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ACTIVITY AND MECHANISM OF ACTION OF HISTONE DEACETYLASE INHIBITORS IN SYNOVIAL SARCOMA

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Activity and mechanism of action of histone deacetylase inhibitors in synovial sarcoma
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To my mother

致我敬爱的母亲

ABSTRACT

Synovial sarcoma is a rare but aggressive paediatric soft tissue sarcoma that is driven by the genomic rearrangement t(X;18)(p11.2;q11.2). The resulting fusion protein, SS18-SSX, exhibits oncogenic properties through protein-protein interactions that alter gene transcriptions and chromatin remodelling. Synovial sarcomas respond poorly to the conventional chemotherapies. Hence, it is necessary to develop novel therapeutic targets to improve the outcome of the treatment in synovial sarcoma. Considering that the expression of the fusion protein SS18-SSX is specific and essential for synovial sarcoma development and survival, targeting this chimeric oncoprotein or its functional protein network is a potential therapeutic strategy. To date there are no known compounds that directly target SS18-SSX, the driving fusion protein of synovial sarcoma.

SS18 is a transcriptional activator, member of the SWI/SNF complex that is ubiquitously expressed in normal cells. In the SS18-SSX fusion protein, the last nine amino acids of SS18 are replaced by 78 C-terminal amino acids of SSX. The resultant fusion protein therefore inherits oncogenic activities that are independent of the wild type SS18 and SSX. It recruits proteins that are involved in epigenetic gene regulation such as histone deacetylases and polycomb group proteins to form a fusion protein complex that regulates gene transcription and subsequently drives malignant transformation.

The work in this thesis explores the role of two class III histone deacetylases, SIRT1 and SIRT2, in the proliferation and survival of synovial sarcoma. We found that SIRT1 is overexpressed in primary synovial sarcomas. Both siRNA mediated and pharmacological knock down of SIRT1 and SIRT2 in synovial sarcomas and rhabdomyosarcomas impairs cell proliferation and autophagy flux. The overexpression of SIRT1 in synovial sarcomas was not associated with the clinical outcome of patients. However, in experimental *in vitro* cell assays, we observed that nutrient deprivation enhanced the sensitivity of synovial sarcoma and rhabdomyosarcoma cells to sirtuin inhibition. These results suggest a role of SIRT1 and SIRT2 in tumor cell survival under conditions of nutrient deficiency.

Mechanistically, we found that SIRT1 and SIRT2 form a complex with SS18-SSX and their histone substrate H4K16ac in a synovial sarcoma cell line and a patient-derived synovial sarcoma. This complex is disrupted shortly after exposure to the sirtuin inhibitors tenovin-6 and AGK2. These findings indicate that the interactions between SIRT1/SIRT2 and SS18-SSX may contribute to the malignant phenotype of synovial sarcoma by modifying acetylation of the SIRT1 and SIRT2 substrate, H4K16. The acetylation of H4K16 is associated with autophagy. Future studies should address whether autophagy genes are targets of the SS18-SSX/SIRT1/SIRT2 complex.

We set up a proximity ligation assay (PLA) as a screening method to search for small compounds that disrupt the SS18-SSX fusion protein complex. We first validated the method and showed that the association of SS18-SSX with TLE1 is specific for synovial sarcoma and can be visualised by PLA. We then screened a library of 16000 molecules and identified class I HDAC inhibitors and a novel compound, SXT1596, as agents that are able to dissociate the SS18-SSX/TLE1 complex and induce apoptosis in synovial sarcomas. We further showed that the disruption of the SS18-SSX/TLE1 complex by SXT1596 in synovial sarcoma released repression of EGR1 and rescued normal signaling.

The studies in this thesis provide direct evidences that SS18-SSX interacts with proteins that regulate transcription by epigenetic modification of targets, such as class I histone deacetylases and sirtuins. The disruption of the SS18-SSX protein complex with small molecular HDAC inhibitors induces rapid death of synovial sarcoma cells proving that targeting the driving complex of synovial sarcoma may give an opportunity to develop effective therapies for synovial sarcoma patients. Furthermore, PLA based drug screening is shown to be a reliable and valuable technique to identify lead compounds that disrupt protein-protein interactions and could be applied in other cancer types that are driven by fusion transcription factors.

LIST OF SCIENTIFIC PAPERS

I. **SIRT1 and SIRT2 inhibition impairs paediatric soft tissue sarcoma growth.**

Limin Ma, Maruwge W, Strambi A, D'Arcy P, Pellegrini P, Kis L, de Milito A, Lain S, Brodin B

Cell Death and Disease (2014) 5, e1483; doi:10.1038/cddis.2014.385

II. **Identification of cytotoxic agents disrupting synovial sarcoma oncoprotein interactions by proximity ligation assay.**

Aimée N Laporte, Jennifer X Ji, **Limin Ma**, Torsten O Nielsen and Bertha Broidn

Oncotarget, 2016 Apr 21. doi: 10.18632/oncotarget.8882

III. **Studies on the clinical relevance and mechanism of activity of sirtuin inhibitors in synovial sarcoma.**

Limin Ma, Lingjing Chen and Bertha Brodin

Manuscript

RELATED PUBLICATION

Oncogenic functions of the cancer-testis antigen SSX on the proliferation, survival, and signaling pathways of cancer cells

Padraig D'Arcy, Wessen Maruwge, Barry Wolahan, **Limin Ma**, Bertha Brodin:

PLoS ONE, 2014; 9(4): e95136.

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ARMS	Alveolar rhabdomyosarcoma
ATP	Adenosine triphosphate
CR	Calorie restriction
CRE	Cyclic adenosine monophosphate-response element
CTAs	Cancer testis antigens
DNA	Deoxyribonucleic acid
Dnmt1	DNA-methyltransferase 1
DSB	Double-strand break
EGR1	Early growth response 1
ERMS	Embryonal rhabdomyosarcoma
FOXO1	Forkhead box O1
GIST	Gastrointestinal stromal tumor
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HDI	Histone deacetylase inhibitor
HDM2	Human double minute 2 homolog
HPC2	Human polycomb 2
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
kb	Kilobase
kDa	Kilodalton
KRAB	Krüppel-associated box
LOH	Loss of heterozygosity
MSC	Mesenchymal stem cell
mRNA	Messenger RNA
NAD^+	Oxidised nicotinamide adenine dinucleotide
NAM	Nicotinamide

PAX	Paired box gene family
PcG	Polycomb-group
PLA	Proximity ligation assay
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RAB3IP	RAB3A interacting protein
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
shRNA	Short hairpin RNA
Sir2	Yeast silent information regulator 2
siRNA	Small interfering RNA
SIRT	Sirtuin
SS	Synovial Sarcoma
SS18	Synovial sarcoma translocation chromosome 18
SSX	Synovial sarcoma translocation chromosome X
SSX2IP	SSX2 interacting protein
SSXDD	SSX divergent domain
SSXRD	SSX repression domain
TERT	Telomerase reverse transcriptase gene
TLE1	Transducer-like enhancer 1
TSA	Trichostatin A

1 INTRODUCTION

1.1 SARCOMA

Our body can be viewed as a complicated machine in which numerous cells form different modules and perform specialized functions [1]. All cells arise through the division of pre-existing cells and then differentiating to various specialized tissues [2]. This system is not foolproof and mistakes are being made during the cell division process, DNA mutations accumulate and the cell fails to recognise them causing cells to mutate. The mutated cells therefore gain malignant traits such as proliferating unlimitedly, resisting cell death, escaping from immune surveillance, inducing angiogenesis, activating metastases and enabling replicative immortality [3]. Dependent on the origin of the cell, human cancers can be categorized into 5 major groups. i) Carcinoma is the most commonly diagnosed cancer that originates in epithelial tissues such as the skin; ii) Sarcoma is the cancer that arises in transformed cells of mesenchymal origin; iii) Leukaemia, originates in blood and bone marrow; iv) Lymphoma & myeloma, cancers of lymphocytes; and v) brain & spinal cord cancer that occur in the central nervous system. The work in this thesis is limited to sarcoma, which is therefore the focus of the following section.

Sarcomas are aggressive tumors that develop in the connective tissue, including bones, muscles, fat, blood vessels, nerves and cartilage. The incidence is slightly higher in males than females. Sarcomas are rather common in teenagers where it makes up 15% of all the cancers, while for adults it accounts for only 1% of all cancers. Sarcoma can occur in any part of the body. It is subdivided into soft tissue sarcoma and bone sarcoma depending on the tissue of origin.

Soft tissue sarcomas are a heterogeneous group of tumors that consist of more than 50 different histological subtypes. Unlike other types of cancer, which are usually named for the part of the body where the cancer began, the terminology of soft tissue sarcomas is often dependent on the normal tissue cells they most closely resemble. Soft tissue sarcoma comprises less than 1% of all cancers diagnosed each year. Such low morbidity might be because of that the connective/soft tissue cells are not continuously dividing compared with for example epithelial tissues that give rise to the majority of the human cancers. The most common sites of soft tissue sarcomas are extremities, chest, or abdomen, but they can be found anywhere in the body and behave clinically different.

The most common primary malignant bone cancers are osteosarcoma, chondrosarcoma and Ewing's sarcoma. They derive from different parts of bones and metastasize to multiple sites in the body. Osteosarcoma accounts for 35% of all bone cancers and arises from primitive mesenchymal osteoid tissue, often in the knee and upper arms of children, the most susceptible metastatic site is the lung [4]. Twenty-five percent of the bone cancers are diagnosed as chondrosarcoma, which originates in cartilaginous tissue and is often seen in the pelvis, upper legs and shoulders. Ewing's sarcoma is also called Ewing family of tumours, it

consists of several different tumor types and accounts for 16% of bone cancers. Ewing's sarcoma usually forms in the pelvis, femur, ribs and clavicle. The 5-year overall survival rate of primary Ewing's sarcoma is around 70%. For patients with metastatic diseases, this survival rate is only 20-30%. The genetic alteration that underlines most of Ewing's sarcomas is the chromosome translocation between chromosome 22 and 11, which results in the fusion gene EWS/FLI1 [5].

Table 1. **Sarcomas and related chromosome translocations**

Tumor type	Translocation	Fusion product
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) [6]	PAX3-FOXO1A
	t(1;13)(q36;q14) [6]	PAX7-FOXO1A
	t(2;2)(p23;q35) [7]	PAX3-NCOA1
	t(2;8)(q35;q13) [7]	PAX3-NCOA2
	t(8;13;9)(p11.2;q14;9q32) [8]	FGFR1-FOXO1
Alveolar soft part sarcoma	t(X;17)(p11;q25) [9]	TFE3-ASPL
Clear cell sarcoma	t(12;22)(p13;q12) [10]	EWS-ATF1
Desmoplastic small-round cell tumour	t(11;22)(p13;q12) [11]	EWS-WT1
	t(21;22)(q22;q12) [12]	EWS-ERG
Ewing sarcoma (ES)	t(11;22)(q24;q12) [13]	EWS-FLI1
	t(21;22)(q22;q12) [14]	EWS-ERG
	t(7;22)(p22;q12) [15]	EWS-ETV1
	t(17;22)(q21;q12) [16]	EWS-ETV4
	t(2;22)(q33;q12) [17]	EWS-FEV
	t(2;16)(q35;p11) [18]	FUS-FEV
	t(1;22)(q36.1;q12) [19]	EWS-ZSG
	t(4;19)(q35;q13) [20]	CIC-DUX4
Inflammatory myofibroblastic tumour	t(1;2)(q25;q23) [21 22]	TPM3-ALK
	t(2;19)(q23;q13) [22]	TPM4-ALK
	t(2;17)(q23;q23) [21]	CLTC-ALK
	t(2;2)(p23;q13) [23]	RANBP2-ALK
Low grade fibromyxoid sarcoma	t(7;16)(q33;q11) [24]	FUS-CREB3L2
	t(11;16)(q11;q11) [25]	FUS-CREB3L1
Myxoid liposarcoma	t(12;16)(q13;q11) [26]	FUS-DDIT3
	t(12;22)(q13;q12) [19]	EWSR1-DDIT3
Synovial sarcoma	t(X;18)(p11;q11) [27]	SS18-SSX1
	t(X;18)(p11;q11) [27]	SS18-SSX2
	t(X;18)(p11;q13) [28]	SS18-SSX4

The etiology of sarcomas is not clear. Nearly 30% of all sarcomas are characterized by specific genetic alterations. They either carry specific oncogenic mutations like somatic mutations of c-KIT in gastrointestinal stromal tumors (GIST), or are characterized by certain chromosomal translocations resulting in the expression of chimeric transcription factors (Table 1) [29]. The sarcomas with non-specific genetic abnormalities often have unbalanced karyotypes and numerous aberrations (such as chromosomal losses and gains), which have been identified as driving events.

1.1.1 Rhabdomyosarcoma

The most common peadiatric soft tissue sarcoma is rhabdomyosarcoma (RMS), it originates from striated or skeletal muscle cells and develops, most commonly, in the head, neck, genitals and extremities [30]. RMS is highly aggressive and has a strong tendency for recurrence and metastasis. Common metastatic sites are lung, bones and bone marrow [31]. It has been suggested that in children, below the age of 3, the development of RMS might be related with germline mutation of p53 [32]. Studies also revealed that Beckwith-Wiedemann syndrome, a rare genetic disorder due to abnormalities on chromosome 11p15, is related with the tumourigenesis of RMS [33].

RMS is divided into different histological subtypes, in which embryonal RMS (ERMS) and alveolar RMS (ARMS) are the most common ones, accounting for ~60% and ~20% of cases, respectively. ERMS is characterized by loss of heterozygosity (LOH) on chromosome 11, at the location of p15.5. The expression of the genes within this region is epigenetically affected by genomic imprinting, which means that one of the alleles inherited from one of the parents is silenced. The genes located on the active allele might be inactivated by point mutation, leading to the loss of the entire gene. It is postulated that there are tumor suppressor genes located in this region. When both alleles of the tumor suppressor are inactive due to a combination of genetic imprinting and allelic loss, oncogenic effect is promoted.

The majority of ARMS carry the chromosomal translocation t(2;13)(q35;q14) which fuses the PAX3 gene (paired box 3) located on chromosome 2 to FOXO1 (fork-head box O1 family, formerly called FKHR) which is located on chromosome 13. Another common ARMS translocation is t(1;13)(p36;q14), which fuses PAX7, a PAX family gene located on chromosome 1, with FOXO1 [6]. PAX3-FOXO1 is expressed in approximately 70% of the cases while PAX7-FOXO1 in 10-15% and they are referred as P3F and P7F, respectively. These fusing proteins play pivotal roles in initiating or enhancing tumourigenesis [34]. The expression of PAX-FOXO1 fusion protein in ARMS influences the prognosis of the patient. For example, the fusion gene-negative ARMS has similar molecular profile with ERMS and is less aggressive compared to the ARMS that carries the fusion gene [35,36]. Meanwhile, the ARMS that carries PAX7-FOXO1 fusion transcript is associated with better prognosis compared with the more common PAX3-FOXO1 variant [37,38].

Still, there are 20-30% of the ARMS that show no sign of PAX-FOXO1 translocations. The study of a rare ARMS case has shown that the PAX3 gene is fused with transcriptional coactivator NCOA1 or NCOA2 because of the translocations t(2;2)(p23;35) or t(2:8)(q35;q13) [7]. Another ARMS case reported in 2011 was identified to have a special t(8;13;9)(p11.2;q14;9q32) three-way translocation [8].

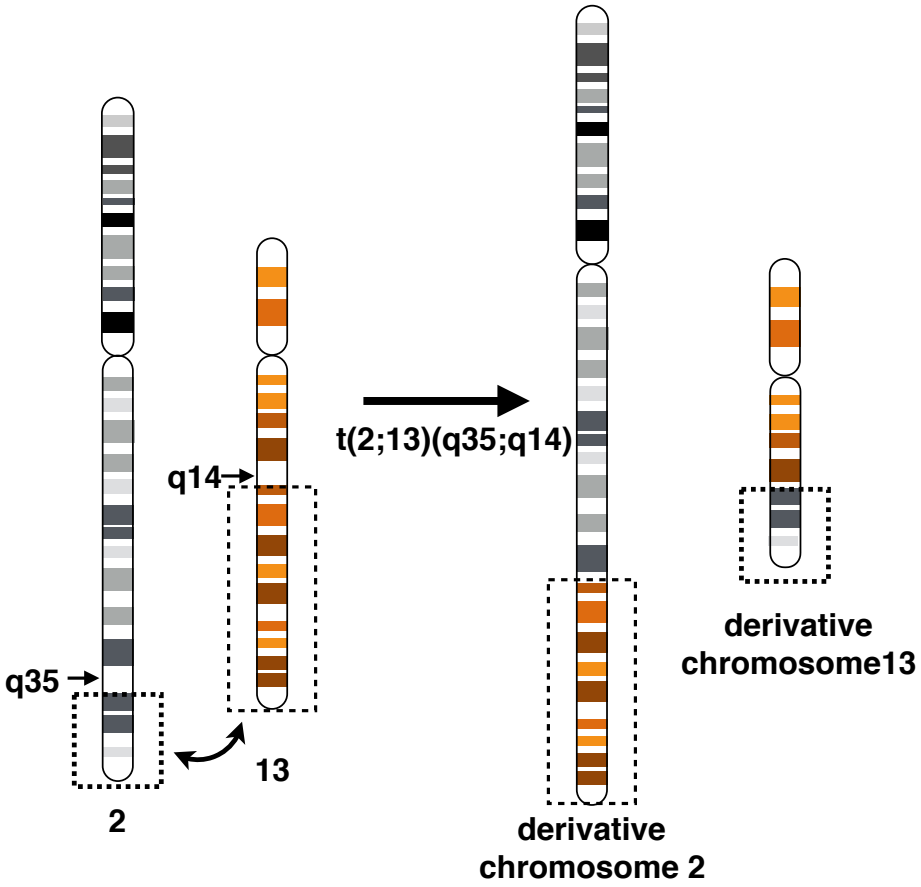

Figure 1. **Chromosomal translocation t(2;13)(q35;q14) in ARMS.**

1.1.2 Synovial sarcoma

Synovial sarcoma (SS) is the second most commonly diagnosed paediatric soft tissue sarcoma. It accounts for 10% of all soft tissue sarcomas and occurs predominantly in teenagers and young adults between the age of 15 and 40 [39]. Despite its name, SS neither originates from synovial cells nor differentiates toward synovium [40,41]. Instead, it derives from myoblastic precursor cells and is able to develop anywhere in the body [42,43]. Most SS develops in deep soft tissues around the large joints of the extremities, with 60 % -70 % occurring around knees. The prognosis of SS is normally poor because of recurrence and metastatic diseases.

Histologically, SSs are divided into biphasic, monophasic and poorly differentiated types. The monophasic SS shows the morphology of spindle shaped mesenchymal cells and appears to be most frequent in adults (72%). Biphasic SS exhibits both spindle cell morphology and foci of epithelial differentiation. However, in children, these 2 subtypes are more equally distributed (55% monophasic and 45% biphasic) [43,44]. The poorly differentiated variant of SS is less common (20%) but normally more aggressive and indicates poor prognosis compared to other types. Sometimes small round cells can be observed in poorly differentiated SS, which makes it difficult to distinguish this type from other small round cell tumors by histology. Therefore extra genotypic analyses are necessary to confirm the diagnosis.

1.2 SS18-SSX, THE FUSION ONCOPROTEIN IN SYNOVIAL SARCOMA

SS is one of the most well understood translocation-associated sarcomas. The characteristic translocation t(X;18)(p11.2;q11.2) involves the SS18 gene on chromosome 18 and SSX1, SSX2 or SSX4 gene on chromosome X [45], which results in the expression of the aberrant fusion protein, SS18-SSX in nearly all SS. It is not known whether this genetic alteration happens randomly or follows certain events, but it has been proven that it is necessary and sufficient for SS tumor initiation, therefore SS18-SSX is considered as a driver oncogene [27,46 47]. In more than 30% of SS, SS18-SSX is the only cytogenetic anomaly and it remains detectable in metastatic diseases. Studies have shown that knock down of the SS18-SSX fusion protein decreases SS cells' viability and induces apoptosis [48]. On the other hand, the conditional expression of the fusion protein SS18-SSX in a mouse model induces SS with 100% penetrance [42]. Several Studies have shown that the SSX fusion type has impact on the clinical behaviour of SS. Patients with tumours expressing the SS18-SSX1 fusion gene have worse clinical outcomes. However, there is one study showing contradictory results where the fusion type shows no impact on the survival of SS patients [49].

SS18

Wild type SS18 (formerly named SSXT, SYT) is an ubiquitously expressed gene located on the long arm of chromosome 18 (at position q11.2). It encodes a transcriptional activator that contains 418 amino acids. The highly conserved N-terminal domain of SS18, which is designated as SNH domain, is composed of 54 amino acids and is responsible for the nuclear localisation of SS18 [50]. The C-terminal of SS18 is designated as the QPGY domain, which represents the amino acid glutamine, proline, glycine, and tyrosine in this region. The mutants bearing the QPGY deletion show a decreased transcriptional activation potential of SS18 [23,51]. SS18 has no DNA binding motifs, but contains many potential protein-protein interaction sites and functions as a transcriptional activator by interacting with DNA binding proteins. These proteins are involved in chromatin modification and gene expression [50], such as members of the SWI/SNF complex, histone acetyl transferases p300, components of mSin3A/HDAC complex [52], AF10 [53], and SIP/CoAA [54]. Most of these interactions occur within or close to the N-terminal SNH domain. Some of these interactions share competitive overlapping binding sequences (Figure 2).

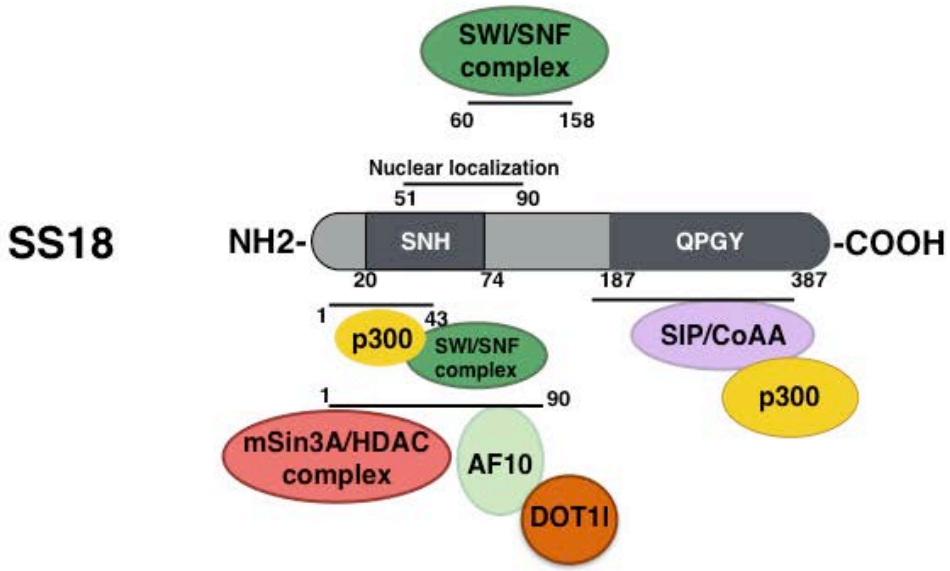

Figure 2. **Domain structure of SS18 and protein-protein interaction sites**

SSX

The SSX family of Cancer-Testis Antigens (CTAs) is comprised by 9 highly homologous members, which have been named sequentially based on their discovery as SSX1-9. SSX genes are localised on chromosome X and have their expression restricted to spermatogonia in testis [55] and fetal mesenchymal stem cells. SSX expression is down-regulated following mesenchymal stem cell differentiation, suggesting that SSX plays a role in the repression of cell differentiation [56] [57]. Although SSX genes are aberrantly expressed in vast human tumor types, no clear correlation between SSX expression and tumor prognosis has been found except for very few reports which show that the expression of SSX in late stage tumors is higher compared to early stage tumors [58]. Studies have reported that SSX increases the invasive potential and represses E-cadherin expression in melanoma [57] and breast cancer cells [59].

Most of the SSX proteins consist of 188 amino acids [27] and contain three functional domains. The N–terminal Krüppel–associated box (KRAB) domain and the highly conserved C–terminal dominant repressor domain (SSXRD) are the major domains [50,60]. They can directly bind to DNA and show strong repressive effect. The SSX proteins are strong transcriptional repressors due to the activities of KRAB and SSXRD domain. Some studies suggest that the KRAB domain enhances the activity of the C-terminal SSXRD domain instead of showing intrinsic activity of its own [23], which suggests that the SSX mediated repression of gene regulation is mostly related with the activity of the SSXRD domain. A third functional domain of SSX refers to SSX divergent domain (SSXDD). Little is known about the function of SSXDD [61].

Proteins, such as RAB3IP (RAB3 interacting protein) and SSX2IP (SSX2 interacting protein) [62], interact with SSX mainly within the KRAB domain. Although the KRAB domain is considered as the main functional domain, lots of protein-protein interactions occur outside of this region. For instance, there are several proteins that compose polycomb chromatin remodelling complex (PcG complex) colocalise with SSX through interacting with SSXRD domain, including BMI1 (B cell-specific moloney murine leukaemia virus insertion site 1 protein), HPC2 (human polycomb group protein 2), RING1 (ring finger protein 1) and RING2. The transcription activator LHX4 was also found to be colocalised with SSX in this region [63] (Figure 3).

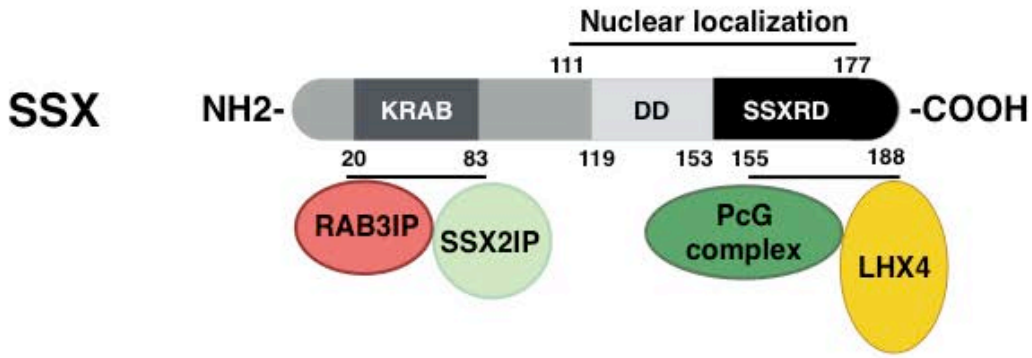

***Figure 3*. Domain structure of SSX and protein-protein interaction sites**

1.2.1 SS18-SSX and transcriptional repression

In the SS18-SSX fusion protein, the last 8 amino acids of SS18 (N-terminal) is replaced by 78 C-terminal residues of SSX containing the repressor domain. The resulting chimeric protein contains both transcriptional activating and repressing domain (Figure 4). Hence the fusion protein harvests oncogenetic activities that are independent of the wild type SS18 and SSX proteins. It recruits new interacting partners that involve in gene transcription modulation and epigenetic regulation, such as transducer-like enhancer of split (TLE1), activating transcription factor 2 (ATF2), histone deacetylases (HDACs) and polycomb group members (PcG) [64].

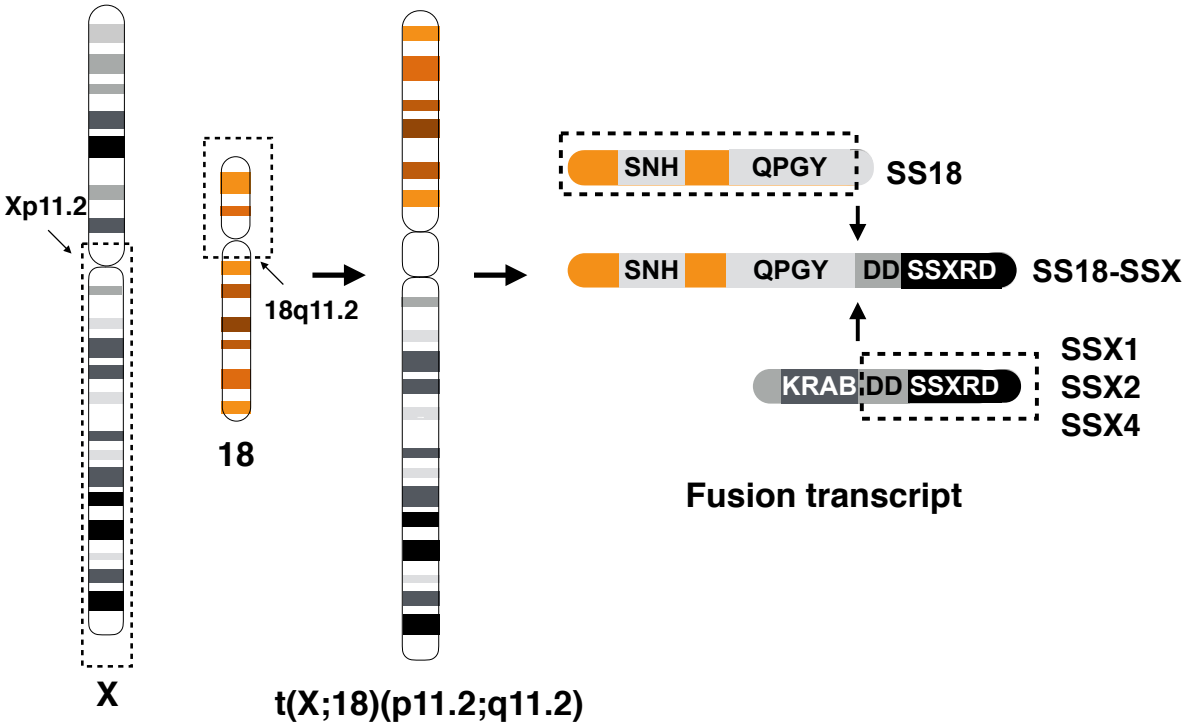

Figure 4. **Chromosome translocation t(x;18)(p11.2;q11.2) and aberrantly transcribed fusion gene SS18-SSX in synovial sarcoma.**

The transcription factor ATF2 carries a DNA-binding domain that promotes the transcription by recognising the cyclic AMP-response element (CRE) and recruiting histone acetyltransferases (HATs) [65]. TLE genes are transcriptional corepressors, they interact with transcriptional activators and function negatively to inhibit the transcription of target genes [66]. TLE1 involves in Wnt/β-catenin signaling pathway and is expressed during embryogenesis similar to other 3 TLE genes. Knösel et al. have reported that the TLE1 gene is overexpressed in 96% of the SS (249 out of 259 SS specimens) [67]. Positive immunohistochemical staining of TLE1 is a strong indication of SS. Therefore TLE1 has been recognised as an excellent diagnosis marker for SS. However, some studies indicate limited specificity of TLE1 expression in SS. Kosemehmetoghu et al. found positive TLE1 expression in 37% of the tested non-SS mesenchymal tumours[68,69].

With the assistance of these master transcriptional regulators, the fusion oncoprotein SS18-SSX functions predominantly in transcriptional regulation in spite of lacking apparent DNA binding motif [70-72]. Le Su et al. have reported that SS18-SSX forms a functional endogenous complex with recruited ATF2 and TLE1, which leads to the repression of ATF2 target genes and abnormal transcriptional activities that drive the malignant transformation in SS. The recruitment of ATF2 and TLE1 are independent processes and involve different protein domains of SS18-SSX. The oncoprotein SS18-SSX serves like a scaffold to link these proteins together [64].

The tumor suppressor early growth response 1 gene (EGR1) is an identified target of SS18-SSX in SS. The fusion protein complex (more specifically, polycomb proteins) directly binds to the EGR1 promoter and results in the down regulation of the EGR1 expression [73,74]. It has been reported that reintroduction of EGR1 in human tumor impairs tumor development and induces the expression of some tumor suppressors located downstream, like p53, transforming growth factor-β, phosphatase and tensin homolog (PTEN) and others. Small molecules that can reactivate EGR1 expression in SS cells such as romidepsin have been studied to find a way to reverse the transcriptional repression effect of the fusion oncogene [74].

More molecules that are involved in the SS18-SSX-mediated transcriptional repression have been identified, including polycomb group complexes, trimethylated histone H3 lysine 27 (H3K27me3) [75], enhancer of zeste 2 (EZH2), histone deacetylase 1 (HDAC1) , embryonic ectoderm development (EED) protein, suppressor of zeste 12 homolog (SUZ12) and insulin-like growth factor 2 (IGF2) [31]. These molecules are recruited to the target [74]promoters of the fusion protein SS18-SSX independently [64]. Immunohistochemical examine of SS tissues has confirmed the expression of cyclin D1, β-catenin, cytokeratin, vimentin, BCL2, IGF-1R and KIT [32-34].

1.2.2 SS18-SSX and p53 function

The human TP53 gene is located on chromosome 17 and encodes one of the mostly studied tumor suppressor proteins, p53, which is also called the guardian of the genome. The activation of p53 happens in response to various cellular stresses such as DNA damage, oxidative stress and osmotic shock [76]. Activated p53 shows a great anti-cancer character by holding the cell cycle at the G1/S stage through its downstream targets, activating DNA repair proteins, or initiating apoptosis when there are irreparable DNA damages. However, as the most frequently mutated gene in human cancer, TP53 mutations were identified in almost all types of cancer at rates of 10-100% [77] [78]. This indicates that point mutations induced disruption of p53-mediated tumor surveillance is a common strategy in tumourigenesis.

SS cells carry intact copies of wild type TP53 genes and they fail to activate p53 functions in response to cellular stress such as DNA damage and deregulated oncogene expression. Gene expression profile studies of SS cells show that the inhibition of the SS18-SSX fusion gene reactivates p53 checkpoint pathway and induces growth arrest or apoptosis [79,80]. Our group explained this observation by demonstrating that the SS18-SSX oncoprotein promotes TP53 ubiquitination and degradation through a mechanism involving HDM2 (human double minute 2 homolog). In healthy cells, p53 is kept in a low level because of the p53-HDM2 auto-regulatory loop, in which HDM2 acts as a negative regulator to promote the ubiquitination and degradation of p53 and prevents the aberrant activation. Ubiquitinized p53 can be rapidly stabilised to maintain the genome stability when the cell senses cellular stress. However, in SS, the fusion protein SS18-SSX sustains HDM2 expression and promotes HDM2 stabilisation by inhibiting its auto-ubiquitination [81]. In our study, we confirmed that HDM2 has an effect on p53 in a SS cell line that carries the SS18-SSX2 fusion gene.

Exposure of SYO-1 cells to the HDM2 antagonist nutlin-3 increases p53 stability, activates the expression of p53 target genes, thereby inducing growth arrest and apoptosis [82].

1.2.3 SS18-SSX and Wnt/β-catenin signaling

Studies have demonstrated that the Wnt/β-catenin signaling pathway is frequently activated in SS, indicating a unique function of the Wnt/β-catenin pathway in this cancer type [83]. β-catenin is a downstream target of the SS18-SSX fusion protein as well as an important component of the Wnt/β-catenin signaling pathway; The interference of β-catenin-mediated signaling results in the initiation of different types of tumours [84]. Tadashi H et al. have observed that β-catenin is accumulated in the nuclei of primary and metastatic SS [85]. The nuclear accumulation of β-catenin is important in maintaining the morphology of spindle cells in SS [83]. It was further shown that nuclear accumulation of β-catenin promotes tumor progression and leads to short term survival of SS patients [85]. By screening genetic alterations in series of 49 SS samples, it was found that 30-60% of SS cases contain mutations both in adenomatous polyposis coli (APC) and β-catenin [86].

Functional evidence for a critical role of Wnt/β-catenin signaling in SS has been recently shown. Using SS tumor xenograft and a SS18-SSX2 transgenic mouse model, Whitney B et al. have found that the Wnt/β-catenin pathway is activated by the fusion oncoprotein SS18-SSX2, which is reflected by the upregulation of Wnt/β-catenin cascade. This finding was confirmed in further studies that detected downstream targets of Wnt/β-catenin using immunohistochemistry staining in a cohort of 30 primary SS biopsies; Transfection of SS18-SSX into HEK293 cells shows a similar activating function. The inhibition of Wnt/β-catenin signaling by knocking out of β-catenin prevents SS cell proliferation and tumour formation [87].

1.3 EPIGENETICS

Epigenetics is the study of inheritable changes in gene expression that does not affect the DNA sequence. It is the study of phenotypical but not of genotypical changes. Histone modification, together with DNA methylation and non-coding RNA-associated gene silencing, initiate and sustain epigenetic changes. Compared with transcriptional regulation, epigenetic modulation is a fast reaction and takes place after proteins are synthesised, which allows the cell to respond fast to environmental changes.

1.3.1 Histones, the core proteins of the nucleosome

Each human diploid cell accommodates about 6 billion base pairs (bp) of DNA within 23 pairs of chromosomes. The length of 1 bp DNA is about 0.34 nm, which means that there are approximately 2 metres of DNA in a single cell [88]. Eukaryotic chromatins are highly efficient structures that compact such long DNA sequences to fit in the nucleus of the relatively small cell.

The basic chromatin structure, a nucleosome, is the primary level of DNA compaction. It consists of 146 bp of the DNA that is on average tightly wrapped 1.65 times around a histone

octamer, like thread wrapped around a spool. Histones can tightly bind with DNA due to the negatively charged phosphate-sugar backbone of DNA. The histone octamer consists of a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers [89]. The linker histone H1 is located close to the DNA entry and exit region in a nucleosome unit. Strings of nucleosomes are further coiled into even shorter and thinner structures, which are called 30-nanometre fibres (Figure 5).

The nucleosomal structures of eukaryotic chromatin form a physical barrier to the enzymes that unwind and copy DNA. DNA replication and gene transcription can therefore not happen since such processes require unwound DNA strands to allow polymerases access to the DNA template. Remodelling of highly compacted chromatin and modification of histones are temporarily reversible processes that control the accessibility of DNA. Once one or both of these processes are completed, the remodelled chromatin or modified histone returns to its compact state to keep the stability of the genome [90,91].

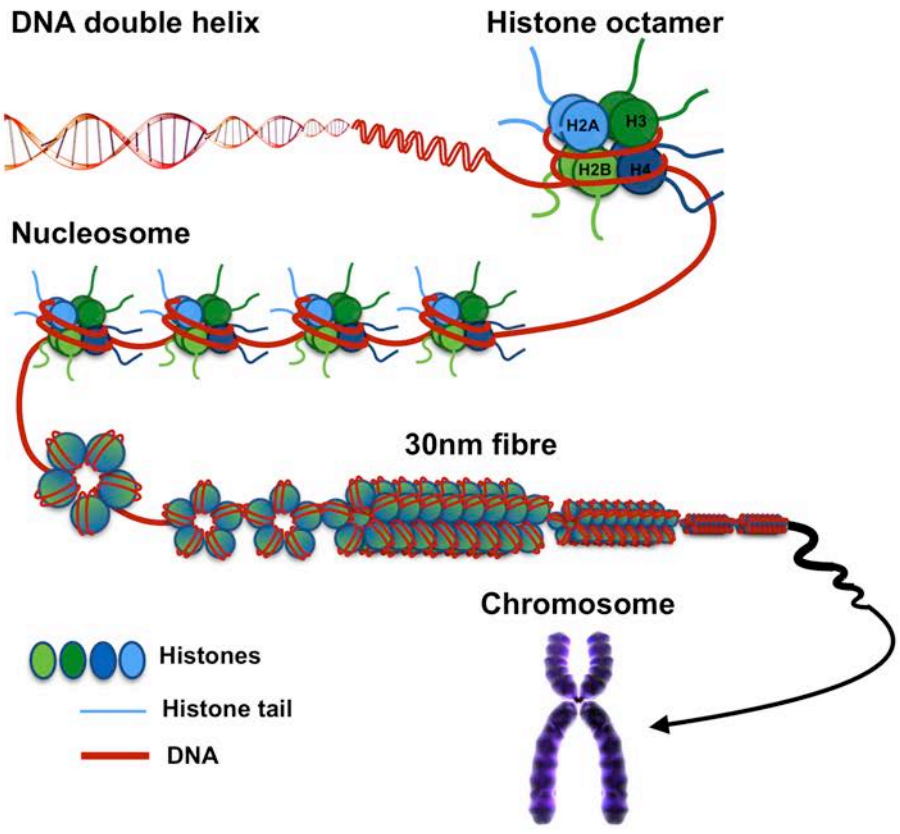

Figure 5. **Chromosome structure**

1.3.2 Histone modification

A histone protein is constructed by a structured core and an exposed tail domain. The histone tail comprises more than 25% of the mass of a single histone. It protrudes from the histone core and provides an accessible surface for potential reactions with other proteins. Most of

the histone modifications occur on the amino acid residues in its tail in the form of methylation, phosphorylation, acetylation, ubiquitylation and sumoylation [92].

Histone modification can help to partition the genome into distinct domains such as euchromatin that is under active transcriptions and heterochromatin that is inaccessible for transcription. Histone modification can facilitate DNA-based functions like DNA-replication, DNA repair and chromosome condensation. Histone acetylation and deacetylation are the most studied histone modifications and are reversible mechanisms. Histone acetylation depends on the activity of histone acetyltransferases (HATs), which move the acetyl groups onto lysine residues. Histone deacetylation depends on the activity of histone deacetylases (HDACs), which take the acetyl groups away (Figure 6). This post-translational modification can happen on any of the core histone components (H2A, H2B, H3 and H4) in a histone octamer, but is most common on H3 and H4. The marks of H3 that are most acetylated are lysine 9, 14, 18, 23 and 56 in most species. The acetylation of H4 normally takes place at lysine 5, 8, 12 and 16 [93]. Generally, the acetylation of certain lysine residues in the histone tail is associated with gene activation whereas the deacetylation is associated with a repressed gene expression. The balance between acetylation and deacetylation is crucial for the development of healthy cells.

Histone acetyltransferases (HATs)

There are 2 types of HATs according to their cellular localisation and biological function. Type A HATs acetylate nuclear histones that are closely related to gene transcription. While, type B HATs are often found to acetylate newly synthesised histones in the cytoplasm until they are transferred into the nucleus and bind to freshly constituted DNA sequence. More than 20 HATs have been identified, such as type A HATs Gcn5p and SAGA, which were discovered in yeast and target on H3 and nuclear H2B; p300, a human type A HATs that targets all the core histones; Hat1p, a yeast type B HATs that targets free H4 [94]. The human proteins that show HATs activities are classified as lysine (K) acetyltransferases (KATs) [95].

Histone deacetylases (HDACs)

The removal of acetyl groups from histones is accomplished by histone deacetylases (HDACs). So far there have been 18 mammalian HDACs identified [96]. These isoforms can be further divided into 4 distinct classes according to their localisation, number of catalytic pockets and size. Class I HDACs include the enzymes HDAC 1, 2, 3 and 8, which primarily localise in the nucleus. The members of class II HDACs (HDAC 4, 6, 7, 9, 10) are believed to be able to deacetylate both non-histone as well as histone proteins according to their subcellular localisation between nucleus and cytoplasm. Both class I and class II HDACs are zinc-dependent and can be inhibited by the drug trichostatin A. The class IV HDACs, which contain only one isoform, HDAC11, has features of both class I and II. Interestingly, the class III HDACs is a group consisting of unique types of enzymes that are underlined by a special mechanism. Different from the Zn^{2+}-dependent activity of other classes, these enzymes require NAD^{+} (oxidised nicotinamide adenine dinucleotide) as a cofactor and are not

sensitive to compounds that inhibit Zn^{2+} dependent enzymes. Class III HDACs are known as sirtuins (SIRT1-7) and are homologous with the yeast silent information regulator 2 (Sir2).

HDACs regulate not only histones, but also a wide range of non-histone proteins. These substrates are key elements of crucial cellular processes. HDACs are therefore involved in numerous signaling pathways and play a pivotal role in chromatin remodelling and epigenetic regulation of gene expression. Abnormal expression of HDACs and related aberrant chromatin deacetylation displays significant effects on cell cycle and cell survival and contributes in oncogenesis. For example, it was reported that the expression of HDAC1 was upregulated in prostate, colon, gastric and breast carcinomas [97 98 99 100]. Overexpression of HDAC 2, 3 and 6 were observed in different cancer specimens as well [101,102]. Our previous study showed that some members of the class III HDACs, namely sirtuins, were overexpressed in SS [103]. The hypoacetylation of lysine 16 on H4 was described as a hall mark of cancer [3]. Studies also demonstrated that histone hypoacetylation is related with tumor invasion and metastasis [104]. Different from DNA mutation, epigenetic modifications are reversible. This provides a possible strategy for the treatment of cancer by reversing the distorted epigenome to a normal epigenome in the affected cells. HDACs have therefore become interesting potential targets for cancer treatment.

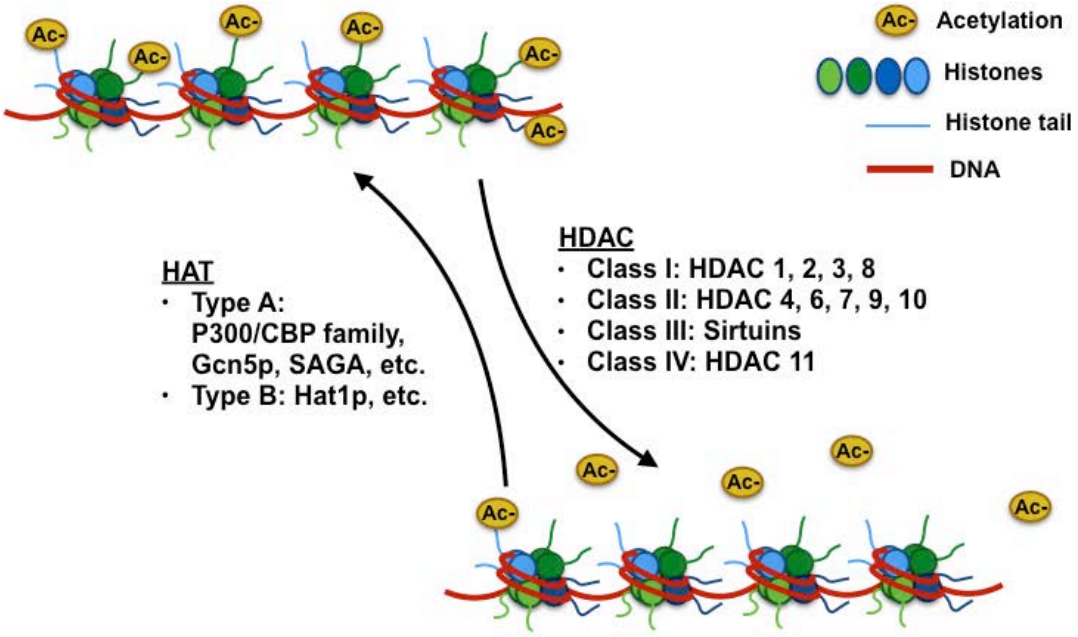

Figure 6. **Histone acetyltransferases (HDIs) and deacetylases (HDACs)**

1.4 CLASS III HDACS: SIRTUINS

Sirtuins are a group of phylogenetically conserved proteins that have been found in archaea, bacteria, eukaryote and even viruses [105]. It was first discovered as a transcriptional repressor of the mating type yeast, named as Silent Information Regulator 2 (SIR2). In the late 1990s, several studies showed that deletion of SIR2 shortens the lifespan of Saccharomyces cerevisiae, while increased SIR2 gene dosage extends its lifespan [106]. Similar effects were

observed subsequently in Caenorhabditis elegans and Drosophila melanogaster [107,108]. These findings triggered great interest for finding similar effects in mammals and in particularly humans.

Since 1999, seven mammalian sirtuin paralogues (SIRT1-7) have been identified [109]. They share a highly conserved catalytic and binding domain (also called sirtuin corn domain), which consists of a typical Rossmann fold and a small zinc-binding domain composed by approximately 250 amino acids. The structures of amino- and carboxy-terminal extensions of the 7 mammalian sirtuins are quite divergent, which may endow them diverse biological functions, distinct subcellular localisations, special enzymatic activities and various unique substrates during evolution.

SIRT1 and SIRT6 are found predominantly in the nucleus, SIRT3, 4 and 5 are localised in the mitochondria, whereas SIRT2 primarily resides in the cytosol and SIRT7 in the nucleolus. Sometimes SIRT1 and SIRT2 can be observed both in the nucleus and cytoplasm which suggests that they shuttle between different compartments within the cell [110]. The subcellular localisation of mammalian sirtuins is probably also dependent on cell type, stress status and molecular interactions. SIRT1 and SIRT2 are the most studied mammalian sirtuins [109].

***Table 2*. Mammalian sirtuins' properties**

Mammalian sirtuins	Cellular localisation	Enzymatic activities
SIRT1	Nucleus	Deacetylase
	Cytoplasm	Deacetylase
SIRT2	Cytoplasm	Deacetylase
	Nucleus	Deacetylase
SIRT3	Mitochondia	Deacetylase
	Cytoplasm	Deacetylase
SIRT4	Mitochondia	ADP-ribosyl-transferase
SIRT5	Mitochondia	Deacetylase, Deacylase
SIRT6	Nucleus	Deacetylase, ADP-ribosyl-transferase
SIRT7	Nucleolus	Deacetylase
	Cytoplasm	Deacetylase

1.4.1 Sirtuin substrates and activities

Sirtuins harbour 2 types of different but related enzymatic activities: deacetylase activity and ADP-ribosal transferase activity as described in Hawse and Du J's papers [111 112]. However, the current understanding of the latter is very limited.

Histones are the most conserved and basic substrates of the sirtuin family. Sirtuins transfer acetyl groups from certain substrates to an ADP-ribose molecule in assistance of NAD^+, which is a key factor involved in the cellular metabolism. Sirtuins' activities are strongly dependent on the availability of cellular NAD^+.

Sirtuins act as sensors of the balance between metabolism and energy and thereby coordinates cellular maintenance of the genome integrity [113,114]. In the past decade, a lot of

research has been done to investigate the correlations between sirtuins and metabolic pathways. Sirtuins, especially SIRT1, are involved in cellular metabolism regulation under the condition of calorie restriction (CR). Numerous studies have shown that in many organisms, like yeast, worms, mice and flies, CR prolongs the lifespan up to 50%. NAD^+ levels are also observed to be increased as well as upregulated SIRT1 expression in some tissues during CR [115].

Sirtuins target histones and a wide range of non-histone substrates. Many of these substrates are important enzymes that are involved in different biological pathways and cellular functions that regulate the stress response, chromatin machinery, transcription, genome stability, metabolism, etc. Examples include the chromatin related protein p300; stress related proteins p53, FOXO1, HIF1a; DNA repair protein Ku70; metabolic components ACS1, ALDH2, GDH [116,117]. By regulating the activity of various substrates, sirtuins are involved in many human diseases, especially age-related diseases such as cancer, diabetes and cardiovascular disease. Despite the diversity of sirtuin substrates, Gil Blander and his colleagues showed in their study that substrate recognition of SIRT1 does not depend on the amino acid sequence close to the acetylated lysine [118]. Instead, sirtuins preferentially deacetylate acetyl-lysine within unstructured regions, which suggests that conformational requirement might be a general feature of substrate recognition in the sirtuin family [119].

In animal models, the transgenic SIRT6 mouse exhibits a 10-15% increased lifespan compared with their wild-type littermates [120]. However, the normal human cells that are studied show no sign of prolonged lifespan when the expression of a single sirtuin protein is increased [121]. Several reports indicate that deregulated expression of sirtuins is involved in the development of various malignancies [122]. Apart from SIRT1 and SIRT2, the molecular mechanisms underlying sirtuin-mediated biological functions and the particular pathways remain poorly understood.

1.4.2 The oncogenic role of SIRT1 and SIRT2

Cancer is such an incredibly heterogeneous disease for which every single sample is unique. Even in several specimens of the same tumour, the mutated genes are widely diverse. For most cancer related genes, including sirtuins, it is not easy to simply classify them into either tumor prompters or tumor suppressors. Multiple issues have to be considered, such as the type of cancer, the stage of its development, tissue of origin, experimental conditions. The most investigated members in the sirtuin family for their role in regulation of cancer metabolism are SIRT1 and SIRT2, which are also the main study targets of two constituent papers of this thesis.

SIRT1

With the extraordinary boost of interest in investigating sirtuins during the past decade, the list of sirtuin targets were rapidly identified and extended and their cellular functions became better understood. Evidence suggests that SIRT1 plays a dual role in different types of cancer. Initially, most of the studies suggest that SIRT1 acts as a tumor promoter because of its

overexpression in breast cancer, colon cancer, leukaemia, prostate cancers, etc. compared to the corresponding normal tissues. Inhibition or down regulation of SIRT1 with siRNA impairs tumor cell proliferation [123]. The first identified substrate of SIRT1 was the tumour suppressor p53. SIRT1 deacetylates lysine 382 of p53 and therefore inhibits its activity, allowing tumor cells to bypass p53-mediated apoptosis, resulting in a higher risk of accumulating mutations and developing cancer [124]. Some *in vivo* studies carried out by Herranz et al. demonstrated that the crossing of SIRT1 transgenic mice to PTEN deficient mice leads to SIRT1 overexpressed offsprings that are prone to develop thyroid carcinoma and lung metastasis. These mice also show a much higher incidence of developing prostate cancer, which is in agreement with SIRT1 levels being higher in murine and human prostate carcinomas [125]. It has been reported that gain of SIRT1 expression by siRNA transfection decreases the sensitivity of some cancer cells to certain anti-cancer compounds. Similarly, loss of SIRT1 activity leads to opposite effects on apoptosis and chemosensitivity [126]. Moreover, SIRT1 inhibits the FOXO- and P53- related apoptosis or transcription through its deacetylase activity [127]. All together, these studies suggest that overexpression of SIRT1 plays a direct role in promoting tumourigenesis.

However, there are numerous contradictory findings about the role of SIRT1 in cancer. For instance, SIRT1 expression is significantly reduced in certain cancer types, including bladder carcinoma, breast cancer, glioblastoma, ovarian cancer and hepatic carcinoma, relative to their non-transformed counterparts. Reactivation of SIRT1 in these tumors leads to impaired cell proliferation [128]. BRCA1-mutated human breast cancer has a lower SIRT1 level compared to non-BRCA1-related breast cancer and normal breast tissue. Activation of SIRT1 by resveratrol in BRCA1 cancer cells leads to decreased cell viability [129]. Yuan et al. have found several c-MYC binding sites at SIRT1 promoter region. The over expression of c-MYC leads to upregulated SIRT1 expression[130]. However, SIRT1 negatively regulates c-MYC stability through deacetylating the lysine 323 of c-MYC, resulting in decreased c-MYC downstream gene expression, such as telomerase reverse transcriptase gene (TERT), and enhanced expression of tumor suppressors like p21 [130]. These findings indicate a tumor suppressor role of SIRT1.

SIRT2

Analogous with the dual role of SIRT1 in cancer, SIRT2 also shows both tumor promoting and suppressing activity in different cancer types. Danielle et al. have found that SIRT2 expression is greatly upregulated in melanomas compared to in normal melanocytes [131]. Hiratsuka et al. have reported that SIRT2 gene was down-regulated in human glioma, where a colony formation assay showed that the abnormal expression of SIRT2 in glioma cells leads to significant reduction of stable clones [132]. Mechanistical studies aiming to understand the functions of SIRT2 have been performed. Hyun-Seok Kim et al. knocked out SIRT2 gene in mice and observed higher level of mitotic regulators and mitotic cell death. As a consequence, the SIRT2 deficient mice showed a greater chance to develop tumours in various organs [133]. These results indicate a tumour suppressor role of SIRT2 in certain cancer

types. Nevertheless, several studies have provided convincing evidences that SIRT2 promotes oncogenic phenotypes. Dan et al. reported significant SIRT2 upregulation in primary acute myeloid leukaemia (AML) cells in comparison to normal bone marrow cells. Inhibition of SIRT2 results in impaired cell proliferation and increased apoptosis in both AML cell lines and primary cells [134]. In the same manner, upregulated SIRT2 in neuroblastoma and pancreatic cancer cells stabilises the Myc oncoprotein through repressing Myc ubiquitination and degradation resulting in promotion of cancer cell growth [135].

The debates about whether SIRT1 and SIRT2 are tumour promoters or suppressors have not been solved to date. All the data so far suggest that both SIRT1 and SIRT2 play a dual role in different cancers, which is not unprecedented. For instance, the expression of TERT significantly increases tumor cell adhesion and migration [136]. TERT induced telomere dysfunction decreases genomic stability; this indicates a tumourigenesis promoter function of TERT. However, it has also been shown that telomere dysfunction could be recognised by DNA damage machinery and hence activates tumour suppressor pathways [137]. The genomic hypomethylation by Dnmt1 (DNA methyltransferase 1) promotes tumor initiation by destabilising the genome and silencing tumor suppressor genes, yet the Dnmt1-induced DNA hypomethylation might also reduce the risk for cancer as shown in the study of the Dnmt1 transgenic mouse model [138]. Similar with those genes, the specific activity of SIRT1 and SIRT2 in tumour development may correspondent with certain cellular and molecular context, and specific tumour microenvironment. Further studies of the functions of sirtuins should take these factors into consideration. A better understanding of the activities and functional roles of sirtuins will be beneficial for the further investigations of sirtuins as therapeutic targets.

1.5 HDAC INHIBITORS

1.5.1 Class I, II and IV HDIs

There are many natural and synthetic compounds have been identified as class I, II and IV HDAC inhibitors (HDIs). They are categorized into different groups according to their different chemical nature, mechanism of action and heterogeneous biochemical structure. However, all of the Zn^{2+} dependent HDIs share three pharmacophoric groups. They contain an aromatic cap, which is also a surface recognition unit, a functional Zn^{2+} binding domain that can chelate the cation at the bottom of the HDAC catalytic pocket, and a hydrophobic linker that connects the top hat and the bottom part. Modifications on any of the groups will affect the potency and activity of the inhibitor (Figure 7).

For example, TSA (trichostatin A) is a representative HDI that belongs to the group of hydroxamic acid. It is one of the first HDAC inhibitors that has been described and well studied. The disadvantage of TSA is its unspecific HDAC targeting and strong toxicity. However, the structure of TSA provides lots of inspiration for developing synthetic drugs that are less toxic and more specific. An example of this is suberoylanilide hydroxamic

acid (SAHA) [139], also called vorinostat, the earliest HDI that has been approved by the U.S. Food and Drug Administration (FDA) for use in cutaneous T cell lymphoma treatment. Other chemicals belonging to this group like LBH589 (panobinostat), PXD101 (belinostat), SB939 (pracinostat) have also been evaluated in clinical trials for different types of cancer [140] [141] [142]. Hydroxamic acids are the most studied HDIs.

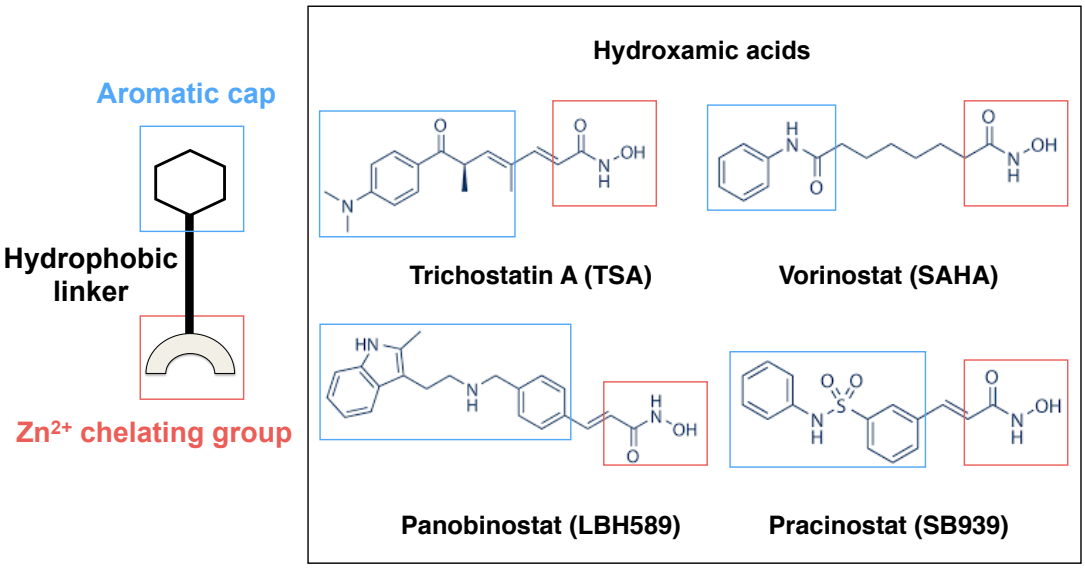

Figure 7. **Hydroxamic acids as HDAC inhibitors**

Short chain fatty acid is a novel class of HDIs that includes valproic, 4-phenylbutanoic and butanoic acid. Valproic acid is a very old drug that has been previously used to treat epilepsy. Studies have shown that valproic acid induces the differentiation and/or apoptosis of carcinoma cells and patient-derived myeloid leukaemic blasts *in vitro*. It also suppresses tumor proliferation and metastasis *in vivo* [143,144]. Similarly, 4-phenylbutyric acid is a drug that has been approved for the treatment of urea cycle disorders, but has recently been repositioned as an anti-cancer drug. The activity of 4-phenylbutyric acid has been studied in malignant glioma and acute myeloid leukaemia [145]. In a clinical trial, the response was observed in a patient with metastatic non-small cell lung cancer treated with butanoic acid [146].

1.5.2 Sirtuin inhibitors

Because of the complexity and dual roles that SIRT1 and SIRT2 play in cancer, small molecules that target sirtuins were considered to have potential therapeutic benefit for cancer patients. Sirtuin activators that have been developed were mainly targeting SIRT1. There are more than 3000 SIRT1-activating compounds that have been synthesised and studied since the last decade, including SRT1720, SRT2183, SRT2104 [147] [148], and some analogs of nicotinamide (NAM) [149] [150], most of them were used for the treatments of ageing and age-related diseases like diabetes, cardiovascular disease and certain cancer types [151]. However, in cancer field, studies are more focused on the development of sirtuin inhibitors.

Nicotinamide (NAM)

NAM is one of the first sirtuin inhibitors and the only physiological sirtuin inhibitor that has been found [152]. Benzamide is a sirtuin inhibitor that has a very similar structure with NAM [153] (Figure 8). Analogs of NAM and benzamide have been studied. The anti-cancer activities of these compounds have been observed in human leukaemia, colon cancer [154], prostate cancer [155], oral cancer [156], etc.

***Figure 8*. Chemical structure of 3 sirtuin inhibitors.**

Thioacyllysine-containing compounds

The first thioacyllysine-containing compound was synthesised by incorporating a N-thioacetyllysine into a peptide that is derived from the 372-389 amino acid residue region of human p53. The mechanism of the action of this molecule has been studied extensively. It can form a strong stable covalent intermediate with sirtuins and sirtuin-targets at early stages of the deacetylation, therefore the active site of the sirtuin is occupied and the deacetylation reaction can not be processed [157,158]. The earliest thioacyllysine-containing compounds are peptide-based. The potency and specificity of these peptides have been tested intensively and the IC50 of the most potent SIRT1 inhibitor was 0.2 μM [159]. However, the disadvantages of peptide-based inhibitors are that they are not so stable and have poor cellular permeability, which represents a problem for *in vitro* studies. Therefore, efforts were made to replace the peptides with pseudopeptides in thioacyllysine-containing compounds. Mellini and colleagues developed thioacetylated pseudopeptides with *in vitro* activity against SIRT1-3 in multiple cancer cells [160].

Indole derivatives

A group of indole derivatives represented by EX527 were discovered as potent sirtuin inhibitors [161]. Solomon J et al. have shown that EX527 inhibits the catalytic activity of SIRT1 and increased the acetylation of p53 in several types of cancer [162]. Other members of this group, such as AC-93253 and inauhzin, also show cytotoxic activities on multiple cancer types, like prostate cancer, pancreatic cancer and leukaemia [163,164].

Tenovin and its analogs

Tenovins, including tenovin-1 and tenovin-6, is a group of bioactive molecules discovered by their ability to re-activate the function of the tumor suppressor p53. Tenovin-6 inhibits SIRT1

mediated deacetylation of lysine 382 of P53 in cancer cells [165], and restores p53-mediated DNA repair or apoptotic function. The cytotoxic effect of tenovin-1 has been shown in melanoma, lymphoma and breast cancer cells *in vitro*. Tumours with wild type p53 show better response upon tenovin-1 treatment compared to the ones that carry mutant p53 [165]. Tenovin-6 is water soluble and more active in impairing tumor growth compared to its analogue tenovin-1[166]. The cytotoxic activity of tenovin-6 has been reported for many cancer types, like uveal melanoma, acute lymphoblastic leukaemia, SS, RMS, gastric cancer and cutaneous T-cell lymphoma [103,167 168 169,170].

Other sirutin inhibitors

AGK2

AGK2 doesn't belong to any of the groups mentioned above. It was discovered as a potent SIRT2 inhibitor that shows protective activity against Parkinson's disease both *in vitro* and *in vivo* [171]. AGK2 induces apoptosis and necrosis in C6 glioma cells *in vitro* [172] and it is the most selective SIRT2 inhibitor to this date [173].

Sirtinol and its analogues

The SIRT2 inhibitor Sirtinol was identified from a cell-based high throughput drug screening. Sirtinol and its analogues inhibit cancer cell proliferation and induce p53-mediated apoptosis [174]. Salermide and cambinol are SIRT1 and SIRT2 inhibitors that have been developed based on the structure of sirtinol. The anti-proliferative activities of these 2 groups of molecules have been reported in cancer cells [175].

Despite the discovery of a big amount of pharmacological sirtuin modulators, they are in general not specific and potent enough due to the complexity of the sirtuin activities. Moreover, most of them only target on SIRT1 and SIRT2. Studies that focus on other mammalian sirtuins are very limited. Further research is therefore crucial to improve the understanding of individual sirtuin functions and the development of more specific sirtuin inhibitiors.

1.6 TREATMENT STRATEGIES FOR SS

SS is a rare and aggressive tumor. There is no consensus among different oncological centres for what is the best standard treatment for SS. The primary treatment normally involves complete surgical resection of the tumor and adjuvant radiotherapy before or after surgery. However, the recurrence is very common and more than 50% of the patients develop metastases. The 5-year overall survival rate for the patients with local recurrence and metastatic disease is 76% and 10%, respectively [176]. 74-81% of metastatic diseases occur in the lungs and approximately 20% in lymph nodes and bones [177]. Similar to the primary SS, surgical resection is the main approach to treat local recurrence. For metastatic SS, the routine use of chemotherapy has limited benefit due to the therapeutic toxicity and eventual progression of the disease [178].

Lots of efforts have been made for the purpose of developing new therapeutic options to improve SS outcomes. For example, immunotherapy againsts the cancer-testis antigens NY-ESO-1 and SSX, two antigens expressed in sarcomas and other tumors but not in normal cells, have been considered as potential therapeutic options [179] [24]. FZD10 (frizzled homologue 10), a cell surface receptor, is present in SS cells but not in normal tissue, and is a promising therapeutic target. Antibodies that recognise this protein have been tested in SS xenografts and showed encouraging results [180].

The expression of the fusion protein SS18-SSX is unique in SS, the following composition of the oncoprotein complex leads to aberrant transcriptional activity and dysregulated gene expression which are necessary to initiate the tumourigenesis and sustain tumor cell survival. Direct targeting of the SS18-SSX complex is therefore an attractive approach for the discovery and development of molecules for SS treatment. Compounds that are able to dissociate the SS18-SSX complex or target constituent proteins of the complex might be beneficial for SS patients.

2 AIMS OF THE THESIS

The general aim of the thesis was to look for a novel therapeutic target for synovial sarcoma. The work mainly focuses on investigating the biochemical activity between the constituent proteins in chimeric SS18-SSX complex and potential compounds that can disrupt the chimeric fusion protein.

The specific aims of these studies were:

Paper I: To investigate the role of sirtuins in the proliferation and survival of two paediatric soft tissue sarcomas: rhabdomyosarcoma and synovial sarcoma.

Paper II: To set up a high-throughput screening assay to screen for small molecules that disrupt the association between SS18-SSX and TLE1 in synovial sarcoma by using proximity ligation assay (PLA).

Paper III: To investigate the clinical relevance of the over-expressed SIRT1 and SIRT2 proteins in a cohort of 30 primary synovial sarcomas and obtain insight into the epigenetic mechanisms associated with the action of sirtuin inhibitors in SS.

3 RESULTS AND DISCUSSION

PAPER I

SIRT1 and SIRT2 inhibition impairs paediatric soft tissue sarcoma growth

Sirtuins are a class of conserved proteins that shows deacetylases or ADP-ribosyl transferases activities on histone and non-histone substrates. They are involved in numerous cellular processes including cellular metabolism, cell cycle, DNA integrity maintenance and tumourigenesis. SIRT1 and SIRT2, the most studied mammalian sirtuins, have been associated with different cancer processes, however, their role in cancer development is still controversial.

In paper I we investigated the possible oncogenic roles of SIRT1 and SIRT2 in two paediatric sarcoma models: alveolar rhabdomyosarcoma and synovial sarcoma.

We showed that SIRT1 was significantly upregulated in both SS biopsies and cell lines compared to normal mesenchymal cells. To investigate possible roles of SIRT1 and SIRT2 on the proliferation of RMS and SS, we inhibited the activity of SIRT1 and SIRT2 with a sirtuin inhibitor, tenovin-6. We observed that tenovin-6 showed a fast and significant effect in impairing the sarcoma cells' proliferative capacity but not in primary mesenchymal stem cells. By measuring the level of caspase 3/7, we found that the sarcoma cells appeared to undergo apoptosis only at high concentrations of tenovin-6.

Tenovin-6 was discovered as a p53 activator. It reactivates p53 through inhibiting the deacetylation activities of sirtuins, which target the lysine 382 residue of p53. To investigate whether the activity of tenovin-6 in sarcoma cell lines was associated with p53 reactivation, we analysed the expression levels of acetylated $^{k\text{-}382}$-p53 and p21. We found that in tenovin-6 exposed cells, the expression of p21, a downstream target of p53, was upregulated with no changes in the overall expression of SIRT1, SIRT2 or p53. This upregulated expression of p21 is independent of the mutation status of the p53 gene. Interestingly, the enzymatic activity of sirtuins was however decreased in all of the cell lines investigated. This result indicates that the inhibition of the de-acetylating function of SIRT1/SIRT2 with tenovin-6 is p53 independent.

The cytotoxic activity of tenovin-6 has been associated with dysregulated autophagy in melanoma cell lines. With this in mind, we assessed the autophagic flux in several sarcoma cell lines in the presence of tenovin-6 treatment. We found that tenovin-6 induced accumulation of LC3II and p62 in SS and RMS cell lines. This pointed to a connection of SIRT1 and autophagic flux and was subsequently confirmed by the accumulated autophagosomes in the treated cell lines using a tandem probe RFP-GFP-LC3, which can visualise the autophagosome and autolyosome with different colours in transfected cells. Our

investigations confirmed that the inhibition of sirtuins by tenovin-6 in sarcoma cells blocks the autophagy process and promotes cell death.

Sirtuins are involved in the regulation of the cellular metabolism. Increased NAD^+ levels and sirtuin activities were observed under the condition of calorie restriction. In order to enquire into the activity of tenovin-6 in starving conditions, we cultured a SS cell line, SYO-1, and a RMS cell line, RD, in a nutrient-deprived medium which did not contain any glucose and essential amino-acids. We treated the cells with 2μM tenovin-6 and found that the anti-tumour activity of tenovin-6 was increased in the nutrient-deprived condition in sarcoma cell lines. This result indicates that the activity of SIRT1 and SIRT2 is crucial for SS and RMS in the condition of nutrient deprivation, a condition observed in fast-growing tumors such as sarcomas.

The pivotal role of SIRT1 and SIRT2 in tumor cell survival was confirmed using siRNA assays in the RMS cell line RD. We found that the proliferation of the tumour cells was impaired due to the siRNA silencing of SIRT1 and SIRT2. The cytotoxic effect was observed 48 hours after transfection and increased gradually through out the 120 hrs of the assay during which the nutrient supply becomes deficient in the culture medium. Our result shows that the expression of SIRT1 and SIRT2 is crucial for the survival of SS and RMS cells.

We further evaluated the activity of tenovin-6 *in vivo*. SCID mice were xenografted with the RMS cell line RD and treated with tenovin-6 at the time tumour xenografts were palpable. After 10 days of treatment with tenovin-6, we found decreased tumour volumes in tenovin-6 treated mice compared to the placebo group. However at later times, tumour growth was evident in both tenovin-6 and placebo treated groups. This study shows that tenovin-6 has a weak anti-tumor effect *in vivo*, and suggests that drug combinations may enhance tenovin-6 activity.

PAPER II

Identification of cytotoxic agents disrupting synovial sarcoma oncoprotein interactions by proximity ligation assay

Synovial sarcoma is one of the most studied translocation-associated sarcomas. In this tumor, the fusion protein SS18-SSX forms a functional complex with proteins that are involved in epigenetic regulation and chromatin remodelling to reprogramme gene transcription and drive malignant transformation in synovial sarcomas. The known component proteins in this complex include ATF2, TLE1, HDACs and polycomb group (PcG) members.

Today there is no effective systemic treatment for patients with synovial sarcoma. We hypothesise that dissociating the SS18-SSX protein complex will specifically induce synovial sarcoma cell death. Therefore, small molecules that are capable to disrupt the protein-protein interactions within the oncoprotein complex may have selective anti-tumor activity in patients with synovial sarcoma.

In order to develop a high-throughput drug screening assay to search for molecules that disrupt the SS18-SSX driver complex, we have set up a proximity ligation assay (PLA) to visualise the interaction of SS18-SSX with its partner TLE1 in synovial sarcoma cell lines.

Using an antibody that recognise both wild type SS18 and the fusion protein SS18-SSX and a TLE1 specific antibody, we could detect specific SS18/TLE1 PLA signals in SS cell lines. On the contrary, the SS18-TLE1 proximity ligation signal was very weak or negative in the cell lines of other cancer types. This result was confirmed by co-immunoprecipitation, which further demonstrated that the PLA signals we detected in synovial sarcoma cell lines are due to the co-localisation of SS18-SSX with TLE1.

To confirm the specificity of the PLA assay, we knocked down SS18-SSX with siRNA molecules and shRNA vectors that specifically target the SSX domain in the fusion transcript. PLA signals were clearly decreased in SS18-SSX silenced cells. We therefore concluded that the interaction of SS18 with TLE1 is specific for synovial sarcomas and involves only the N-terminal SS18 part of the fusion protein, but not the wild type SS18.

We also showed that the proximity ligation assay can detect the SS18-SSX and TLE1 interactions in formalin-fixed paraffin embedded synovial sarcoma tumor samples.

We next investigated whether the PLA could be used to screen for molecules that disrupt the SS18-SSX complex in synovial sarcoma. It has been previously shown that synovial sarcomas are very sensitive to class I HDAC inhibitors due to the dissociation of the SS18-SSX/TLE1 complex. To investigate whether the disruption of the SS18-SSX/TLE1 complex is detectable using PLA, we treated the synovial sarcoma cell line SYO-1 with different HDAC inhibitors. The proximity ligation signals of the SS18-SSX/TLE1 complex were quantified and compared shortly after treatment. As expected, the class I HDAC inhibitor romidepsin (FK228) efficiently dissociated SS18-SSX molecule from TLE1 resulting in increased apoptosis, while other types of HDAC inhibitor didn't. These results were further

confirmed by co-immunoprecipitation. Our results indicate that only class I HDAC inhibitors can disrupt the SS18-SSX/TLE1 fusion complex in synovial sarcomas.

Having proved the utility of the PLA as a phenotypical assay to screen for molecules that disrupt the SS18-SSX TLE1 interaction, we then screened a library containing 16,000 compounds (Maybridge) against the synovial sarcoma cell line SYO-1 and used PLA to find cytotoxic compounds that are capable to dissociate the SS18-SSX/TLE protein complex. One of the compounds, designated SXT1596, showed a particular anti-proliferative activity associated with complete loss of SS18-SSX/TLE proximity ligation signals and onset of apoptosis in the synovial sarcoma cell line SYO-1.

The SS18-SSX/TLE1/ATF2 complex directly binds to the EGR1 promoter and represses its transcription. The compound SXT1596 reactivates EGR1 transcription in SS cells, indicating that disruption of the SS18-SSX complex releases the repression of EGR1 and restores normal signaling in synovial sarcoma.

This study proves that the proximity ligation assay is a reliable and valuable method to screen for compounds that disrupt the fusion oncoprotein associations in SS. It also shows the possibility of using the proximity ligation assay to identify compounds with therapeutic potential in other cancer types that are driven by fusion transcription factors.

PAPER III

Studies on the clinical relevance and mechanism of activity of sirtuin inhibitors in synovial sarcoma

Sirtuins are class III HDACs that play important roles in a wide range of cellular processes like transcriptional gene silencing, DNA repair, cell cycle arrest, apoptosis and ageing. The most studied mammalian sirtuins, SIRT1 and SIRT2, were shown to have both tumour promoter and suppressor roles in cancer. Small molecules that target SIRT1 and SIRT2 were therefore considered to be beneficial for cancer patients, especially SIRT1 and SIRT2 inhibitors. However, the mechanisms underlining most of the sirtuin inhibitors are still poorly understood.

Dysregulated expression of both SIRT1 and SIRT2 were reported in different cancer types. In order to assess the expression of SIRT1 and SIRT2 in synovial sarcoma, we performed mRNA quantification of the both targets in a cohort consisting of 30 primary synovial sarcomas. We found a significant 10-fold increment of SIRT1 mRNA levels in 57% of the samples compared to the SIRT1 mRNA levels in normal cells.

In order to further explore whether this overexpression is associated with clinical outcomes of the patients, we performed wilcoxon nonparametric tests based on our sample size. The clinical and molecular characteristics analysed include the patient's gender, age, SSX type, tumour size and metastatic site(s). No significant correlation was found between these parameters and SIRT1/SIRT2 overexpression. This result may be explained by the small sample size and low statistical power of the experiment. Because synovial sarcoma is a very rare disease, the chances of obtaining patient material are very limited. The amount of our primary synovial sarcoma samples was quite small. For further and more accurate statistical analyses, a bigger collection of the tumour samples should be evaluated.

Small molecules that are able to inhibit the activities of SIRT1 and SIRT2 have been developed and investigated as novel therapeutics for cancer. In this study, we evaluated tenovin-6 (an inhibitor of both SIRT1 and SIRT2) and AGK2 (a potent SIRT2 inhibitor) in different SS cell lines and a patient-derived primary SS culture 83SS that was established and characterized in our lab. We found that AGK2 inhibited the viability of SS cells at an IC50 concentration of 5-6μM. Tenovin-6 on the other hand was more toxic than AGK2, it impaired the viability of SS cells at an IC50 concentration of 2.5μM. The cytotoxicity of both compounds was also demonstrated in real time proliferation curves of the SS cell lines SYO-1 and 83SS.

The fusion oncoprotein SS18-SSX recruits additional interacting partners that are involved in gene transcription and epigenetic regulation to form a functional complex. The SS18-SSX complex predominantly regulates gene transcription and tumour cell survival in synovial sarcomas. In our previous paper, we have demonstrated that synovial sarcoma was sensitive to class I HDAC inhibitors due to the disruption of the SS18-SSX/TLE1 fusion complex. However, there are still no studies that fully explain how the synovial sarcoma fusion

complex works and what the constituent proteins are. Based on the dysregulated expression and necessity of SIRT1 and SIRT2 in the survival of synovial sarcoma cells, we assumed that these two proteins are subunits of the SS18-SSX complex since the dissociations of SS18-SSX/SIRT1 and SS18-SSX/SIRT2 by small molecular inhibitors are lethal for synovial sarcoma cells.

In order to prove this, we performed co-immunoprecipitation in total cell extracts of synovial sarcomas. We found that the fusion protein SS18-SSX was pulled down with SIRT1 in synovial sarcoma cell line SYO-1 and the primary synovial sarcoma culture 83SS. Moreover, we found that H4K16ac and H3K9me3 were able to precipitate with SIRT1 as well. These results indicate that SIRT1, together with its histone targets H4K16ac and H3K9me3, share interactions with the fusion protein SS18-SSX in synovial sarcoma cells. Interestingly, a dissociation of the SS18-SSX/SIRT1 induced by 2μM tenovin-6 could already be observed after 2 hours of treatment, as well as the association between SIRT1 and H4K16ac/H3K9me3. However, H3K9me3 and H4K16ac gradually re-associated with SIRT1 in the absence of the fusion protein. These results were further confirmed by PLA.

To determine the mechanism of action of compound AGK2, we performed similar experiments. Protein-protein interactions of SS18-SSX/SIRT2, SIRT2/H3K9me3 and SIRT2/H4K16ac were observed in SYO-1. A concentration of 5μM AGK2 showed the activity of dissociation of these interactions in a time dependent manner. The effect of AGK2 peaked at 8 hours of treatment and the interactions were completely disrupted at this time point.

The results of this study are still preliminary, but indicate a possible role of SIRT1 and SIRT2 in synovial sarcoma tumourigenesis that is driven by the SS18-SSX complex induced transcriptional dysregulation and epigenetic modulation.

4 CONCLUSIONS

Synovial sarcoma is a rare disease driven by the aberrantly expressed fusion oncoprotein SS18-SSX and the related fusion oncoprotein complex. In the first paper, the expression, activity and necessity of the NAD^+ dependent class III HDACs, sirtuins, were examined with the focus on SIRT1 and SIRT2 in SS and RMS. We concluded from the study that:

- SIRT1 and SIRT2 expression and activity are crucial for the survival of sarcoma cells.
- The pharmacological inhibition of SIRT1 and SIRT2 by tenovin-6 impairs tumor cell proliferation, induces apoptosis and impairs the autophagy flux in sarcoma cells.

The fusion protein SS18-SSX is unique and necessary for the survival of synovial sarcoma. It recruits interacting proteins and forms an endogenous fusion protein complex in SS cells that regulate gene transcriptions. Therefore, the fusion oncoprotein complex is a potential therapeutic target in synovial sarcoma. Small molecular compounds that are capable to disrupt the fusion protein complex may have selective anti-tumor activity. TLE1 is a known core subunit of the SS18-SSX fusion protein complex. In the second paper, we applied a proximity ligation assay to screen for novel compounds that can disrupt the interaction of SS18-SSX with TLE1. The following findings were demonstrated:

- The interaction of SS18-SSX with TLE1 is specific for synovial sarcomas.
- SS18-SSX/TLE1 interactions are sensitive to class I HDAC inhibitors and a novel compound designated SXT1596.
- SXT1596 re-establishes normal cell signaling in synovial sarcoma.
- PLA can be utilised in drug screenings to assess the capability of drugs to disrupt the relevant functional protein-protein interaction.

In the third paper, we further evaluated the activity of sirtuin inhibitor tenovin-6 and AGK2 in SSs and explored the mechanism of action of these 2 compounds. We concluded from the study that:

- SIRT1 and SIRT2 are involved in synovial sarcoma tumourigenesis that is driven by the SS18-SSX fusion protein complex.
- Tenovin-6 and AGK2 dissociate the interactions between SIRT1/SIRT2 and the fusion protein SS18-SSX, the dissociation activities of the 2 compounds are time dependent.

5 FUTURE PERSPECTIVES

The translocation t(X;18) was first described and linked with synovial sarcoma in 1986. Since then a lot of effort have been made to investigate the role of this translocation in synovial sarcoma. It has been elucidated nowadays that the fusion product SS18-SSX is the driving event for synovial sarcoma tumourigenesis. It functions predominantly in transcriptional regulation due to protein-protein interactions and associations with important chromatin remodelling complexes, resulting in aberrant gene expressions and oncogenic properties. Considering the limited benefit of the current therapies for the patients with synovial sarcoma, targeting the fusion oncoprotein SS18-SSX and the related oncoprotein complex shows great therapeutic potential. Therefore, continued studies that identify component proteins and target genes of the SS18-SSX complex are essential for the development of novel therapeutics for synovial sarcoma.

Apart from PLA and co-immunoprecipitation, there are several methods that assess protein-protein interactions, such as pull-down assay, label transfer, bimolecular fluorescence complementation (BiFC) and fluorescence resonance energy transfer (FRET). Each of the approaches has its own strengths and weaknesses. In the short-term perspective of our project, we expect that combination of different methods can be used to confirm the interactions of SIRT1/SIRT2 and SS18-SSX in synovial sarcoma. Mass spectrophotometry can be used to elucidate the components of SS18-SSX complex. Furthermore, chromatin immunoprecipitation and DNA sequencing (Chip-seq assay) could be performed to identify the genes directly targeted by the SS18-SSX transcriptional complex. The identified proteins and genes can be evaluated in an in vitro transfection model using CRISPR/Cas 9 system. Protein-protein interaction based drug screening should elucidate more compounds that target specific SS18-SSX complex components. The potential of the new agents can be further tested in an in vivo model.

In the long-term perspective, we believe that by identifying the aberrantly recruited proteins to the SS18-SSX fusion oncoprotein, our understanding of the synovial sarcoma will continue to increase. Combining the information gained from this thesis study with the knowledge to be gained from future studies as mentioned above, assisted with the technical advances in detecting and visualising protein-protein interactions and our ability to design new therapeutic strategies for synovial sarcoma patients will likely lay down a fruitful foundation for finding effective novel therapeutic agents.

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7 REFERENCES

1 Alberts, B. *et al. Molecular Biology of the Cell, 4th edition*. (Garland Science, 2002).

2 Weinberg, R. A. *The Biology of Cancer, 2nd Edition*. (Garland Science, 2013).

3 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).

4 Vander Griend, R. Osteosarcoma and its variants. *Orthop Clin North Am* **27**, 575-581 (1996).

5 Anderson, J. L., Denny, C. T., Tap, W. D. & Federman, N. Pediatric sarcomas: translating molecular pathogenesis of disease to novel therapeutic possibilities. *Pediatr Res* **72**, 112-121, doi:10.1038/pr.2012.54 (2012).

6 Shern, J. F. *et al.* Comprehensive genomic analysis of rhabdomyosarcoma reveals a landscape of alterations affecting a common genetic axis in fusion-positive and fusion-negative tumors. *Cancer Discov* **4**, 216-231, doi:10.1158/2159-8290.CD-13-0639 (2014).

7 Sumegi, J. *et al.* Recurrent t(2;2) and t(2;8) translocations in rhabdomyosarcoma without the canonical PAX-FOXO1 fuse PAX3 to members of the nuclear receptor transcriptional coactivator family. *Genes Chromosomes Cancer* **49**, 224-236, doi:10.1002/gcc.20731 (2010).

8 Liu, J. *et al.* FOXO1-FGFR1 fusion and amplification in a solid variant of alveolar rhabdomyosarcoma. *Mod Pathol* **24**, 1327-1335, doi:10.1038/modpathol.2011.98 (2011).

9 Ladanyi, M. *et al.* The der(17)t(X;17)(p11;q25) of human alveolar soft part sarcoma fuses the TFE3 transcription factor gene to ASPL, a novel gene at 17q25. *Oncogene* **20**, 48-57, doi:10.1038/sj.onc.1204074 (2001).

10 Mrozek, K., Karakousis, C. P., Perez-Mesa, C. & Bloomfield, C. D. Translocation t(12;22)(q13;q12.2-12.3) in a clear cell sarcoma of tendons and aponeuroses. *Genes Chromosomes Cancer* **6**, 249-252 (1993).

11 Rekhi, B., Basak, R., Desai, S. B. & Jambhekar, N. A. A t (11; 22) (p13; q12) EWS-WT 1 positive desmoplastic small round cell tumor of the maxilla: an unusual case indicating the role of molecular diagnosis in round cell sarcomas. *J Postgrad Med* **56**, 201-205, doi:10.4103/0022-3859.68628 (2010).

12 Shibasaki, M. *et al.* Mandibular Ewing sarcoma with chromosomal translocation t(21;22)(q22;q12). *J Craniofac Surg* **24**, 1469-1472, doi:10.1097/SCS.0b013e31829030ed (2013).

13 J. R. Downing *et al.* Detection of the (11;22)(q24;q12) translocation of Ewing's sarcoma and peripheral neuroectodermal tumor by reverse transcription polymerase chain reaction. *AM J PATHOL* **143**, 1294-1300 (1993).

14 Sorensen, P. H. *et al.* A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. *Nat Genet* **6**, 146-151, doi:10.1038/ng0294-146 (1994).

15 Jeon, I. S. *et al.* A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1. *Oncogene* **10**, 1229-1234 (1995).

16 Rougemont, A. L. *et al.* A t(17;22)(q21;q12) with partial ETV4 deletion in a soft tissue Ewing sarcoma. *Cancer Genet* **205**, 55-60, doi:10.1016/j.cancergen.2012.01.007 (2012).

17 Wang, L. *et al.* Undifferentiated Small Round Cell Sarcomas with Rare EWS Gene Fusions. *J Mol Diagn* **9**, 498-509 (2007).

18 Ng, T. L. *et al.* Ewing sarcoma with novel translocation t(2;16) producing an in-frame fusion of FUS and FEV. *J Mol Diagn* **9**, 459-463, doi:10.2353/jmoldx.2007.070009 (2007).
19 Mackintosh, C., Madoz-Gurpide, J., Ordonez, J. L., Osuna, D. & Herrero-Martin, D. The molecular pathogenesis of Ewing's sarcoma. *Cancer Biol Ther* **9**, 655-667 (2010).
20 Choi, E. Y. *et al.* Undifferentiated small round cell sarcoma with t(4;19)(q35;q13.1) CIC-DUX4 fusion: a novel highly aggressive soft tissue tumor with distinctive histopathology. *Am J Surg Pathol* **37**, 1379-1386, doi:10.1097/PAS.0b013e318297a57d (2013).
21 Armstrong, F. *et al.* Differential effects of X-ALK fusion proteins on proliferation, transformation, and invasion properties of NIH3T3 cells. *Oncogene* **23**, 6071-6082, doi:10.1038/sj.onc.1207813 (2004).
22 Lawrence, B. *et al.* TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors. *Am J Pathol* **157**, 377-384, doi:10.1016/S0002-9440(10)64550-6 (2000).
23 Thaete, C. *et al.* Functional domains of the SYT and SYT-SSX synovial sarcoma translocation proteins and co-localization with the SNF protein BRM in the nucleus. *Hum Mol Genet* **8**, 585-591 (1999).
24 Jungbluth, A. A. *et al.* Monophasic and biphasic synovial sarcomas abundantly express cancer/testis antigen NY-ESO-1 but not MAGE-A1 or CT7. *Int J Cancer* **94**, 252-256, doi:10.1002/ijc.1451 (2001).
25 Mertens, F. *et al.* Clinicopathologic and molecular genetic characterization of low-grade fibromyxoid sarcoma, and cloning of a novel FUS/CREB3L1 fusion gene. *Lab Invest* **85**, 408-415, doi:10.1038/labinvest.3700230 (2005).
26 Knight, J. C., Renwick, P. J., Dal Cin, P., Van den Berghe, H. & Fletcher, C. D. Translocation t(12;16)(q13;p11) in myxoid liposarcoma and round cell liposarcoma: molecular and cytogenetic analysis. *Cancer Res* **55**, 24-27 (1995).
27 Clark, J. *et al.* Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. *Nat Genet* **7**, 502-508, doi:10.1038/ng0894-502 (1994).
28 Brodin, B. *et al.* Cloning and characterization of spliced fusion transcript variants of synovial sarcoma: SYT/SSX4, SYT/SSX4v, and SYT/SSX2v. Possible regulatory role of the fusion gene product in wild type SYT expression. *Gene* **268**, 173-182 (2001).
29 Antonescu, C. R. The role of genetic testing in soft tissue sarcoma. *Histopathology* **48**, 13-21, doi:10.1111/j.1365-2559.2005.02285.x (2006).
30 Dagher, R. & Helman, L. Rhabdomyosarcoma: an overview. *Oncologist* **4**, 34-44 (1999).
31 Koscielniak, E. *et al.* Metastatic rhabdomyosarcoma and histologically similar tumors in childhood: a retrospective European multi-center analysis. *Med Pediatr Oncol* **20**, 209-214 (1992).
32 Diller, L., Sexsmith, E., Gottlieb, A., Li, F. P. & Malkin, D. Germline p53 mutations are frequently detected in young children with rhabdomyosarcoma. *J Clin Invest* **95**, 1606-1611, doi:10.1172/JCI117834 (1995).
33 Matsumoto, T. *et al.* Molecular analysis of a patient with Beckwith-Wiedemann syndrome, rhabdomyosarcoma and renal cell carcinoma. *Jpn J Hum Genet* **39**, 225-234, doi:10.1007/BF01876842 (1994).
34 Fredericks, W. J. *et al.* The PAX3-FKHR fusion protein created by the t(2;13) translocation in alveolar rhabdomyosarcomas is a more potent transcriptional activator than PAX3. *Mol Cell Biol* **15**, 1522-1535 (1995).

35 Williamson, D. *et al.* Fusion gene-negative alveolar rhabdomyosarcoma is clinically and molecularly indistinguishable from embryonal rhabdomyosarcoma. *J Clin Oncol* **28**, 2151-2158, doi:10.1200/JCO.2009.26.3814 (2010).
36 Malempati, S. & Hawkins, D. S. Rhabdomyosarcoma: review of the Children's Oncology Group (COG) Soft-Tissue Sarcoma Committee experience and rationale for current COG studies. *Pediatr Blood Cancer* **59**, 5-10, doi:10.1002/pbc.24118 (2012).
37 Sorensen, P. H. *et al.* PAX3-FKHR and PAX7-FKHR gene fusions are prognostic indicators in alveolar rhabdomyosarcoma: a report from the children's oncology group. *J Clin Oncol* **20**, 2672-2679 (2002).
38 Davis, R. J., D'Cruz, C. M., Lovell, M. A., Biegel, J. A. & Barr, F. G. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. *Cancer Res* **54**, 2869-2872 (1994).
39 McKinney, C. D., Mills, S. E. & Fechner, R. E. Intraarticular synovial sarcoma. *Am J Surg Pathol* **16**, 1017-1020 (1992).
40 Fisher, C. Synovial sarcoma. *Ann Diagn Pathol* **2**, 401-421 (1998).
41 Smith, M. E. F., Fisher, C., Wilkinson, L. S. & Edwards, J. C. W. Synovial sarcomas lack synovial differentiation. *Histopathology* **26**, 279-281, doi:10.1111/j.1365-2559.1995.tb01444.x (1995).
42 Haldar, M., Hancock, J. D., Coffin, C. M., Lessnick, S. L. & Capecchi, M. R. A conditional mouse model of synovial sarcoma: insights into a myogenic origin. *Cancer Cell* **11**, 375-388, doi:10.1016/j.ccr.2007.01.016 (2007).
43 Thway, K. & Fisher, C. Synovial sarcoma: defining features and diagnostic evolution. *Ann Diagn Pathol* **18**, 369-380, doi:10.1016/j.anndiagpath.2014.09.002 (2014).
44 Kerouanton, A. *et al.* Synovial sarcoma in children and adolescents. *J Pediatr Hematol Oncol* **36**, 257-262, doi:10.1097/MPH.0000000000000154 (2014).
45 Kawai, A. *et al.* SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. *N Engl J Med* **338**, 153-160, doi:10.1056/NEJM199801153380303 (1998).
46 Ladanyi, M. Fusions of the SYT and SSX genes in synovial sarcoma. *ONCOG* **20**, 5755-5762 (20010).
47 Crew, A. J. *et al.* Fusion of SYT to two genes, SSX1 and SSX2, encoding proteins with homology to the Kruppel-associated box in human synovial sarcoma. *EMBO J* **14**, 2333-2340 (1995).
48 Carmody Soni, E. E., Schlottman, S., Erkizan, H. V., Uren, A. & Toretsky, J. A. Loss of SS18-SSX1 inhibits viability and induces apoptosis in synovial sarcoma. *Clin Orthop Relat Res* **472**, 874-882, doi:10.1007/s11999-013-3065-9 (2014).
49 ten Heuvel, S. E., Hoekstra, H. J., Bastiaannet, E. & Suurmeijer, A. J. The classic prognostic factors tumor stage, tumor size, and tumor grade are the strongest predictors of outcome in synovial sarcoma: no role for SSX fusion type or ezrin expression. *Appl Immunohistochem Mol Morphol* **17**, 189-195, doi:10.1097/PAI.0b013e31818a6f5c (2009).
50 dos Santos, N. R., de Bruijn, D. R., Kater-Baats, E., Otte, A. P. & van Kessel, A. G. Delineation of the protein domains responsible for SYT, SSX, and SYT-SSX nuclear localization. *Exp Cell Res* **256**, 192-202, doi:10.1006/excr.2000.4813 (2000).
51 Lange, S. E. S., Kremer, J. C., Schenone, A. D., Schultze, M. B. & Van Tine, B. A. Current Management and Molecular Targets of Synovial Sarcoma. *Journal of Cancer and Clinical Research* (2014).

52 Ito, T. *et al.* SYT, a partner of SYT-SSX oncoprotein in synovial sarcomas, interacts with mSin3A, a component of histone deacetylase complex. *Lab Invest* **84**, 1484-1490, doi:10.1038/labinvest.3700174 (2004).
53 de Bruijn, D. R. H. *et al.* The synovial sarcoma associated protein SYT interacts with the acute leukemia associated protein AF10. *Oncogene* **20**, 3281-3289 (2001).
54 Perani, M. *et al.* The proto-oncoprotein SYT interacts with SYT-interacting protein/co-activator activator (SIP/CoAA), a human nuclear receptor co-activator with similarity to EWS and TLS/FUS family of proteins. *J Biol Chem* **280**, 42863-42876, doi:10.1074/jbc.M502963200 (2005).
55 Ren, Y., Li, X., Cao, Y., Chen, Y. & Cui, X. Absence of 19 known hotspot oncogenic mutations in soft tissue clear cell sarcoma: two cases report with review of the literature. *International journal of clinical and experimental pathology* **7**, 5242-5249 (2014).
56 Lim, J. *et al.* OCT2, SSX and SAGE1 reveal the phenotypic heterogeneity of spermatocytic seminoma reflecting distinct subpopulations of spermatogonia. *J Pathol* **224**, 473-483, doi:10.1002/path.2919 (2011).
57 Cronwright, G. *et al.* Cancer/testis antigen expression in human mesenchymal stem cells: down-regulation of SSX impairs cell migration and matrix metalloproteinase 2 expression. *Cancer Res* **65**, 2207-2215, doi:10.1158/0008-5472.CAN-04-1882 (2005).
58 Naka, N. *et al.* Quantification of SSX mRNA Expression in Human Bone and Soft Tissue Tumors Using Nucleic Acid Sequence-Based Amplification. *The Journal of Molecular Diagnostics* **7**, 187-197, doi:10.1016/s1525-1578(10)60545-4 (2005).
59 Chen, L. *et al.* Cancer/testis antigen SSX2 enhances invasiveness in MCF-7 cells by repressing ERalpha signaling. *Int J Oncol* **40**, 1986-1994, doi:10.3892/ijo.2012.1369 (2012).
60 Gure, A. O. *et al.* SSX: A multigene family with several members transcribed in normal testis and human cancer. *International Journal of Cancer* **72**, 965-971, doi:10.1002/(sici)1097-0215(19970917)72:6<965::aid-ijc8>3.0.co;2-n (1997).
61 Zollner, S. K., Rossig, C. & Toretsky, J. A. Synovial sarcoma is a gateway to the role of chromatin remodeling in cancer. *Cancer Metastasis Rev* **34**, 417-428, doi:10.1007/s10555-015-9575-z (2015).
62 de Bruijn, D. R. *et al.* The cancer-related protein SSX2 interacts with the human homologue of a Ras-like GTPase interactor, RAB3IP, and a novel nuclear protein, SSX2IP. *Genes Chromosomes Cancer* **34**, 285-298, doi:10.1002/gcc.10073 (2002).
63 de Bruijn, D. R., van Dijk, A. H., Willemse, M. P. & van Kessel, A. G. The C terminus of the synovial sarcoma-associated SSX proteins interacts with the LIM homeobox protein LHX4. *Oncogene* **27**, 653-662, doi:10.1038/sj.onc.1210688 (2008).
64 Su, L., Sampaio, A. V. & Jones, K. B. Deconstruction of the SS18-SSX Fusion Oncoprotein Complex: Insights into Disease Etiology and Therapeutics. *Cancer Cell* **21** 333-347 (2012).
65 Kawasaki, H. *et al.* ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature* **405**, 195-200, doi:10.1038/35012097 (2000).
66 Terry, J. *et al.* TLE1 as a diagnostic immunohistochemical marker for synovial sarcoma emerging from gene expression profiling studies. *Am J Surg Pathol* **31**, 240-246, doi:10.1097/01.pas.0000213330.71745.39 (2007).

67 Knosel, T. *et al.* TLE1 is a robust diagnostic biomarker for synovial sarcomas and correlates with t(X;18): analysis of 319 cases. *Eur J Cancer* **46**, 1170-1176, doi:10.1016/j.ejca.2010.01.032 (2010).
68 Kosemehmetoglu, K., Vrana, J. A. & Folpe, A. L. TLE1 Expression Is Not Specific for Synovial Sarcoma: A Standard Section Study of 155 Soft Tissue and Bone Neoplasms. *Laboratory Investigation* **89**, 16A-16A (2009).
69 Chuang, H. C. *et al.* Reappraisal of TLE-1 immunohistochemical staining and molecular detection of SS18-SSX fusion transcripts for synovial sarcoma. *Pathol Int* **63**, 573-580, doi:10.1111/pin.12113 (2013).
70 Lim, F. L., Soulez, M., Koczan, D., Thiesen, H. J. & Knight, J. C. A KRAB-related domain and a novel transcription repression domain in proteins encoded by SSX genes that are disrupted in human sarcomas. *Oncogene* **17**, 2013-2018, doi:10.1038/sj.onc.1202122 (1998).
71 dos Santos, N. R., de Bruijn, D. R. & van Kessel, A. G. Molecular mechanisms underlying human synovial sarcoma development. *Genes Chromosomes Cancer* **30**, 1-14, doi:10.1002/1098-2264(2000)9999:9999<::aid-gcc1056>3.0.co;2-g (2001).
72 Kadoch, C. *et al.* Proteomic and bioinformatic analysis of mammalian SWI/SNF complexes identifies extensive roles in human malignancy. *Nat Genet* **45**, 592-601, doi:10.1038/ng.2628 (2013).
73 Laporte, A. N., Ji, J. X., Ma, L., Nielsen, T. O. & Brodin, B. A. Identification of cytotoxic agents disrupting synovial sarcoma oncoprotein interactions by proximity ligation assay. *Oncotarget*, doi:10.18632/oncotarget.8882 (2016).
74 Lubieniecka, J. M. *et al.* Histone deacetylase inhibitors reverse SS18-SSX-mediated polycomb silencing of the tumor suppressor early growth response 1 in synovial sarcoma. *Cancer Res* **68**, 4303-4310, doi:10.1158/0008-5472.CAN-08-0092 (2008).
75 Cao, R. *et al.* Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**, 1039-1043, doi:10.1126/science.1076997 (2002).
76 Han, E. S. *et al.* The in vivo gene expression signature of oxidative stress. *Physiol Genomics* **34**, 112-126, doi:10.1152/physiolgenomics.00239.2007 (2008).
77 Peller, S. & Rotter, V. TP53 in hematological cancer: low incidence of mutations with significant clinical relevance. *Hum Mutat* **21**, 277-284, doi:10.1002/humu.10190 (2003).
78 Ahmed, A. A. *et al.* Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. *J Pathol* **221**, 49-56, doi:10.1002/path.2696 (2010).
79 Spillane, A. J., A'Hern, R., Judson, I. R., Fisher, C. & Thomas, J. M. Synovial sarcoma: a clinicopathologic, staging, and prognostic assessment. *J Clin Oncol* **18**, 3794-3803 (2000).
80 Xie, Y. *et al.* Gene expression profile by blocking the SYT-SSX fusion gene in synovial sarcoma cells. Identification of XRCC4 as a putative SYT-SSX target gene. *Oncogene* **22**, 7628-7631, doi:10.1038/sj.onc.1207153 (2003).
81 D'Arcy, P., Maruwge, W., Ryan, B. A. & Brodin, B. The oncoprotein SS18-SSX1 promotes p53 ubiquitination and degradation by enhancing HDM2 stability. *Mol Cancer Res* **6**, 127-138, doi:10.1158/1541-7786.MCR-07-0176 (2008).
82 D'Arcy, P., Ryan, B. A. & Brodin, B. Reactivation of p53 function in synovial sarcoma cells by inhibition of p53-HDM2 interaction. *Cancer Lett* **275**, 285-292, doi:10.1016/j.canlet.2008.10.030 (2009).

83 Saito, T. *et al.* Nuclear beta-catenin correlates with cyclin D1 expression in spindle and pleomorphic sarcomas but not in synovial sarcoma. *Hum Pathol* **37**, 689-697, doi:10.1016/j.humpath.2006.01.017 (2006).
84 Pretto, D. *et al.* The synovial sarcoma translocation protein SYT-SSX2 recruits beta-catenin to the nucleus and associates with it in an active complex. *Oncogene* **25**, 3661-3669, doi:10.1038/sj.onc.1209413 (2006).
85 Hasegawa, T., Yokoyama, R., Matsuno, Y., Shimoda, T. & Hirohashi, S. Prognostic significance of histologic grade and nuclear expression of beta-catenin in synovial sarcoma. *Hum Pathol* **32**, 257-263, doi:10.1053/hupa.2001.22764 (2001).
86 Saito, T. *et al.* APC mutations in synovial sarcoma. *J Pathol* **196**, 445-449, doi:10.1002/path.1066 (2002).
87 Barham, W. *et al.* Targeting the Wnt pathway in synovial sarcoma models. *Cancer Discov* **3**, 1286-1301, doi:10.1158/2159-8290.CD-13-0138 (2013).
88 McGraw-Hill. *McGraw-Hill Encyclopedia of Science & Technology*. 5000 (1997).
89 Biswas, M., Voltz, K., Smith, J. C. & Langowski, J. Role of histone tails in structural stability of the nucleosome. *PLoS Comput Biol* **7**, e1002279, doi:10.1371/journal.pcbi.1002279 (2011).
90 Dong, X. & Weng, Z. The correlation between histone modifications and gene expression. *Epigenomics* **5**, 113-116, doi:10.2217/epi.13.13 (2013).
91 Smith, C. L. & Peterson, C. L. ATP-dependent chromatin remodeling. *Curr Top Dev Biol* **65**, 115-148, doi:10.1016/S0070-2153(04)65004-6 (2005).
92 Strahl, B. D. & Allis, C. D. The language of covalent histone modifications. *Nature* **403**, 41-45, doi:10.1038/47412 (2000).
93 Thorne, A. W., Kmiciek, D., Mitchelson, K., Sautiere, P. & Crane-Robinson, C. Patterns of histone acetylation. *Eur J Biochem* **193**, 701-713 (1990).
94 Wapenaar, H. & Dekker, F. J. Histone acetyltransferases: challenges in targeting bi-substrate enzymes. *Clin Epigenetics* **8**, 59, doi:10.1186/s13148-016-0225-2 (2016).
95 Sadoul, K., Wang, J., Diagouraga, B. & Khochbin, S. The tale of protein lysine acetylation in the cytoplasm. *J Biomed Biotechnol* **2011**, 970382, doi:10.1155/2011/970382 (2011).
96 Dokmanovic, M., Clarke, C. & Marks, P. A. Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res* **5**, 981-989, doi:10.1158/1541-7786.MCR-07-0324 (2007).
97 Choi, J. H. *et al.* Expression profile of histone deacetylase 1 in gastric cancer tissues. *Jpn J Cancer Res* **92**, 1300-1304 (2001).
98 Halkidou, K. *et al.* Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. *Prostate* **59**, 177-189 (2004).
99 Wilson, A. J. *et al.* Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem* **281**, 13548–13558 (2006).
100 Zhang, Z. *et al.* Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast*. *Breast Cancer Res Treat* **94**, 11-16, doi:10.1007/s10549-005-6001-1 (2005).
101 Zhang, Z. *et al.* HDAC6 expression is correlated with better survival in breast cancer. *Clin Cancer Res* **10**, 6962-6968, doi:10.1158/1078-0432.CCR-04-0455 (2004).
102 Huang, B. H. *et al.* Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell Death Differ* **12**, 395-404, doi:10.1038/sj.cdd.4401567 (2005).

103 Ma, L. *et al.* SIRT1 and SIRT2 inhibition impairs pediatric soft tissue sarcoma growth. *Cell Death Dis* **5**, e1483, doi:10.1038/cddis.2014.385 (2014).
104 Yasui, W. *et al.* Histone acetylation and gastrointestinal carcinogenesis. *Ann N Y Acad Sci* **983**, 220-231 (2003).
105 Bheda, P., Jing, H., Wolberger, C. & Lin, H. The Substrate Specificity of Sirtuins. *Annu Rev Biochem* **85**, 405-429, doi:10.1146/annurev-biochem-060815-014537 (2016).
106 Kaeberlein, M., McVey, M. & Guarente, L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev* **13**, 2570-2580 (1999).
107 Tissenbaum, H. A. & Guarente, L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* **410**, 227-230, doi:10.1038/35065638 (2001).
108 Rogina, B. & Helfand, S. L. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A* **101**, 15998-16003, doi:10.1073/pnas.0404184101 (2004).
109 Frye, R. A. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* **273**, 793-798, doi:10.1006/bbrc.2000.3000 (2000).
110 Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C. & Horikawa, I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol Biol Cell* **16**, 4623-4635, doi:10.1091/mbc.E05-01-0033 (2005).
111 Hawse, W. F. & Wolberger, C. Structure-based mechanism of ADP-ribosylation by sirtuins. *J Biol Chem* **284**, 33654-33661, doi:10.1074/jbc.M109.024521 (2009).
112 Du, J., Jiang, H. & Lin, H. Investigating the ADP-ribosyltransferase activity of sirtuins with NAD analogues and 32P-NAD. *Biochemistry* **48**, 2878-2890, doi:10.1021/bi802093g (2009).
113 Bosch-Presegue, L. & Vaquero, A. Sirtuin-dependent epigenetic regulation in the maintenance of genome integrity. *FEBS J* **282**, 1745-1767, doi:10.1111/febs.13053 (2015).
114 Vaquero, A. The conserved role of sirtuins in chromatin regulation. *Int J Dev Biol* **53**, 303-322, doi:10.1387/ijdb.082675av (2009).
115 Cohen, H. Y. *et al.* Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* **305**, 390-392, doi:10.1126/science.1099196 (2004).
116 Michan, S. & Sinclair, D. Sirtuins in mammals: insights into their biological function. *Biochem J* **404**, 1-13, doi:10.1042/BJ20070140 (2007).
117 Martinez-Redondo, P. & Vaquero, A. The diversity of histone versus nonhistone sirtuin substrates. *Genes Cancer* **4**, 148-163, doi:10.1177/1947601913483767 (2013).
118 Blander, G. *et al.* SIRT1 shows no substrate specificity in vitro. *J Biol Chem* **280**, 9780-9785 (2005).
119 Khan, A. N. & Lewis, P. N. Unstructured conformations are a substrate requirement for the Sir2 family of NAD-dependent protein deacetylases. *J Biol Chem* **280**, 36073-36078, doi:10.1074/jbc.M508247200 (2005).
120 Kanfi, Y. *et al.* The sirtuin SIRT6 regulates lifespan in male mice. *Nature* **483**, 218-221, doi:10.1038/nature10815 (2012).
121 Igci, M. *et al.* High-throughput screening of Sirtuin family of genes in breast cancer. *Gene* **586**, 123-128, doi:10.1016/j.gene.2016.04.023 (2016).

122 Yuan, H., Su, L. & Chen, W. Y. The emerging and diverse roles of sirtuins in cancer: a clinical perspective. *Onco Targets Ther* **6**, 1399-1416, doi:10.2147/OTT.S37750 (2013).
123 Chen, L. Medicinal chemistry of sirtuin inhibitors. *Curr Med Chem* **18**, 1936-1946 (2011).
124 Luo, J. *et al.* Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* **107**, 137-148 (2001).
125 Herranz, D. *et al.* SIRT1 promotes thyroid carcinogenesis driven by PTEN deficiency. *Oncogene* **32**, 4052-4056, doi:10.1038/onc.2012.407 (2013).
126 Liang, X. J. *et al.* SIRT1 contributes in part to cisplatin resistance in cancer cells by altering mitochondrial metabolism. *Mol Cancer Res* **6**, 1499-1506, doi:10.1158/1541-7786.MCR-07-2130 (2008).
127 Brunet, A. *et al.* Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011-2015, doi:10.1126/science.1094637 (2004).
128 Wang, R. H. *et al.* Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. *Cancer Cell* **14**, 312-323, doi:10.1016/j.ccr.2008.09.001 (2008).
129 Wang, R. H. *et al.* Interplay among BRCA1, SIRT1, and Survivin during BRCA1-associated tumorigenesis. *Mol Cell* **32**, 11-20, doi:10.1016/j.molcel.2008.09.011 (2008).
130 Yuan, J., Minter-Dykhouse, K. & Lou, Z. A c-Myc-SIRT1 feedback loop regulates cell growth and transformation. *J Cell Biol* **185**, 203-211, doi:10.1083/jcb.200809167 (2009).
131 Halfed, D. G. *et al.* SIRT2 Expression Is Higher in Uveal Melanoma than In Ocular Melanocytes. *Ocul Oncol Pathol* **2**, 100-104, doi:10.1159/000439309 (2015).
132 Hiratsuka, M. *et al.* Proteomics-based identification of differentially expressed genes in human gliomas: down-regulation of SIRT2 gene. *Biochem Biophys Res Commun* **309**, 558-566 (2003).
133 Kim, H. S. *et al.* SIRT2 maintains genome integrity and suppresses tumorigenesis through regulating APC/C activity. *Cancer Cell* **20**, 487-499, doi:10.1016/j.ccr.2011.09.004 (2011).
134 Dan, L. *et al.* The role of sirtuin 2 activation by nicotinamide phosphoribosyltransferase in the aberrant proliferation and survival of myeloid leukemia cells. *Haematologica* **97**, 551-559, doi:10.3324/haematol.2011.055236 (2012).
135 Liu, P. Y. *et al.* The histone deacetylase SIRT2 stabilizes Myc oncoproteins. *Cell Death Differ* **20**, 503-514, doi:10.1038/cdd.2012.147 (2013).
136 Chin, L. *et al.* p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* **97**, 527-538, doi:10.1016/s0092-8674(00)80762-x (1999).
137 Cosme-Blanco, W. & Chang, S. Dual roles of telomere dysfunction in initiation and suppression of tumorigenesis. *Exp Cell Res* **314**, 1973-1979, doi:10.1016/j.yexcr.2008.03.011 (2008).
138 Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L. & Jaenisch, R. DNA hypomethylation leads to elevated mutation rates. *Nature* **395**, 89-93, doi:10.1038/25779 (1998).
139 Richon, V. M. *et al.* Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. *Proc Natl Acad Sci U S A* **93**, 5705-5708 (1996).

140 Su, G. H., Sohn, T. A., Ryu, B. & Kern, S. E. A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. *Cancer Res* **60**, 3137-3142 (2000).
141 Plumb, J. A. *et al.* Pharmacodynamic response and inhibition of growth of human tumor xenografts by the novel histone deacetylase inhibitor PXD101. *Mol Cancer Ther* **2**, 721-728 (2003).
142 Atadja, P. *et al.* Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824. *Cancer Res* **64**, 689-695 (2004).
143 Gottlicher, M. *et al.* Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* **20**, 6969-6978, doi:10.1093/emboj/20.24.6969 (2001).
144 Villar-Garea, A. & Esteller, M. Histone deacetylase inhibitors: understanding a new wave of anticancer agents. *Int J Cancer* **112**, 171-178, doi:10.1002/ijc.20372 (2004).
145 Lea, M. A. & Tulsyan, N. Discordant effects of butyrate analogues on erythroleukemia cell proliferation, differentiation and histone deacetylase. *Anticancer Res* **15**, 879-883 (1995).
146 Patnaik, A. *et al.* A phase I study of pivaloyloxymethyl butyrate, a prodrug of the differentiating agent butyric acid, in patients with advanced solid malignancies. *Clin Cancer Res* **8**, 2142-2148 (2002).
147 Cote, C. D. *et al.* Resveratrol activates duodenal Sirt1 to reverse insulin resistance in rats through a neuronal network. *Nat Med* **21**, 498-505, doi:**10.1038/nm.3821** (2015).
148 Shin, D. H., Choi, Y. J. & Park, J. W. SIRT1 and AMPK mediate hypoxia-induced resistance of non-small cell lung cancers to cisplatin and doxorubicin. *Cancer Res* **74**, 298-308, doi:10.1158/0008-5472.CAN-13-2620 (2014).
149 Smith, J. J. *et al.* Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo. *BMC Syst Biol* **3**, 31, doi:10.1186/1752-0509-3-31 (2009).
150 Sauve, A. A., Moir, R. D., Schramm, V. L. & Willis, I. M. Chemical activation of Sir2-dependent silencing by relief of nicotinamide inhibition. *Mol Cell* **17**, 595-601, doi:10.1016/j.molcel.2004.12.032 (2005).
151 Hubbard, B. P. & Sinclair, D. A. Small molecule SIRT1 activators for the treatment of aging and age-related diseases. *Trends Pharmacol Sci* **35**, 146-154, doi:10.1016/j.tips.2013.12.004 (2014).
152 Harris, L. J. Nicotinamide and pellagra; a review of the development of knowledge and of the present position. *Practitioner* **168**, 57-59 (1952).
153 Mai, A. *et al.* Design, synthesis, and biological evaluation of sirtinol analogues as class III histone/protein deacetylase (Sirtuin) inhibitors. *J Med Chem* **48**, 7789-7795, doi:10.1021/jm050100l (2005).
154 Audrito, V. *et al.* Nicotinamide blocks proliferation and induces apoptosis of chronic lymphocytic leukemia cells through activation of the p53/miR-34a/SIRT1 tumor suppressor network. *Cancer Res* **71**, 4473-4483, doi:10.1158/0008-5472.CAN-10-4452 (2011).
155 Jung-Hynes, B., Nihal, M., Zhong, W. & Ahmad, N. Role of sirtuin histone deacetylase SIRT1 in prostate cancer. A target for prostate cancer management via its inhibition? *J Biol Chem* **284**, 3823-3832, doi:10.1074/jbc.M807869200 (2009).
156 Alhazzazi, T. Y. *et al.* Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. *Cancer* **117**, 1670-1678, doi:10.1002/cncr.25676 (2011).

157 Smith, B. C. & Denu, J. M. Acetyl-lysine analog peptides as mechanistic probes of protein deacetylases. *J Biol Chem* **282**, 37256-37265, doi:10.1074/jbc.M707878200 (2007).
158 Hawse, W. F. *et al.* Structural insights into intermediate steps in the Sir2 deacetylation reaction. *Structure* **16**, 1368-1377, doi:10.1016/j.str.2008.05.015 (2008).
159 Kiviranta, P. H. *et al.* N(epsilon)-thioacetyl-lysine-containing tri-, tetra-, and pentapeptides as SIRT1 and SIRT2 inhibitors. *J Med Chem* **52**, 2153-2156, doi:10.1021/jm801401k (2009).
160 Mellini, P. *et al.* Screen of pseudopeptidic inhibitors of human sirtuins 1-3: two lead compounds with antiproliferative effects in cancer cells. *J Med Chem* **56**, 6681-6695, doi:10.1021/jm400438k (2013).
161 Napper, A. D. *et al.* Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. *J Med Chem* **48**, 8045-8054, doi:10.1021/jm050522v (2005).
162 Solomon, J. M. *et al.* Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol Cell Biol* **26**, 28-38, doi:10.1128/MCB.26.1.28-38.2006 (2006).
163 Pasco, M. Y. *et al.* Characterization of sirtuin inhibitors in nematodes expressing a muscular dystrophy protein reveals muscle cell and behavioral protection by specific sirtinol analogues. *J Med Chem* **53**, 1407-1411, doi:10.1021/jm9013345 (2010).
164 Zhang, Y. *et al.* Identification of a small molecule SIRT2 inhibitor with selective tumor cytotoxicity. *Biochem Biophys Res Commun* **386**, 729-733, doi:10.1016/j.bbrc.2009.06.113 (2009).
165 Lain, S. *et al.* Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. *Cancer Cell* **13**, 454-463, doi:10.1016/j.ccr.2008.03.004 (2008).
166 McCarthy, A. R. *et al.* Synthesis and biological characterisation of sirtuin inhibitors based on the tenovins. *Bioorg Med Chem* **20**, 1779-1793, doi:10.1016/j.bmc.2012.01.001 (2012).
167 Hirai, S. *et al.* Antitumor effects of a sirtuin inhibitor, tenovin-6, against gastric cancer cells via death receptor 5 up-regulation. *PLoS One* **9**, e102831, doi:10.1371/journal.pone.0102831 (2014).
168 Dai, W., Zhou, J., Jin, B. & Pan, J. Class III-specific HDAC inhibitor Tenovin-6 induces apoptosis, suppresses migration and eliminates cancer stem cells in uveal melanoma. *Sci Rep* **6**, 22622, doi:10.1038/srep22622 (2016).
169 Jin, Y. *et al.* Tenovin-6-mediated inhibition of SIRT1/2 induces apoptosis in acute lymphoblastic leukemia (ALL) cells and eliminates ALL stem/progenitor cells. *BMC Cancer* **15**, 226, doi:10.1186/s12885-015-1282-1 (2015).
170 Nihal, M., Ahmad, N. & Wood, G. S. SIRT1 is upregulated in cutaneous T-cell lymphoma, and its inhibition induces growth arrest and apoptosis. *Cell Cycle* **13**, 632-640, doi:10.4161/cc.27523 (2014).
171 Outeiro, T. F. *et al.* Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science* **317**, 516-519, doi:10.1126/science.1143780 (2007).
172 He, X. *et al.* SIRT2 activity is required for the survival of C6 glioma cells. *Biochem Biophys Res Commun* **417**, 468-472, doi:10.1016/j.bbrc.2011.11.141 (2012).

173 Mangas-Sanjuan, V. *et al.* Tubulin acetylation promoting potency and absorption efficacy of deacetylase inhibitors. *Br J Pharmacol* **172**, 829-840, doi:10.1111/bph.12946 (2015).
174 Kalle, A. M. *et al.* Inhibition of SIRT1 by a small molecule induces apoptosis in breast cancer cells. *Biochem Biophys Res Commun* **401**, 13-19, doi:10.1016/j.bbrc.2010.08.118 (2010).
175 Rotili, D. *et al.* Discovery of salermide-related sirtuin inhibitors: binding mode studies and antiproliferative effects in cancer cells including cancer stem cells. *J Med Chem* **55**, 10937-10947, doi:10.1021/jm3011614 (2012).
176 Deme, D. & Telekes, A. Therapeutic options for synovial sarcoma. *Orv Hetil* **156**, 875-880, doi:10.1556/650.2015.30166 (2015).
177 Hajdu, S. I., Shiu, M. H. & Fortner, J. G. Tendosynovial sarcoma: a clinicopathological study of 136 cases. *Cancer* **39**, 1201-1217 (1977).
178 Al-Hussaini, H. *et al.* Clinical features, treatment, and outcome in 102 adult and pediatric patients with localized high-grade synovial sarcoma. *Sarcoma* **2011**, 231789, doi:10.1155/2011/231789 (2011).
179 Dallos, M., Tap, W. D. & D'Angelo, S. P. Current status of engineered T-cell therapy for synovial sarcoma. *Immunotherapy* **8**, 1073-1080, doi:10.2217/imt-2016-0026 (2016).
180 Nagayama, S. *et al.* Therapeutic potential of antibodies against FZD 10, a cell-surface protein, for synovial sarcomas. *Oncogene* **24**, 6201-6212, doi:10.1038/sj.onc.1208780 (2005).