Sirt1-mediated deacetylation of Werner syndrome ATP-dependent helicase (WRN helicase) promotes efficient repair through HR [151-155].

In response to DNA damage, **Sirt6 KO** mice show impaired BER [52, 156, 157]. Although no direct correlation of Sirt6 has been established with any member of the BER pathway, it was shown that Sirt6 increases BER efficiency in a poly-ADP ribose polymerase 1 (PARP1)-dependent manner. It is speculated that Sirt6 might indirectly regulate BER by affecting chromatin accessibility through its capability to deacetylate H3K56Ac and hence allow DNA repair machinery to operate efficiently [49, 158, 159]. In addition, Sirt6 is recruited to DSB

sites with the help of lamin A. At the damage site, Sirt6 interacts with and mono-ADP-ribosylates PARP1 (which is the key enzyme for BER and DSB signaling), hence stimulating the repair [160-168]. Sirt6 is also involved in NHEJ repair by associating with DNA-PKcs and Ku70/80. Sirt6 localizes on chromatin leading to H3K9Ac deacetylation which stabilizes the association of DNA-PKcs to the chromatin, thus enabling the repair factors to access the lesion [51].

Sirt7 depletion has been associated with genomic instability and increased sensitivity to various DNA damaging agents [133]. Sirt7 overexpression increases the efficiency of NHEJ repair by 1.5 fold and of HR by 2.8 fold in human fibroblasts treated with paraquat toxin [160]. Recently, the mechanism by which Sirt7 enhances NHEJ efficiency was described. Sirt7 is recruited to DNA damage sites in a PARP1-dependent manner. Once localized to the damage sites, Sirt7 increases NHEJ by deacetylation of H3K18Ac, which in turn facilitates the recruitment of 53BP1 protein for repair [133]. However counterintuitively, it has been shown that translocation of Sirt7 from the nucleus to cytoplasm and the resulting increase in H3K18Ac is also important for chromatin relaxation and DNA repair. This suggests that H3K18Ac requires a dynamic change to ensure efficient DNA repair [169]. Sirt7 is also recruited to DSBs, where it transiently forms compact chromatin through H1K22suc desuccinylation and promotes DSB repair by activating upstream DDR signaling [16, 170].

Besides Sirt1, Sirt6 and Sirt7 the remaining sirtuin family members are also involved in DDR. **Sirt2** binds to PR-Set7 in response to stress leading to an increase in H4K20me1, which causes cell cycle arrest at G2/M phase promoting DNA repair [83, 106, 171-175]. Sirt2 also deacetylates H4K16Ac, which is important for the recruitment of 53BP1 to double strand breaks in response to DNA damage, thus facilitating NHEJ repair [176]. Establishment of H4K20me1 is promoted by H4K16Ac deacetylation. Interestingly, H4K20me1 is also required for the recruitment of 53BP1 at the repair foci, hence making formation of H4K20me1 a necessity for DNA damage response [72, 83, 106, 176, 177]. Lastly, it has been shown that Sirt2 is important to maintain genomic integrity by deacetylating cyclin-dependent kinase 9 (CDK9) and ataxia telangiectasia-mutated and Rad3-related interacting protein (ATRIP) increasing their activity and contributing to cell recovery from replication stress [178, 179].

Sirt3 plays an important role in cellular response to DNA damage and aging. As mentioned earlier, Sirt3 is primarily a mitochondrial sirtuin however, a small fraction of full length Sirt3 also resides in the nucleus. Consistently, Sirt3 has been implicated in mechanisms of DNA repair of genomic and mitochondrial DNA (mtDNA). Sirt3 promotes mtDNA repair by deacetylating 8-oxoguanine-DNA glycosylase 1 (OGG1), a DNA repair enzyme involved in DDR through BER. Loss of Sirt3 is associated with higher acetylation and degradation of OGG1 and a decrease in its incision activity [180]. In the nucleus, Sirt3 interacts with and deacetylates the DNA repair protein Ku70 in response to DNA damage, suggesting its possible role in Ku70-dependent pathway [181]. Sirt3 also promotes DNA repair by deacetylating H3K56Ac in the nucleus [58, 182, 183]. Sirt3-dependent deacetylation of H3K56Ac is important for 53BP1 localization to DSB and efficient repair through NHEJ [184].

Sirt4 is the most highly induced sirtuin in response to DNA damage. Sirt4 represses glutamine consumption resulting in a decrease in the incorporation of glutamine derived intermediates into the tricarboxylic acid (TCA) cycle, which contributes to cell cycle arrest of damaged cells and promotes DNA repair. Therefore, Sirt4 KO MEFs exhibit increased genomic instability due to accumulation of DNA damage [185].

Till date there is no known role of Sirt5 in DDR pathways. **Fig.1.5** summarizes the mechanisms by which different sirtuin family members promote the cellular adaptation to stress stimuli.

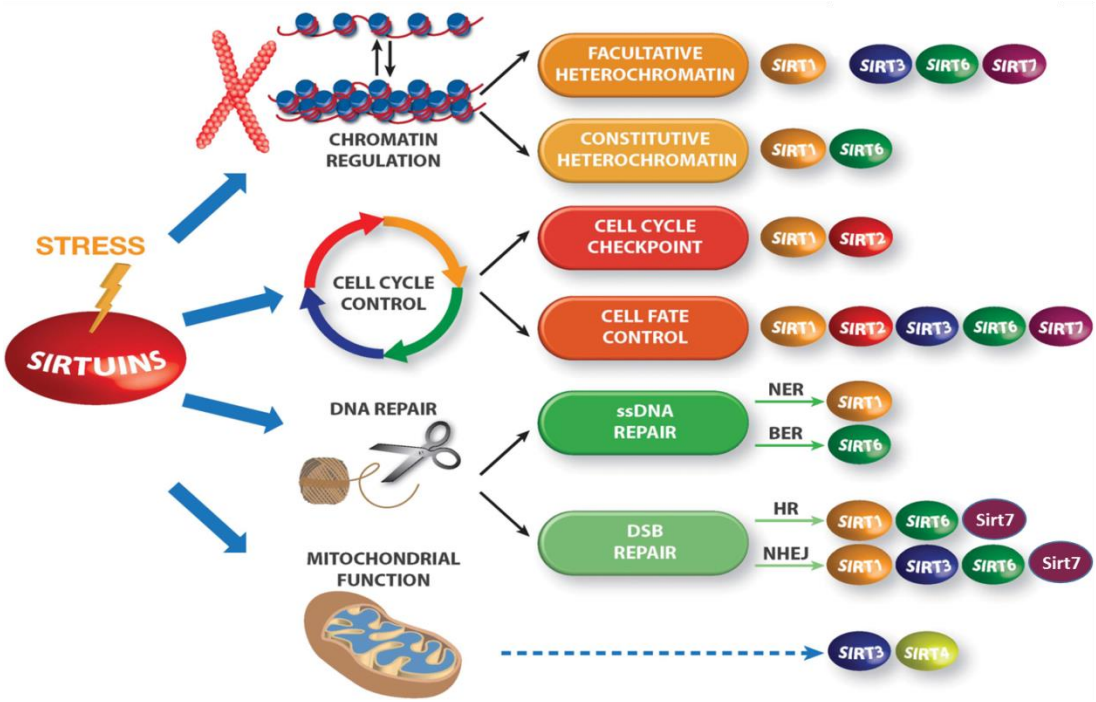


Fig.1.5. Mammalian sirtuins in the cellular stress response. The picture depicts the involvement of sirtuins in different molecular mechanisms that control the cellular response to stress stimuli (Figure adapted from Bosch-Presegue et al.[31]).

1.5 Mammalian sirtuins in apoptosis

Sirtuins play major role in regulating cellular stress resistance and assigning threshold for cell death. Sirtuins in general promote cellular survival in response to different kind of stressors by regulating the expression of various apoptosis related genes, e.g. BAX, BAK1 and BCL2 [186, 187]. However, sirtuins can also lead to induction of apoptosis in specific cell types or under particular physiological conditions.

Sirt1 has predominantly an anti-apoptotic role in response to DNA damage and oxidative stress by deacetylating several apoptosis related proteins such as p53, NFκβ, FOXO4, Ku70, AKT, MAPK and NRF2 [188, 189]. Deacetylation of p53 by Sirt1 inhibits its transactivation activity resulting in impaired p53-mediated transcription of pro-apoptotic genes and hence stimulates cell survival [25, 190]. As mentioned above, Sirt1 also binds and deacetylates Ku70

leading to inhibition of Bax-mediated apoptosis by preventing Bax translocation to the mitochondria [109]. Sirt1 inhibits the pro-apoptotic proteases caspase3 and caspase7 in transformed epithelial cells, enhancing cancer cell survival through suppression of the transcription factor FOXO4 [191]. However, Sirt1 has also been shown to promote apoptosis in cancer cells as in case of lung adenocarcinoma [192].

Sirt2 has a pro-survival role by deacetylating and antagonizing p53-dependent apoptosis in response to DNA damage, similarly to Sirt1 [193]. Additionally, Sirt2 inhibits apoptosis in cholangiocarcinoma by increasing cMYC expression [194, 195]. Despite this, also pro-apoptotic roles have been attributed to Sirt2. For instance, Sirt2 inhibition reduces cochlear cell apoptosis in inner ear by attenuating oxidative stress-induced damage [196]. Moreover, Sirt2 also promotes apoptosis by deacetylating and increasing nuclear translocation of FOXO3a, which activates caspase3 and caspase8 expression [197].

Sirt3 has a pro-apoptotic role by controlling the BCl2/p53 and JNK-regulated apoptotic pathways [198-200]. Cells lacking Sirt3 were demonstrated to have decreased stress-induced apoptosis [117]. In contrast, Sirt3 can also possess anti-apoptotic functions. Sirt3 binds to and deacetylates Ku70 preventing apoptosis similarly to Sirt1 [181, 201, 202]. In addition, Sirt3 protects cells from death under nutrient deficient condition by maintaining mitochondrial NAD^+ levels. This process is mediated through nicotinamide phosphoribosyltransferase (NAMPT), an enzyme involved in the biosynthesis of NAD^+, although through mechanisms that still remain uncharacterized [203].

Sirt4 protects cells against hypoxia-induced apoptosis by inhibiting BAX translocation to the mitochondria. Sirt4 also has an anti-apoptotic role through NAMPT, similarly to Sirt3 [203]. However, in case of colorectal cancer cells treated with 5- fluorouracil (5-FU), Sirt4 has a pro-apoptotic function [204, 205].

Sirt5 mostly protects cells from programmed cell death. In case of hepatocellular carcinoma, Sirt5 inhibits apoptosis by deacetylating cytochrome C (cyt C) and reducing thereby its mobilization to the cytosol. This phenomenon prevents formation of the apoptosome and hence blocks apoptosis [206]. In neuroblastoma, Sirt5 protects cells against staurosporine-induced apoptosis [207, 208]. However, the only instance where Sirt5 has been shown to be pro-apoptotic is when it is localized in the mitochondria of cerebellar granule neurons [209].

Sirt6 overexpression induces apoptosis in cancer cells but not in normal cells. In response to DNA damage and oxidative stress, Sirt6 can regulate apoptosis by deacetylating molecules such as Ku70, Bax, and p53. Sirt6 inhibits apoptosis by deacetylating Ku70 similarly to Sirt1 and Sirt3 [210]. On the other hand, Sirt6 stimulates apoptosis in various kinds of tumor cells by modulating different pathways. Sirt6 induces cell death in colorectal cancer by increasing phosphatase and tensin homolog (PTEN) expression and stability and by enhancing extracellular signal-regulated kinases1/2 (ERK1/2) signaling pathway in hepatocellular cancer [211-213].

Sirt7 has an anti-apoptotic role in response to DNA damage and oxidative stress by modulating molecules such as FOXO3, DNA damage binding protein 1 (DDB1) and p53 [214-216]. Additionally, Sirt7 protects cells against apoptosis by regulating several signaling pathways such as MYC, NFκB, mammalian target of rapamycin/insulin growth factor2 (mTOR/IGF2) and p38 mitogen-activated protein kinases (p38MAPK) [217-220]. Sirt7 inhibits apoptosis by repressing miR-34a expression through H3K18Ac deacetylation at its promoter [221]. Consistently with its pro-apoptotic role, Sirt7 knockdown in gastric cancer cells was associated with upregulation of pro-apoptotic genes (Bax, cleaved caspase3, cleaved PARP and Bim) and downregulation of anti-apoptotic genes (Bcl2 and Mcl1) [221].
All evidences suggest that sirtuins in general can have a pro- or an anti-apoptotic role depending on cell type, cellular localization or in response to different kinds of stressors.

1.6 Characterization of sirtuins knock out phenotypes

Given the critical role of sirtuins as key players in the maintenance of cellular homeostasis, it is not surprising that ablation of sirtuins expression in cells and mice is associated with an aberrant phenotype. The knock out (KO) mice for six of the seven sirtuins Sirt1-4, Sirt6 and Sirt7 display increased levels of genomic instability, resulting in chromosomal aberrations, high levels of DNA damage and defective DNA repair. Moreover, KO mice for four of these sirtuins, i.e. Sirt2, Sirt3, Sirt4 and Sirt6, show spontaneous tumor formation [31]. These phenotypes impressively document the critical role of the sirtuins in the cellular protection and their importance for the organismal homeostasis. The most relevant phenotypic characteristics of sirtuins KO mice are listed below.

Sirt1 KO embryos show chromosomal aberrations and impaired DNA repair [142]. Sirt1 controls telomere maintenance by significantly attenuating age-dependent telomere shortening [222, 223]. As a consequence, Sirt1 overexpression in mice results in higher stability of the telomeres while, telomere fragility and breakages were reported in Sirt1 depleted cells [224]. Sirt1-overexpressing mice show fewer signs of aging and are protected from severe damage induced by high fat diet and liver carcinogenesis [225]. In addition, since Sirt1 is critical for chromosomes condensation in metaphase, Sirt1 KO cells show aberrant progression in mitosis, unequal chromosome segregation, aneuploidy and accumulation of chromosome bridges and breaks [142].

Sirt2 KO cells show genomic instability, centrosome amplification and cell death during mitosis. As a consequence, ablation of Sirt2 in mice is associated with spontaneous tumor formation in multiple tissues [83, 226].

Sirt3 KO cells show abnormal mitochondrial phenotype, increased levels of ROS, genomic instability and spontaneous transformation, suggesting a possible link between aberrant ROS levels and tumor permissive phenotype [117]. As a consequence, Sirt3 KO mice develop spontaneous mammary tumors later in life while decreased levels of Sirt3 are observed in this

kind of cancer [117]. Additionally, depletion of Sirt3 has been associated with metabolic syndrome, a precondition for obesity and cardiovascular diseases [227, 228].

Sirt4 takes part in cellular metabolic response to DNA damage as described earlier. Depletion of Sirt4 in mice results in accumulation of genomic instability and enhanced tumorigenesis, which results in spontaneous lung tumor formation later in age [185].

Sirt5 KO mice develop normally, however they show global protein hypersuccinylation and hypermalonylation which results in altered mitochondrial homeostasis [11, 229]. Sirt5 KO mice show increased mortality and impaired oxidative metabolism following cardiac pressure overload [230].

Sirt6 KO mice display premature aging phenotype, shortened lifespan, severe metabolic defects, genomic instability and hypersensitivity to DNA damage [52, 156, 157]. Consistently, ablation of Sirt6 is associated with premature cellular senescence and enhanced tumorigenesis [50, 52, 231, 232]. In addition, Sirt6 acts as a tumor suppressor through its crucial role in maintenance of glucose homeostasis. This process is achieved through epigenetic suppression of several glycolytic genes via deacetylation of H3K9Ac. Loss of this metabolic control in absence of Sirt6 leads to increased anaerobic glycolysis which promotes cancer development [126, 232].

Sirt7 KO mice show signs of accelerated aging associated with multiple organ dysfunction such as cardiac hypertrophy and lipodystrophy [216, 233]. Sirt7 KO cells possess enhanced replication stress, increased sensitivity to various DNA damaging agents, higher mutation rate and elevated apoptosis levels. Despite this, no evidences for spontaneous tumor development has been reported in these mice so far [133, 216, 234, 235].

Sirtuins have a wide range of functions ranging from metabolism to DNA repair. It is very interesting to observe that several members of sirtuin family share common targets as discussed above. In this work, I showed that two mammalian sirtuins, Sirt1 and Sirt7, share a common target: the methyltransferase Suv39H1. I demonstrate that Sirt7 indirectly inhibits Suv39H1 by preventing the autocatalytic activation of Sirt1, a prominent stimulator of Suv39H1 activity. Through this mechanism, Sirt7 controls the dynamic organization of constitutive heterochromatin both under normal conditions and following genotoxic stress.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Cell lines

The following table provides the details of the cell lines used in this study along with the identification number provided by the supplier.

Cell Line	Supplier
Human embryonic kidney cell line (293T-HEK)	ATCC-CRL-11268^{TM}
Human osteosarcoma cell line (U2OS)	ATCC-HTB-96^{TM}

Table.2.1.1. List of cell lines.

2.1.2 Culture materials

The following table provides the detail of cell culture media and solutions used in this work and their respective supplier.

Material	Supplier
Dulbecco's modified eagle's medium-low glucose	Sigma-Aldrich-D6046
Dulbecco's modified eagle's medium-high glucose	Sigma-Aldrich-D5796
Fetal calf serum (FCS)	Sigma-Aldrich-F7524
100X Penicillin+streptomycin+L-glutamine solution (PSG)	Sigma-Aldrich-G1146
2.5% Trypsin	Gibco-150090046
Opti-MEM	Gibco-51985

Table.2.1.2. List of cell culture materials.

2.1.3 Buffers and solutions

The list of the buffers and solutions used in the study is provided in the following table.

Buffer	Composition/Supplier
1X Phosphate buffered saline (PBS)	2.7mM KCl, 2mM KH_2PO_4, 10MM Na_2HPO_4, 137mM NaCl (pH-7.4)
5X Laemmli sample buffer without glycerol	66mM Tris-HCl (pH-6.8), 2%SDS
5X Laemmli sample buffer with glycerol	66mM Tris-HCl (pH-6.8), 2%SDS,27% Glycerol (V/V)

MES running buffer for western blot	Invitrogen-NP0002
MOPS running buffer for western blot	Invitrogen-NO0001
Transfer buffer for western blot	12.5mM Bicine, 12.5mM Bis-Tris, 0.8mM EDTA, 20% Methanol
Radio-Immunoprecipitation assay buffer (RIPA)	50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.25% Na-deoxycholate (pH 7.4)
1X Tris buffered saline + Tween 20 (TBST) buffer	20mM Tris, 140mM NaCl, 0.1% Tween-20
Immunoflourescence solution (IF)	0.5% BSA, 0.1% Triton X-100, 5% FCS in PBS
Chromatin immunoprecipitation (ChIP) cell lysis buffer	5mM HEPES (pH-8.0), 85mM KCl, 0.5% NP-40
Chromatin immunoprecipitation (ChIP) dilution buffer	0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA (pH-8.0), 16.7mM Tris-HCl (pH-8.1), 167mM NaCl
Chromatin immunoprecipitation (ChIP) nuclear lysis buffer	50mM Tris-HCl (pH-8.1), 10mM EDTA (pH-8.0), 1% SDS
Chromatin immunoprecipitation (ChIP) high salt buffer	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH-8.1), 500mM NaCl
Chromatin immunoprecipitation (ChIP) low salt buffer	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH-8.1), 150mM NaCl
Chromatin immunoprecipitation (ChIP) Lithium Chloride (LiCl) buffer	10mM Tris-HCl (pH-8.0), 250mM LiCl, 1mM EDTA, 1% NP-40, 1% deoxycholic acid
Tris EDTA (TE) buffer	10mM Tris-HCl (pH 8.1), 1mM EDTA (pH-8.0)
Chromatin immunoprecipitation (ChIP) elution buffer	1%SDS, 100mM $NaHCO_3$, 10mM DTT
Mini preparation solution A	25mM Tris-HCl (pH-8.0), 10mM EDTA
Mini preparation solution B	0.2M NaOH, 1% w/v SDS
Mini preparation solution C	3M NaOAc (pH-5.6)
Chromatin Fractionation buffer A	1mM HEPES (pH7.9),10mM KCl, 1.5mM $MgCl_2$, 0.34M Sucrose, 10% Glycerol, 1mM DTT
Chromatin Fractionation buffer B	3mM EDTA, 0.2mM EGTA, 1mM DTT

Table.2.1.3. List of buffers and solutions.

2.1.4 Antibodies

The primary and secondary antibodies used in this work and their supplier information are listed below.

Primary antibody	Supplier
Anti-Tag (CGY)FP	Evrogen-Ab122
Anti-FLAG tag	Sigma-Aldrich-F1804
Ral A	BD Trans. Laboratories-R23520
H3K9me3	Abcam-ab8898
H3	Cell Signaling Technology -9715
HP-1 alpha	Millipore-MAB3584
Mouse non-immune IgG	Cell Signaling Technology -5415S
Rabbit non-immune IgG	Diagenode-C15410206
Sirt1 (IF3)	Cell Signaling Technology -8469
Sirt7	Cell Signaling Technology -5360
Suv39H1	Cell Signaling Technology -8729S
Suv39H1	Novus Biologicals-NB120-12405
Anti-HA.11 tag	Roche-11867423001
Anti-Myc tag	Cell Signaling Technology -2278
B23 (NPM)	Santa Cruz biotechnology- sc6013-R
Cleaved Caspase 3	Cell Signaling Technology-9661
PARP	Cell Signaling Technology -9542

Secondary antibody	Supplier
Anti-mouse IgG HRP-conjugated	Pierce-1858413
Anti-rabbit IgG HRP-conjugated	Thermo Fisher Scientific-31460
Anti-mouse IgG HRP-conjugated True blot™	eBioscience-18-8877
Anti-rabbit IgG HRP-conjugated True blot™	eBioscience-18-8816
Anti-mouse IgG Alexa-Fluor 488 conjugated	Invitrogen-A11001
Anti-mouse IgG Alexa-Fluor 594 conjugated	Invitrogen-A11005
Anti-rabbit IgG Alexa-Fluor 488 conjugated	Invitrogen-A1107

Anti-rabbit IgG Alexa-Fluor 594 conjugated	Invitrogen-A11012

Table.2.1.4. List of antibodies.

2.1.5 Chemicals

The following table provides the name of chemicals and their supplier used in this study.

Chemicals	Supplier
Agarose	Biozym-840004
Bovine serum albumin fraction V (BSA)	Merck Millipore-112018
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich-D4540
Formaldehyde 37%	Sigma-Aldrich-F1635
Glycine	Sigma-Aldrich-G8898
Paraformaldehyde (PFA)	Merck Millipore-104005
Skim milk powder	Sigma-Aldrich-70166
4'-6-dimidino-2-phenylindole (DAPI)	Invitrogen-A1306
Mowiol®	Merck Millipore-475904
Adriamycin HCl-solution (Doxorubicin)	Sigma-Aldrich-D1515-10MG
Anti-flag M2 affinity gel	Sigma-Aldrich-A2220
Flag peptide	Sigma-Aldrich-F3290
Protein G sepharose beads	GE Healthcare-17-0618
Protein A beads	Diagenode-C03020002
Chelex 100 resin	Bio-Rad-143-2832
10X Red Alert (Western blot stain)	Merck Millipore-71078
Trizol lysis reagent	Qiazol
Blasticidin	Thermo Fischer Scientific-R21001
Turbofectamin	Thermo Fischer Scientific-R0531
Bromophenol blue	Merck-1081220005
Ethidium bromide	Applichem-A1152,0100
Protein marker VI pre-stained	PanReac Applichem-A8889
Ammonium persulfate (APS)	Sigma-Aldrich-A3678
N,N,N',N'-Tetramethyl ethylenediamine (TEMED)	Sigma-Aldrich-T9281
ROTIPHORESE®Gel 30 (37,5:1) Polyacrylamid (PAA)	Roth-3029.1
Ex-527	Sigma-Aldrich-E7034

Polybrene (Hexadimethrine bromide)	Sigma-Aldrich-H9268
Puromycin dihydrochloride	Sigma-Aldrich-9620
KAPA Sybr FAST	KAPABiosystems-KM4104
Sodium fluoride (NaF)	Sigma-Aldrich-201154
Sodium orthovandate (Na_3VO_4)	Sigma-Aldrich-S6508
Phenylmethanesufonyl fluoride (PMSF)	Sigma-Aldrich-P7626
Aprotinin	Sigma-Aldrich-10820
Leupeptin	Sigma-Aldrich-L2884
Benzamidine	Acros-organics-401790050
Super signal west femto maximum sensitivity substrate	Thermo Fischer Scientific-34095
Westernbright chemiluminescent substrate sirius	Biozym-541020
Super signal west pico plus maximum sensitivity substrate	Thermo Fischer Scientific-34578

Table.2.1.5. List of chemical used.

2.1.6 Materials and machines

The machines and materials used and their supplier are listed in the following table.

Material/Machines	**Supplier**
Blotting paper	Roth-CL66.1
Microscope glass slides	Menzel-Gläser-631-0413
Coverslips	VWR-631-0148
Cell culture dishes	Greiner Bio-one
Nitrocellulose membranes (Amershan Protran)	GE Heathcare-10600002
Nucleobond Xtra Midi/Maxi	Macherey-Nagel-740414
QIAEX II Gel extraction kit	Qiagen-20021
Qiagen miRNeasy Mini Kit	Qiagen-217004
BioDocAnalyze transilluminator	Biometra
Mithras LB940 microplate reader	Berthold Technologies
Bioruptor Sonicator	Diagenode
iCycler Real-time PCR detection system	Bio-RAD
Mastercycler PCR machines	Eppendorf
Chemidoc MP Imaging system	Bio-RAD

Z1 fluorescence microscope	Carl Zeiss Jena GmbH
TCS SP2 confocal microscope	Leica microsysteme vertrieb GmbH, Bensheim
CM10 electron microscope	Philips
Nanodrop 2000/2000c spectrophotometer	Thermo Fischer Scientific
Cross linker UVP Cx-2000	Fisher Scientific
Incubator	Thermo Fischer Scientific-Heracell 150i
Hood	Thermo Fischer Scientific-HerasafeKS
Centrifuge	Hettich- Universal320R

Table.2.1.6. List of materials and machines.

2.1.7 Softwares

The following table provides the details of software used in this study for data acquisition and analysis together with the relative supplier.

Software	Supplier
DNASTAR Lasergene 11	DNASTAR
Image J	National institute of health, Bethesda, Maryland
Image Lab version 5.0	Bio-RAD
Leica confocal software 2.61	Leica microsystems, Heidelberg GmbH
AxioVision 4.8	Carl Zeiss Microscopy GmbH
Prism 5 version 5.0	GraphPad Inc.
Zen	Carl Zeiss Microscopy GmbH

Table.2.1.7. List of softwares.

2.1.8 Enzymes

The Enzymes used in this study and their relative supplier are listed below.

Enzyme	Supplier
Benzonase	Sigma-Aldrich-E1014
T4 DNA ligase	Promega-M180
DNase I	Roche-04716728001
Proteinase K	Roth- 7528.1

RNase A	Sigma-Aldrich-R4875

Table.2.1.8. List of enzymes.

2.1.9 Plasmids

The following table provides details of the plasmids used and their supplier.

Plasmid	Supplier
Empty FLAG: 2x Flag-Tag cloned into the pcDNA5/To vector	Kindly provided by Dr. Christian Smolka
Empty YFP: pTagYFP-N vector	Evrogen (FP132)
Sirt1 FLAG: pCMV-sport6-mSirt1-Flag	Kindly provided by Prof. Leo, Bruxelles, Belgium
Sirt1 HA: Mouse Sirt1 ORF in the pCMV-Sport6 vector fused with 3X HA Tag.	Kindly provided by Dr. Christian Smolka (MPI, Bad Nauheim)
Sirt7 FLAG: Human Sirt7 ORF cloned into the pcDNA 3.1+ vector	Kindly provided by Dr. Alejandro Vaquero (JCI, Barcelona)
Sirt7 YFP: Mouse Sirt7 ORF cloned into the pTagYFP-N vector	Kindly provided by Dr. Christian Smolka (MPI, Bad Nauheim)
Suv39H1 EGFP: pcDNA4-TO_GFP_mouse_Suv39H1	Kindly provided by Prof. Alejandro Vaquero (JCI, Barcelona)
Suv39H1 MYC: mSuv39H1 in mycTag-pCS2+	Kindly provided by Dr. Christian Smolka (MPI, Bad Nauheim)
Envelop vector: VSV-G/pMD2.G	Kindly Provided by Dr. Johnny Kim (MPI, Bad Nauheim)
Packaging vector: pCMV-R8.74psPAX2	Kindly Provided by Dr. Johnny Kim (MPI, Bad Nauheim)
Scramble PLKO.1: PLKO.1 plasmid with shRNA insert : 5´CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3´	Sigma-Aldrich

Sirt7 KD: PLKO.1 plasmid with shRNA insert : 5´CCGGGTCCAGCCTGAAGGTTCTAAACTCGAGTTTAGA ACCTTCAGGCTGGACTTTTTG-3´	Sigma-Aldrich
Scramble pGPIZ: pGPIZ plasmid with shRNA insert: 5´- TGCTGTTGACAGTGAGCGATCTCGCTTGGGCGAGAGTAA GTAGTGAAGCCACAGATGTACTTACTCTCGCCCAAGCGA GAGTGCCTACTGCCTCGGA-3´	Sigma-Aldrich
Sirt1 KD: pGPIZ plasmid with shRNA insert: 5´TGCTGTTGACAGTGAGCGAGGTGATGAAATTATCACT AATTAGTGAAGCCACAGATGTAATTAGTGATAATTTCAT CACCGTGCCTACTGCCTCGGA-3´	Sigma-Aldrich
Suv39H1 KD: pGPIZ plasmid with shRNA insert: 5´- TGCTGTTGACAGTGAGCGACGGG CCTTCGTGTACATCAATTAGTGAAGCCACAGATGTAATT GATGTACACGAAGGCCCGCTGCCTACTGCCTCGGA-3´	Sigma-Aldrich

Table.2.1.9. List of plasmids.

2.1.10 Mouse strains

The following table provides the details of the mouse strains used in this work.

Mouse strains	Provider
C57BL/6J (WT)	Harlan-Winkelmann, Paderborn
Sirt7 Knock out	Kindly provided by Vakhrusheva O.[216]

Table.2.1.10. List of mouse strains.

2.1.11 Media and antibiotics for bacterial culture

Bacterial culture medium and antibiotics used in this study and their relative supplier are listed in the table below.

Substance	Supplier
LB Agar (Lennox) 35g/l	Roth-6671
LB medium (Lennox) 20g/l	Roth-X964
Ampicillin sodium salt	Roth-K029
Kanamycin sulphate	Roth-T832

Table2.1.11. List of media and antibiotics used for bacterial culture.

2.1.12 List of primers

The following table provides the list of primers used for sequencing or RT-qPCR together with their relative sequence.

RT-qPCR Primer	Sequence
Human satellite DNA	Forward: 5'-CATCGAATGGAAATGAAAGGAGTC-3' Reverse: 5'- ACCATTGGATGATTGCAGTCAA -3'
Mouse satellite DNA	Forward: 5'-GACGACTTGAAAAATGACGAAATC-3' Reverse: 5'- CATATTCCAGGTCCTTCAGTGTGC-3'
Human β actin	Forward: 5'-CATGTACGTTGCTATCCAGGC-3' Reverse: 5'-CTCCTTAATGTCACGCACGAT-3'
Mouse β actin	Forward: 5'-CAACGAGCGGTTCCGATG-3' Reverse: 5'-GCCACAGGATTCCATACC-3'
Human MMP9	Forward: 5'- GATGCGTGGAGAGTCGAAAT -3' Reverse: 5'- CACCAAACTGGATGACGATG -3'

Sequencing Primer	Sequence
BGH reverse	Reverse: 5'-TAGAAGGCACAGTCGAGG -3'
CMV forward	Forward: 5'-CGCAAATGGGCGGTAGGCGTG-3'
Mouse Sirt7 begin	Forward: 5'-CTGGAATCAGCACAGCAGC-3'
Human Sirt7 middle	Forward: 5'-GGATCCAATCTTCTCCTTGG-3'
Mouse Suv39H1 middle	Forward: 5'-TATCGAGTTGGTGAGGGCA-3'
Mouse Suv39H1	Reverse: 5'-CTTGTTCCCAACGCTGAAGT-3'
SP6	Forward: 5'-ATTTAGGTGACACTATAGAA-3'
T7	Forward: 5'-TAATACGACTCACTATAGGG-3'
Mouse Sirt1 5' overhang	Forward: 5'-CGAGGGCGGCTGACGACTTC-3'
Mouse Sirt1 3' overhang	Forward: 5'-ATCCCCTCCATCAGCTCCAAATCC-3'
Mouse Sirt1 exon4	Forward: 5'-GCCTTGCGGTGGACTTC-3'

Table.2.1.12. List of primers.

2.2 Methods

2.2.1 Cell culture

293T-HEK and osteosarcoma cell line (U2OS) were cultured in high glucose DMEM (4.5g/L glucose) supplemented with 10% FCS and 1X PSG [Penicillin (100U/mL)/ Streptomycin (0.1mg/ml)/ L-Glutamine (2mM)] at 37°C in humidified atmosphere with 5% carbon dioxide. For passaging, sub confluent plates were washed once in 1X PBS and incubated with 1X trypsin for 5 min at 37°C. DMEM was added to the plates in a 1:1 ratio to neutralize the trypsin. Cells were collected by centrifugation at 1200 rpm for 5 min. Cell pellets were re-suspended in fresh DMEM and transferred into fresh cell culture dishes.

2.2.2 Cell freezing and thawing

To freeze cells, 60-70% confluent plates were washed in PBS once and trypsinized. After neutralization of trypsin, cell pellets were collected by centrifugation at 1200 rpm for 5 min. The pellet was re-suspended in 1ml of freezing medium (90% FCS + 10% DMSO) and transferred into cryo vials. Cells were stored either at -80°C for short-term storage or in liquid nitrogen for long-term storage (longer than two weeks).

For thawing of cryopreserved cells, the cryo vials were quickly incubated in a 37˚C water bath for 10 min. The cells were added dropwise into 5ml of fresh medium and collected by centrifugation at 1200 rpm for 5 min. Cells were re-suspended in fresh medium and plated into cell culture dishes.

2.2.3 Transfection of cells

293T-HEK cells were transfected using calcium phosphate method as already described [236]. Briefly, 293T-HEK cells were plated in a 10 cm petri dish at approximately 20-30% confluency. On the next day, the transfection solution was prepared as following: (I) 61µl of $CaCl_2$ (2M), 10µg of plasmid and milli-Q water to a final volume of 500µl. (II) The resultant mix was added dropwise into 500µl of 2X HBS. (III) This solution was added dropwise to the plate and the cells were incubated with the transfection solution for 16-24 hours. Following incubation, the medium was then changed to fresh DMEM and the cells were incubated for additional 48 hours before being harvested.

U2OS cells were transfected using TurboFect reagent following manufacturer's guidelines. Briefly, cells were plated in 6cm petri dish at 90% confluency overnight. On the next day, the transfection solution was prepared as following: 6µg of plasmid DNA and 12µl of TurboFect transfection reagent were diluted in 600µl of Opti-MEM medium and the mixture was incubated at room temperature for 15 min. Following incubation, the solution was added dropwise to the cells. 5 hours post transfection, the medium was replaced with fresh DMEM. The cells were harvested 24-48 hours post-transfection as indicated in each experiment.

2.2.4 Lentivirus-based generation of stable knock down cell lines

Stable knock down cell lines were generated as already described [236]. Briefly, 293T-HEK cells were transfected with 10µg of plasmid carrying the shRNA, 9 µg of packaging vector (pCMV-R8.74psPAX2) and 1µg of envelope vector (VSV-G/pMD2.G) using the calcium phosphate method as described previously. 24 hours post-transfection, the medium was changed to 5ml of fresh DMEM and the cells were incubated for an additional 24 hours. The medium containing lentiviral particles was collected 24 and 48 hours after medium change. Polybrene was added to the lentivirus-containing medium to a concentration of 8µg/ml. Additionally, FCS was added to the medium to a final concentration of 30%. The lentiviral particles were filtered through 0.2µm filter to remove contaminant cell and either stored at -20°C or directly used for target cells transduction.

For lentiviral transduction, target cells were trypsinized and re-suspended in the lentivirus medium and transferred to new cell culture dishes. The cells were cultured in a humidified incubator at 37°C and 5% CO_2 for 24 hours. After incubation with the virus, cells were washed twice in PBS and supplemented with normal DMEM medium. Cells were allowed to recover for additional 24 hours in fresh DMEM medium prior to selection with 5µg/µl of puromycin. To ensure optimal selection of the cells, cells were grown for 1 week in puromycin-containing medium and then further cultured in normal DMEM.

2.2.5 Immunofluorescence (IF)

For immunofluorescence, cells were grown on 13mm glass cover slips in a 24-well cell culture plate. Cells were washed 3 times with 1X PBS and fixed in 4% paraformaldehyde in 1X PBS for 5 min. After this, the coverslips were washed 2 times in 1X PBS for 5 min. Specific primary antibodies diluted in IF solution were added to the cover slips and incubated overnight at 4°C in a humid chamber. The next day, cells were washed 3 times in 1X PBS and incubated with appropriated fluorophore-conjugated secondary antibodies diluted in IF solution for 1 hour at room temperature. After incubation, samples were washed 3 times in 1X PBS. Cell nuclei were counterstained with 2µg/ml DAPI diluted in PBS for 5 min. The coverslips were washed twice in 1X PBS (5 min each) and mounted on glass microscope slides using the Mowiol® mounting medium. The glass slides were allowed to dry at 4°C for at least 1 hour before imaging. Fluorescence pictures were taken using Z1 fluorescent microscope or a SP6 confocal microscope as indicated in the figure legends.

2.2.6 Electron microscopy

Cells and tissues for electron microscopy were fixed in 0.1nM/L sodium cacodylate/7.5% sucrose/3% glutaraldehyde for 4 hours. The tissue slides were washed 3 times in 1X PBS for 5 min. Cells were further fixed in 1% osmium tetroxide for 1 hour. The samples were dehydrated by washing in a series of gradient ethanol dilution and embedded in Epon. The sections were stained with uranyl acetate and Reynolds lead citrate. Images were captured using the Philips CM10 electron microscope.

2.2.7 Maintenance of animals

Mice were housed in hygienic environmental conditions with 22.5°C (+/- 1°K) temperature, 50% of humidity (+/- 5%) and 11/13 hours of light/dark cycle. Cages were supplied with sterile woodchips for bedding, water bottle and food container. Food and water were provided *ad libitum*. All experiments were conducted according to the German animal welfare law.

2.2.8 Generation of primary mouse embryonic fibroblasts (MEFs)

Primary MEFs were isolated from embryos collected following the mating of Sirt7 heterozygous mice. Pregnant mice 13.5 days post-coitum (DPC) were sacrificed by cervical dislocation. The uterine horn was collected and placed into a petri dish with PBS. Individual embryos were separated from the placenta and from the embryonic sac. The head and red organs were separated from the embryos and the remaining part was transferred into a petri dish containing 0.25% trypsin. The embryos were then minced with a scalpel. Individual cells were separated by extensive pipetting and further incubation at 37°C. 2 volumes of DMEM were added to the mixture to neutralize the trypsin. Cells were then collected by centrifugation (1200 rpm for 5 min), re-suspended in fresh medium and plated into petri dishes. Finally, cells were frozen after 48 hours and considered to be at passage 0 at this stage. For all the experiments in this work, primary MEFs were used until passage 2 (P2).

2.2.9 Western blot

Cells were washed twice in 1X PBS and collected by scraping in 5X Laemmli buffer supplemented with protease and phosphatase inhibitors as described in the material section. Cell lysates were sonicated to shear the DNA (3cycles at 45% energy, 10sec each). Cell debris was removed by centrifugation at 14000 rpm for 15 min. Following centrifugation, the supernatant was collected into fresh tubes. The protein concentration was measured using the Biorad® DC protein assay kit following manufacturer´s instructions. Samples were then diluted to a concentration of 1µg/µL in 5X Laemmli buffer containing a final concentration of 15% glycerol, 50mM DTT and bromophenol blue. Samples were boiled at 95°C for 5 min and protein lysates were resolved by electrophoresis into 9% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes by wet transfer at 30 Volts for 90 min. Membranes were blocked in 5% skimmed milk in 1X TBST for 1 hour at room temperature followed by washing 3 times (5 min each) in 1X TBST and incubated with specific primary antibodies overnight at 4°C on a shaking platform. On the next day, the membranes were washed 5 times in 1X TBST and incubated with secondary antibodies diluted in 3% skimmed milk in 1X TBST for 1 hour at room temperature. After incubation with secondary antibodies, samples were extensively washed in 1X TBST. Pictures were captured with ChemiDoc imaging system (Biorad) using Super signal Femto or Pico substrate (Thermofisher).

2.2.10 SDS polyacrylamide gel

The SDS page gels used for western blotting were prepared using 1.0mm gel cassettes from Novex. In all experiments, 9% polyacrylamide running gels and 5% stacking gels were used.

The gel composition used is as described in the table. The gels were stored in humidified packages at 4°C up to a month.

Constituents	Separating gel (9%)	Stacking gel (5%)
PAA 30% (37.5:1)	2.1ml	0.29ml
3.5X Bis-Tris(pH 6.5-6.8)	2ml	0.5ml
MilliQ H_2O	2.9ml	0.96ml
10% APS	25µl	8µl
TEMED	7µl	3µl

Table.2.2.10. List of components of SDS polyacrylamide gel.

2.2.11 Co-Immunoprecipitation (CO-IP)

Cells were washed 2 times in 1X PBS and harvested in RIPA buffer supplemented with phosphatase and protease inhibitors. Cell lysates were incubated for 30 min at 4°C on a rotating wheel and then clarified by centrifugation (14000 rpm for 15 min). 1000-2000µg of protein lysates were precleared for 1 hour with 30µl of protein G Sepharose 4 Fast Flow beads. After preclearing, beads were removed by centrifugation at 3000 rpm for 1 min and the supernatants were collected in fresh tubes. For the immunoprecipitation, equal amounts of protein lysates were incubated with 2µg of primary antibody or non-immune IgG control overnight. On the next day, the lysates were incubated for 2 hours at 4°C with pre-blocked G Sepharose 4 Fast Flow beads (incubated overnight in 5% BSA in TBST and then washed three times in RIPA buffer). Following incubation of the beads with the lysates, the beads were washed 5 times 10 min each in RIPA buffer on a rotating wheel and collected each time by centrifugation at 3000 rpm for 1 min. After the last wash, the beads were re-suspended in 20µl of 5X Laemmli buffer containing 15% glycerol, 50mM DTT and bromophenol blue and boiled at 95°C for 5 min. After this step, beads were separated from the samples by centrifugation at 14000 rpm for 1 min. The supernatants were resolved by western blotting as described above.

Co-immunoprecipitation experiments of FLAG tagged proteins were carried out using anti-FLAG M2 Affinity beads. In these experiments, clarified cellular lysates were directly incubated with 20µl of pre-blocked anti-FLAG beads overnight. On the next day, the beads were washed 5 times in RIPA buffer and collected by centrifugation at 3000 rpm for 1 min. The immunoprecipitates were eluted from the beads by addition of 20µl of RIPA buffer supplemented with 0.4µg/µl of FLAG peptide (Sigma-Aldrich) for 30 min at 30°C. Following incubation, the supernatants were collected by centrifugation and diluted in 5X Laemli extraction buffer with glycerol containing bromophenol blue and 50mM DTT. The immunoprecipitates were boiled at 95°C for 5 min prior to western blot analysis.

2.2.12 Mass Spectrometry

Post-translational modifications of Suv39H1 were analyzed using mass spectrometry. 293T-HEK cells were transfected with GFP-tagged Suv39H1, alone or in combination with Flag-tagged Sirt7 using calcium phosphate method as described previously. 48 hours post-transfection, cells were treated with the histone deacetylase inhibitor TSA (5µM for 5 hours) to increase the levels of acetylation [237]. After incubation, the protein lysates were subjected to immunoprecipitation using an anti-GFP antibody to immmunoprecipitate Suv39H1. The immunoprecipitates were resolved by electrophoresis and the gel was stained using Novex® colloidal blue staining Kit according to manufacturer's instructions. The bands corresponding to the molecular weight of GFP-Suv39H1 were cut into small pieces and subjected to in-gel digestion method as already described [238]. Briefly, gel pieces were de-stained using 50mM ammonium bicarbonate in 50% ethanol for 20 min at room temperature. The samples were then dehydrated by 10 min incubation in 100% ethanol 2 times and collected by centrifugation for 5 min using Speedvac™. Peptides were reduced by incubation with 10mM dithiothreitol (DTT) at 56°C for 45 min followed by alkylation in 55mM iodoacetamide (IAA)/50mM ammonium bicarbonate for 30 min. Peptides were washed sequentially in: 50mM ammonium bicarbonate for 15 min, 100% ethanol for 15 min, 50mM ammonium bicarbonate for 15 min and finally two times in 100% ethanol for 15 min. The gel pieces were dried by centrifugation at 30°C in Speedvac™ and then separated in individual peptides by digestion with 4.6ng/µl of trypsin in 50mM ammonium bicarbonate over night at 37°C. The supernatant containing the peptides was collected in a separate tube. In order to collect remaining peptides, the gel pieces were treated with increasing concentration of acetonitrile: 30% acetonitrile/ 3% trifluoroacetic acid for 20 min, 70% acetonitrile for 20 min and finally two times in 100% acetonitrile for 20 min. After each incubation, the supernatants were collected together in the same tubes. The derived samples were mixed with 5% acetonitrile and 1% trifluoroacetic acid in a 1:1 ratio and loaded on pre-equilibrated C18 stage tips prior to mass spectrometry analysis. The peptides were eluted from C18 stage tips and subjected to reversed phase liquid chromatography and tandem mass spectrometry (MS/MS) and analyzed by MaxQuant software [239-242].

2.2.13 Chromatin Fractionation

Fractionation of cellular compartments was carried out as described by Mendez and Stillman [243] with minor modifications. Briefly, cells were washed twice in 1X PBS, scraped and collected by centrifugation at 3000 rpm for 2 min. The pellets were re-suspended in 200µl chromatin fractionation buffer A supplemented with protease and phosphatase inhibitors containing 0.1 % (v/v) of TritonX-100 and incubated on ice for 5 min. Samples were separated into nuclear (pellet) and cytoplasmic fraction (supernatant) by centrifugation at 1300g at 4°C for 4 min. The cytoplasmic fraction (S1) was further cleared of cellular debris by centrifugation at 20,000g for 15 min.

The nuclear pellet was washed with 200µl of chromatin fractionation buffer A, collected by centrifugation at 1700g for 5 min at 4°C and dissolved in 100µl of chromatin fractionation

buffer B supplemented with protease and phosphatase inhibitors. The samples were then incubated on ice for 30 min and centrifuged at 1700g for 5 min at 4°C. The pellet obtained in this step, which represents the chromatin fraction, was washed once again in 200 µl chromatin fractionation buffer B and resuspended in 5X Laemmli buffer containing 15% glycerol, 50mM DTT and bromophenol blue. Cytoplasmic fractions were also diluted in 5X Laemli extraction buffer containing glycerol, DTT and bromophenol blue as above. Samples were boiled at 95°C for 5 min and resolved by western blotting.

2.2.14 Chromatin immunoprecipitation (ChIP)

For ChIP, cells were grown on 15cm cell culture plates, treated and harvested as indicated in each experiment. Cells were washed once in 1X PBS and the DNA-proteins complexes were cross-linked by adding 18ml of 1% formaldehyde in 1X PBS for 10 min at room temperature on a shaking platform. The reaction was quenched by addition of 2ml of glycine to a final concentration of 125mM and incubated for 5 min on a rotating platform. After incubation, cells were washed twice with ice-cold PBS and collected by scraping and centrifugation at 2500 rpm for 5 min. Cells were lysed in 500µl of ChIP cell lysis buffer supplemented with proteases and phosphatases inhibitors for 10 min on ice. The suspension was then centrifuged at 5000 rpm for 5 min to collect the cellular nuclei. Nuclear pellets were re-suspended in 250µL of ChIP nuclear lysis buffer (with proteases and phosphatases inhibitors). The chromatin was sheared into 200-500bp fragments by sonication using the Bioruptor (3 cycles, 30sec on/off for 15 min at 4°C) and cleared by centrifugation at 14000 rpm for 15 min. Before proceeding to next step, chromatin size was verified by running 10µl of sheared chromatin on a 2% agarose gel. The chromatin was diluted to a final concentration of 200ng/µl in ChIP dilution buffer. 30-100µg of sheared chromatin were used for immunoprecipitation, while 20µl of diluted chromatin was collected as inputs. 2µg of primary antibody or non-immune IgG were added to the samples and incubated overnight at 4°C on rotating wheel. In parallel, protein A-agarose beads were pre-blocked overnight in 0.1% BSA/0.2mg/ml sonicated salmon sperm DNA in ChIP dilution buffer. On the next day, pre-blocked beads were washed 3 times in ChIP dilution buffer and 20µl of beads were then added to the ChIP samples followed by incubation for 2 hours at 4°C. The beads were washed 2 times for 10 min in the following buffers: (I) ChIP low salt buffer, (II) ChIP high salt buffer (III), ChIP LiCl buffer and (IV) TE buffer and collected each time by centrifugation at 1000 rpm for 30sec. After the last wash, the supernatant was carefully removed and 270µl of 10% BT Chelex® 100 resin were added to the beads. In parallel, 180µl of 10% BT Chelex® 100 resin were added to the inputs. Both the ChIP samples and the inputs were incubated for 10 min at 95°C on a shaker at 1350 rpm to reverse the crosslinking. The samples were cooled shortly on ice and supplemented with 2µl of 10mg/ml protein kinase (PK). The proteins were digested by incubation at 56°C for 30 min at 1350 rpm and the PK was denaturated by further incubation at 95°C for 10 min. Finally, the ChIP samples were centrifuged at 1000 rpm for 30sec and the supernatants were collected in fresh tubes. The immunoprecipitated chromatin was diluted 1:100 in deionized water and analyzed by qPCR

using specific primers. The quantification was performed by the ΔΔCt method as already described [244] using the inputs as internal control for normalization.

2.2.15 RNA extraction and cDNA synthesis

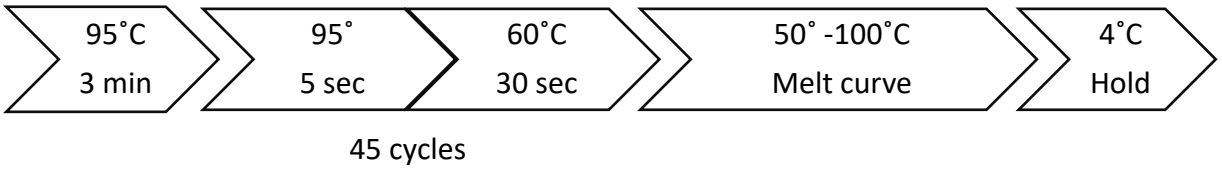
RNA extraction was performed using the TriFast™ reagent kit following the manufacturer's instructions. Briefly, cells were washed once in PBS and collected in 1mL of TriFast reagent. Samples were either processed immediately or stored at -80°C. 2µl of glycogen (20µg/µl) followed by 200µl of chloroform were added to the samples. Samples were vigorously mixed by inversion and incubated for 2-3 min at room temperature. After incubation, samples were centrifuged at 12000g for 15 min at 4°C centrifuge. After centrifugation, the upper aqueous phase was collected in fresh tubes and 500µl of isopropanol was added. The samples were extensively mixed by inversion and incubated at room temperature for 10 min. The RNA was collected by centrifugation at 12000g for 10 min at 4˚C. The RNA pellet was washed twice with 1ml of 75% ethanol and collected by centrifugation at 7500g for 5min. After washing, the pellet was allowed to dry under a chemical hood for 10 min. The dried RNA was finally re-suspended in 20µL of deionized water and incubated at 56°C for 10 min. RNA concentration was measured using a Nanodrop reader and stored at -20°C.

For cDNA synthesis, equal amounts of RNA were treated for 30 min at 37°C with 2 units of RQ1 RNAse-free DNase (Promega) following manufacturer´s instructions to digest any gDNA contaminants. The reaction was stopped by addition of 2µl of stop solution and incubation at 65°C for 5 min. After DNase digestion, cDNA synthesis was performed using the SuperScript II Reverse Transcriptase kit following manufacturer's protocol. Briefly, 1 µg of DNase treated RNA was mixed with 1µl of oligo dT primers, 1µl of random primers and 1µl of dNTPs and water was added to a final volume of 12µl. The mixture was then heated to 65˚C for 5 min and quickly chilled on ice. The tubes were briefly centrifuged to collect the samples and 4µl of 5X first stand buffer, 2µl of 0.1M DTT, 1µl of RNase out and 1µl of Superscript II enzyme were added. The reaction was carried out at 42˚C for 50 min followed by incubation at 70˚C for 15 min. cDNA was then diluted 1:20 in deionized water prior to qPCR reaction.

2.2.16 qPCR

For q-PRC analysis, 5µl of diluted cDNA was amplified using the KAPA SYBR FAST reagent following manufacturer's protocol in the iCycler real time PCR detection system (Biorad). Briefly, 10µl of 2X KAPA SYBR mix, 0.8µl of forward and reverse primers and 3.4µL of water were mixed together with 5µl of diluted cDNA. For each sample, the reaction was performed in triplicate. The quantification of the expression of the gene of interest was performed using the ΔΔCt method as described earlier [244]. β-actin was used as loading control for normalization of the gene expression.

The qPCR cycles were the following:



2.2.17 RNA isolation for RNA sequencing

Total RNA for sequencing was isolated using the Qiagen miRNeasy Mini Kit using manufacturer's protocol. Briefly, sub-confluent 6cm cell culture dishes were washed twice in PBS and collected in 700uL of QIAzol lysis reagent. The samples were vortexed for 1 min and incubated at room temperature for 5 min. 140µl of chloroform was added to the tubes and mixed vigorously for approximately 15 sec and the samples were further incubated at room temperature for 2-3 min followed by centrifugation at 12000g for 15 min at 4˚C. After centrifugation, aqueous phase was transferred into fresh RNAse-free tubes and 525µl of 100% ethanol were added to the samples and mixed by inversion. 700µl of the suspension was transferred to the RNeasy mini spin columns in a 2ml supplied collection tube. The column was then centrifuged two times at 8000g for 15 sec at room temperature and the RNA suspension collected in the column was transferred into fresh tubes. The suspension was further cleared by another round of centrifugation. The column was washed with 350µl of buffer RWT following by centrifugation for 15 sec. 80µl of DNase I incubation mix (10µl of DNase I stock and 70µl of buffer RDD) was added to each column and incubated for 15 min at room temperature. After incubation, the column was further washed with 350µl of buffer RWT, 500µl of buffer RPE (2X) and finally dried by centrifugation at 8000g for 2 min. The columns were placed in a fresh 1.5ml collection tube and 30µl of RNase free water was added to the columns. The DNase treated RNA was eluted and collected by centrifugation at 8000g for 1 min.

2.2.18 Generation of competent cells

XL1-Blue bacteria were rendered competent for transformation using the CaCl2 method as already described with minor modification [245]. Briefly, bacteria were grown overnight in 100ml of LB medium. On the next day, the Optical density (OD) at 600nm of the bacterial culture was measured and adjusted to a value of 0.3-0.6. The cells were collected by centrifugation at 5000 rpm for 15 min at 4°C, re-suspended in 25ml of 50mM $CaCl_2$ and incubated for 20 min on ice. After incubation, the suspension was centrifuged at 3000g for 5 min. The pellet was re-suspended in 50mM $CaCl_2$/ 15% glycerol, aliquoted and stored at -80°C.

2.2.19 Transformation

Bacteria were transformed using the heat shock method. Briefly, 5 ng of plasmid DNA was mixed with 100µl of competent cells and incubated on ice for 30 min. After incubation, the samples were incubated for 1.5 min at 42 °C and rapidly transferred on ice for an additional 2 min. The transformed bacteria were inoculated in 1ml of LB medium and allowed to grow in

a bacterial shaker for 1 hour at 37°C. Cells were finally plated on LB agar plates containing appropriate antibiotics and allowed to grow overnight at 37°C. Single bacterial colonies were picked and inoculated for mini or maxi preparation of plasmid DNA.

2.2.20 Mini preparation

For mini preparation of plasmid DNA, a single bacterial colony was inoculated in 3ml of LB medium with appropriate antibiotics and incubated overnight at 37˚C with vigorous shaking. On the following day, 1.5 ml of culture were centrifuged at 14000 rpm for 5 min to collect the bacteria. The pellet was re-suspended in 100µl of solution A (re-suspension buffer) followed by addition of 200 µl solution B (lysis buffer). The solution was mixed by inversion and then incubated at room temperature for 5 min. After incubation, 150µl of solution K (neutralizing buffer) was added to solution and the samples were mixed by inversion followed by incubation at room temperature for 3 min. The bacterial debris were removed by centrifugation at 14000 rpm for 10 min and the supernatant was transferred into fresh tubes. The plasmid DNA was precipitated by the addition of 800µl of isopropanol, incubation for 10 min at room temperature and centrifugation at 14000 rpm for 10 min. The DNA pellet was washed in 300µl of 70% ethanol and air dried for 10 min at room temperature. The plasmid DNA was finally re-suspended in 50µl of deionized water containing 0.5µl of 10mg/ml RNaseA and incubated at 37°C for 30 min.

2.2.21 Maxi preparation

Maxi preparation was done using the Nucleobond Xtra Midi/Maxi kit following the manufacturer's instructions. Briefly, single bacterial colonies were grown overnight in 150ml of LB medium at 37˚C with vigorous shaking. On the next day, bacteria were collected by centrifugation at 5000g for 15 min at 4˚C. The pellet was re-suspended in 12ml of re-suspension buffer containing RNaseA (66.7µg/µl). 12ml of lysis buffer were added to the suspension and mixed by inversion. The lysates were incubated at room temperature for 5 min. During incubation, the separation columns were equilibrated with 25ml of equilibration buffer. 12ml of neutralization buffer were added to the suspension and mixed by inversion. The suspensions were loaded on the equilibrated columns and filtered through the columns. After filtration, 15ml of equilibration buffer was added into the columns. Columns were washed with washing buffer and the DNA was eluted and precipitated in 10.5 ml of isopropanol. The DNA was collected by centrifugation for 15 min at 5000g at room temperature. After precipitation, the plasmid DNA was washed with 5ml of 70% ethanol and collected again by centrifugation at 5000g for 30 min at room temperature. The plasmid DNA was air dried and re-suspended in 300µl of deionized water.

All Plasmids were verified by sequencing by the Seqlab company (Göttingen, Germany).

Chapter 3: Results

3.1 Sirt7 forms a molecular complex with Suv39H1

Sirt7 is the only member of the sirtuin family which is primarily enriched in the nucleolus, although it is also present in other sub-cellular compartments such as the nucleoplasm and cytoplasm [246, 247]. Sirt7 is highly associated with chromatin but its role in global chromatin organization remains largely uncharacterized. Former studies in our laboratory demonstrated that Sirt7 is a potent inhibitor of Sirt1 enzymatic activity by preventing its autocatalytic activation. In absence of Sirt7, hyperactive Sirt1 suppresses adipocytes differentiation resulting in lipodystrophy in Sirt7 deficient mice [233]. Besides the role of Sirt1 in the inhibition of adipocytes differentiation, Sirt1 promotes constitutive heterochromatin assembly through direct deacetylation of different histone marks such as H3K9 and H1K26 and through deacetylation-dependent activation of the methyltransferase Suv39H1 [22, 48]. I hypothesized that Sirt7 might regulate constitutive heterochromatin organization by modulation of the Sirt1/Suv39H1 axis. To corroborate this assumption, I first aimed to prove whether Sirt7 and Suv39H1 form a molecular complex performing co-immunoprecipitation experiments. I expressed EGFP-tagged Suv39H1 alone or in combination with Flag-tagged Sirt7 in 293T-HEK cells. After transfection, cell lysates were subjected to immunoprecipitation using an anti-EGFP antibody and the immunoprecipitates were analyzed by Western blotting using anti-EGFP and anti-Flag antibodies as indicated in (**Fig 3.1.A**). I could demonstrate that Sirt7 efficiently co-precipitates with Suv39H1, indicating that the two molecules belong to the same molecular complex (**Fig.3.1.A**). This was further confirmed by co-immunoprecipitation experiments performed in primary mouse embryonic fibroblasts (MEFs) demonstrating an interaction between endogenous Sirt7 and Suv39H1 (**Fig.3.1.B**). A pre-requisite for protein-protein interaction is that the two molecules co-localize in the same cellular compartment. To assess this, I transfected U2OS osteosarcoma cell line with MYC-tagged Suv39H1 and YFP-tagged Sirt7 and performed immunostaining for exogenous proteins using anti-MYC and anti-YFP antibodies. As expected, I could demonstrate that Sirt7 and Suv39H1 co-localize in the nucleus, predominantly in proximity of the nucleoli (**Fig.3.1.C**). These results confirmed that Sirt7 and Suv39H1 form a molecular complex within the nuclei in different cells.

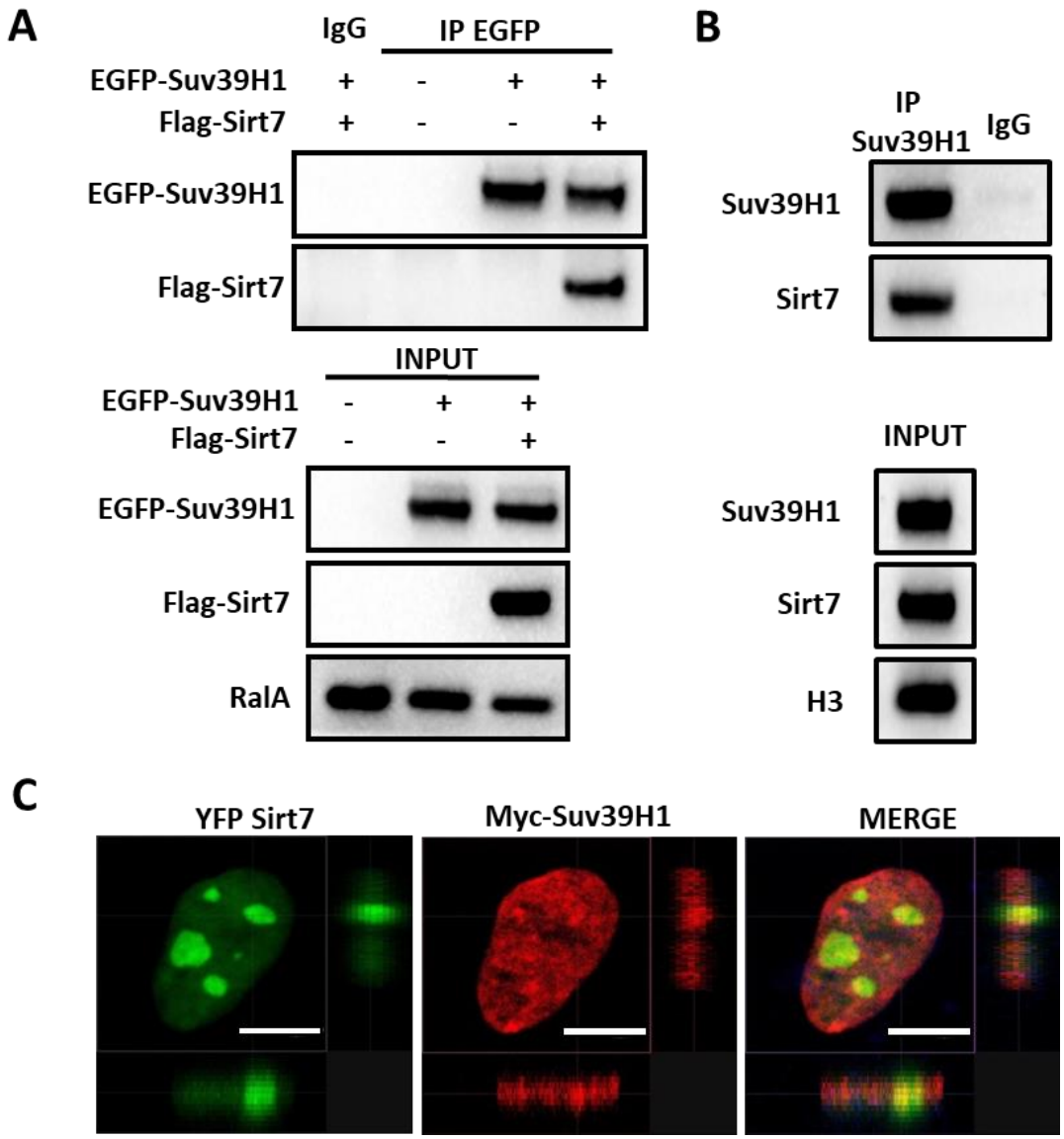


Fig.3.1. Sirt7 forms a molecular complex with Suv39H1. A. 293T-HEK cells were transfected with EGFP-Suv39H1 and Flag-Sirt7 as indicated. Cells were harvested 48 hours post-transfection and subjected to immunoprecipitation using anti-EGFP tag or non-immune IgG antibodies (negative control). The co-immunoprecipitation is shown in the upper panel. The lower panel shows a representative Western blot of the inputs of the immunoprecipitation. Antibodies used for the immuno-blot are shown on the left side. **B.** Coupled immunoprecipitation (anti-Suv39H1 antibody) and western blot analysis (anti-Suv39H1 and anti-Sirt7 antibodies) of primary mouse embryonic fibroblasts. Non-immune IgG antibody was used as a negative control. The upper panel shows a representative immunoblot of the co-immunoprecipitation while the lower panel indicates the inputs. In A and B representative pictures out of three independent experiments are shown **C.** U2OS cells were transfected with YFP-tagged Sirt7 and Myc-tagged Suv39H1. 48 hours post-transfection, cells were used for immuno-fluorescence analysis using anti-YFP tag (green) and anti-Myc tag (red) antibodies. Cell nuclei were counterstained with DAPI. Scale bar 10µm.

3.2 Sirt7 deficiency results in increased Suv39H1 enzymatic activity

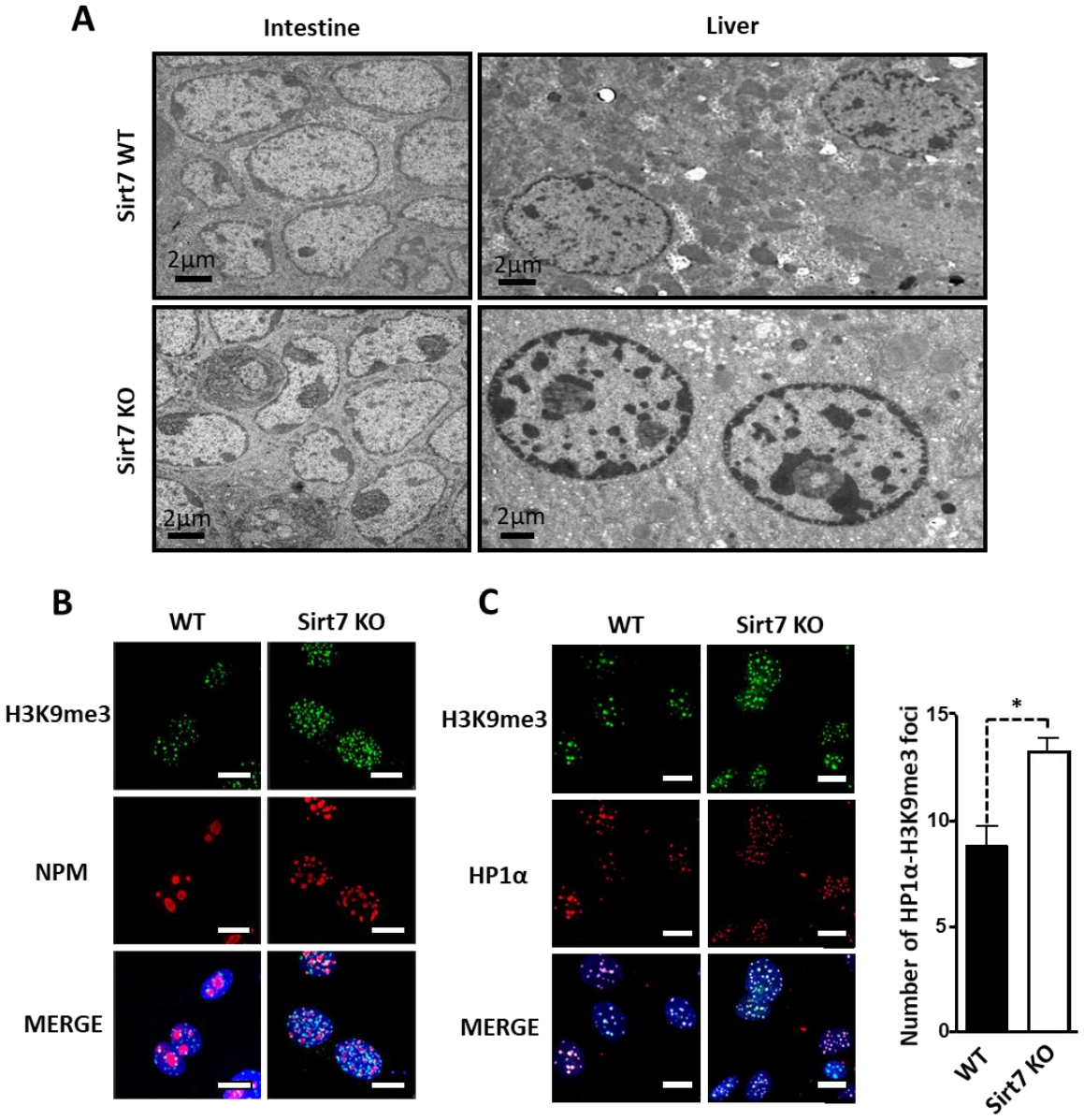


Fig.3.2. Sirt7 deficiency promotes aberrant heterochromatin accumulation. A. Representative electron microscopy images of intestine and liver tissue sections derived from WT and Sirt7 KO mice. Note the aberrant accumulation of heterochromatin foci (dark spots) in Sirt7 KO mice as compared with WT littermates. Scale bar 2µm. **B**. Representative Immunofluorescence pictures of the heterochromatin marker H3K9me3 (green) and the nucleolar marker nucleophosmin (NPM; red) in primary MEFs derived from WT and Sirt7 KO mice. Cell nuclei were counterstained with DAPI. Scale bar 10µm. **C.** Immunofluorescence staining for the heterochromatin markers H3K9me3 (green) and HP1α (red) in MEFs isolated from WT and Sirt7 KO mice. Cell nuclei were counterstained with DAPI. Scale bar 10µm. The quantification of the number of H3K9me3/HP1α double positive foci is shown in the histogram on the right. *p<0.05.

In the next step, I aimed to determine the functional significance of Sirt7/Suv39H1 interaction. As outlined in the introduction, Suv39H1 is a major promoter of heterochromatin formation. In order to understand whether the interaction of Sirt7 is important to control Suv39H1-

mediated formation of constitutive heterochromatin, I first analyzed the chromatin organization in tissues derived from WT and Sirt7 KO mice using electron microscopy. Strikingly, I could demonstrate a dramatic increase in electron dense regions in tissues obtained from Sirt7 KO animals as compared to WT littermates (**Fig. 3.2.A**). These data suggested that in absence of Sirt7, Suv39H1 might be more active, thereby promoting aberrant accumulation of heterochromatin foci. To corroborate the hypothesis that Sirt7 deficiency results in hyperactivation of Suv39H1, I first analyzed the accumulation of the major Suv39H1-dependent histone mark, H3K9me3, in WT and Sirt7 KO MEFs by immunofluorescence analysis. Interestingly, I observed a significant increase in the number of H3K9me3 foci in Sirt7 deficient cells as compared with cells derived from WT littermates. In addition, consistently with already published data, I observed a fragmented nucleolus in Sirt7 KO cells as shown by concomitant staining for the nucleolar marker NPM (**Fig.3.2.B**) [79, 80]. To further confirm that Sirt7 deficiency results in enhanced accumulation of heterochromatin foci, I performed immunofluorescence analysis for another well-established marker of heterochromatin, i.e. heterochromatin protein 1α (HP1 α), in WT and Sirt7 KO MEF cells. (**Fig.3.2.C**). Consistently with an increased number in H3K9me3 foci (**Fig.3.2.B**), I could also observe a significant increase in the number of HP1α foci. Taken together these data unequivocally demonstrate that Sirt7 deficiency results in aberrant accumulation of constitutive heterochromatin.

3.3 Sirt7 inhibits Suv39H1 activity

The subsequent experiments were designed to prove whether the aberrant accumulation of heterochromatic foci in Sirt7 deficient cells is caused by enhanced activation of Suv39H1. I generated stable U2OS cell lines expressing Sirt7 or Suv39H1 targeting shRNAs, either alone or in combination using a lentivirus driven system (as described in the material and methods section). I then assessed the levels of expression of H3K9me3 (the main target of Suv39H1 activity) in these cells using western blotting analysis. As expected, Suv39H1 knockdown cells showed a significant decrease in the expression of H3K9me3 as compared with cells expressing a scramble shRNA. In line with my hypothesis, I could further prove that Sirt7 knock down cells displayed a significant increase in H3K9me3 expression as compared to control cells. However, the higher levels of H3K9me3 in Sirt7 deficient cells could be reverted to the levels observed in scramble cells by concomitant knock down of Suv39H1 (**Fig.3.3.A, B**). These data clearly indicate that the aberrant accumulation of H3K9me3 in Sirt7 deficient cells is caused by enhanced activation of Suv39H1. A comparable levels of Suv39H1 in control and in Sirt7 deficient cells indicated that the enhanced H3K9me3 deposition in Sirt7 deficient cells depends on Suv39H1 hyperactivation and is not a consequence of differential expression of Suv39H1 (**Fig.3.3.C**).

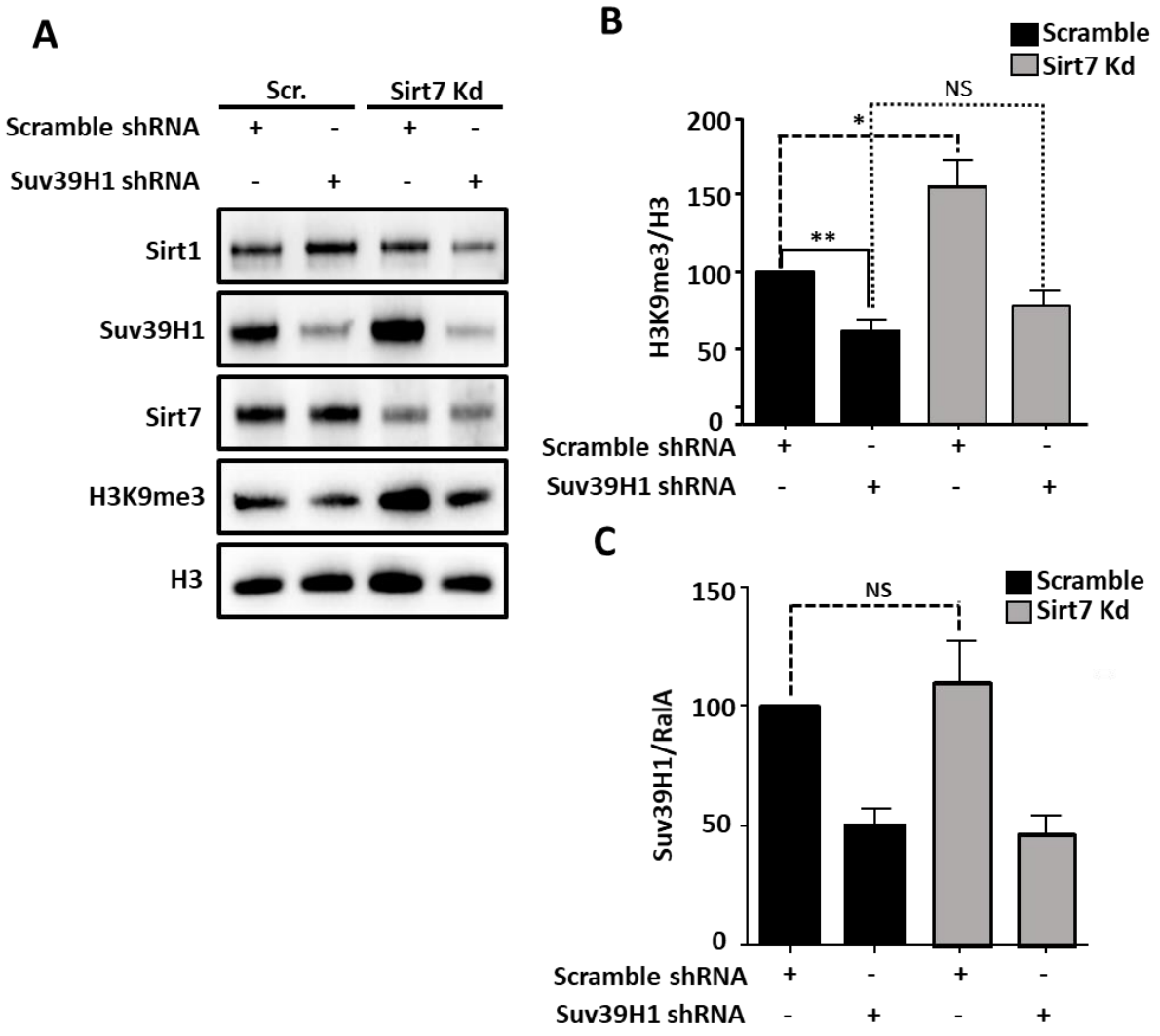


Fig.3.3. Sirt7 inhibits Suv39H1 activity. A. Stable Sirt7 and Suv39H1 knockdown U2OS cell lines were generated by lentivirus-driven delivery of shRNAs targeting Sirt7 and Suv39H1. Stable cell lines were subjected to western blot analysis for H3K9me3 expression. Total histone 3 (H3) was used as a loading control. A representative western blot picture out of four independent experiments is shown. Antibodies used in the WB analysis are shown on the left side. **B, C.** A quantification of H3K9me3 and Suv39H1 levels is shown in the histograms. The graphs report the average ± SD of the normalized protein levels of the indicated markers. *P<0.05, **P<0.01, NS: not-significant.

3.4 Suv39H1 inhibition by Sirt7 is a result of restricted Sirt1 autocatalytic activation

As discussed in the introduction, Sirt1 is a major activator of Suv39H1 mainly through direct deacetylation at lysine 266(K266) [23] [248]. Since Sirt7 also exhibits deacetylation activity, I reasoned that Sirt7 might inhibit Suv39H1 through direct deacetylation. In order to prove this hypothesis, 293T-HEK cells transfected with EGFP-tagged Suv39H1 alone or in combination with Flag-Sirt7 were immunoprecipitated for Suv39H1 and analyzed for Suv39H1 acetylation using mass spectrometry. I could not identify any specific lysine, which was directly deacetylated by Sirt7. In contrast, this analysis revealed that the overexpression of Sirt7 results in increased acetylation of Suv39H1 at K266 **(Fig.3.4)**. Based on the evidence that K266 in Suv39H1 protein is the primary target of Sirt1-mediated deacetylation, I reasoned that Sirt7 might inhibit Suv39H1 by reducing Sirt1 activity [249]. Previous experimental evidence obtained in our laboratory indeed indicated that Sirt7 blocks Sirt1 activation by preventing its autodeacetylation at K230 [233]. To investigate whether Sirt7 might block Suv39H1 activation through a similar mechanism, I first analyzed whether the deacetylation of Sirt1 at K230 impacts Sirt1 capacity to bind Suv39H1. I thereby generated plasmids containing Sirt1 cDNA in which K230 was mutated either to arginine (Sirt1 230R) or to glutamine (Sirt1 230Q) to mimic deacetylation and acetylation at this residue, respectively. I then performed co-immunoprecipitation experiments in the 293T-HEK cells to assess the binding capacity of Sirt1 acetylation and deacetylation mutants to Suv39H1. I could clearly demonstrate that the acetylation of Sirt1 at K230 impairs its binding to Suv39H1 while deacetylated Sirt1 binds more efficiently to Suv39H1. These data indicate that Sirt7-dependent inhibition of Sirt1 autodeacetylation activity at K230 might be responsible for impaired activation of Suv39H1 **(Fig.3.5.A)**.

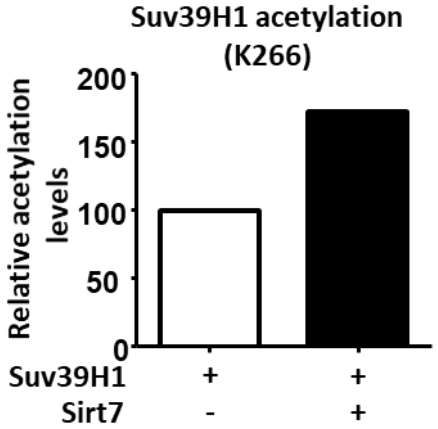


Fig3.4. Acetylation of Suv39H1 in presence of Sirt7. 293T-HEK cells were transfected with EGFP-tagged Suv39H1 alone or in combination with Flag-Sirt7. 48 hours post-transfection, cell lysates were immunoprecipitated with anti-EGFP antibody and analyzed by mass spectrometry. The levels of Suv39H1 acetylation at K266 (lable free quantification; LFQ) normalized to the total levels of Suv39H1 are indicated in the graph.

To further verify that the hyperactivation of Suv39H1 in Sirt7 deficient cells is caused by an aberrant increase in Sirt1 activity, I generated stable U2OS cell lines expressing Sirt7-targeting shRNA either alone or in combination with Sirt1-targeting shRNA. Then, I performed ChIP analysis to assess the enrichment of the main Suv39H1 target, H3K9me3, at the satellite DNA repeats (the major constituent of constitutive heterochromatin) in these cells. I observed a significant increase in the levels of H3K9me3 associated with pericentric heterochromatin in Sirt7-depleted cells. Interestingly, this increase was reverted to the levels of scramble cells upon depletion of Sirt1. Since the observed differences in H3K9me3 might result from enhanced accumulation of histone 3 at these chromosomal loci, I concomitantly performed ChIP analysis for total histone 3 (H3). I could not detect any change in the global levels of H3 in the analyzed conditions, proving that the observed differences in H3K9me3 do not depend on aberrant enrichment of histones in the pericentric heterochromatin **(Fig. 3.5.B)**.

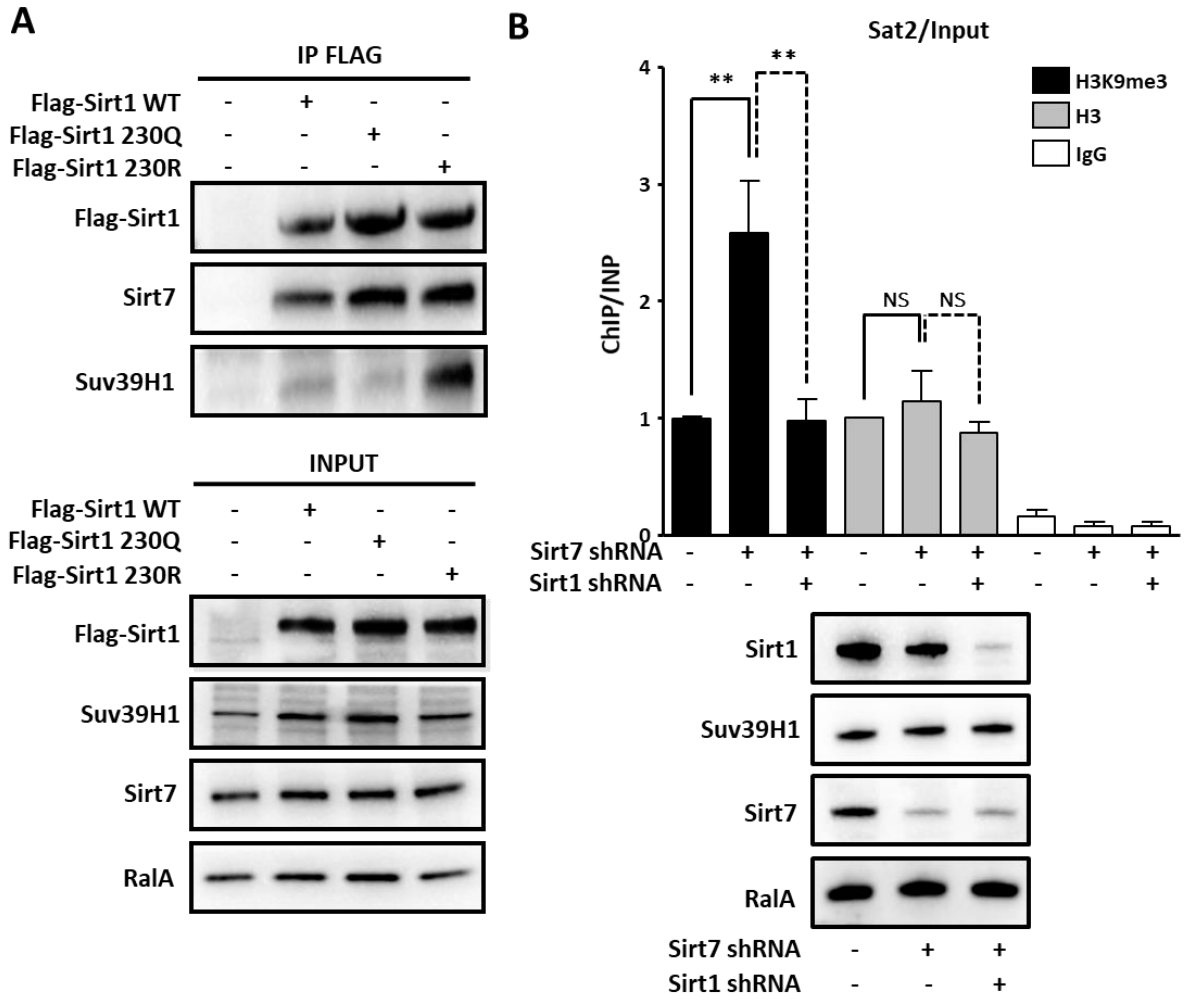


Fig.3.5. Sirt7 inhibits Suv39H1 by blocking Sirt1 autodeacetylation activity. A. 293T-HEK cells were transfected with Flag-tagged Sirt1 WT, Sirt1-230Q (acetylation mimetic mutant) or Sirt1-230R (deacetylation mimetic mutant). Cells were harvested 48 hours post-transfection and immunoprecipitated using an anti-Flag antibody. The amount of endogenous Suv39H1 and Sirt7 co-precipitated with Sirt1 was analyzed by western blotting (upper panel). The lower panel shows the inputs of the immunoprecipitation. A representative western blot of three independent experiments

is shown. **B.** ChIP analysis of H3K9me3 and total H3 associated with the pericentric heterochromatin performed in stable U2OS cell lines expressing a specific Sirt7-targeting shRNA alone or in combination with Sirt1 shRNA. Non-immune immunoglobulin (IgG) antibody was used as a negative control. The graph represents a quantification of six independent experiments for H3K9me3 and three experiments for H3. **p<0.01, NS: not-significant. Efficient knock down of Sirt7 and Sirt1 was assessed by western blot, as shown in the lower panel.

To get additional evidence that Sirt7 blocks Suv39H1 activity by inhibiting Sirt1, I analyzed the levels of Suv39H1 target H3K9me3 upon overexpression of Suv39H1 alone or in combination with Sirt1 and Sirt7 in 293T-HEK cells using western blot. As expected, I could demonstrate that overexpression of Suv39H1 leads to increased levels of H3K9me3. Furthermore, in line with the positive effect of Sirt1 on Suv39H1 activation [48], I could prove that the concomitant overexpression of Sirt1 with Suv39H1 increases Suv39H1-mediated deposition of H3K9me3. Strikingly, Sirt7 overexpression along with Suv39H1 and Sirt1 dramatically reduced the levels of H3K9me3, unequivocally demonstrating that Sirt7 blocks Suv39H1 activation through Sirt1 [250] **(Fig.3.6.A)**. Finally, to support these data, I analyzed the levels of H3K9me3 in WT and Sirt7 KO primary MEFs using a specific Sirt1 inhibitor, Ex527. I could clearly observe that the inhibition of Sirt1 activity in Sirt7 deficient cells significantly inhibits the levels of Suv39H1-mediated deposition of H3K9me3 **(Fig.3.6.B)**.

Taken together, these data clearly demonstrate that the enhanced formation of heterochromatin foci in Sirt7 deficient cells is caused by an aberrant Sirt1-dependent hyperactivation of Suv39H1. Furthermore, I could demonstrate that this phenomenon is dependent on Sirt7-mediated inhibition of Sirt1 autodeacetylation at K230 (**Fig.3.5**).

The possible mechanism of Sirt7-dependent regulation of Suv39H1 activity is schematically summarized in the **Fig.3.7**.

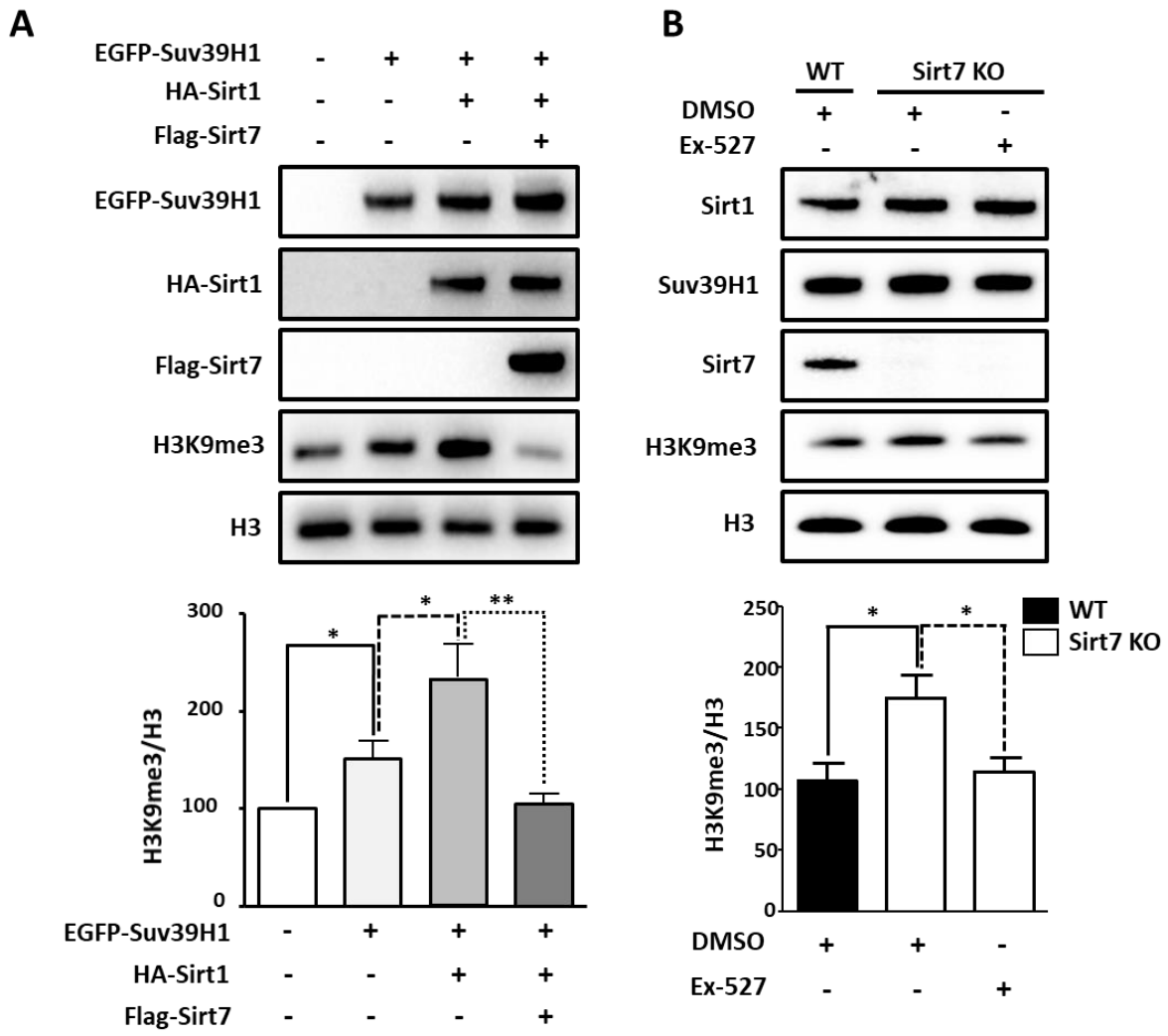


Fig.3.6. Sirt7 inhibits Suv39H1 by blocking Sirt1. A. 293T-HEK cells were transfected with EGFP-Suv39H1, HA-Sirt1 and Flag-Sirt7 as indicated. Cells were harvested 48 hours post-transfection and cellular lysates were subjected to western blot analysis for H3K9me3 expression to evaluate the activity of Suv39H1 (upper panel). Quantification of H3K9me3 normalized to the total H3 levels (H3K9me3/H3) is shown in the histogram below. The graph represents the average ± SD of three independent experiments.*p<0.05, **p<0.01. **B.** Wild type and Sirt7 KO primary MEFs were either treated with DMSO (vehicle) or with 10nM Ex-527, a specific inhibitor of Sirt1 activity for 24 hours. H3K9me3 levels, as marker of Suv39H1 activity, were assessed by western blots analysis. Antibodies used for WB analysis are indicated on the left side of the blots. Quantification of H3K9me3 levels normalized to the global levels of histone 3 (H3K9me3/H3) is shown in the lower panel. n=3, *p<0.05, **p<0.01.

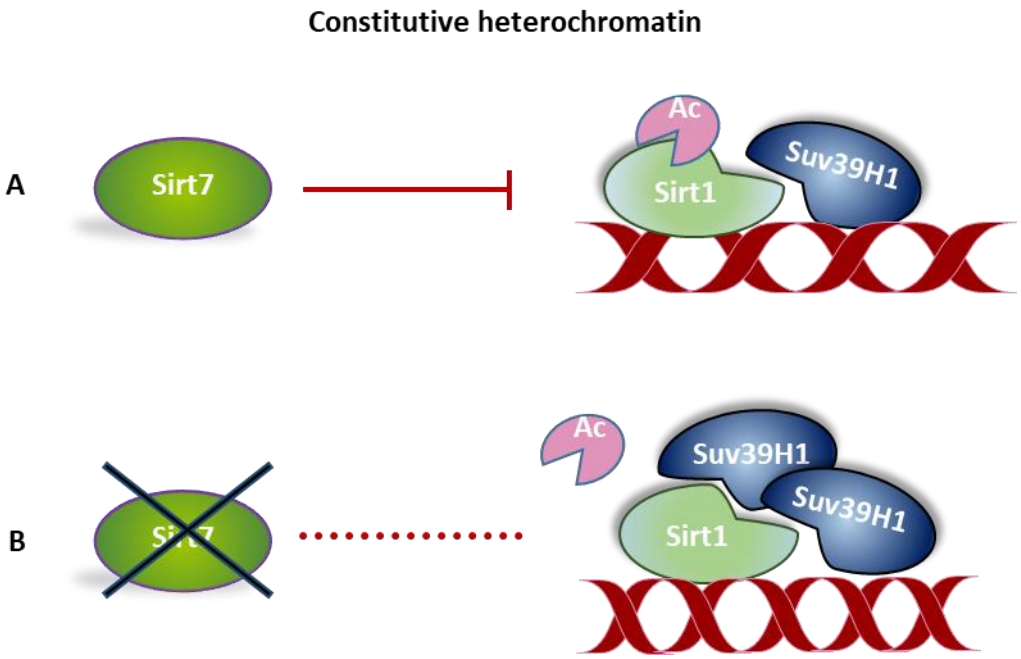


Fig.3.7. Scheme depicting the putative role of Sirt7 in the regulation of the Sirt1/Suv39H1 axis. A. Sirt7 prevents the autodeacetylation of Sirt1 at K230 inhibiting Sirt1 catalytic activity and thereby resulting in suppression of Suv39H1 activity [233]. **B.** In absence of Sirt7, hyperactive Sirt1 promotes Suv39H1 activation and enhances its recruitment to constitutive heterochromatin causing an aberrant accumulation of H3K9me3 heterochromatin mark.

3.5 Sirt7-mediated inhibition of Suv39H1 promotes relaxation of the constitutive heterochromatin following genotoxic stress

In the last decade, growing experimental evidence demonstrated that Sirt7 plays a pivotal role in the maintenance of cellular homeostasis expecially in response to stress [251]. Interestingly, under genotoxic stress, Sirt7 relocalizes out of nucleoli and accumulates in the cytoplasm where it promotes the activation of specific signaling patways involved in the maintenance of cellular homeostasis [169]. Genotoxic stress also modulates the localization and activation of Suv39H1. Inactivation of Suv39H1 following DNA damage is pivotal for the relaxation of the constitutive heterochromatin to allow thereby DNA repair of repetitive elements, thus ensuring the maintenance of genomic stability [46].

Since my results so far provided evidence for Sirt7 mediated inhibition of Suv39H1, I wondered whether this inhibition might play a role in the constitutive heterochromatin relaxation induced by genotoxic stress. I firstly used electron microscopy to analyze the heterochromatin organization in primary MEFs derived from WT and Sirt7 KO mice, either under normal condition or in response to treatment with DNA damaging agents, such as UV or doxorubicin.

My previous results already demonstrated an aberrant heterochromatin accumulation in tissues of Sirt7 deficient animals (**Fig. 3.2.A).** Consistent with these results I observed an

increased number of heterochromatin foci in Sirt7 KO as compared with WT cells. When I exposed these cells to genotoxic stress, a complete relaxation of the heterochromatin was apparent in wild type cell. In sharp contrast, Sirt7 KO cells maintained a higher number of heterochromatin foci (**Fig.3.8**), strongly indicating that Sirt7 promotes heterochromatin relaxation under cellular stress conditions. To get further support for such a role of Sirt7, I performed additional experiments. A well-established marker of heterochromatin relaxation is the expression of the satellite DNA [252]. Thus, I analyzed the expression of these elements of the genome (alpha sat and sat2) in WT and Sirt7 KO mouse embryonic fibroblasts either under control conditions or following exposure to UV- and doxorubicin-induced genotoxic stress (**Fig.3.9.A**). As expected, I could demonstrate that genotoxic stress dramatically increases expression of the satellite DNA as previously reported [253, 254]. Strikingly, Sirt7 deficient cells showed a significant reduction of the expression of satellite DNA. Furthermore, I could support these data by analyzing the expression of satellite DNA repeats upon overexpression of Sirt7 in U2OS cancer cell line exposed to genotoxic stress. In agreement with the previous results, a significant increase in α-sat and sat2 expression was observed upon Sirt7 overexpression (**Fig.3.9.B**). Altogether, these results demonstrate that Sirt7 is essential for heterochromatin relaxation following genotoxic stress.

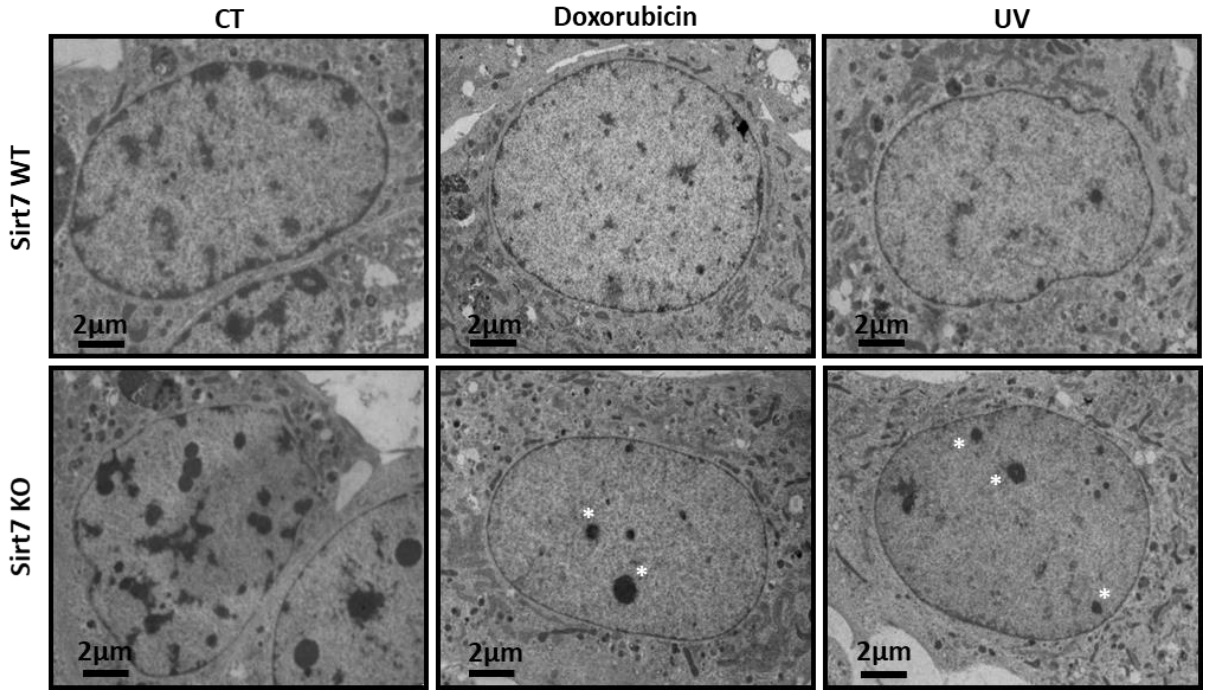


Fig. 3.8. Lack of Sirt7 impairs heterochromatin relaxation in response to genotoxic stress. Primary MEF cells from wild type (WT) and Sirt7 knock out (Sirt7 KO) mice were either left untreated or exposed to 1µM doxorubicin for 24 hours or irradiated with 80 J/m^2 UVC and harvested 12 hours post-irradiation. Heterochromatin organization was analyzed by electron microscopy. The electron dense regions indicate heterochromatin foci. White asterisks represent the unresolved heterochromatin in Sirt7 KO cells upon genotoxic stress. Scale bar 2µM.

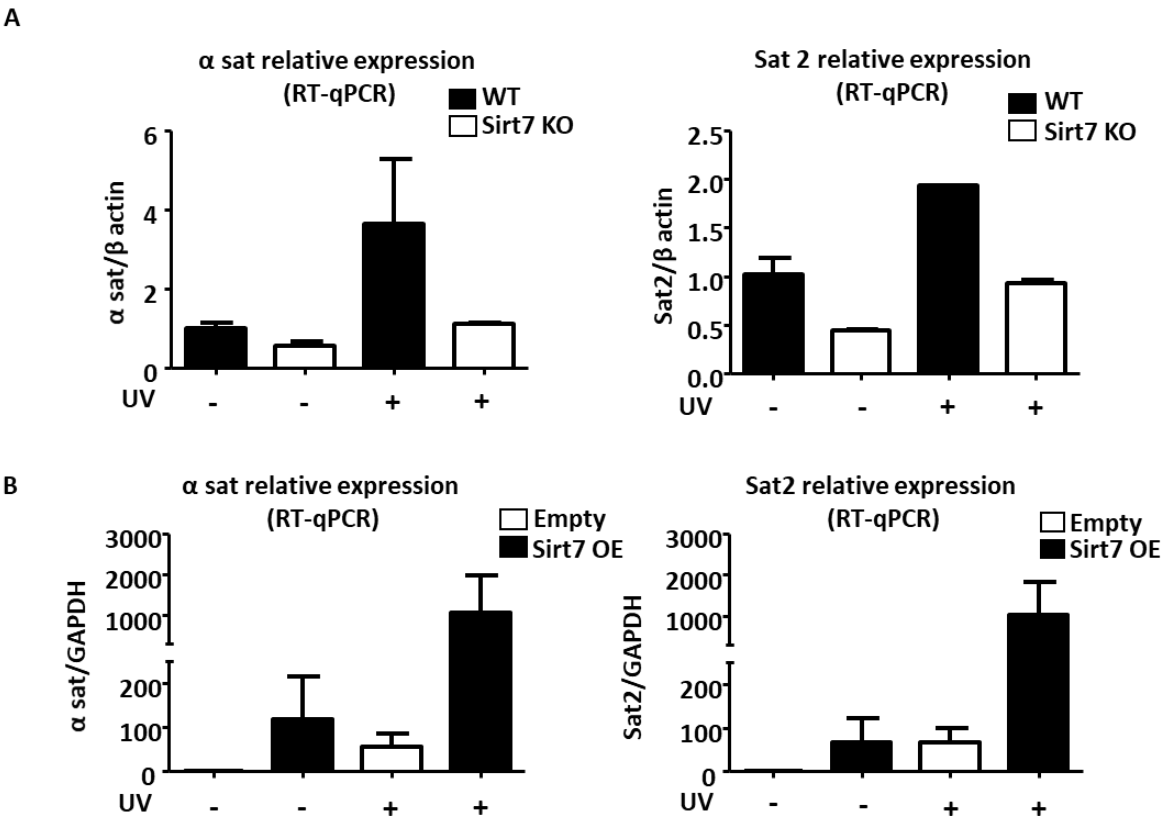


Fig. 3.9. Lack of Sirt7 impairs heterochromatin relaxation in response to genotoxic stress. A. Primary MEF cells from wild type and Sirt7 knock out mice were either left untreated or exposed to 80 J/m^2 UVC irradiation. 12 hours post-treatment, expression of sat2 and alpha sat satellite DNA was analyzed by RT-qPCR. n=3. **B.** U2OS cells were transiently transfected with an empty vector or with Flag-tagged Sirt7 (Sirt7 overexpression; Sirt7 OE) as indicated. 48 hours post-transfection, cells were then exposed to UVC irradiation (40J/m^2) and harvested 12 hours post-treatment. The expression of sat2 and alpha sat was analyzed using RT-qPCR. n=3.

3.6 Absence of Sirt7 leads to higher occupancy of Suv39H1 at the constitutive heterochromatin

Since I have already demonstrated that Sirt7 is a potent mediator of heterochromatin relaxation under normal conditions by inhibiting the methyltransferase Suv39H1, I next aimed to demonstrate whether Sirt7 promotes heterochromatin relaxation by the same mechanism also under genotoxic stress. First, I analyzed the amount of Suv39H1 associated with the satellite DNA repeats in WT and Sirt7 KO MEF cells exposed to genotoxic stress by chromatin immunoprecipitation (ChIP). I observed that the amount of Suv39H1 localized on satellite DNA repeats was significantly higher in primary MEFs derived from Sirt7 KO as compared to WT cells already under normal conditions, further confirming my previous results (**Fig. 3.5.B**).

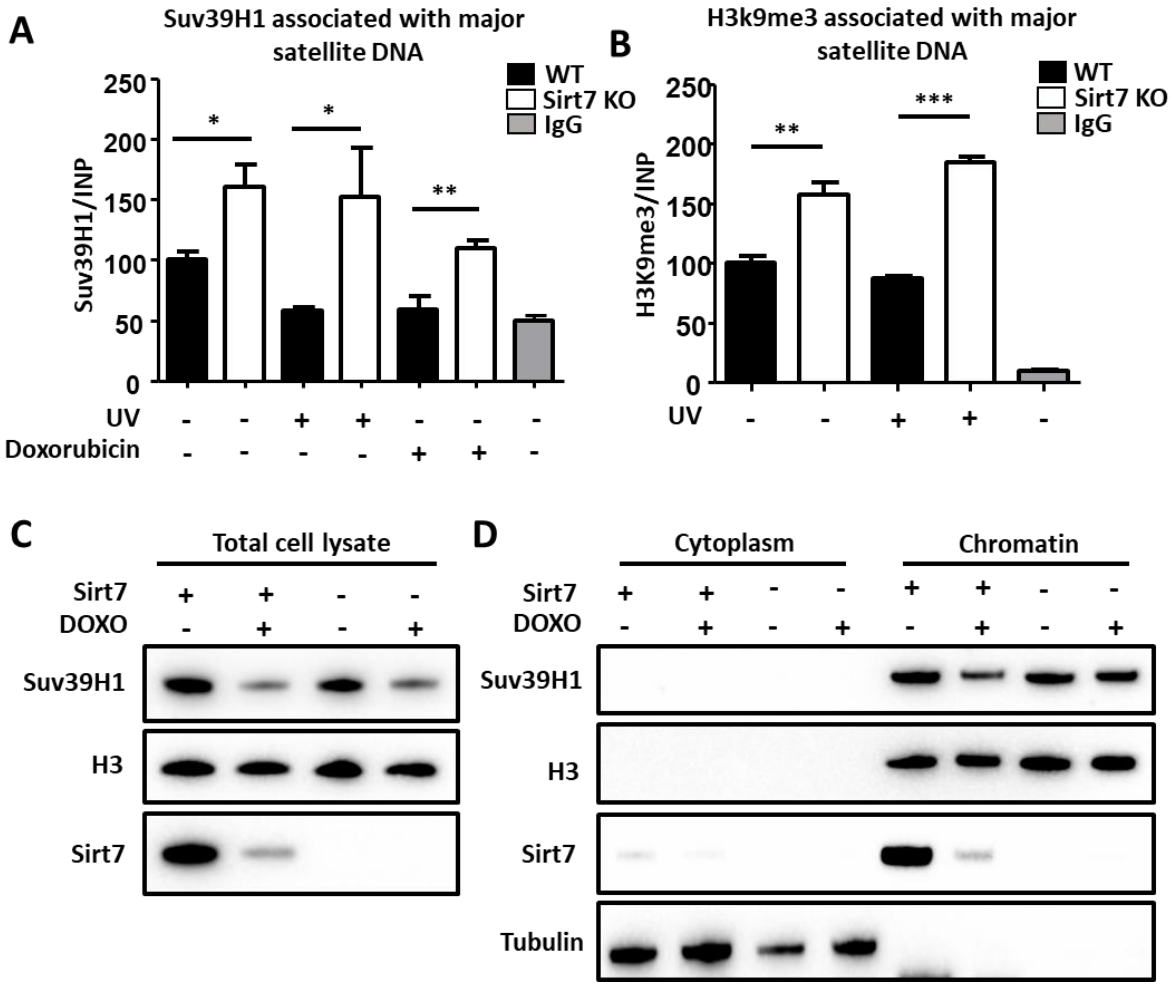


Fig.3.10. Lack of Sirt7 impairs relaxation of constitutive heterochromatin following genotoxic stress. A. Primary MEF cells derived from wild type (WT) and Sirt7 knock out (Sirt7 KO) mice were either left untreated or exposed to 1µM doxorubicin for 24 hours or exposed to 40J/m^2 UVC irradiation and harvested 12 hours post-treatment. Cells were subjected to chromatin immunoprecipitation using anti-Suv39H1 antibody. The immunoprecipitates were analyzed by RT-qPCR using primers specific for the satellite DNA repeats and normalized to the inputs. **B.** Primary WT and Sirt7 KO MEFs were either left untreated or exposed to UVC irradiation as in A. Cell lysates were subjected to chromatin immunoprecipitation using anti-H3K9me3 antibody. Chromatin immunoprecipitates were analyzed by RT-qPCR using specific primers for the satellite DNA repeats. The inputs of the ChIP were used for normalization. *P<0.05, **P<0.01, ***p<0.001 **C.** Primary MEF cells from wild type and Sirt7 knock out animals were either left untreated or exposed to 1µM doxorubicin for 24 hours. Cells were harvested and subjected to western blot using the indicated antibodies. A representative WB out of three independent experiments is shown. **D.** Primary MEFs were harvested from WT and Sirt7 Knock out animals as in A and subjected to chromatin fractionation. Chromatin and cytoplasmic fractions were analyzed by western blot using the indicated antibodies. A representative WB out of three independent experiments is shown.

Notably, I could additionally demonstrate that treatment with the DNA damaging agents UVC and doxorubicin caused a significant decrease in Suv39H1 occupancy at the satellite DNA

repeats in WT cells. This effect was dramatically blunted in Sirt7 deficient cells (**Fig.3.10.A**). Furthermore, I also performed additional ChIP analysis to assess the enrichment of H3K9me3, the primary target of Suv39H1, at the satellite DNA repeats in primary WT and Sirt7 KO MEFs. As expected, already under normal conditions, I observed a higher deposition of H3K9me3 at the satellite DNA repeats in Sirt7 KO cells as compared with WT cells. Upon UVC irradiation, the levels of H3K9me3 were significantly reduced in cells harboring Sirt7 (WT) as compared to Sirt7 deficient cells (Sirt7KO), which maintained higher H3K9me3 levels (**Fig.3.10.B**). These data indicate that Sirt7 is responsible for efficient removal of Suv39H1 from the constitutive heterochromatin and its deactivation following genotoxic stress. However, Suv39H1 expression in WT and Sirt7 KO cells was strongly reduced under genotoxic stress independently of Sirt7 expression (**Fig.3.10.C**). Interestingly, when I analyzed the amount of Suv39H1 associated with chromatin using biochemical separation of cytoplasmic and chromatin fractions, I could clearly demonstrate that lack of Sirt7 resulted in enhanced Suv39H1 association to the chromatin under stress conditions in contrast to WT cells (**Fig.3.10.D**). This experimental finding supports the assumption that Sirt7 indeed acts to remove Suv39H1 from the chromatin as a part of stress response. Altogether, these data demonstrate that Sirt7 is a critical factor that promotes heterochromatin relaxation following genotoxic stress by instigating Suv39H1 inactivation.

3.7 Sirt7 promotes heterochromatin relaxation following genotoxic stress by inhibiting Sirt1-dependent activation of Suv39H1

In the next experiments, I aimed to further characterize the molecular mechanism by which Sirt7 promotes Suv39H1 inactivation following genotoxic stress. Since I previously demonstrated that under normal conditions Sirt7 inhibits Suv39H1 through Sirt1, I next wanted to ascertain whether a similar mechanism is also responsible for Suv39H1 inhibition in response to genotoxic stress. To prove this hypothesis, I analyzed the rate of constitutive heterochromatin relaxation in stable U2OS cancer cell lines expressing Sirt7-targeting shRNA alone or in combination with Sirt1-targeting shRNA using RT-qPCR analysis of satellite DNA expression. Interestingly, I could further demonstrate that knock down of Sirt7 resulted in lower satellite DNA repeats expression in response to genotoxic stress (**Fig.3.11**). This finding additionally corroborates my previous data obtained in WT and Sirt7 KO MEFs (**Fig.3.9.A**), suggesting generally lower chromatin relaxation during the stress response in Sirt7 deficient cells. Furthermore, the inhibition of Sirt1 in Sirt7 deficient cells efficiently rescues the impaired satellite DNA expression, indicating that hyperactive Sirt1 in Sirt7 knockdown cells is responsible for blunted relaxation of heterochromatin (**Fig.3.11**).

Altogether, these experiments strongly suggest that Sirt7 promotes constitutive heterochromatin relaxation following genotoxic stress by associating more efficiently to Sirt1 and leading, consequently, to a higher inhibition of the Sirt1-Suv39H1 binding (**Fig.3.9**).

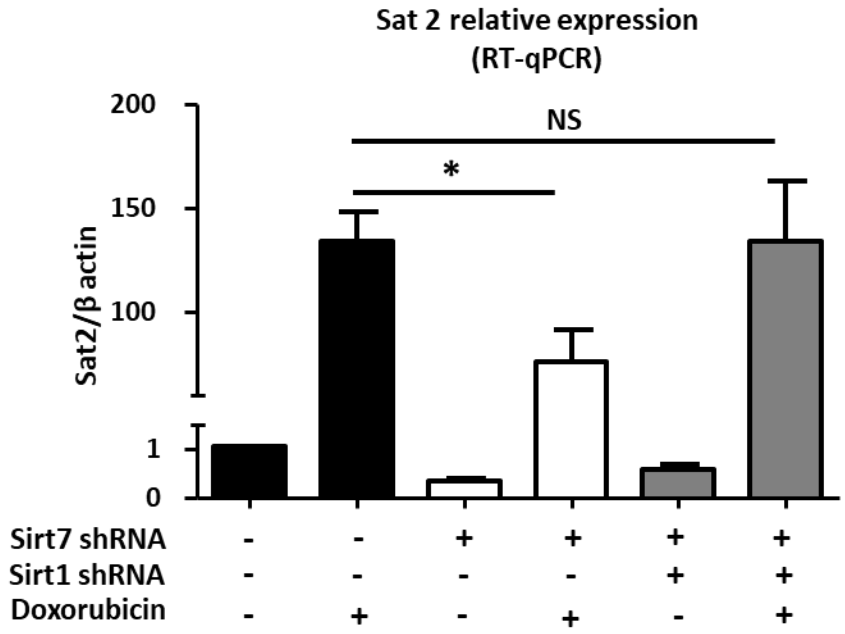


Fig. 3.11. Absence of Sirt7 blunts genotoxic stress-induced heterochromatin relaxation via Sirt1. Stable U2OS cells expressing a specific Sirt7-targeting shRNA alone or in combination with Sirt1-targeting shRNA, as indicated, were either left untreated or exposed to 1μM doxorubicin for 24 hours. After treatment cells were subjected to RT-qPCR of sat2 expression. n=4; *p<0.05; NS: not significant.

3.8 Sirt7 controls the transcription of a subset of target genes in a Suv39H1-dependent manner

The data shown till now clearly demonstrate that Sirt7 is pivotal for the removal of Suv39H1 from the constitutive heterochromatin following genotoxic stress. I wondered whether Sirt7 might regulate the activity of Suv39H1 also at specific euchromatin loci, controlling thereby the expression of Suv39H1 target genes. Indeed, besides its role in the maintenance of the constitutive heterochromatin structure, Suv39H1 has been shown to regulate transcription [255, 256]. In order to understand whether Sirt7 affects Suv39H1-dependent gene expression, I performed RNA sequencing analysis in stable U2OS cell lines expressing a Sirt7-targeting shRNA alone or in combination with Suv39H1 shRNA after treatment with 1μM doxorubicin. Interestingly we identified a subset of genes that were highly expressed in Sirt7 knockdown cells but were dramatically reduced upon concomitant inhibition of Suv39H1. We found that some of these genes are involved in extracellular matrix organization (LUM and EMILIN1) [257-260] and in cell apoptosis (MMP9, mir30A and TRIML2 [261-265] (**Fig.3.12.A, B**).

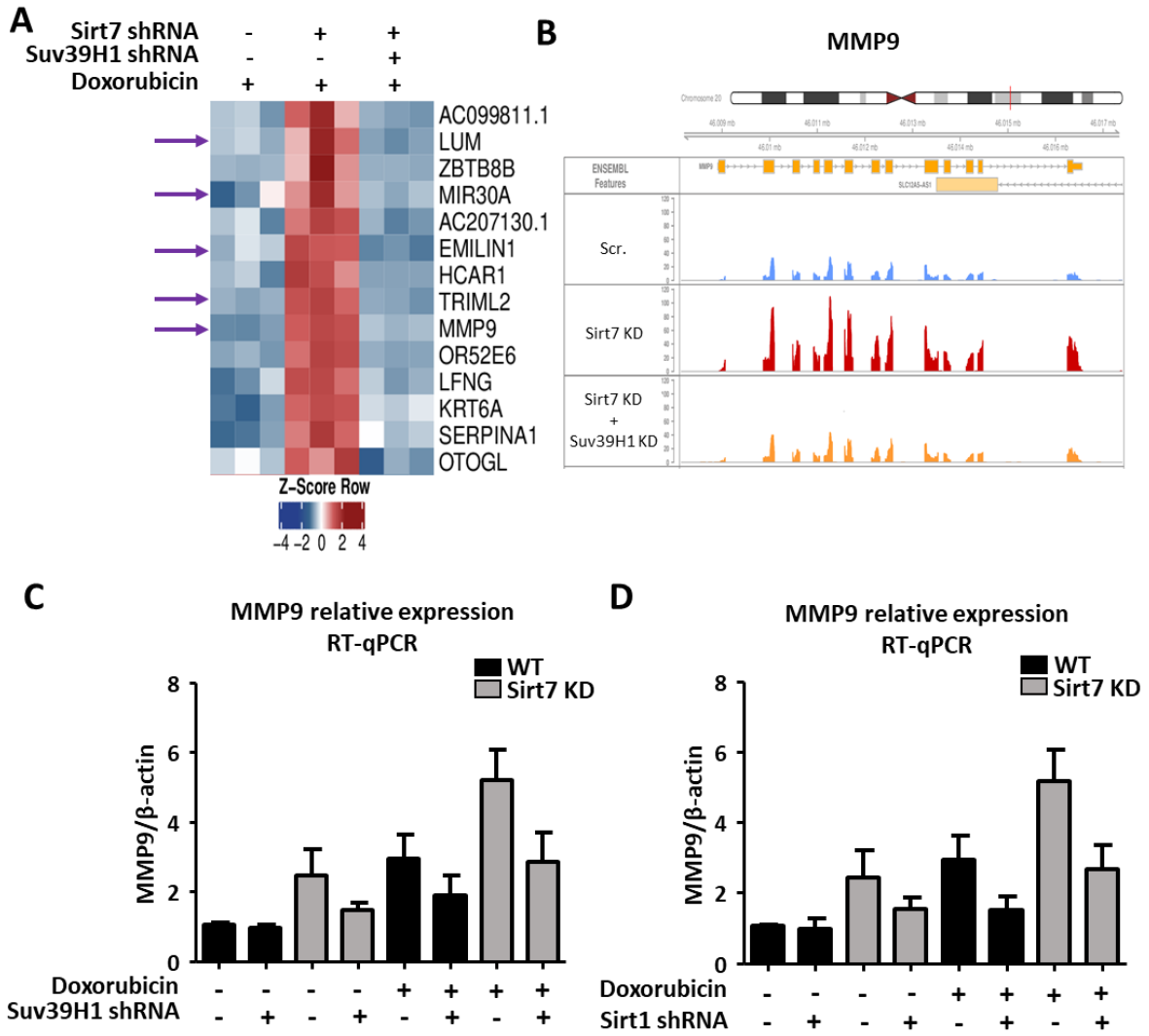


Fig.3.12. Hyperactive Suv39H1 stimulates MMP9 expression following doxorubicin treatment. A. Stable U2OS cells expressing Sirt7-targeting shRNA alone or in combination with Suv39H1 shRNA were treated with 1μM doxorubicin for 24 hours and subjected to RNA sequencing analysis. The heat map on the left indicates genes which I found to be up-regulated in Sirt7 KD cells in a Suv39H1-dependent manner. **B.** Right panel shows the coverage plot for one of the identified genes, i.e. MMP9. **C.** RT-qPCR analysis for the expression of MMP9 in stable scramble, Sirt7 knockdown and Sirt7/Suv39H1 double knockdown cells. **D.** RT-qPCR analysis for the expression of MMP9 in stable scramble, Sirt7 knockdown and Sirt7/Sirt1 double knockdown cells.

In order to validate the sequencing data, I performed RT-qPCR analysis of MMP9 expression in scramble, Sirt7 knockdown and Sirt7-Suv39H1 double knockdown cells either under normal conditions or in response to doxorubicin-induced genotoxic stress. These data confirmed that MMP9 is highly expressed in Sirt7 knockdown cells as compared with scramble cells upon doxorubicin treatment. Furthermore, shRNA-mediated inhibition of Suv39H1 in Sirt7 knockdown cells reduces the MMP9 expression to the levels observed in scramble control cells. These data clearly indicate that Sirt7 deficiency enhances Suv39H1-mediated stimulation of MMP9 expression (**Fig.3.12.C**). Finally, since we demonstrated that enhanced Suv39H1 activity in Sirt7 deficient cells is caused by hyperactivation of Sirt1, we also analyzed the

expression of MMP9 in Sirt7 knockdown cells upon concomitant shRNA mediated inhibition of Sirt1. In line with our expectation, we could further demonstrate that Sirt1 inactivation restores the expression of MMP9 in Sirt7 knockdown cells (**Fig.3.12.D**).

Taken together these data indicate that the overactive Sirt1/Suv39H1 axis in Sirt7 deficient cells following genotoxic stress is not only involved in impaired constitutive heterochromatin relaxation but also plays a pivotal role in the expression of a subset of genes involved in the cellular response to genotoxic stress.

3.9 Sirt7 promotes cellular survival under stress in a Suv39H1-dependent manner

Since RNA sequencing data suggested that Sirt7 might control cellular apoptosis following genotoxic stress, I further wanted to investigate whether depletion of Sirt7 is involved in cell survival in response to stress. Western blot analysis for the apoptosis markers cleaved caspase 3 and PARP in stable scramble and Sirt7 KD cells clearly demonstrated that the depletion of Sirt7 increases cellular apoptosis upon exposition of cells to UVC-induced genotoxic stress (**Fig.3.13**). Interestingly, depletion of Sirt7 enhanced apoptosis, despite the fact that the levels of p53, one of the major mediators of this process, appeared to be reduced in Sirt7 depleted cells. These data confirm our previous findings demonstrating a critical role of Sirt7 in stabilizing p53 under genotoxic stress (Ianni, Kumari et al. under revision in PNAS). I could further substantiate these findings in overexpression experiments as visualized by a diminished expression of the cleaved caspase 3. I could clearly demonstrate that the overexpression of Sirt7 suppresses apoptosis following UV-irradiation. Also under these condition Sirt7 apparently contributed to p53 stabilization (**Fig.3.14.A**).

These data suggest that Sirt7 may promote cellular survival in a p53 independent manner under genotoxic stress. To corroborate this assumption, I analyzed the apoptotic response in p53 deficient cells H1299 exposed to genotoxic stress upon depletion of Sirt7. Ablation of Sirt7 increased cellular apoptosis in these cells univocally demonstrating that Sirt7 acts as a negative regulator of apoptosis in response to stress through a mechanism that does not require p53 expression (**Fig.3.14.B**).

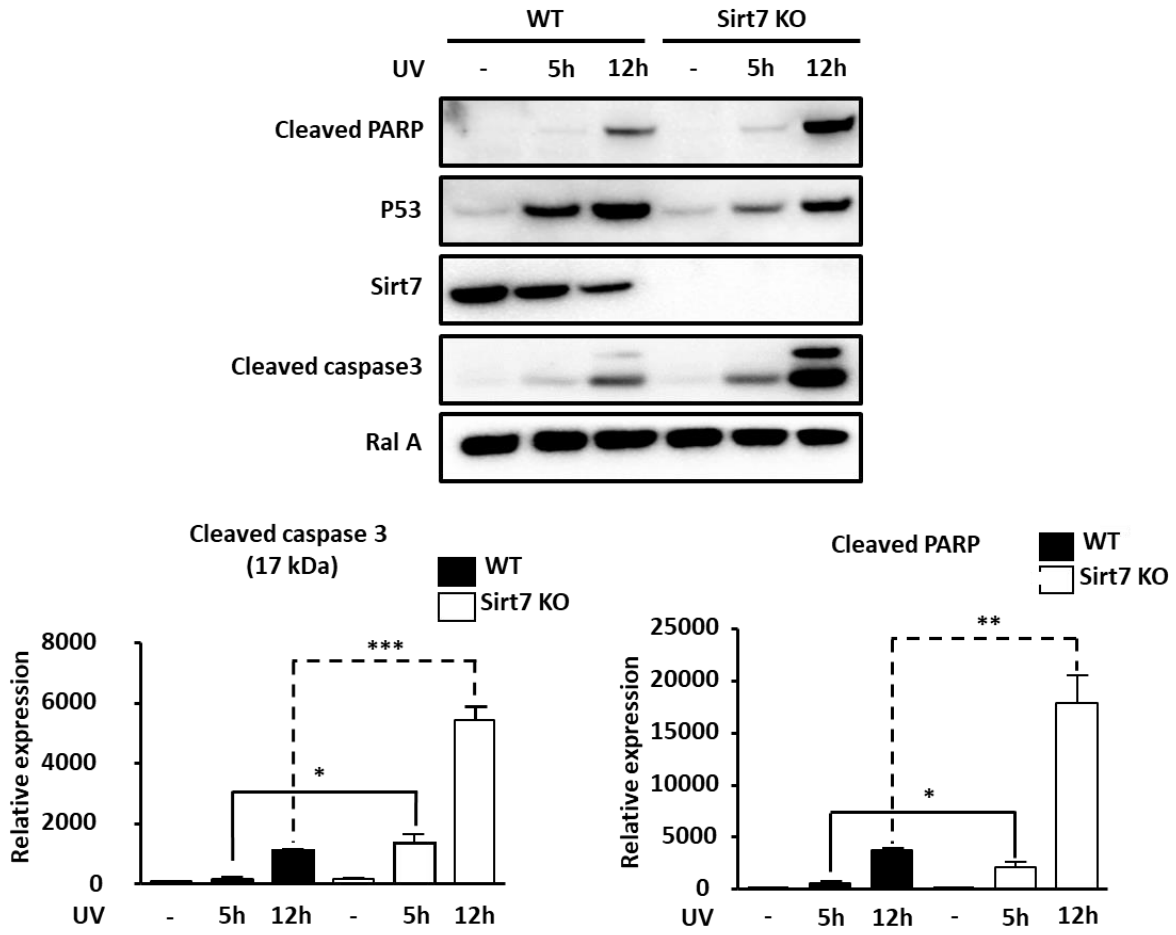


Fig.3.13. Increased apoptosis in Sirt7 deficient cells in response to stress. Primary mouse embryonic fibroblasts from wild type and Sirt7 knock out mice were either left untreated or exposed to UVC-irradiation and harvested 5 and 12 hours after treatment, as indicated . Cellular lysates were subjected to western blot analysis for the apoptotic markers, cleaved caspase3 and PARP. RalA was used as a loading control. A representative western blot out of four independent experiments is shown in the figure. The histograms below represent quantification of the relative expression of the apoptosis makers ±SD (n= 4; *p<0.05, **P<0.01, ***p<0.001).

I further wanted to investigate whether the aberrant activity of Suv39H1 in Sirt7 depleted cells is responsible for enhanced apoptosis. I thereby generated stable U2OS cell lines expressing a combination of Sirt7- and Suv39H1-targeting shRNAs and analyzed the apoptotic response in these cells upon treatment with UVC-stress. Interestingly, I observed that depletion of Suv39H1 in Sirt7 deficient cells abolished the enhanced apoptotic response following irradiation suggesting that aberrant activation of Suv39H1 is responsible for increased apoptosis in Sirt7 deficient cells (**Fig.3.15.A**). Similar results were obtained following treatment with doxorubicin (**Fig.3.15.B**), demonstrating that the role of Sirt7/Suv39H1 axis in apoptosis is not a specific response to UVC-irradiation but represents a more general mechanism induced by genotoxic stress.

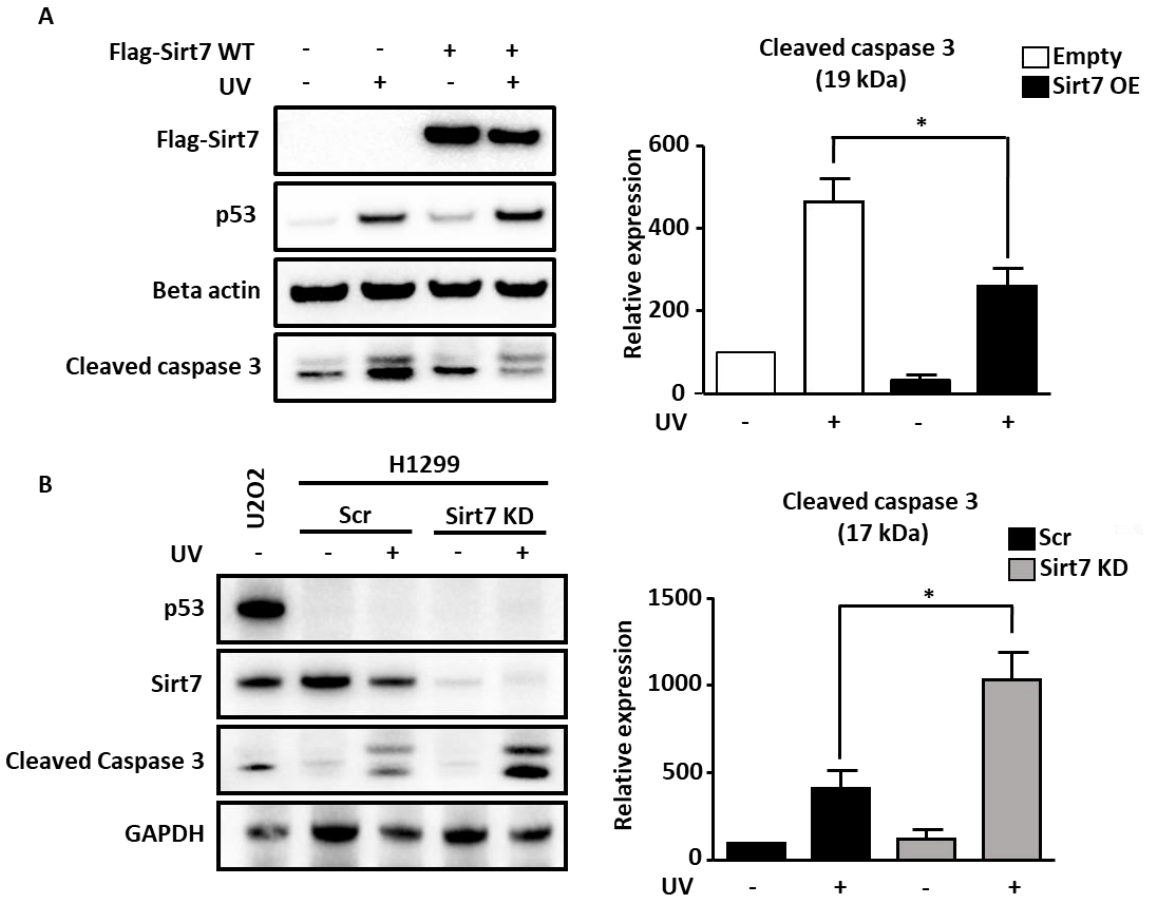


Fig.3.14. Sirt7 suppresses apoptosis in a p53-independent manner. A. U2OS cells were transfected with an empty vector or with Flag tagged-Sirt7. 48 hours post-transfection, cells were either left untreated or exposed to UVC-irradiation and harvested 5 hours after irradiation. Cellular lysates were subjected to western blot analysis for the indicated markers. Beta actin was used as a loading control. A representative blot of four independent experiments is shown in the figure. The graph on the right shows the quantification of the relative expression of cleaved caspase3 ±SD. **B.** H1299 cells stably expressing scramble or Sirt7-targeting shRNA were either left untreated or exposed to UVC irradiation. 12 hours post-irradiation, cellular lysates were collected and analyzed by western blot for the expression of the indicated markers. A representative western blot out of four independent experiments is shown in the figure. The quantification for cleaved caspase3 is shown in the histogram on the right. n= 4; *p<0.05.

To prove that this process is independent of p53, I generated Suv39H1-Sirt7 double knockdown H1299 cells and analyzed the induction of apoptosis in response to UVC-stress. As expected, I could show that increased apoptosis in stressed Sirt7 deficient cells could be reverted after inhibition of Suv39H1 (**Fig.3.16**). Taken together these data provide compelling evidence that hyperactivation of Suv39H1 in Sirt7 deficient cells is responsible for enhanced apoptosis under stress.

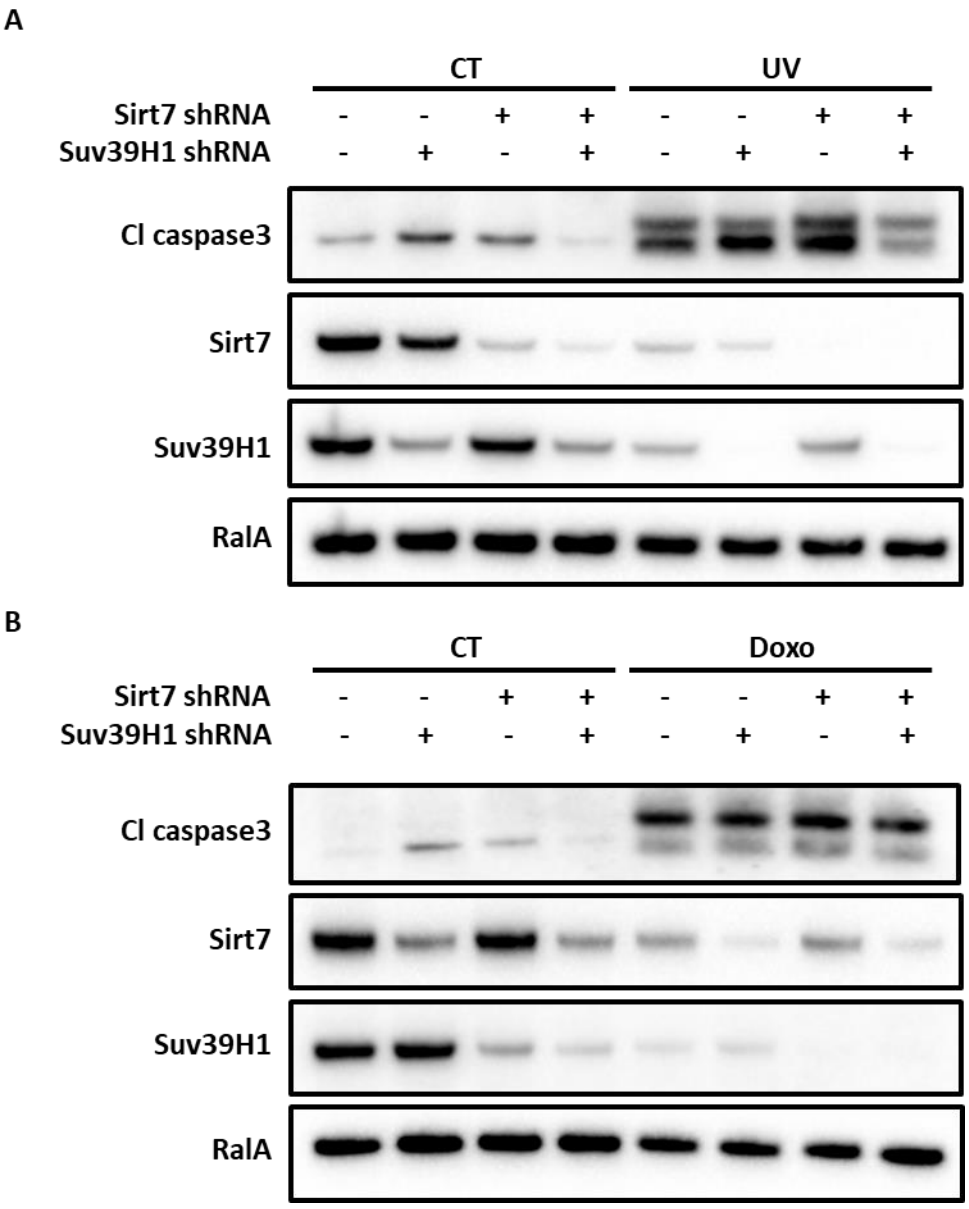


Fig.3.15. Sirt7 promotes cellular survival by inhibiting Suv39H1 activity. A. U2OS cells stably expressing Sirt7 and Suv39H1-targeting shRNAs alone or in combination were either left untreated or exposed to UVC-irradiation as indicated. 16 hours post treatment, cellular lysates were collected and analyzed by western blot for the apoptosis marker, cleaved caspase 3. A representative blot of four independent experiments is shown in the figure. **B.** Stable U2OS cells as in A were either left untreated or treated with doxorubicin for 16 hours as indicated. Cell lysates were collected and analyzed by western blot for the indicated markers. RalA was used as a loading control. A representative blot out of four independent experiments is shown in the figure.

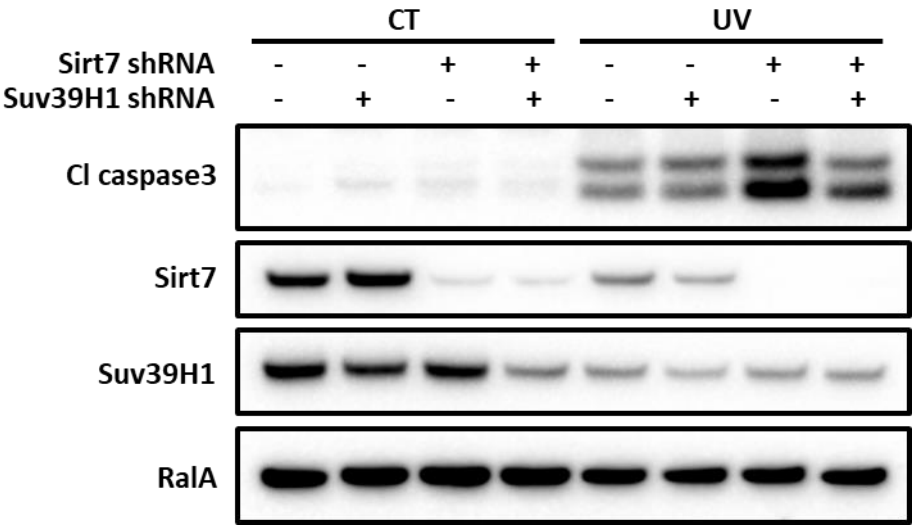


Fig.3.16. Sirt7 inhibits apoptosis following genotoxic stress in a p53-independent and Suv39H1-dependent manner. p53 deficient cell lines H1299 stably expressing Sirt7 and Suv39H1-targeting shRNAs alone or in combination were either left untreated or exposed to UVC-irradiation as indicated. 16 hours post treatment, cellular lysates were collected and analyzed by western blotting for the expression of the apoptotic marker cleaved caspase 3. A representative western blot out of four independent experiments is shown in the figure.

Chapter 4: Discussion

The major finding reported in this thesis is the characterization of the crosstalk between two members of sirtuin family, Sirt1 and Sirt7 and how this crosstalk controls constitutive heterochromatin dynamics through the regulation of a downstream target of Sirt1: the methyltransferase Suv39H1. I showed that Sirt7-mediated inhibition of Sirt1 catalytic activity is required for constitutive heterochromatin relaxation under normal conditions [250] and following genotoxic stress. Furthermore, I demonstrated that the Sirt7/Sirt1/Suv39H1 axis is fundamental to control gene expression in cancer cells exposed to genotoxic stress. This work highlights the importance of mammalian sirtuins in the tight regulation of cellular homeostasis following genotoxic stress. The above findings will be discussed in more detail in the following sections in context of the relevant literature.

4.1 The crosstalk between Sirt7 and Sirt1

Sirtuins constitute a family of enzymes which principally act as protein deacetylases although some members also possess mono-ADP ribosyl transferase as well as other enzymatic activities [21]. Sirtuins play major role in the control of several biological processes such as transcriptional regulation, senescence, aging, metabolism, genomic stability, DNA repair and apoptosis among others [21]. The involvement of sirtuins in such a variety of biological processes is mainly achieved by the capacity of these molecules to deacetylate both histone and non-histone targets. In the last decade, growing experimental evidence revealed that mammalian sirtuins might be involved in a complex network of reciprocal regulation thus adding another layer of complexity in the mechanisms by which sirtuins promote cellular homeostasis. For instance, it has been shown that Sirt1 inhibits Sirt3 expression through the AMPK (AMP-activated protein kinase)-PGC1 (proliferator-activated receptor γ-coactivator-1) pathway leading to an increased ROS generation, decrease in blood-brain barrier permeability and cell apoptosis under normal and ischemic conditions [266-269]. Sirt1 also inhibits Sirt3 by deacetylating zinc finger 5 (ZF5). Deacetylated ZF5 binds more efficiently to specificity protein 1 (SP1), which in turn cannot act as a transcriptional activator of Sirt3 expression [270]. Inversely, Sirt1 can also positively stimulate Sirt3 expression to promote mitochondrial biogenesis and adaptation to sepsis [271]. Another regulatory crosstalk is also documented between Sirt1 and Sirt6. Sirt1 positively regulates the expression of Sirt6 by deacetylating the transcription factor FOXO3a, which has higher affinity to bind to Sirt6 promoter increasing its expression [272]. Furthermore, it was recently reported that Sirt1 can directly deacetylate Sirt6 at K33 increasing its mobilization to DSBs for efficient DDR [273]. Lastly, Sirt4 absence has been shown to enhance Sirt1 expression in hepatocytes through increased NAD^+ levels [274]. The cases reported so far show that the sirtuins can act both in a synergistic and antagonistic manner depending on cellular requirement. Previous work in our laboratory disclosed that Sirt7 inhibits Sirt1 activity [233], uncovering another layer of complexity in the crosstalk between mammalian sirtuins. We showed that Sirt7 represses Sirt1 by preventing

the autocatalytic activation at K230 and that this process is required to promote adipogenesis and fat deposition in mice. In absence of Sirt7, lower fat deposition is observed in mice due to hyperactive Sirt1 that blocks PPARγ activity and hence adipocytes differentiation [250, 255]. In this work, I demonstrate that Sirt7-mediated inhibition of Sirt1 affects other biological functions of Sirt1. I found out that Sirt7 inhibits Suv39H1 activity through Sirt1 repression, thereby controlling heterochromatin dynamics under normal conditions [250] and in response to stress. However, other work described cooperative functions of Sirt1 and Sirt7. Opposite to our results, it was shown that Sirt1 and Sirt7 act synergistically in epithelial and mesenchymal tumors to repress E-cadherin expression and promote acquisition of metastatic phenotype [275]. In addition, it was recently demonstrated that the interaction between Sirt1 and Sirt7 acts in collaboration to maintain heterochromatin organization at rDNA repeats thus maintaining nucleolar chromatin structure and preventing genomic instability [79]. In another work, Li and collaborators showed that in thyroid cancer Sirt7 stimulates Sirt1 activity by epigenetically attenuating the expression of a well-established inhibitor of Sirt1, the deleted in breast cancer 1 (DBC1) [276]. These data indicate that the interaction of Sirt7 and Sirt1 is cell specific and can differentially activate dissimilar molecular pathways according to cellular context. DBC1 for example appears to be regulated by Sirt7 in a cell-specific manner. Our unpublished data demonstrated that depletion of Sirt7 increases DBC1 expression in some cancer cell lines but Sirt7 failed to do so in the U2OS cell line used in my study. Similar duality might also explain the contradictory results concerning heterochromatin organization in Sirt7 deficient cells. Opposite to our results, it was demonstrated that Sirt7 promotes heterochromatin formation in human mesenchymal stem cells [277]. However, whether the basis of this mechanism depends on the differential regulation of DBC1 protein by Sirt7, remains to be investigated. The molecular mechanism by which Sirt7 controls Sirt1/Suv39H1 axis is complex and requires further research. First it remains to be addressed how Sirt1 autodeacetylation is inhibited by Sirt7. We have previously demonstrated that Sirt7 enhances binding of Set7/9 to Sirt1 [233]. Since, Set7/9 is also known to directly methylate and inhibit Suv39H1, an increased Sirt1-Set7/9 binding might interfere with Suv39H1 activation by Sirt1 [46]. Moreover, our unpublished data identified Set7/9 as an interacting partner of Sirt7. In part Sirt7 might control Suv39H1 activity by inhibiting Sirt1 but also by recruiting more Set7/9 to promote Suv39H1 methylation and inhibition. Further work is necessary to elucidate this mechanism.

4.2 Sirt7 might act as an oncogene by promoting heterochromatin destabilization through inhibition of Suv39H1

Maintenance of proper heterochromatin structure is pivotal to control numerous cellular functions both under normal conditions and following genotoxic stress. As highlighted in the introduction, the heterochromatin can be broadly divided into two main sub-types, facultative heterochromatin and constitutive heterochromatin. Facultative heterochromatin is found at specific locations that normally contain genes involved in development and might be

regulated under particular circumstances [278]. They are responsible for processes like genomic imprinting, inactivation of the X chromosome and silencing of immunoglobulin genes. Ideally facultative heterochromatin is characterized by accumulation of specific histone marks such as higher methylation and lower acetylation [33, 36]. Constitutive heterochromatin corresponds to more compact part of chromatin, which mainly contains repetitive DNA elements like satellite sequences and transposable elements at centromeres and telomeres [279]. Constitutive heterochromatin plays crucial role in cell division and silencing of the transposons. Since constitutive heterochromatin is highly enriched for repetitive DNA elements, its preservation is critical to ensure genomic stability. Lack of maintenance of constitutive heterochromatin during DNA replication and repair can result in recombination of the repetitive elements and hence their exclusion leading to genomic instability [44, 280]. Furthermore, transposons are highly mutagenic due to their capacity to target protein-coding genes for insertion. Therefore, relaxation of constitutive heterochromatin can result in chromosomal breakage and promote illegitimate genome rearrangements [281]. Due to its fundamental role in the process of maintaining genomic stability, it is not surprising that insufficient constitutive heterochromatin condensation can be associated with ageing and cancer progression. Loss of heterochromatin in cancer cells especially at pericentromeric and telomeric regions is associated with higher genomic instability that favors generation of aneuploidy giving rise to a panel of diverse tumor cells [254, 282].

H3K9me3 and H3K27me3 are important for formation, propagation and maintenance of heterochromatin [283, 284]. There is a battery of histone methyltransferases and demethylases that dynamically regulate the methylation at these lysine residues, signifying that there is a tight regulation of heterochromatin dynamics in the cell. Tri-methylation at H3K9 is the most important H3 modification associated with constitutive heterochromatin formation. Several methyltransferases and demethylases have been discovered that mediate either formation or removal of H3K9me3, respectively. It is thereby not surprising that reduced levels of H3K9me3 caused by downregulation and/or mutations in methyltransferases or enhanced expression of demethylases have been associated with cancer progression predominantly due to increased genomic instability [285, 286]. The methyltransferases Suv39H1 and Suv39H2 are the major mediators of H3K9me3 deposition and depletion of Suv39H1/2 in mice is linked with increased genomic stability and development of B cell lymphomas, due to significant loss of H3K9me3 [71]. Moreover, mutation in Suv39H2 which resulted in lower H3K9me3 has been associated with enhanced risk of lung cancer [287]. Histone methyltransferases Suv39H1/2 and polycomb repressive complex2 (PRC2) are responsible for H3K9me3 and H3K27me3 formation, respectively and are deregulated in several different cancers [71, 288, 289]. At the same time, upregulation of demethylases like KDM4 and KDM6A is also known to be important for sustenance of cancer phenotype [290]. Consistently, higher mutation rates are observed in human cancers both in constitutive and facultative heterochromatin [291, 292]. Therefore, expression profiles of methyltransferases and demethylases are of great focus in cancer studies.

However, in addition to the observed altered expression of methyltransferases and demethylases, an altered enzymatic activity of these enzymes might equally contribute to aberrant heterochromatin organization that can ultimately lead to cancer progression [287].

PTMs like phosphorylation, ubiquitination, acetylation, among several others play an instrumental role in the protein function [293]. Several decades of research elaborates on the importance of PTMs in stability and efficacy of proteins. Furthermore, PTMs in part control the transcriptional and epigenetic-driven changes in gene expression of numerous cellular events [294, 295]. Suv39H1 has been shown to undergo several post translational modifications which regulate its enzymatic activity and stability. Besides deacetylation by Sirt1 which increases Suv39H1 activity, MDM2-mediated ubiquitination downregulates Suv39H1 stability by directing it for proteasome-mediated degradation [48, 72]. Suv39H1 is also phosphorylated during metaphase which enhances its interaction with metaphase centromeres for correct cell cycle progression [296]. Finally, a recent report demonstrated that Suv39H1 is methylated by Set7/9 and this PTM is required for inactivation of Suv39H1 following genotoxic stress for efficient heterochromatin decondensation to allow DNA repair. In addition, Sirt1 inhibits MDM2-mediated ubiquitination and the subsequent degradation of Suv39H1 during oxidative stress, which, in turn, increases the turnover of Suv39H1 at the heterochromatin and protects genome stability [72]. Therefore, investigating potential PTMs of Suv39H1 in response to DNA damage can help to further elucidate the functions of Suv39H1.

Sirtuins promote the formation of constitutive and facultative heterochromatin by different means. They reinforce heterochromatin compaction either through direct deacetylation of different histone marks or by controlling numerous histone modifiers. The best characterized mechanism involving both means of regulation of heterochromatin formation is executed by Sirt1. Sirt1 deacetylates different histone marks such as H1K26, H3K9 and H4K16 to promote heterochromatin formation [67, 81]. At the same time, Sirt1 enhances deposition of the histone mark H3K9me3 mainly by activating Suv39H1 through direct deacetylation [48]. Heterochromatin formation by Sirt1 is mutually dependent on both functions, as H3K9 deacetylation is prerequisite for its subsequent methylation by Suv39H1 [56]. Additional examples of histone modifiers regulated by Sirt1 are represented by EZH2, P300 and MOF among others [81, 95, 99]. It was recently demonstrated that Sirt6 also plays a role in the formation of constitutive heterochromatin mainly by deacetylation of H3K18Ac [64]. Surprisingly, Sirt7, a known mediator of deacetylation of H3K18Ac failed to reduce acetylation of H3K18 at pericentric chromatin [64]. Interestingly, my work suggests that Sirt7 acts rather as a negative regulator of heterochromatin formation through an unexpected mechanisms: the indirect inhibition of Suv39H1.

Interestingly, Sirt7 is considered an oncogene by its role in cancer growth through mechanisms such as stimulation of ribosome biogenesis and deacetylation at the promoter of tumor suppressors to inhibit their expression [26]. Consistently, Sirt7 upregulation has been reported in majority of human cancers such as hepatocellular, thyroid, colorectal, gastric, breast,

bladder, ovarian and prostate cancer, where it promotes cancer sustenance, growth and metastasis via different mechanisms [221, 275, 297-304]. Although further studies are required to demonstrate the involvement of Sirt7 in cancer, my work suggests that Sirt7 might contribute to cancer progression at least in part by promoting constitutive heterochromatin relaxation and thus stimulating genomic instability in cancer through inhibition of Suv39H1 activity.

4.3 Sirt7 promotes heterochromatin relaxation following genotoxic stress: possible implications in DNA repair mechanisms

In this work, I further demonstrated that Sirt7-mediated inhibition of Suv39H1 activity is fundamental not only to ensure proper constitutive heterochromatin formation under normal conditions [250] but also plays a critical role in heterochromatin relaxation following genotoxic stress. Interestingly, numerous reports have demonstrated an essential role of different sirtuin family members in several molecular mechanisms involved in maintenance of genomic stability following genotoxic stress such as modulation of chromatin structure and activation of different mechanisms of DNA repair. All three nuclear sirtuins Sirt1, Sirt6 and Sirt7 are crucial for DNA damage response and repair [31]. Sirt1 and Sirt6 are recruited to the DSBs immediately after damage while Sirt7 is recruited later, signifying role of Sirt1 and Sirt6 in initial stage of DDR while Sirt7 has a more distal role [305]. In particular, Sirt7 plays a critical role in DSB repair by promoting NHEJ repair [133]. In addition, under genotoxic stress Sirt7 partially relocates from nucleolus to cytoplasm through its interaction with Dicer, which results in an increase of H3K18Ac leading to chromatin relaxation, facilitating DNA repair [169, 306]. Hence, relaxation of the constitutive heterochromatin due to Suv39H1 relocation might represent an additional mechanism by which Sirt7 participates in DNA repair mechanisms.

In response to DNA damage heterochromatin undergoes several dynamic changes to facilitate DDR. For instance, it has been shown that following DNA damage at pericentromeric heterochromatin DSBs are relocated to nuclear periphery in *Drosophila melanogaster* [307-310]. Relocation of DSBs in pericentromeric heterochromatin has been extensively studied. The repositioning of DSBs relies at least in part on the activation of DNA damage checkpoint kinases in *Drosophila melanogaster* as well as in mouse cells and requires functional DNA end-resection [308, 311]. However, how DNA end resection drives mobility of DSBs is still vague. The importance of DNA lesion relocation could be highlighted by the study showing that DNA damage detection and repair is partially impaired in the heterochromatin region [312]. Therefore, their relocation to periphery might make the lesions more accessible to repair factors. Additionally, relocation of DSBs also ensures relatively safer DDR by avoiding aberrant HR among sister chromatids due to presence of several copies of template DNA for repair [313]. At the same time, it is widely believed that heterochromatin is partially protected from DNA damage due to its compact structure and presence of chromatin associated proteins like HP1α and HP1β [314]. However, this can negatively affect the integrity of heterochromatin by

acting as a barrier for damage detection and repair [312]. There are several studies confirming that heterochromatin domains indeed undergo slower repair and hence have higher mutation rates [315-317]. Subsequently, numerous evidence clearly demonstrated that efficient relaxation of the constitutive heterochromatin is required to allow the recruitment of the DNA repair machinery and to promote thereby the repair of this genomic loci. For instance, the damaged pericentromeric heterochromatin is decondensed in response to nuclease or radiation induced breaks in flies and in mouse embryonic fibroblasts (MEFs) [46, 308, 311, 318]. Similarly, evidence from *Caenorhabditis elegans* indicates that the depletion of H3K9/K36 trimethyl lysine demethylase JMJD2A contributes to delayed DNA repair [319]. This data suggest that a decrease in H3K9me3 is important for DNA damage response. Indeed, it has been reported that the DNA repair by NER was considerably slower in H3K9me3 enriched heterochromatin domains [315]. The mechanisms that control the heterochromatin relaxation following genotoxic stress still remain poorly characterized. One of the mechanisms that has been described demonstrates that KAP1 (ATM-dependent phosphorylation of the KRAB-domain associated protein 1), a factor involved in heterochromatin formation, is critical for DNA damage response and facilitates the repair of DSBs at pericentromeres [320, 321]. Phosphorylation of KAP1 leads to removal of the chromatin remodeler chromodomain helicase DNA binding protein 3 (CHD3) which helps in chromatin relaxation [322].

Interestingly, inhibition of Suv39H1 has been demonstrated to play a fundamental role in heterochromatin relaxation following genotoxic stress [46]. In this work, I demonstrate that Sirt7 plays a critical role in deactivation of Suv39H1 and heterochromatin relaxation following stress by controlling the association of Suv39H1 to the constitutive heterochromatin. Although further experiments are required to specifically address whether lack of Sirt7 results in increased accumulation of DNA damage in cells exposed to genotoxic stress, based on the current research, it is reasonable to speculate that Sirt7 might promote the repair of constitutive heterochromatin. However, it remains to be addressed which signals are responsible to stimulate the Sirt7/Sirt1/Suv39H1 axis resulting in heterochromatin relaxation. Recent reports and our unpublished data demonstrated that Sirt7 undergoes different PTMs such as phosphorylation and ADP-ribosylation after stress [30, 323]. I speculate that these PTMs might increase the capacity of Sirt7 to interact with Sirt1 and hence to inhibit more efficiently the enzymatic activity of Sirt1, thus resulting in impaired Suv39H1 activity and heterochromatin relaxation. Further experiments are required to understand this mechanism in detail.

4.4 Sirt7 controls gene expression by modulating Suv39H1 activity

Mammalian gene expression is highly impacted by epigenetic machinery. Combination of different modifications on histone tails at the promoter of a gene, specify the outcome of gene expression. Besides its indisputable role as main mediator of constitutive heterochromatin formation, the histone mark H3K9me3 localizes also in euchromatin regions. Although a vast majority of literature has demonstrated that H3K9me3 mainly acts as a suppressor of gene

expression, a stimulatory effect of this histone mark have been also reported [324, 325]. The association of H3K9me3 to euchromatin loci together with other histone marks might play a stimulatory role not by affecting the heterochromatin condensation but rather by the recruitment of specific transcription factors and/or RNA polymerase II, thus endorsing stimulation of gene transcription [324]. For example, H3K9me3 has been shown to colocalize with activating histone marks H3K9Ac and H3K4me2 where it aids gene transcription [325].

In this work, I could further prove that the Sirt7/Sirt1/Suv39H1 axis controls the expression of genes involved in cellular apoptosis. Using RNA sequencing, I could demonstrate that Sirt7 controls gene expression in a Suv39H1-dependent manner. Specifically, following genotoxic stress, lack of Sirt7 is associated with upregulation of a subset of genes involved in cellular apoptosis such as Mir30a, Lumican, Emilin1, MMP9 and TRIML2 [326-328] [329, 330]. MicroRNA 30a (MiR30a) induces apoptosis in different types of cancer like hepatocellular carcinoma by targeting several molecules involved in apoptosis like IGF1 (Insulin like growth factor 1), AEG1 (Astrocyte elevated gene 1), etc. [326-328]. Lumican (Lum) induces apoptosis in a Fas-dependent manner in normal conditions as well as in cancer cells [329, 330]. Emilin1 is also implicated to promote apoptosis, even though the mechanism of action is not well characterized yet [260, 331, 332]. Tripartite motif family like 2 (TRIML2) is a relatively new target of p53 that has been shown to increase apoptosis by transactivation of a subset of p53 target genes that are known to induce apoptosis [265]. MMP9 is a well-established regulator of apoptosis through several different mechanisms and in a variety of different cell types like neuronal cells, thymocytes and cardiac stem cells among others [333-335].

Interestingly, ablation of Suv39H1 in Sirt7 deficient cells was able to revert the increased levels of gene expression to normal levels and, consistently, ablation of Suv39H1 in Sirt7 deficient cells exposed to genotoxic stress prevented the enhanced apoptosis. These data indicate that aberrant activation of Suv39H1 in Sirt7 deficient cells is responsible for increase in cellular death following genotoxic stress. It still remains to be investigated how Suv39H1 exerts these effects in Sirt7 deficient cells. On one hand, hyperactive Suv39H1 in Sirt7 depleted cells might promote enhanced recruitment of Suv39H1 at the downstream of transcription start site (TSS) of the target genes [336, 337]. This could lead to the enhanced deposition of H3K9me3 and act as a stimulatory signal to induce transcription of these genes, as it has been shown for other gene targets like homeobox A9 (HOXA9) and orthodenticle homeobox 2 (OTX2) [325]. Alternatively, Sirt7 might act in a different manner in constitutive heterochromatin than in facultative heterochromatin, promoting the removal of Suv39H1 from the constitutive heterochromatin but enhancing the recruitment of Suv39H1 to target genes through mechanisms that still warrant further research. In this case, the deposition of H3K9me3 at these promoters might act as an inhibiting signal. As a consequence, Sirt7 deficient cells would have a decrease in Suv39H1 recruitment at the gene promoter of pro-apoptotic genes which would increase their expression. ChIP analysis for H3K9me3 and Suv39H1 recruited at these genes will shed light on the mechanisms controlling this phenomenon.

The role of Sirt7 as an inhibitor of apoptosis following genotoxic stress has already been described [16, 31, 133, 216, 235]. However, the main function of Sirt7 in promoting cell survival following genotoxic stress is the capability of Sirt7 to deacetylate the tumor suppressor p53 and hence reduce its transcriptional activity on targets genes responsible for apoptosis induction [338-340]. Consistently, inhibitors of Sirt7 have been designed as potential anticancer drugs to induce cell apoptosis by enhancing the p53 signaling [341]. My work provides compelling evidence that Sirt7 promotes cell survival in a p53-independent manner and through the control of Suv39H1 activity. Further understanding of this function will be relevant for the development of innovative therapeutic strategies against cancer.

Additionally, further research will demonstrate if the control of Sirt1 activity by Sirt7 might control acetylation status of other Sirt1 targets, extending thereby our understanding in the complexity of the regulation of sirtuin targets.

4.5 Dual role of Sirt7 in cancer

Mammalian sirtuins are recognized as critical factors that ensure maintenance of cellular homeostasis in response to different stress signals. They facilitate mechanisms like metabolic regulation, cell cycle control, chromatin regulation and DNA repair among others to help cells adapt to adverse conditions [31]. The importance of their function is highlighted by the fact that ablation of several sirtuin family members is associated with impaired stress responses, genomic instability, accumulation of DNA damage and predisposition to cancer development [31, 342]. Contradictorily, sirtuins are also known to promote cancer development by aiding various mechanisms like cancer growth and metastasis, inflammation, angiogenesis and evasion from apoptosis [342]. The observation that the sirtuins are often upregulated in a variety of cancer types supports their oncogenic role. Currently, the mechanisms governing the dual nature of sirtuins remain poorly understood.

Sirt7 still remains the least investigated member of sirtuin family, however accumulating evidence points that, similar to other sirtuins, Sirt7 may also possess a dual role in cancer progression. Sirt7 plays important role in maintaining genomic stability by controlling the heterochromatin organization at the rDNA repeats and by participating in different mechanisms of DNA repair mainly through deacetylation of H3K18Ac and desuccinylation of H3K122 [16, 133]. On the other hand, Sirt7 acts as an oncogene by promoting different tumorigenic functions such as cell proliferation, metastasization and evasion from apoptosis by controlling numerous signaling pathways [26].

My work suggests that Sirt7 might possess such a duality in cancer at least in part by controlling the functions of Suv39H1. Sirt7 might act as a tumor suppressor in response to genotoxic stress by controlling heterochromatin relaxation, thus facilitating DNA repair mechanism. In contrast, through the inhibition of Suv39H1, Sirt7 might contribute to cancer development by different means. The upregulation of Sirt7 observed in several cancers might

promote genomic instability and contribute to chromosomal aberration in cancer by destabilizing the constitutive heterochromatin in a Suv39H1-dependent manner. Another characteristic of cancer cells that might render them resistant to therapeutic treatment is represented by the acquisition of the capacity to escape from programmed cell death [342]. In this work, I demonstrated that Sirt7 might act as an oncogene by promoting cancer cell survival through a mechanism that requires Suv39H1 and is independent of p53.

Further work is necessary to confirm whether the Sirt7/Suv39H1 axis might explain at least in part such duality in cancer.

Appendix

List of abbreviations

°C: Celsius degree	**µm:** micrometer
µg: microgram	**µl:** microliter
53BP1: p53 binding protein 1	**5-FU:** 5-fluorouracil
A: adenine	**ADP:** adenosine diphosphate
ATM: ataxia-telangiectasia mutated	**ATR:** ataxia telangiectasia and Rad3 related
ATRIP: ATR-interacting protein	**AKT:** serine/threonine kinase
BAX: Bcl-2-associated X protein	**BAK1:** Bcl-2 homologous antagonist killer
BCL2: B-cell lymphoma 2	**BER:** base excision repair
BIM: bcl-2-interacting mediator of cell death	**bp:** base pair
BSA: Bovine serum albumin	**BRCA1:** Breast cancer type 1 susceptibility protein
C: cytosine	**CDK9:** cyclin-dependent kinase 9
cDNA: complementary DNA	**Chk2:** Checkpoint kinase 2
co-IP: Co-immunoprecipitation	**ChIP:** chromatin immunoprecipitation
DAPI: 4´-6-dimidino-2-phenylindole	**DBC1:** deleted in breast cancer gene 1 protein
DDB2: DNA damage binding protein 1	**DDR:** DNA damage response
DMEM: Dulbecco modified eagles medium	**DMSO:** dimethyl sulfoxide
DNA: deoxyribonucleic acid	**Dnmt1:** DNA (cytosine-5)-methyltransferase 1
DNA-PKcs: DNA-dependent protein kinase catalytic subunit	**DOXO:** doxorubicin
DOT1: disruptor of telomeric silencing 1	**DSBs:** DNA double-strand breaks
DTT: Dithiothreitol	
EGFP: enhanced green fluorescent protein	**ELK4:** ETS-like transcription factor 4
EM: electron microscopy	**eNoSC:** energy dependent nucleolar silencing complex
ERK: extracellular signal-regulated kinase	**EZH2:** Enhancer Of Zeste 2 Polycomb Repressive Complex 2
FCS: fetal calf serum	**FOXO:** forkhead transcription factors of the O-class
g: gram	**G:** guanine
GPDH: glucose-6-phosphate dehydrogenase	
H1: histone 1	**H1K26:** lysine 26 of histone 1
H2AX: X isoform of histone H2A	**H2A.Z:** Z isoform of histone H2A
H3: histone 3	**H3K9Ac:** acetylated lysine 9 of histone 3

H3K18Ac: acetylated lysine 18 of histone 3	**H3K56Ac:** acetylated lysine 56 of histone 3
H3K79Ac: acetylated lysine 79 of histone 3	**H3K122:** lysine 122 of histone 3
H3K9me2: di-methylation of lysine 9 of histone 3	**H3K9me3:** tri-methylation of lysine 9 of histone 3
H4K16Ac: acetylated lysine 16 of histone 4	**H4K20me1/2/3:** mono-/di-/tri-methylation of lysine 20 of histone 4
HA: human influenza hemagglutinin	**HAT:** histone acetyl transferase
HDAC: histone deacetylase	**HDM:** histone demethylase
HEK: human embryonic kidney	**HIF1α:** hypoxia-inducible factor α
HMT: histone methyl transferase	**HOXA9:** homeobox A9
HP1: heterochromatin protein 1	**HR:** homologous recombination
HSF1: Heat shock factor 1	
IAA: iodoacetamide	**IDH:** isocitrate dehydrogenase
IF: immunofluorescence	**IgG:** immunogluobulin G
IGF2: insulin growth factor 2	**IP:** immunoprecipitation
JNK: c-Jun N-terminal kinase	
kb: kilobase	**Kd:** knockdown
KDa: Kilodalton	**KO:** knockout
Kdm1A/LSD1: lysine demethylase 1A	
LFQ: label free quantification	**LB:** Lysogeny broth
mADPRT: mono-ADP-ribosyl transferase	**MAPK:** mitogen-activated protein kinase
MCL1: Myeloid cell leukemia 1	**MDM2:** Mouse double minute 2 homolog
MEFs: mouse embryonic fibroblast	**min:** minute
ml: milliliter	**MLL1:** mixed-lineage leukemia 1
mM: millimolar	**MMP9:** matrix metalloproteinase 9
MnSOD: Manganese superoxide dismutase	**MOF:** males absent on the first
MPI: Max-Planck Institute	**MPS:** Max Planck Society
mRNA: messenger RNA	**MS:** mass-specreometry
MSL: male specific lethal	**MRN:** MRE11/RAD50/NBS1 complex
mTOR: mammalian target of rapamycin	
NAD^+: oxidized nicotinamide adenine dinucleotide	**NADH:** reduced nicotinamide adenine dinucleotide
NAF: sodium fluoride	**NAM:** nicotinamide
NAMPT: NAM phospho ribosyl transferase	**NBS1:** Nijmegen breakage syndrome 1
NER: nucleotide excision repair	**NFκB:** nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NHEJ: non-homologous end joining	**NRF2:** nuclear factor erythroid 2-related factor 2
NoRC: Nucleolar remodeling complex	**NPM:** nucleophosmin
OAADPr: O-acetyl-ADP-ribose	**OE:** over expression

OGG1: 8-oxoguanine-DNA glycosylase 1	
PARP1: Poly-ADP ribose polymerase 1	**PBS:** phosphate buffered saline
PCR: polymerase chain reaction	**PFA:** paraformaldehyde
PK: protein kinase	**Pol I-III:** RNA polymerase 1-3
PMSF: phenylmethanesulfonyl fluoride	**pre-rRNA:** pre-ribosomal RNA
PRC2: polycomb repressive complex 2	**PR-SET7:** PR/SET domain containing protein 7
PSG: penicillin streptomycin L-glutamine	**PTEN:** Phosphatase and tensin homolog
Q: glutamine	**qPCR:** quantitative PCR
R: arginine	**RalA:** ras-related protein Ral-A
rDNA: ribosomal DNA	**RNA:** ribonucleic acid
RIPA: radio-immunoprecipitation assay	**ROS:** reactive oxygen species
RPA32: Replication protein A 32 kDa subunit	**rpm:** revolution per minute
rRNA: ribosomal RNA	**RT-qPCR:** quantitative reverse transcription PCR
s: second	**S. cerevisiae:** Saccharomyces cerevisiae
SDS: sodium dodecyl sulfate	**shRNA:** short hairpin RNA
Sirt1-7: NAD-dependent protein deacetylase sirtuin 1-7	**SNF2H:** Sucrose nonfermenting *protein* 2 homolog
SSBs: single-strand breaks	**Suv39H1:** suppressor of variegation 3-9 homolog 1
T: thymidine	**TBST:** tris-buffered saline-tween20
TCA: tricarboxylic acid	**TIP60:** TAT-interactive protein 60
TSA: trichostatin A	
U2OS: human osteosarcoma	**Ub:** ubiquitin
UVC: ultra violet radiation C	
WB: western blot	**WRN:** Werner syndrome ATP-dependent helicase
WT: wild type	
XPA: xeroderma pigmentosum comlementation group A	**XPC:** xeroderma pigmentosum comlementation group C
Y: tyrosine	**YFP:** yellow fluorescent protein

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Scientific Publications

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