

Investigating the roles of SMARCA4 and EP300 in synovial sarcoma

A thesis submitted to Trinity College Dublin for the degree of Doctor in Philosophy

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Declaration

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Summary

Synovial sarcoma is a rare and aggressive cancer that accounts for approximately 10% of all soft tissue sarcomas diagnosed annually. This malignancy is caused by a characteristic fusion protein known as SS18-SSX and is particularly prevalent in adolescents and young adults. The disease-causing SS18-SSX fusion protein integrates into ATP-dependent chromatin remodelling SWI/SNF complexes; where it induces their redistribution along the chromatin fibre, driving oncogenic gene expression. Previous work from our lab and others has demonstrated that targeting SWI/SNF complexes in this disease using small molecules can yield significant therapeutic responses. Discoveries which have led to the initiation of clinical trials in synovial sarcoma patients.

This PhD thesis aimed to extend our initial findings to examine additional opportunities to therapeutically target oncogenic SS18-SSX-containing SWI/SNF complexes. Firstly, publicly available DepMap CRISPR screening data was utilised to explore the genetic dependencies of all SWI/SNF subunits, aiming to identify novel vulnerabilities that exhibit a selective impact on synovial sarcoma cells. Through this approach, the catalytic SWI/SNF subunit SMARCA4 emerged as crucial in synovial sarcoma cell lines, with these cells displaying heightened sensitivity to SMARCA4 loss. Despite synovial sarcoma being traditionally considered a classic SWI/SNF-perturbed cancer, comprehensive research into the functional role of SMARCA4 in this disease and the assessment of SMARCA4 targeting as a potential therapeutic strategy have not been addressed in published studies to date. Following on from the identification of SMARCA4 as a promising genetic dependency in synovial sarcoma cells, the novel concept of complete protein degradation was embraced and utilised to characterise the biochemical consequences of SMARCA4 degradation. Secondly, having characterised the biochemical consequences of SMARCA4 degradation it was pertinent to examine the transcriptional effects of SMARCA4 loss and to examine the chromatin dynamics associated with SMARCA4

degradation. Addressing these key aims was important because mammalian SWI/SNF complexes are ATP-dependent chromatin remodelers that regulate DNA accessibility and control gene expression dynamics and SMARCA4/2 are the two mutually catalytic subunits found in all three SWI/SNF subclasses. In the initial study from our lab which identified BRD9 as a critical functional dependency in synovial sarcoma, the E1A-binding protein p300 (EP300) was also identified as a promising therapeutic target in synovial sarcoma cells. The goal of the third research chapter was to follow up on this finding and to investigate the effect of perturbing EP300 function on the transcriptional program of synovial sarcoma cells.

Publications, Poster Presentations and Awards

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I dedicate this work to my beloved father, George Doherty, whose strength, courage, and diligent work ethic inspire me to strive for the same.

“When nothing seems to help, I go and look at a stonecutter hammering away at his rock perhaps a hundred times without as much as a crack showing in it. Yet at the hundred and first blow it will split in two, and I know it was not that blow that did it, but all that had gone before.”

— Jacob Riis

List of abbreviations

Ac Acetylation.

AML acute myeloid leukemia.

ASU Asunaprevir.

ATAC-seq assay for transposase-accessible chromatin with sequencing.

ATP Adenosine triphosphate.

BAF Brahma-Associated Factor.

BAP Brahma-associated protein.

BRD9 Bromodomain Containing 9.

cBAF canonical BAF.

CHD Chromodomain-Helicase-DNA binding.

ChIP chromatin immunoprecipitation.

CpG 5'—C—phosphate—G—3'.

CREBBP Cyclic adenosine monophosphate response element binding protein binding protein.

CRISPR clustered regularly interspaced short palindromic repeats.

CUT&RUN Cleavage Under Targets and Release Using Nuclease.

DepMap Dependency Map.

DMSO Dimethylsulfoxide.

DNA Deoxyribonucleic acid.

DNase-seq DNase I hyper-sensitivity sequencing.

EP300 E1A-associated protein 300.

EZH2 Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit.

FAIRE-seq Formaldehyde-Assisted Isolation of Regulatory Elements sequencing.

GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase.

GBAF/ncBAF noncanonical BAF.

HAT Histone acetyltransferase.

HEK293T Human Embryonic Kidney 293T cells.

iBAQ intensity-based absolute quantification.

IgG immunoglobulin G.

IN080 human Inositol auxotrophy 80.

INF Interferon.

IP immunoprecipitation.

IP-MS immunoprecipitations coupled with mass spectrometry.

ISWI Imitation switch.

KAT Lysine acetyltransferase.

KDM2B Lysine Demethylase 2B).

Me1/2/3 mono-, di- and tri-methylation.

mESCs Mouse embryonic stem cells.

mRNA messenger RNA.

MS mass spectrometry.

pAG-MNase protein A/G fused Micrococcal Nuclease.

PBAF polybromo-associated BAF.

PBAP Polybromo-associated BAP.

PBS Phosphate-buffered saline.

PRC1 Polycomb Repressive Complex 1.

PROTAC Proteolysis Targeting Chimera.

PTM Post Translational Modification.

RING Really Interesting New Gene.

RRPM reference-adjusted reads per million.

RSC remodels the structure of chromatin.

SCNT somatic cell nuclear transfer.

shRNA Small hairpin RNA.

SS Synovial sarcoma.

SS18 SS18 Subunit Of BAF Chromatin Remodeling Complex.

SSX1 SSX Family Member 1.

SSX1 SSX Family Member 2.

SSX1 SSX Family Member 4.

SWI/SNF SWItch/Sucrose Non-Fermentable.

TF Transcription factor.

TPM transcript per million.

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Chapter 1

Introduction

1.1 General Introduction

Cellular biology presents an intriguing enigma: how do cells, sharing identical genetic blueprints, embark on individual journeys to craft the complex composition of multicellular organisms with specialised and molecularly distinct cell types? This phenomenon is even more compelling considering that every cell of a multicellular organism arises from the division of a single cell; the zygote. As cell division proceeds they become increasingly distinct forming specialised cell types through the process of differentiation. Prior to 1970, two possible explanations existed to explain this process. One possible explanation was that as a cell differentiates it loses genetic material it no longer requires. The second scenario was that the cell irreversibly inactivates genes it no longer requires. A landmark experiment addressing this question was performed by Sir John Gurdon (LASKEY & GURDON, 1970). In this experiment the nucleus from a somatic cell of an adult toad was inserted into an unfertilised egg with its nucleus removed, a process termed somatic cell nuclear transfer (SCNT). Using this process it was possible to generate live toads, demonstrating that when cells differentiate their genetic material is not lost or irreversibly changed and in fact maintains the same Deoxyribonucleic acid (DNA) content as the zygote (LASKEY & GURDON, 1970). These experiments revealed that within cells, there exists a regulatory mechanism governing the activation or deactivation of specific genes in different cell types-something that is reversible. Subsequently, this regulatory system was identified as the epigenetic system, orchestrating transcriptional programs in cells.

1.2 Mechanisms regulating chromatin structure

The amazing feat of compacting approximately 2 metres of DNA (for the human genome) into a cell nucleus of less than $10\mu\text{m}$ in diameter is achieved by its interaction with histone proteins, to form a DNA-protein complex termed chromatin. The functional unit of chromatin is the nucleosome core particle, consisting of approximately 146 base pairs of DNA wrapped around a histone octamer composed of two molecules of histone H2A, H2B, H3, and H4 (Luger, Mä Der, Richmond, Sargent, & Richmond, 1997). As the name suggests small amounts of linker DNA connect neighbouring nucleosomes. Chromatin is subjected to additional compaction via the formation of polynucleosomal fibres, stabilised by the binding of histone H1 to each nucleosome and to the linker DNA (Quina, Buschbeck, & Di Croce, 2006). In broad terms, chromatin exists in two distinct forms, heterochromatin and euchromatin (**Figure 1.1**). Heterochromatin is highly condensed and for the most part inaccessible to transcriptional machinery. Heterochromatic regions can be further classified as constitutive or facultative. Constitutive chromatin predominantly assembles at repetitive regions of low gene density such as satellite, centromeric and pericentromeric repeats and as such displays comparable patterns of localisation across cell types (Quina et al., 2006). The patterning of facultative heterochromatin is developmentally regulated and as such its deposition is highly cell-type specific (Quina et al., 2006). In contrast, euchromatin is more open and accessible and contains greater numbers of active genes (**Figure 1.1**). Therefore, rather than solely solving a physical packaging issue, nucleosomes provide a layer of functional organization to the genome, permitting regulation of the fundamentally important processes of DNA replication, repair, and transcription (Bracken, Brien, & Verrijzer, 2019). This functional organisation is controlled by the addition of a wide variety of posttranslational modifications (PTMs) to the unstructured N-terminal histone tails that protrude from the nucleosome and the activity of ATP-dependent chromatin remodelling complexes (CRCs) (Valencia & Kadoch, 2019)

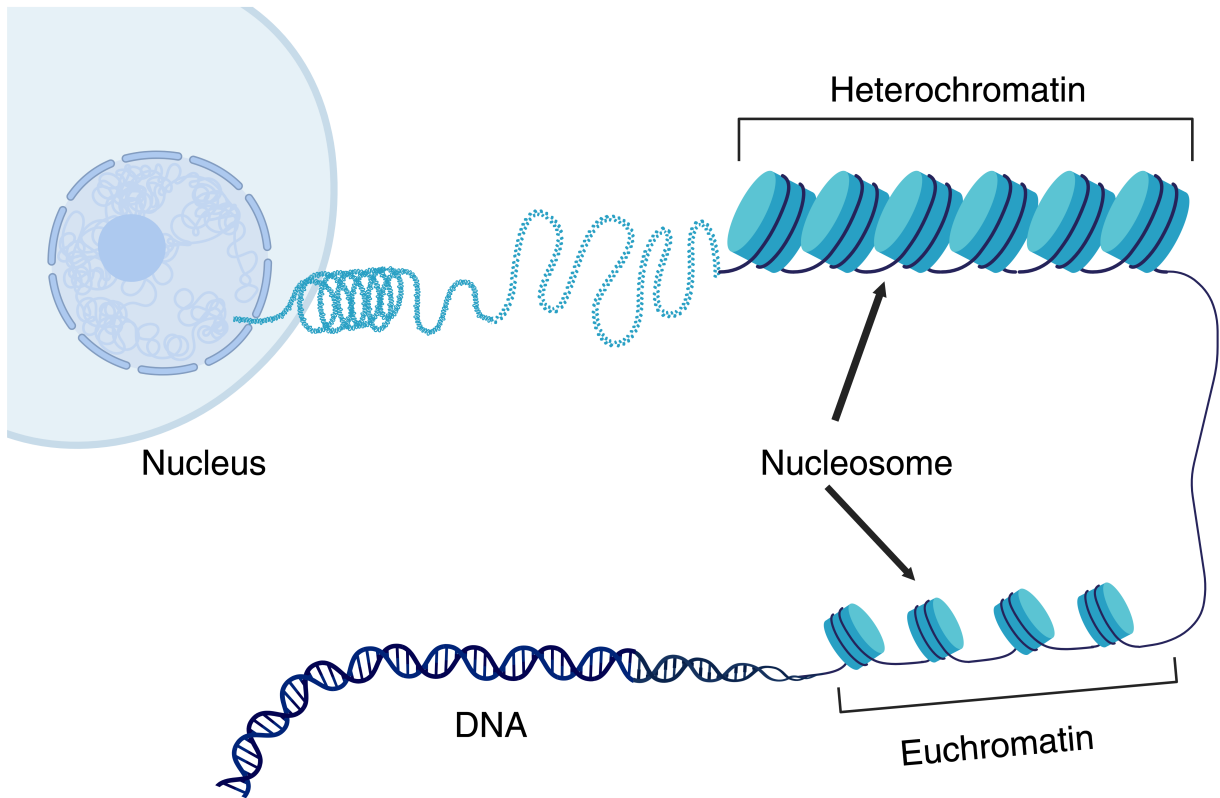


Figure 1.1: Basic chromatin architecture. Schematic representation of chromatin structure in the nucleus depicting nucleosomes, heterochromatin and euchromatin. Regions of heterochromatin are highly condensed and contain inactive genes. Euchromatic regions are open and contain active genes.

Dynamic regulation of chromatin structure via histone modifications involves the carefully balanced interplay between three important groups of proteins termed chromatin “readers”, “writers” and “erasers” (Wimalasena, Wang, Sigua, Durbin, & Qi, 2020) (**Figure 1.2**). Chromatin “writers” catalyse the addition of histone post-translational modifications. These modifications have the ability to affect gene transcription by directly altering the electrostatic interactions within or between nucleosomes (Brien, Valerio, & Armstrong, 2016). This is the case for post translational acetylation of histones where the addition of acetyl groups to histone proteins reduces the positive charge of histones, thus weakening their interaction with negatively charged DNA (Allfrey, Faulkner, & Mirsky, 1964; Brownell et al., 1996). This is thought to permit transcription factors and components of the transcriptional machinery with access to the DNA to facilitate transcription. As such histone acetylation, regardless of the residue modified, is generally found at active chromatin. At the same time, the modifications established by chromatin “writers” also affect transcription more indirectly by serving as binding sites for chromatin “reader” proteins (**Figure 1.2**) (Brien et al., 2016). This class of protein can bind nucleosomes marked by specific modifications via their distinct

binding domains such as chromo-, bromo-, and PHD-finger domains and alter the local chromatin environment by additional intrinsic chromatin modifying activity or the recruitment of additional proteins (Brien et al., 2016). For example, the effects of histone methylation are considered to be primarily exerted by the interplay with chromatin reader proteins, as unlike acetylation histone methylation does not alter the charge of the histone protein (Bannister & Kouzarides, 2011; Hyun, Jeon, Park, & Kim, 2017). In further contrast to acetylation, histone methylation can be associated with both active and repressed chromatin subject to the residue modified (Hyun et al., 2017). Of fundamental importance is the reversibility of histone post translational modifications to allow for dynamic control of gene expression. Key players in this regard are the chromatin “erasers” the class of proteins responsible for catalysing the removal of histone modifications (**Figure 1.2**) (Brien et al., 2016).

The chromatin regulatory proteins described above work in tandem with chromatin remodellers and specific DNA binding transcription factors to establish the chromatin state and control gene expression. Chromatin remodellers are multi-subunit biochemical assemblies that act as molecular motors using the energy of ATP hydrolysis to reposition, eject, slide or alter the composition of nucleosomes (Valencia & Kadoch, 2019) By regulating the accessibility of DNA, these multi-subunit complexes play a fundamentally important role in gene regulation (Bracken et al., 2019; Valencia & Kadoch, 2019). The four families of chromatin remodellers include SWItch/Sucrose Non-Fermentable (SWI/SNF or BAF), ISWI, INO80 and CHD (Bracken et al., 2019).

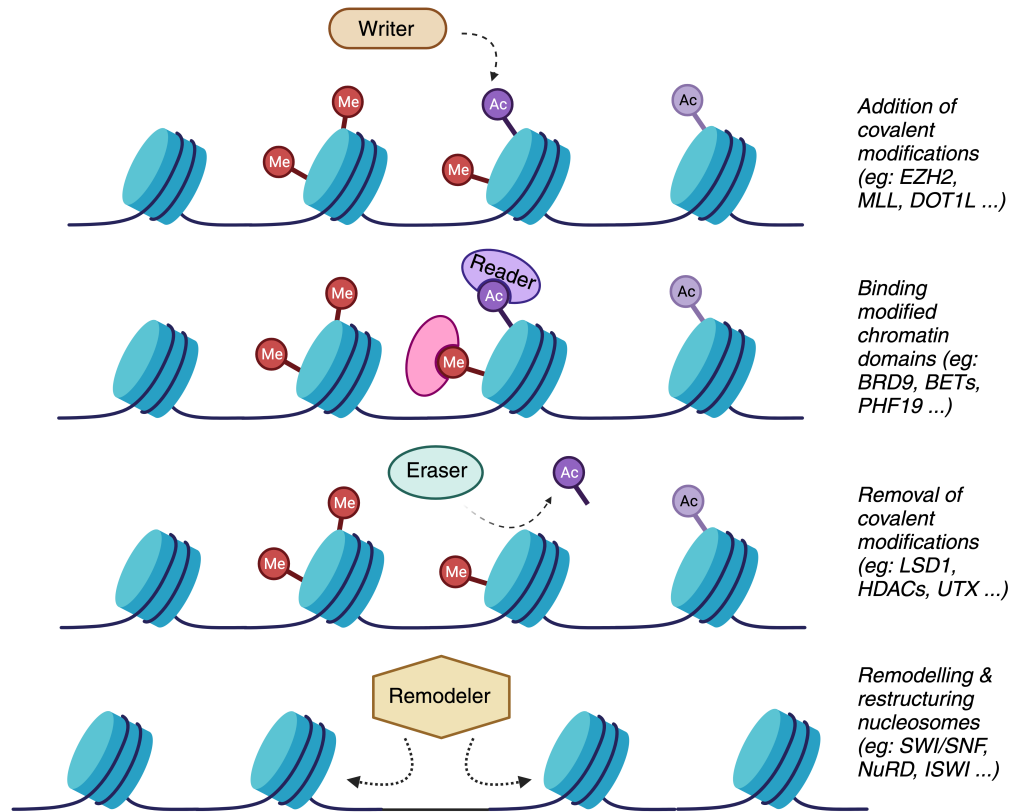


Figure 1.2: Mechanisms controlling chromatin structure in the nucleus. A schematic demonstrating the functional roles of chromatin readers, writers, erasers and remodellers with examples given for each protein class.

In recent years cancer genome sequencing studies have highlighted that disrupting the function of chromatin regulatory proteins is one of the most pervasive disease mechanisms in cancer (Bailey et al., 2018; Gröbner et al., 2018; Vogelstein et al., 2013). More specifically, approximately 30% of all cancer driver genes have been shown to encode proteins involved in chromatin regulation (Gröbner et al., 2018). Within this class of proteins the family of chromatin remodellers of particular importance in this research project SWI/SNF are exceptionally prone to mutation with cancer genome sequencing studies revealing that approximately 25% of all human cancers bear mutations in SWI/SNF member genes (Mittal & Roberts, 2020).

1.3 Mammalian SWI/SNF complex composition and function

Mammalian SWI/SNF complexes are combinatorially assembled from the protein products of 29 genes into three well defined SWI/SNF subclasses termed canonical BAF (cBAF), polybromo-associated BAF (PBAF) and GLTSCR1/1L associated or non-canonical BAF (GBAF/ncBAF) (Centore et al., 2020) (**Figure 1.3**).

Their evolutionary counterparts were first identified in the yeast *Saccharomyces cerevisiae* through genetic screening for genes required for mating type switching and sucrose fermentation (Neigeborn & Carlson, 1984; Stern, Jensen, & Herskowitz, 1984). Two subcomplexes exist in yeast and *Drosophila*; SWI/SNF and RSC and BAP and PBAP respectively (Mohrmann & Verrijzer, 2005). The cBAF and PBAF subcomplexes are broadly conserved among eukaryotes with mammalian cBAF being most similar to the yeast SWI/SNF and *Drosophila* BAP complexes. While, the PBAF complex displays greater homology with yeast RSC and *Drosophila* PBAP (Bracken et al., 2019). Significant diversity exists within the SWI/SNF family in higher eukaryotes where paralogous as well as additional subunits have been added through evolution, with an additional nine subunits existing solely in vertebrates (Kadoch & Crabtree, 2015). This diversity perhaps reflecting the need for more precise or nuanced chromatin regulatory processes during multicellular development. It is important to note that complexes analogous to GBAF have recently been identified in *Drosophila*, indicating that the GBAF complex is also evolutionarily conserved (Barish et al., 2020).

The main role of the SWI/SNF family of chromatin remodellers is ATP-dependent chromatin remodelling and as such all three subclasses crucially comprise one of two mutually exclusive catalytic subunits SMARCA4 (BRG1) or SMARCA2 (BRM) (Centore et al., 2020). The cBAF complex is a biochemical assembly of 12 subunits, defined by the presence of either of the two mutually exclusive at-rich interaction domain (ARID)-domain containing proteins, ARID1A or ARID1B as well as specific incorporation of a tandem PHD domain containing protein, which in most cell types is DPF2 (Centore et al., 2020). The PBAF complex is defined by the unique incorporation of the bromodomain containing subunits BRD7 and PBRM1, the PHD domain containing subunit PHF10 and the specific incorporation of ARID2 (Centore et al., 2020). The most recently characterised GBAF subclass is defined by unique incorporation of the GLTSCR1/1L and BRD9 subunits and lacks the SMARCB1 and SMARCE1 subunits shared by the PBAF and cBAF subclasses (Alps & Dykhuizen, 2018; Michel et al., 2018) (**Figure 1.3**).

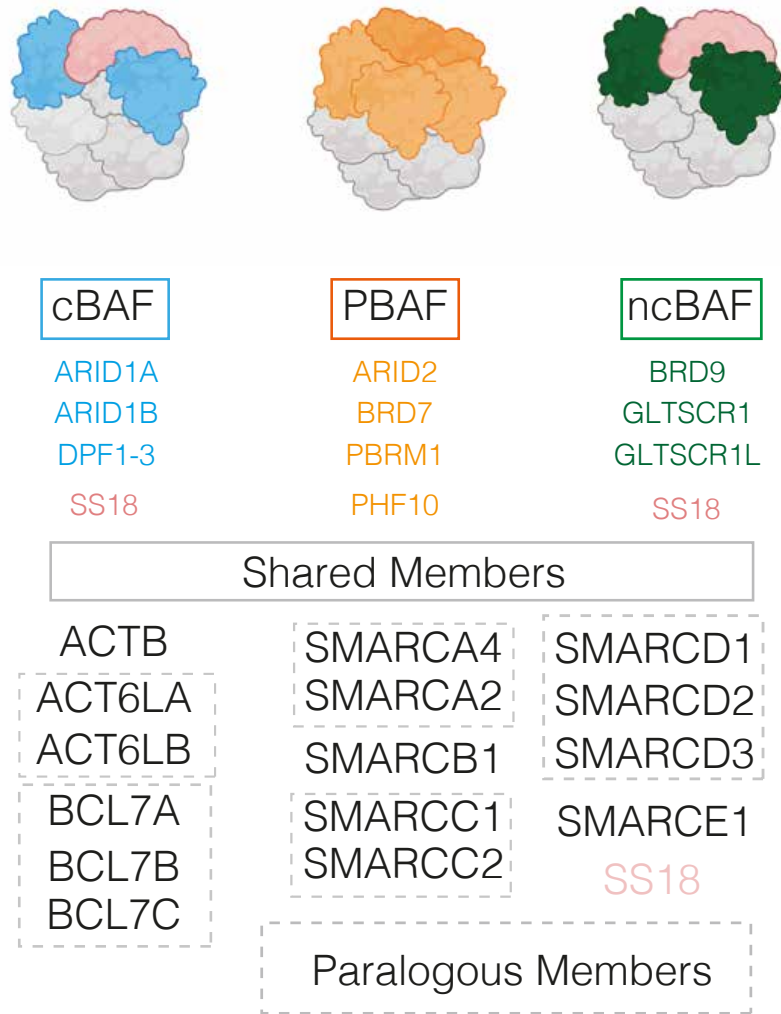


Figure 1.3: Composition of mammalian SWI/SNF subclasses. Schematic representation of the cBAF, PBAF and ncBAF (GBAF) subcomplexes with complex defining subunits highlighted in blue, yellow and green respectively. The SS18 shared subunit is highlighted in pink. All other shared components are coloured grey.

Compounding evidence indicates that the vast diversity among SWI/SNF complexes confers increased functionality and more elaborate regulatory roles. For example, the GBAF and PBAF complexes have recently been reported to play contrasting roles in regards to the regulation of T cell function, with the complexes demonstrating positive and negative regulatory roles respectively (Loo et al., 2020). Moreover, the PBAF complex has also been highlighted as a negative regulator of IFN--responsive genes in cancer cells. SWI/SNF occupancy at target loci is most prominently associated with facilitating and maintaining DNA accessibility to positively regulate gene transcription (Centore et al., 2020). However, the above examples also

demonstrate a unique feature of the more intricate SWI/SNF complexes found in complicated multicellular organisms, which is the fact that they can also remodel nucleosomes to allow binding of repressive transcription factors (TFs) or to establish a repressive chromatin state (Alfert, Moreno, & Kerl, 2019). In line with the above findings is the observation that although the chromatin profiles of all SWI/SNF subclasses demonstrate occupancy at promoter and enhancer regions genome wide, differences in the relative abundances of the different subclasses across these sites has been unmasked. Specifically, cBAF demonstrates the greatest occupancy at active enhancers, PBAF is preferentially enriched at promoters and the GBAF subclass has demonstrated a preference for CCCTC-binding factor (CTCF)-binding sites (J. Li et al., 2021; Michel et al., 2018). This differential genomic targeting is expected to be mediated by two overlapping mechanisms, the direct chromatin binding capabilities of many of the subcomplex defining members and the capacity of these proteins to specifically interact with cell type-specific DNA binding transcription factors or other chromatin regulators, with many studies providing evidence of functional association of SWI/SNF complexes with these proteins in normal and diseased states (Boulay et al., 2017; Dallas et al., 1998; Sandoval et al., 2018).

1.4 Aberrant SWI/SNF complex function and cancer

In the last 10 years, the central role of aberrations in SWI/SNF complex function in cancer has been illuminated with cancer genome sequencing studies revealing that approximately 25% of all human cancers bear mutations in SWI/SNF member genes (Mittal & Roberts, 2020). Despite the observed frequency of mutations, it is recognised that discrete SWI/SNF complex members are associated with the development of distinct cancer subtypes. For example, disruption of the SMARCB1 gene drives oncogenesis in malignant rhabdoid tumours and epithelioid sarcomas but is scarcely disrupted in other cancers (Sullivan, Folpe, Pawel, Judkins, & Biegel, 2013). Analogously, mutation of the PBAF-defining subunit PBRM1 is associated with greater than 40% clear renal cell carcinoma but is rarely mutated in other malignancies (Kadoch et al., 2013). The malignancy under investigation in this study, synovial sarcoma (SS) also epitomises this phenomenon. These findings suggest that certain complexes may have non-redundant, tissue and/or developmental stage-specific tumour protective roles and support the idea that SWI/SNF sub-complexes do in fact play different functional roles.

1.5 Synovial sarcoma

1.5.1 Introduction to synovial sarcoma

Sarcomas constitute a diverse group of cancers classified based on the type of tissue they most closely resemble, derived from either mesoderm or neuroectoderm. This classification includes tissues such as fat, fibrous tissue, smooth and skeletal muscle, endothelium, bone, cartilage, and nerve. These tumors are broadly categorised into two main groups: 1) Soft Tissue Sarcomas - these develop in soft tissues like muscles, tendons, fat, blood vessels, nerves, and deep skin tissues. Soft tissue sarcomas are more common than bone sarcomas; 2) Bone Sarcomas: These sarcomas develop in bone. The two most common types of bone sarcomas are osteosarcoma and Ewing sarcoma (Palmerini, Righi, & Staals, 2020). SS is a rare and aggressive subtype of soft tissue sarcoma. The term "synovial sarcoma" was initially coined for tumours initiating at joints and having some histological likeness to synovial tissue. Despite its name, SS has no connection to synovial joints or synovial fluid. In fact, this malignancy can originate in sites away from joints such as the head, neck, lungs, and heart (Halder, Hancock, Coffin, Lessnick, & Capecchi, 2007). The exact cell of origin of this malignancy remains unknown, however multipotent mesenchymal stem cells are considered the most likely (Fiore et al., 2021). SS manifests histologically in three subtypes: monophasic, biphasic, and poorly differentiated (Gazendam et al., 2021). The monophasic variant is distinguished by its entirely spindle cell composition, featuring elongated shapes with oval nuclei. The biphasic subtype consists of both spindle and epithelial cells with pseudoglandular structures forming within the epithelial component (Gazendam et al., 2021). As implied by its name, the poorly differentiated subtype is characterised by a completely undifferentiated morphology. SS tumours are distinguished by high mitotic activity, indicating rapid cell division and growth (Gazendam et al., 2021).

SS accounts for approximately 5-10% of all soft tissue sarcomas diagnosed annually (Spurrell, Fisher, Thomas, & Judson, 2005). This equates to about 800 new cases each year in the United States (Raney, 2005). SS can arise in patients of all ages but it typically presents in younger individuals aged 15–35 and is therefore part of both paediatric and adult clinical practices (Hale, Sandakly, Shipley, & Walters, 2019). Although a rare cancer, this malignancy is associated with a poor prognosis and high patient mortality (Krieg et al., 2011) and there has been little to no progress in patient survival during the last 30 years, a fact that is directly linked to a lack of rational, mechanistically anchored therapies that directly target core

mechanisms of disease biology.

1.5.2 The genetics of synovial sarcoma

The defining genetic abnormality in SS is a recurrent $t(X; 18)$ chromosomal rearrangement, which fuses the SS18 gene on chromosome 18 to one of three related genes SSX1, SSX2, or rarely SSX4 on the X chromosome (Clark et al., 1994; de Leeuw, Balemans, Weghuis, & van Kessel, 1995; Skytting et al., 1999) (**Figure 1.4**). Several lines of evidence indicate that the resulting SS18-SSX fusion protein is the primary driver of sarcomagenesis. For one, SS tumours are mutationally quiet and the SS18-SSX rearrangement is often the only detectable genetic abnormality in SS tumours (Nielsen, Poulin, & Ladanyi, 2015). The SS mutational burden has been demonstrated to be only minimally higher than that of malignant rhabdoid tumour which has the lowest mutational burden of any cancer sequenced to date (McBride et al., 2018). Furthermore, conditional expression of the fusion protein in muscle progenitor cells of mice leads to the development of tumours that histologically and molecularly resemble the human disease. Importantly cellular context and developmental timing are critical as expression of SS18-SSX in mature muscle lineages causes myopathy without tumorigenesis (Haldar et al., 2007).

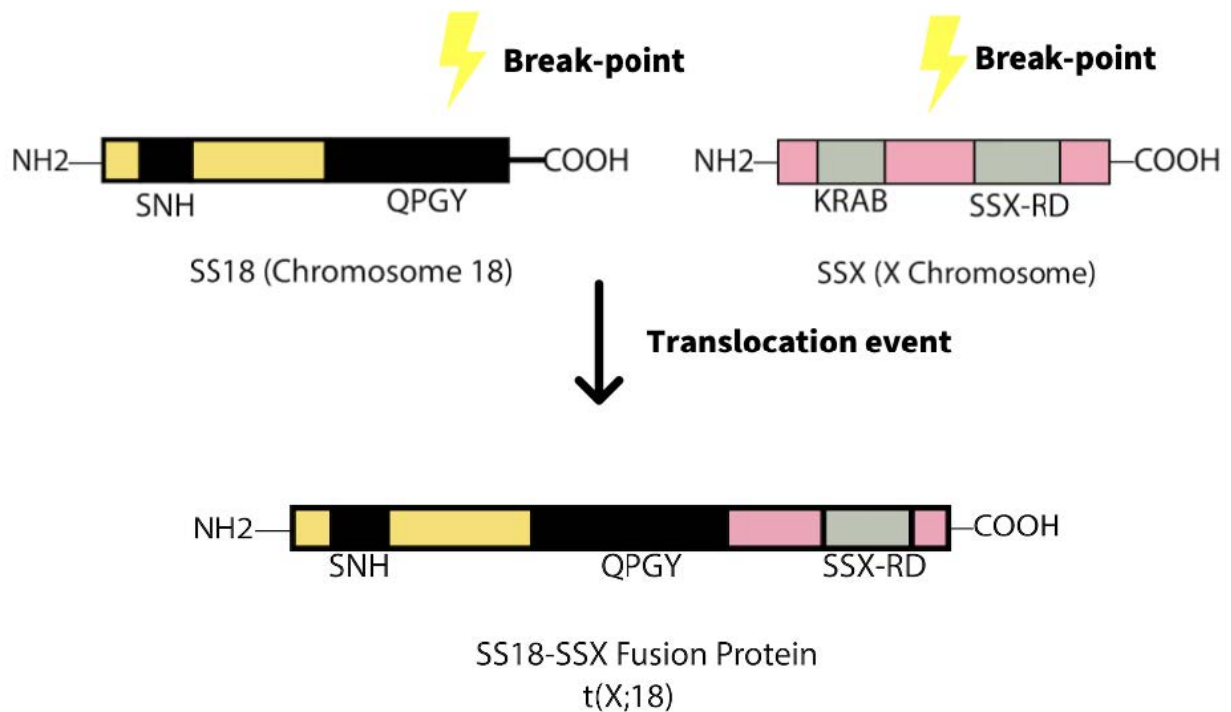


Figure 1.4: Generation of the pathognomonic SS18-SSX fusion protein. Illustration of the events involved in the translocation event between the SS18 gene on chromosome 18 which encodes the cBAF and GBAF dedicated subunit SS18 and a SSX transcriptional repressor located on the X chromosome which can be SSX1, SSX2 or rarely SSX4. Adapted from Lange SES, 2014

1.5.3 Core mechanisms of synovial sarcoma disease biology

It is widely accepted that synovial sarcomagenesis is driven by abnormal SWI/SNF function. In recent years, central mechanisms underpinning SS disease biology have been illuminated. It is understood that the SS18-SSX fusion protein integrates into cBAF complexes and alters its biochemical composition. Specifically, the fusion protein out competes the wild-type SS18 protein and induces the displacement of the tumour suppressor SMARCB1 from the complex and its subsequent proteasomal degradation (**Figure 1.5**) (Kadoch & Crabtree, 2013). Importantly, SS18-SSX does not associate with PBAF but has been shown to also assemble into GBAF complexes (**Figure 1.5**) (Brien et al., 2018; McBride et al., 2018). Early findings suggested that this altered complex assembly binds at the SOX2 gene locus, where it reverses polycomb-mediated gene repression (Kadoch & Crabtree, 2013). More recent genome-wide analyses suggest that the aberrant complex formations instigate a global retargeting of SWI/SNF complexes to repressed Polycomb domains, activating expression of associated genes (McBride et al., 2018; Banito et al., 2018). Importantly, suppression of SS18-SSX, and hence restoration of WT SWI/SNF complex composition and targeting has

been shown to promote a gain in PRC2 mediated H3K27me3, reduced chromatin accessibility at these sites and mesenchymal differentiation (McBride et al., 2018). The mechanisms at the core of this abnormal SWI/SNF complex retargeting are beginning to be unravelled. A complimentary study provided evidence that the association of SS18-SSX with the KDM2B-containing PRC1.1 complex via its C-terminal SSX repressive domain promotes recruitment of SS18-SSX containing SWI/SNF complexes to unmethylated CpG islands ordinarily subject to polycomb mediated repression (Banito et al., 2018). Importantly, KDM2B depletion reverses this paradigm, enabling fusion target gene silencing, reacquisition of a mesenchymal-like expression program, irreversible proliferative arrest and identifying this interaction as a potential therapeutic target (Banito et al., 2018). These findings highlight that targeting mechanisms related to fusion protein recruitment and SWI/SNF complex function may provide a therapeutic opportunity in this disease.

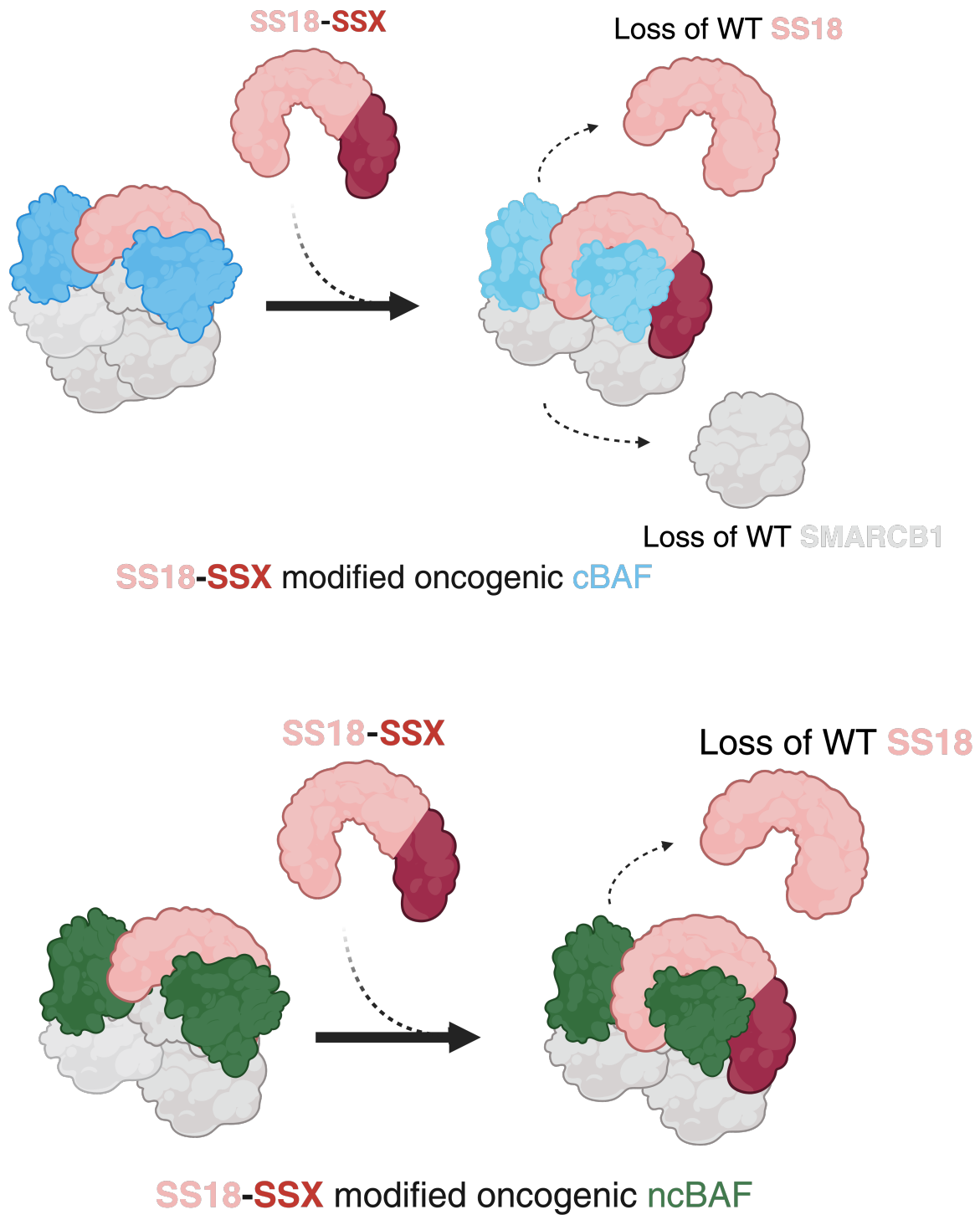


Figure 1.5: Relationship between the wildtype SS18, the fusion protein SS18-SSX and the SWI/SNF complex. The SS18 protein is a dedicated subunit of the cBAF and GBAF (ncBAF) sub-complexes as is the SS18-SSX fusion protein. Incorporation of the fusion protein into cBAF complexes is associated with concomitant loss of SMARCB1 and wild-type SS18. Incorporation of the fusion protein into GBAF (ncBAF) complexes also induces the loss of wild-type SS18 from the complex.

1.5.4 Targeting synovial sarcoma and other SWI/SNF-mutant cancers

The concept of synthetic lethality is highly relevant in terms of therapeutically targeting SWI/SNF mutant cancers, with the term being defined as the combination of two genetic events culminating in a loss of cell fitness (Nijman, 2011; St. Pierre & Kadoch, 2017). This concept in the context of aberrant SWI/SNF complex function was highlighted by genome-wide genetic screening approaches revealing that ARID1A mutant cell lines were uniquely dependent on the paralog ARID1B for survival (Helming et al., 2014) with an analogous scenario pertaining to SMARCA4 mutant cancers (Hoffman et al., 2014). These findings point to the idea that the loss of a SWI/SNF subunit can be partially compensated for by its paralog, making that paralogue a discrete dependency. Interestingly, such intra-complex dependencies are not restricted to paralogous subunits as exemplified by the fact that the dedicated GBAF subunit BRD9 was recently highlighted by work in our lab and others as a promising therapeutic target in SS (Brien et al., 2018; Michel et al., 2018). These studies have led to the initiation of two independent clinical trials (ClinicalTrials.gov identifiers: NCT05355753, NCT05355753). This finding also highlights that in the context of fusion protein-driven malignancies which have proven intractable to direct therapeutic targeting, targeting downstream vulnerabilities is a viable therapeutic rationale.

1.6 EP300 is a functional dependency in synovial sarcoma

The initial study from our lab which identified BRD9 as a critical functional dependency in SS used a domain-focused screening approach targeting known functional regions in 193 chromatin regulatory proteins (Brien et al., 2018). This screen also uncovered the interesting finding that the E1A-binding protein P300 (EP300) but not the highly homologous CREB-binding protein (CREBBP or CBP) is also a critical functional dependency in SS.

EP300 and its closely related paralog CREB-binding protein (CREBBP or CBP) are important transcriptional co-activators and key lysine acetyltransferases (KATs) in multicellular organisms (Attar & Kurdistani, 2017). These large proteins (300 KDa) regulate transcription by interacting with over 400 proteins including DNA binding transcription factors, other chromatin regulators and components of the general transcriptional machinery (Dancy & Cole, 2015). EP300/CBP are appreciated to acetylate not only histone proteins but a vast quantity of non-histone proteins, with the magnitude of this acetylation being elegantly demonstrated

recently with the generation of a high-confidence map of the CBP/EP300 “acetylome” revealing dynamic acetylation of 21,000 lysines on $\approx$ 5,300 proteins (Weinert et al., 2018). EP300/CBP are intimately involved in cancer and depending on the context can function as either tumour suppressors or oncogenes. The first implication of tumour suppressor function by EP300/CBP came from the observation of an increased incidence of cancer in children with the rare congenital developmental disorder Rubenstein-Taybi syndrome, characterised by germline loss of function heterozygous mutations in CBP and less commonly EP300 (Roelfsema & Peters, 2007). Loss of function mutations in EP300/CBP have also been detected in hematologic and solid tumours providing additional support for a tumour suppressor role of EP300/CBP (Attar & Kurdistani, 2017; Dutta, Tiu, & Sakamoto, 2016). On the other hand gain of function mutations involving translocation events are observed in some subtypes of acute myeloid leukaemias and in myelodysplastic syndrome (Attar & Kurdistani, 2017; Dutta et al., 2016). Key functional domains within EP300/CBP include a HAT domain essential for its role as a chromatin “writer” of the H3K27Ac, H3K18Ac and H3K56Ac post translation modifications (among others) (Jin et al., 2011a; Vempati et al., 2010), a bromodomain and a CH2 domain which work in concert to allow EP300/CBP to also function as a chromatin “reader” and two additional cysteine/histidine-rich zinc binding domains (CH1 and CH3) essential for facilitating interactions with a plethora of TFs, additional co-activators and the basal transcriptional machinery (Delvecchio, Gaucher, Aguilar-Gurrieri, Ortega, & Panne, 2013). A key RING domain also exists within the CH2 domain which makes contact with the active site of the HAT domain and plays an inhibitory role (Delvecchio et al., 2013). Interestingly, mutations within this domain are also observed in myelodysplastic syndrome providing additional support for increased HAT activity in its development (Attar & Kurdistani, 2017; Delvecchio et al., 2013).

Although CBP and EP300 have been traditionally considered to function identically several lines of evidence are beginning to indicate that CBP and EP300 function non-redundantly *in vivo*. For one, despite the extensive degree of homology observed within structured domains, much less homology is exhibited outside of these domains and they have been reported to interact with different proteins (Dancy & Cole, 2015). Strong evidence for non-redundancy comes from the finding that both proteins are indispensable for mammalian development (Karamouzis, Konstantinopoulos, & Papavassiliou, 2007; Tanaka et al., 2000; Yao et al., 1998). A recent study has also demonstrated distinct roles for EP300 and CBP in establishing functional chromatin states in mouse embryonic stem cells at a genome-wide scale and that specific gene

sets are transcriptionally dysregulated upon selective depletion of either EP300 or CBP (Martire, Nguyen, Sundaresan, & Banaszynski, 2020). Finally, the data from the genetic screen conducted in our lab which revealed that SS cell lines were selectively dependent on EP300 and not CBP even though robust expression of both genes was confirmed, further suggests that these proteins fulfil non-redundant functions.

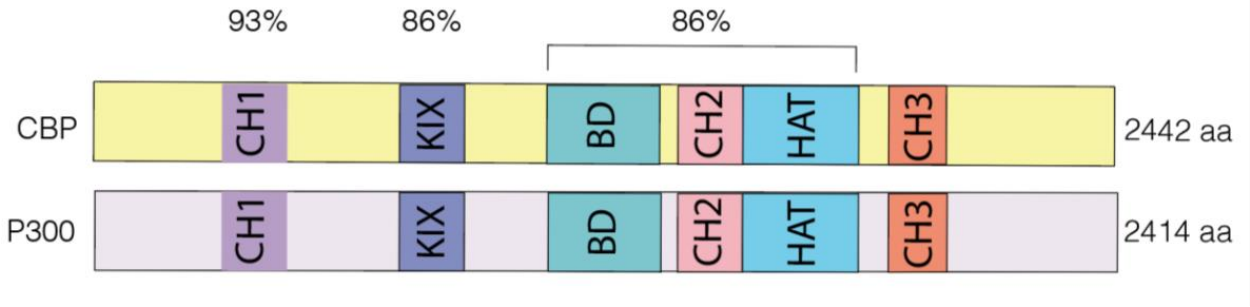


Figure 1.6: The EP300 and CBP proteins share a highly similar structure. Schematic representation of the important structural domains of the EP300 and CBP proteins with the percentage homology within each domain indicated. Original schematic adapted from (Kadoch, 2016; Karamouzis et al., 2007)

1.7 Proteolysis Targeting Chimeras (PROTACs)

The objective of personalised cancer treatment is to create targeted therapeutic strategies tailored to the specific disease, addressing the underlying disease mechanisms. Such an approach holds the promise of improving clinical outcomes for cancer patients while mitigating the debilitating side effects of conventional chemotherapeutics. A hurdle in implementing this approach is identifying specific vulnerabilities associated with each disease, which can serve as practical therapeutic targets for the development of new drugs. Traditional methods of designing small-molecule drugs encounter significant limitations when it comes to deactivating the function of target proteins. This is primarily due to the fact that many potential drug targets lack suitable binding pockets that directly regulate protein function. Additionally, many proteins perform "scaffolding" functions that are resistant to inhibition by small molecule inhibitors. Moreover, the use of drugs often triggers compensatory mechanisms, leading to the increased activity of inhibited proteins in treated cells. These factors combine to create a scenario in which traditional small molecule therapies often have limited effectiveness due to challenges in completely deactivating target protein function. The significance of these challenges is underscored by the fact that there are still very few drugs on the market targeting scaffolding and other non-enzymatic proteins in cells.

Protein degrader drugs or Proteolysis Targeting Chimeras (PROTACs) represent a groundbreaking

paradigm in the field of drug design and targeted therapy. These innovative compounds are engineered to induce the degradation of specific proteins within the cellular environment. Unlike traditional small-molecule inhibitors, protein degraders do not require the identification of precise binding sites on the target protein. PROTACs are composed of three integral components: an E3 ligase ligand, a ligand specific to the protein of interest (POI), and a linker (**Figure 1.7**). This intricate amalgamation leads to the formation of a POI-PROTAC-E3 ligase ternary complex, which, in turn, instigates the ubiquitination and degradation of the POI through the ubiquitin-proteasome system (**Figure 1.7**).

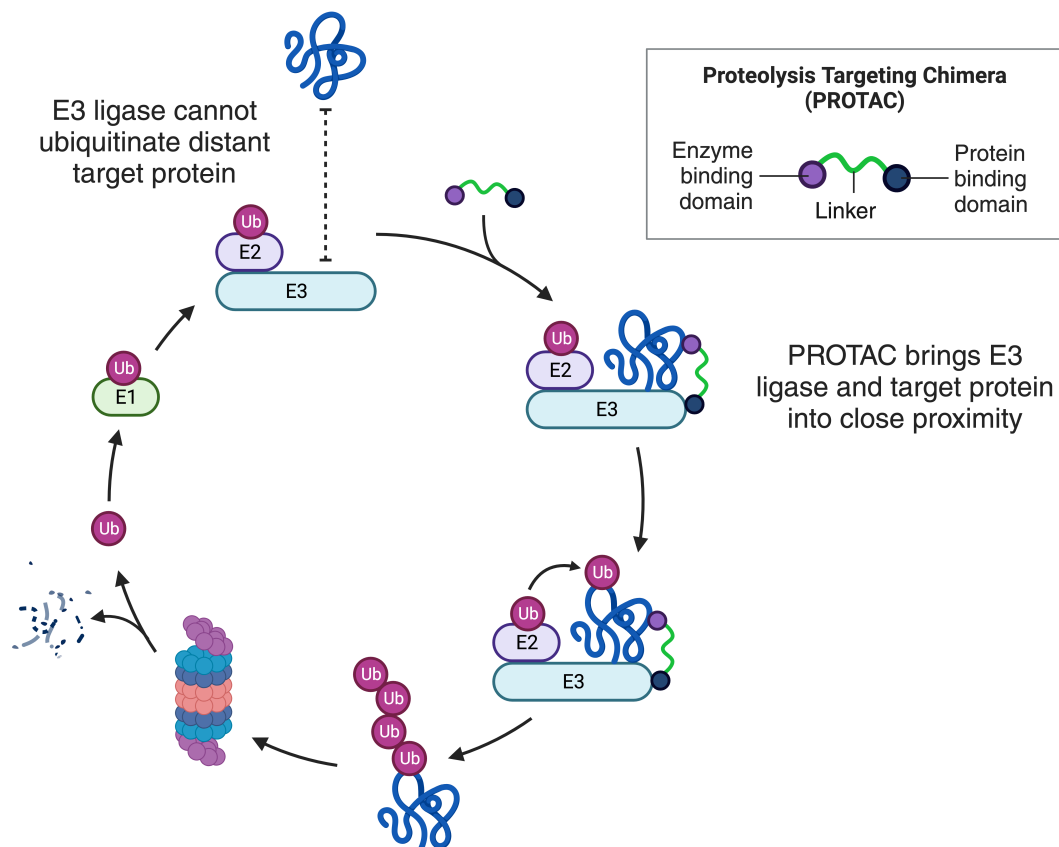


Figure 1.7: Mechanism of action of PROTACs. PROTACs are bivalent compounds that induce the active recruitment of an E3 ligase complex to a specific protein target. This recruitment results in the ubiquitination of the target protein, marking it for elimination by the proteasome. Following this, the compound becomes capable of initiating subsequent cycles of degradation by re-engaging other remaining target proteins. Adapted from Biorender.com

Several seminal studies have illustrated the transformative potential of protein degradation technology in the realm of targeted therapy. In 2014, the anti-tumour activity of the thalidomide-like drug lenalidomide used clinically for the treatment of multiple myeloma was uncovered to be its ability to bind both the cereblon ubiquitin ligase and two specific B cell transcription factors, Ikaros family zinc finger proteins 1 and

3 (IKZF1 and IKZF3) (G. Lu et al., 2014). Since then thalidomide-like drugs have been harnessed to develop PROTACs targeting a wide array of proteins. In 2015, a pioneering investigation in the context of Burkitt’s Lymphoma introduced a PROTAC which effectively targeted the BRD4 protein—a pivotal regulator of cancer gene expression (J. Lu et al., 2015). This innovative approach induced BRD4 degradation, ultimately inhibiting cancer cell growth, and offering a promising avenue for cancer therapy (J. Lu et al., 2015). Notably, a 2020 clinical trial reported on the application of ARV-110, a PROTAC targeting the androgen receptor (AR), in patients with metastatic castration-resistant prostate cancer. This research highlighted the effective degradation of AR, offering a potential therapeutic option for advanced prostate cancer (“Proof-of-Concept with PROTACs in Prostate Cancer”, 2020). A degrader compound capable of selectively degrading the MYCN oncogene, underscored the feasibility of using protein degraders to target specific oncogenic proteins critical in various cancer types (Farrell & Sears, 2014). More recently, oncogenic activity of EP300/CBP in multiple myeloma cells was shown to be further inhibited by complete EP300/CBP degradation compared to inhibition alone (Vannam et al., 2021). These studies collectively represent key milestones in the field of protein degrader drugs. In addition, prominent pharmaceutical companies have actively embraced the PROTAC technology, with industry leaders including Arvinas, Nurix Therapeutics, Kymera Therapeutics, C4 Therapeutics, Bristol-Myers Squibb, and Novartis, among others, adopting the PROTAC platform. By the close of 2021, no less than 15 PROTACs had progressed into clinical trials underscoring the increasing momentum of PROTAC-based therapies in the pharmaceutical landscape. In this research project, the availability of PROTACs targeting proteins of therapeutic relevance in SS were embraced.

1.8 Aim of this thesis

Previous work from our lab and others has demonstrated that targeting SWI/SNF complexes in synovial sarcoma using small molecules can yield significant preclinical responses. These discoveries have led to the initiation of clinical trials in synovial sarcoma patients. The primary aim of this thesis was to extend these findings to examine additional opportunities to therapeutically target oncogenic SS18-SSX-containing SWI/SNF complexes in this malignancy.

In research chapter 1, publicly available CRISPR screening data were harnessed to explore the genetic dependencies of all SWI/SNF subunits in synovial sarcoma cells. This analysis revealed the catalytic SWI/SNF

subunit SMARCA4 as essential in synovial sarcoma cell lines. Following on, the novel concept of complete protein degradation was embraced and utilized to characterize the biochemical consequences of SMARCA4 degradation. In research chapter 2, the transcriptional effects and chromatin dynamics associated with SMARCA4 degradation were evaluated. In the initial study from our lab which identified BRD9 as a critical functional dependency in synovial sarcoma, the E1A-binding protein p300 (EP300) was also identified as a promising therapeutic target. The goal of the third research chapter was to follow up on this finding and to investigate the effect of perturbing EP300 function on the transcriptional program of synovial sarcoma cells.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Cell lines and culture

All cell lines were maintained at 37°C in a humidified incubator with 5% CO₂. HSSYII cells were cultured in DMEM-high glucose (Sigma) supplemented with 10% (v/v) FBS (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). CME1 cells were cultured in RPMI media (Gibco) supplemented with 10% (v/v) FBS (Gibco), 100 U/mL penicillin, and 100 µg/ml streptomycin (Gibco). Trypsin-EDTA (Gibco) was used for passaging all cell lines.

Mouse embryonic stem cells (mESCs) were cultured under 2i/LIF conditions in Glasgow Minimum Essential Medium (GMEM; Sigma) supplemented with 20% v/v Heat-Inactivated FBS, 100 µg/ml penicillin/streptomycin (Gibco), 50 µM β-mercaptoethanol (Sigma), 1:100 Glutamax (Gibco), 1:100 on 0.1% gelatin-coated plates and passaged every two days.

HEK293T cells were cultured in DMEM-high glucose (Sigma) supplemented with 10% (v/v) FBS (Gibco), 100 U/mL penicillin, and 100 µg/ml streptomycin (Gibco).

C2C12 mouse myoblasts were cultured in DMEM-high glucose (Sigma) supplemented with 10% (v/v) FBS (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) and passaged every two days.

2.1.2 Lentivirus production

To generate lentiviral particles HEK293T cells were transected simultaneously with three plasmids, the lentiviral construct plasmid of interest, psPAX2 (Addgene #12259) and pCMV-VSVG (Addgene #8474). A transfection mix containing a 3:1 ratio of Polyethylenimine:DNA was made in Opti-MEM serum-free media (Gibco). Transfection mixes were added directly to HEK293T cell cultures, and lentiviral particles containing media supernatants were collected 72 hours post-transfection and filtered using a 0.45 μ M syringe filter to remove cellular debris. For lentiviral infections, 8.5 μ g/ml polybrene was added to each viral supernatant, which was used directly to infect target cells.

2.1.3 Differentiation of C2C12 cells

Upon reaching 70% confluence Parental and SS18-SSX1 expressing C2C12 cells were divided into three groups. The no differentiation group was treated with DMSO or 2 μ M Asunaprevir for 24 hours. After this 24-hour treatment RNA and protein were harvested. The 4 and 6-day differentiation groups were treated with DMSO or 2 μ M Asunaprevir for 24 hours and maintained exposed to the compounds for the course of the experiment. The next day differentiation was initiated by replacing 10% FBS (Gibco) with 2% horse serum (Gibco) for 4 and 6 days respectively. The differentiation media was replaced every 2 days.

2.2 Cell viability assays

2.2.1 CellTiter-Glo[®] Luminescent Cell Viability Assay

HSSYII and CME1 SS cell lines were seeded in white 96-well flat bottom plates (greiner) at a density of 15,000 cells per well. After 24 hours a freshly prepared stock of 1 μ M ACBI1 or DMSO was added. CellTiter-Glo[®] (CTG) Luminescent Cell Viability Assay was performed following the manufacturer’s instructions at 48 and 72-hours post-treatment.

2.2.2 Manual cell counting

HSSYII and CME1 SS cell lines were seeded in 6-well plates at a density of 100,000 cells per well. After 24 hours a freshly prepared stock of 1 μ M ACBI1 or DMSO was added for up to 8 days. The compound containing media was replaced every 48 hours. Cumulative cell number was calculated at 2, 5 and 8 days.

2.3 DepMap data mining

2.3.1 Accessing and visualising DepMap data

For the SS-specific analysis: The Depmap R package (Laurent Gatto and Theo Killian, 2022) was used to access and prepare visualisations of the 22Q1 release of the DepMap project. This data was filtered for all cell lines denoted as synovial sarcoma and further filtered for known SWI/SNF components. Genes were designated as potential dependencies if the first quartile [Q1] was ≤ -0.5 . For the broader lineage analysis: All lineage subtypes catalogued in DepMap represented by ≥ 2 cell lines were included.

2.4 Protein Methods

2.4.1 Preparation of whole cell protein lysates and immunoblotting

Cell lines were detached from their culture vessels using trypsin and centrifuged at 400G for 3 minutes, followed by two washes with cold Phosphate-buffered saline (PBS). The cell pellets were subsequently lysed with an appropriate volume of RIPA buffer (10mM Tris-HCl pH 8, 1mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, and 140mM NaCl) supplemented with 1X Halt™ protease inhibitor cocktail (Thermo Scientific). Cell lysates were incubated on ice for 30 minutes followed by sonication and an additional 30-minute incubation on ice. Cell lysates were then centrifuged at max speed for 15 minutes at 4°C. Protein concentrations were determined via the Bradford assay and samples normalized to the same concentration.

Protein lysates were separated on pre-cast NuPAGE™ Bis-Tris 4-12% gels (Invitrogen, NP0321BOX) and transferred to PVDF membranes (MilliporeSigma™ Immobilon™-FL PVDF). Membranes were blocked against non-specific binding by incubation with Intercept (PBS) Blocking Buffer (LI-COR) for 1 hour. Membranes were subsequently probed with the primary antibody of interest overnight at 4°C. Following extensive washes, membranes were subsequently incubated with the appropriate secondary antibodies for 1 hour (labeled with near-infrared dye). Following secondary antibody incubation, the membranes were extensively washed and relative protein levels were determined using the Odyssey CLx Imager (LI-COR).

2.4.2 Endogenous protein immunoprecipitation and mass spectrometry

Cells were harvested by scraping and washed twice in cold PBS. Washed whole cell pellets were resuspended in Buffer A (25mM HEPES pH 7.6, 5mM MgCl₂, 25mM KCl, 0.05mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) NP40) supplemented with 1X Halt[®] protease inhibitor cocktail, 1mM PMSF, and 1mM DTT and rotated at 4°C for 10 minutes to extract nuclei. The samples were subsequently centrifuged at 500G 4°C for 10 minutes to pellet nuclei. The supernatant was removed, and pelleted nuclei were resuspended in an appropriate volume of Buffer C (10mM HEPES pH 7.6, 3mM MgCl₂, 100mM KCl, 0.5mM EDTA, 10% (v/v) glycerol supplemented with 1X Halt[®] protease inhibitor cocktail, 1mM PMSF, and 1mM DTT, 300 mM ammonium sulfate (NH₄)₂SO₄) and rotated at 4°C for 20 minutes. Lysed nuclei were ultracentrifuged at 350,000G for 15 minutes at 4°C in a Beckman Coulter Optima L-100XP using a SW 55 Ti rotor. The supernatant was collected, and 300mg of (NH₄)₂SO₄ was added per ml of solution, vortexed, and incubated on ice for 20 minutes to precipitate nuclear extracts. Samples were subjected to the same ultracentrifugation procedure as outlined above; the supernatant was then discarded, and the pelleted nuclear protein was resuspended in an appropriate volume of IP Buffer (300 mM NaCl, 50mM Tris-HCl pH 7.5, 1mM EDTA, 1% (v/v) Triton-X-100) supplemented with 1X Halt[®] protease inhibitor cocktail, 1mM PMSF, and 1mM DTT, and Bradford assays were performed to determine protein concentration. 1mg of protein was prepared in 500 μ l of IP buffer. At this point, input samples were taken. 1 μ l of Benzonase nuclease (Sigma) was added to each sample, and 1 μ g of antibody was used for each IP, and samples were rotated overnight at 4°C in protein-lo bind tubes (Eppendorf).

Following overnight incubation, Protein G Dynabeads (Invitrogen) were washed 5x with IP buffer, and 20 μ l of beads were added to each IP sample and incubated with rotation at 4°C for 2 hours. Beads were collected on a magnetic rack and washed 5 times in IP buffer. Our collaborator Dr Giorgio Oliviero (University College Dublin) conducted the mass spectrometry sample preparation and analysis. The human UniProt database was used for processing the mass spectrometry data with MaxQaunt software. The Differential Enrichment analysis of Proteomics data (DEP) R package (X. Zhang et al., 2018) was used to analyse the tabular output file generated by MaxQuant.

2.4.3 Subcellular fractionation and chromatin mass spectrometry

Cells were harvested and washed twice with 4°C PBS. The volume of the pellet was estimated, resuspended in 2 pellet volumes of Buffer A (25 mM HEPES pH 7.6, 5 mM MgCl₂, 25 mM KCl, 0.05 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) NP40, 1 mM DTT, 1mM Halt[®] and 1 mM PMSF) and incubated on ice for 15 minutes. The suspension was transferred to a dounce homogenizer and dounced 8 times with a type A (loose) pestle. The lysate was centrifuged for 5 minutes at 500G in a 4 °C centrifuge, the resulting supernatant which contains cytoplasmic proteins was collected. The pellet was washed once more in buffer A and the supernatant discarded. To remove any residual supernatant the pellet was centrifuged at max speed and any remaining supernatant carefully removed. The pellet was resuspended in 2 pellet volumes of buffer S1 (120 mM NaCl, 20 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, 1mM Halt[®] and 1 mM PMSF) transferred to a dounce homogenizer and dounced 15 times with a type B (tight) pestle. The lysate was centrifuged for 5 minutes at max speed at 4 °C. The supernatant containing nuclear soluble protein was collected. The pellet was washed once with buffer S1 and any remaining supernatant was carefully discarded. The pellet was resuspended in 2 pellet volumes buffer S2 (420 mM NaCl, 20 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, 1mM Halt[®] and 1 mM PMSF) and incubated for 30 minutes at 4°C with rotation. Buffer S2 was diluted to 210 mM NaCl by adding an equal volume of buffer D (25 mM HEPES, 5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 1mM Halt[®] and 1 mM PMSF) Benzonase nuclease (250 U/ μ l) was added to the solution at a final concentration of (0.15 U/ μ l) and incubated for 1.5 hours at 4°C. The insoluble fraction was separated from the chromatin bound protein extract by centrifugation at max speed for 30 minutes. The resulting supernatant containing the chromatin bound protein fraction was collected. The protein concentration of each respective sub-cellular fraction was calculated using the Bradford assay.

For immunoblotting all samples were normalised to the same concentration. For the purposes of mass spectrometry, 30 μ g of the chromatin bound fraction was utilised. Our collaborator Dr Giorgio Oliviero (University College Dublin) conducted the mass spectrometry sample preparation and analysis. The human UniProt database was used for processing the mass spectrometry data with MaxQuant software. The Differential Enrichment analysis of Proteomics data (DEP) R package (X. Zhang et al., 2018) was used to analyse the tabular output file generated by MaxQuant.

2.5 Cleavage Under Targets and Release Using Nuclease (CUT&RUN)

2.5.1 CUT&RUN-Rx sample preparation

HSSVII cells were harvested using Accutase (STEMCELL Technologies). Cells were counted and mESC's were spiked-in at a 1:10 ratio. Hybrid cell suspensions were cross-linked lightly in the culture medium. 37% formaldehyde was added to achieve a final concentration of 0.1%, and the cells were incubated on a rotator for 1 minute. The crosslinking reaction was quenched by adding 1/20 of the culture volume of 2.5M Glycine and incubating for a further 5 minutes. Fixed cells were washed with PBS and incubated on ice for 10 minutes in nuclear extraction buffer (20mM HEPES pH 7.5, 10mM KCL, 0.1% Triton X-100, 20% Glycerol, 1X protease inhibitor cocktail and 0.5mM spermidine). Extracted nuclei were collected by centrifugation at 4°at 600G. Extracted nuclei were then resuspended in 100 μ l per sample nuclear extraction buffer, and then 100 μ l of the nuclei suspension was added to 10 μ l of ConA bead suspension.

The nuclei:bead slurry was then incubated in PCR strip tubes for 10 minutes at room temperature. The PCR strip tube was then placed into a magnetic stand, and the supernatant was removed. Nuclei:bead conjugates were then resuspended in 50 μ l of cold antibody buffer (20 mMHEPES pH 7.5, NaCl 150 mM, EDTA 2 mM, Spermidine 0.5 mM, Triton X-100 1%, SDS 0.05%, digitonin 0.0025%) and the relevant antibody was added. The samples were incubated overnight on a nutator at 4°C. The following day, the PCR strip tube was placed on a magnetic stand, and the supernatant was removed. The nuclei:bead conjugates were subsequently washed twice in 250 μ l digitonin buffer (20 mM HEPES pH 7.5, NaCl 150 mM, Spermidine 0.5 mM Spermidine, Triton X-100 1%, SDS 0.05%, digitonin 0.0025%) before being resuspended in 50 μ l of the same buffer. 2.5 μ l of CUTANA pAG-MNase (EpiCypher, cat# 15-1016) was added to each sample and incubated at room temperature for 10 minutes. Following this incubation step, the PCR strip was returned to the magnetic stand, the supernatant was removed, and the nuclei:bead conjugates were washed twice with 250 μ l of digitonin buffer prior to resuspension in 50 μ l digitonin buffer. The PCR strip tube was then chilled on ice for 5 minutes prior to the addition of 1 μ l of 100 mM CaCl₂ to each sample. The samples were then incubated for 2 hours on a nutator at 4°C. Following incubation, 33 μ l of stop buffer (340 mM NaCl, 20 mM EDTA, 4 mM EGTA, 50 μ g/ml RNase A, 50 μ g/ml Glycogen) was added to each sample. Samples were

incubated at 37°C for 10 minutes in a thermocycler. The PCR strip tube was then returned to the magnet and the DNA-containing supernatant was added to a 1.5 ml Eppendorf tube. Cross-links were reversed prior to NEB column clean-up (New England Biolabs, cat# T1030S) by adding SDS to a final concentration of 0.09% and Proteinase K to a final concentration of 0.22 $\mu\text{g}/\text{ul}$ and incubating at 55°C overnight.

To facilitate qPCR analysis, a 1% input sample was taken for each condition. This involved lysing an additional 1 million cells in sonication buffer (HEPES 50mM, NaCl 140 mM, EDTA 1mM, EGTA 1mM, Triton X-100 1%, Na-DOC 0.1%, SDS 0.9%) and sonicating under optimized sonication conditions to achieve DNA fragments between 100 and 600 bp in length. The sonicated cell lysate was then incubated at 37°C with RNase A at a final concentration of 0.2 $\mu\text{g}/\text{uL}$ for 2 hours, followed by a second incubation at 55°C with Proteinase K at a final concentration of 0.2 $\mu\text{g}/\text{ul}$ for 2 hours. DNA was then purified using the NEB Monarch DNA Clean-up Kit.

2.5.2 Construction and Sequencing of CUT&RUN Libraries

Illumina compatible CUT&RUN-seq libraries were prepared using the NEBNext Ultra II Library Prep Kit (NEB) as per the manufacturer’s instructions. Quantity and quality of the finished libraries were assessed using the Qubit fluorometer (Invitrogen) and high sensitivity DNA screentape analysis (Agilent Technologies, Inc.) as per the manufacturer’s instructions. Individually indexed libraries were pooled and processed for 75 bp paired-end sequencing on a NextSeq550 sequencer (Illumina) using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404-2005) as per the manufacturer’s instructions.

2.5.3 CUT&RUN-seq Data Analysis

Firstly, the prefix mm10_ was added to the chromosome names of the mm10 genome build. Next, a human/mouse hybrid genome was generated by appending the human hg38 genome build to the mm10 genome build prior to indexing with bowtie2 (Langmead & Salzberg, 2012) as outlined (Brien et al., 2021). Bowtie2 was used to align reads to the hybrid genome in the –very-sensitive mode. SAMtools (H. Li et al., 2009) was employed to only retain reads with a mapping quality ≥ 2 , separate the human and mouse reads, remove duplicate reads and convert SAM files to BAM files. As outlined (Orlando et al., 2014) the spike-in normalisation factor for each target was calculated using the formula for normalised reference-adjusted reads per million (RRPM) (1 per million spike-in reads). The bamCoverage module from the deepTools suite (Ramírez

et al., 2016) was used to generate RRPM normalised bigwig files for genome browser visualisation with the following parameters where SCALEFACTOR is equal to the respective RRPM normalization factor

```
-bs 10 --smoothLength 30 -e 200 -ignore ChrM --scaleFactor $SCALEFACTOR
```

Peaks were called using macs2 (Y. Zhang et al., 2008) with the following parameters using the corresponding IgG sample as the control file:

```
macs2 callpeak -t ${IP_BAM}.dedup.bam -c ${IgG_BAM}.dedup.bam -f BAMPE --broad --broad-cutoff 0.0  
--nomodel --extsize 200
```

The resulting BroadPeak files were converted to bed format using standard bash commands. The bedtools intersect module (Quinlan & Hall, 2010) was used to exclude regions that overlapped with a custom hg38 blacklist. CUT&RUN peaks were annotated with HOMER (Heinz et al., 2010).

The MSPC tool (Jalili, Matteucci, Masseroli, & Morelli, 2018) was used to define consensus peaks as peaks common to at least 2 replicates using the following parameters:

```
-r Bio -w 1e-4 -s 1e-8
```

RRPM normalised bigwig files displaying the average signal for all replicates were generated using custom bash scripts. Specifically, BigWigToBedGraph (Kuhn, Haussler, & Kent, 2013) was used to convert each replicate RRPM bigwig to a bedgraph file. Next, each bedgraph file was intersected with a hg38 bed file divided into 10bp bins. The bedgraph was sorted and converted back to a bigwig file using the bedGraphToBigWig utility (Kuhn et al., 2013). Finally, resulting replicate bigwig files were merged into a single bedgraph using the BigWigMerge module (Kuhn et al., 2013). The merged bedgraph file was sorted and gawk was used to compute the average signal value. The resulting average bedgraph file was converted to an average bigwig file with bedGraphToBigWig utility (Kuhn et al., 2013) utility.

2.6 RNA methods

2.6.1 RNA Extraction, Synthetic RNA Spike-in and Library Preparation

RNA was extracted from 1 million cells using the RNeasy Mini Kit (Qiagen, 74106) as per the manufacturer's instructions. Extracted RNA quality was assessed using the TapeStation (Agilent) with the RNA ScreenTape

assay reagents (Agilent, 5067-5576). A fixed amount of diluted ERCC Spike-in Control Mix #1 was then added to the total RNA yield. The external control spike-ins used were obtained from the ERCC ExFold RNA Spike-In kit (Invitrogen, 4456740). The ERCC RNA Spike-In Control Mixes comprise a set of 92 polyadenylated transcripts that mimic natural eukaryotic mRNAs. The RNAs range in size from 250–2,000 nucleotides in length and span an approximately 106-fold concentration range. For these experiments, 5 μ l of a 1:10 dilution of Mix #1 was added. 1 μ g of total RNA containing ERCC ExFold Mix #1 RNA spike-in controls was used per sample as input material to generate Illumina compatible libraries with the NEBNext Ultra RNA Library Prep kit (NEB, E7530L) as per the manufacturer’s instructions. Quantity and quality of the finished libraries were assessed using the Qubit fluorometer (Invitrogen) and the TapeStation (Agilent) with the D1000 ScreenTape assay reagents (Agilent, 5067–5583). Individually indexed libraries were pooled and processed for 75 bp paired-end sequencing on a NextSeq550 sequencer (Illumina) using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404-2005) as per the manufacturer’s instructions.

2.6.2 RNA sequencing data analysis

FASTQ files were quality controlled via FastQC (*FastQC*, 2015) and RNA-seq reads were aligned to the human reference genome where the sequences of the ERCC synthetic spike-in RNAs had been added, using the Spliced Transcripts Alignment to a Reference (STAR) software (Dobin et al., 2013) Resulting SAM files were converted to sorted indexed BAM files via samtools (H. Li et al., 2009) Read alignments were assigned to gene identifier features annotated in the Ensembl 98 hg38 genome annotation and ERCC92.gtf annotation file using featureCounts (Liao, Smyth, & Shi, 2014). RNA-Seq counts were normalized using size factors calculated from ERCC spike-in controls prior to differential expression testing with the DESeq2 R package (Love, Huber, & Anders, 2014). Differentially expressed genes were detected using a ≥ 1.5 fold change and a < 0.05 Benjamini-Hochberg adjusted P value. Gene ontology analysis was conducted with the clusterProfiler R package (Wu et al., 2021) with a pvalueCutoff = 0.05. Gene set enrichment analysis (GSEA) was carried out with the FGSEA R package (Sergushichev, 2016). The R package EnhancedVolcano (Blighe, Rana, & Lewis, 2022) was used to generate volcano plot visualisations.

2.6.3 QuantSeq 3' mRNA-seq library preparation

Three replicates per condition of total RNA was extracted from cells using the RNeasy kit (Qiagen) as per the manufacturer's instructions. The QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen) was used to prepare Illumina compatible cDNA libraries from 500 ng input RNA as per the manufacturer's protocol. As suggested the PCR Add-on Kit for Illumina (Lexogen) was used to calculate the optimal number of cycles to include in the endpoint PCR. Quantity and quality of the finished libraries were assessed using the Qubit fluorometer (Invitrogen) and high sensitivity DNA screen-tape analysis (Agilent Technologies, Inc.) as per manufacturer's instructions. Individually indexed libraries were pooled and processed for 150 bp paired-end sequencing on a NextSeq550 sequencer (Illumina) using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404-2005) as per the manufacturer's instructions.

2.6.4 QuantSeq 3' mRNA-seq data analysis

The BBDuk module from the BBTools package was used to perform adapter trimming and quality filtering as recommended by the manufacturer with the following parameters:

```
ref=bbmapAdapters.fa
literal=AAAAAAAAA
k=13
ktrim=r
forcetrimleft=11
useshortkmers=t
mink=5
qtrim=t
trimq=10
minlength=20
```

The STAR aligner was used with the following parameters to align filtered reads to the hg38 human genome build:

```
--outFilterType BySJout
--outFilterMultimapNmax 20
```

```

--alignSJoverhangMin 8
--alignSJDBoverhangMin 1
--outFilterMismatchNmax 999
--outFilterMismatchNoverLmax 0.6
--alignIntronMin 20
--alignIntronMax 1000000
--alignMatesGapMax 1000000
--outSAMattributes NH HI NM MD
--outSAMtype BAM SortedByCoordinate

```

The htseq-count tool was used to calculate the number of reads that align to each gene with the following parameters

```

-m intersection-nonempty --nonunique all -s yes -f bam -r pos

```

Differentially expressed genes were detected using a ≥ 1.5 fold change and a < 0.05 Benjamini-Hochberg adjusted P value. Gene ontology analysis was conducted with the clusterProfiler R package (Wu et al., 2021) with a pvalueCutoff = 0.05. Gene set enrichment analysis (GSEA) was carried out with the FGSEA R package (Sergushichev, 2016). The R package EnhancedVolcano (Blighe et al., 2022) was used to generate volcano plot visualisations.

2.7 ATAC-Seq

2.7.1 Omni-ATAC-seq library preparation

Omni-ATAC-seq was carried out as per the established protocol (Corces et al., 2017). In brief, synovial sarcoma cell lines were treated with 1 μ M ACBI1 or DMSO for the indicated time course. Prior to harvesting cell cultures, they were treated with DNase at 200 U/ml for 30 minutes at 37°C. 50,000 viable cells were resuspended in 50 μ l of ATAC-Resuspension-Buffer (ATAC-RSB) (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl₂) + 0.1% Tween-20, 0.1% NP40, and 0.01% Digitonin and incubated at 4°C for 3 minutes. Cells were subsequently washed with 1 ml of (ATAC-RSB) + 0.1% Tween-20. Extracted nuclei were then collected via centrifugation and resuspended in 50 μ l transposition mixture (25 μ l 2x TD buffer, 2.5 μ l transposase

(100nM final), 16.5 μ l PBS, 0.5 μ l 1% digitonin, 0.5 μ l 10% Tween-20, 5 μ l H₂O) and incubated at 37°C for 30 minutes.

On completion of the transposition reaction, transposed DNA fragments were purified using Qiagen MinElute columns. The eluted DNA was used for PCR amplification to add Illumina adapter and index sequences and subsequently purified using AMPure beads. Quantity and quality of the finished libraries were assessed using the Qubit fluorometer (Invitrogen) and the TapeStation (Agilent) with the D1000 ScreenTape assay reagents (Agilent, 5067-5583). Individually indexed libraries were pooled and processed for 75 bp paired-end sequencing on a NextSeq550 sequencer (Illumina) using the NextSeq 500 High Output v2 kit (75 cycle) (Illumina, FC-404-2005) as per the manufacturer’s instructions.

2.7.2 Omni-ATAC-seq data analysis

ATAC-seq reads were quality controlled via FastQC (*FastQC*, 2015) and aligned to the human reference genome (hg38) with Bowtie2 (Langmead & Salzberg, 2012) with the following parameters:

```
--paired --very-sensitive -X 1000
```

The resulting SAM files were converted to sorted indexed BAM files via samtools (H. Li et al., 2009), and quality control analysis was conducted using the ATACseqQC R package (Ou et al., 2018).

The previously described ATAC-seq pipeline optimized for accurate differential accessibility analysis (Reske, Wilson, & Chandler, 2020) was adapted for use with three replicates. In brief, the pipeline was used to remove mitochondrial reads, remove discordant read pairs, subsample the reads to an even molecular complexity, and remove duplicate reads. Peaks were called using MACS2 (Y. Zhang et al., 2008), blacklisted regions were filtered, and differentially accessible regions were identified using csaw (Lun & Smyth, 2016) with loess-normalization in MACS2 only mode.

Chapter 3

Investigating *SMARCA4* dependency and degradation in synovial sarcoma

3.1 Introduction

As explained in the main introduction **subsection 1.5.4** previous work demonstrated that targeting SWI/SNF complexes in synovial sarcoma using small molecules can produce significant therapeutic responses, contributing to the initiation of clinical trials in patients with synovial sarcoma (Brien et al., 2018; Michel et al., 2018) (ClinicalTrials.gov Identifiers: NCT05355753, NCT04965753). The main aim of this chapter was to extend these initial findings to examine additional opportunities to therapeutically target oncogenic SWI/SNF complexes in this disease. To this end, publicly available data generated and released by the DepMap initiative (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.) was leveraged. The DepMap project is a collaborative effort focused on mapping and understanding the genetic dependencies of cancer cells. The project uses CRISPR gene knockout as the primary method to detect genomic dependencies, and at the time of writing (August 2023) > 1800 human cancer cell lines were mapped for dependencies. This large-scale screening approach is one of the main strengths of the initiative. Specifically, DepMap CRISPR screening data was used to investigate all SWI/SNF subunits as genetic dependencies with the ultimate goal of detecting novel vulnerabilities that display a selective effect on SS cells.

From this approach the catalytic SWI/SNF subunit SMARCA4 was identified as a promising therapeutic

target. Although SS has long been regarded as a quintessential SWI/SNF perturbed cancer, to date no published research has performed a thorough investigation into the functional role of SMARCA4 in this disease or evaluated SMARCA4 targeting as a potential therapeutic option (Kadoch & Crabtree, 2013; Brien et al., 2018; McBride et al., 2018; Banito et al., 2018; Michel et al., 2018).

An additional incentive to pursue SMARCA4 targeting was derived from an elegant experiment characterising the composition of endogenous SS18-SSX containing SWI/SNF complexes by combining SS18-SSX immunoprecipitation with mass spectrometry (IP-MS), performed by Dr. Gerard Brien (**Figure 3.1**). In 2 distinct synovial sarcoma cell lines, SMARCA4 was found to be one of the most abundant components of complexes containing SS18-SSX, strongly suggestive of functional importance (**Figure 3.1 C**). This observation gains significance when considering that BRD9, previously identified as a promising therapeutic target in SS (Michel et al., 2018; Brien et al., 2018), is a much less abundant SS18-SSX interactor, co-purifying with the fusion protein in approximately 10% of cases (**Figure 3.1 C**). Identifying and targeting more abundant interactors could potentially offer more impactful and complementary therapeutic opportunities.

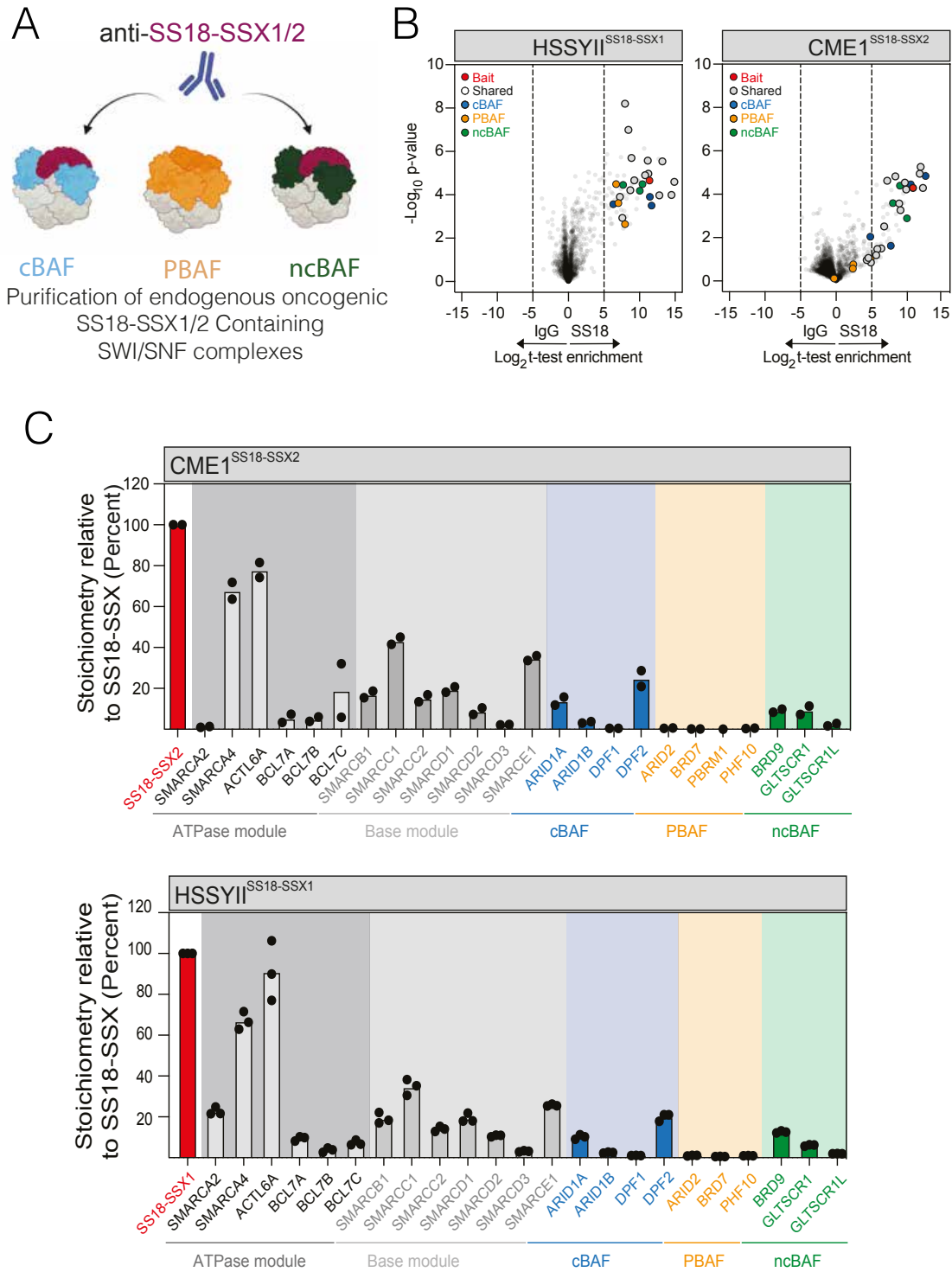


Figure 3.1: SMARCA4 is one of the most abundant components of SS18-SSX containing complexes.

A. Schematic of the fusion protein (SS18-SSX1/2) IP.

B. SS18-SSX1/2 IP coupled with mass spectrometry analysis in HSSYII and CME1 cells treated. The label-free quantification intensity of the SS18 IP over the IgG IP is plotted against the $-\log_{10} p\text{-value}$. SWI/SNF components are colour-coded.

C. Stoichiometry of SS18-SSX interactors in HSSYII (top) and CME1 cell lines (bottom). The intensity based absolute quantification (iBAQ) value of each protein was corrected for the obtained abundance in the control IgG IP. The remaining values were divided by the iBAQ value of SS18-SSX, which was set to 100% ($n = 3$ pulldowns, mean $\pm$ SD).

A revolutionary approach in drug design is the emergence of protein-degrader compounds or PROTACs (Lai & Crews, 2016). The strengths of these compounds over more traditional methods have been outlined in the main introduction **section 1.7**. A secondary objective of this chapter was to evaluate the degrading properties of the SMARCA4 PROTAC, **ACBI1** (Farnaby et al., 2019) and characterise the downstream biochemical consequences in SS cells.

With this goal in mind, the degradation properties of ACBI1 were evaluated and unbiased quantitative time-resolved chromatin proteomic profiling of SS cells was performed after ACBI1 treatment to assess the ability of ACBI1 to degrade chromatin-bound SMARCA4 and to understand the broader effects of the compound. Following on, time-resolved quantitative SMARCC1 immunoprecipitations coupled with mass spectrometry were carried out (IP-MS). Efforts were made to prioritise endogenous SMARCC1 immunoprecipitations over analogous experiments involving an exogenous tag. This approach was chosen because immunoprecipitating endogenous proteins helps prevent any possible interference from introducing an exogenous tag. This precaution ensures that the natural ability of the bait to interact with other proteins remains undisturbed.

3.2 Results

3.2.1 Exploiting the DepMap cancer dependency data

DepMap CRISPR screening data was used to investigate all SWI/SNF subunits as genetic dependencies with the goal of detecting novel vulnerabilities that display a selective effect on synovial sarcoma cells. (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.). Specifically, all SWI/SNF components were grouped by subcomplex and the CRISPR dependency score for all cell lines denoted SS was visualised using a boxplot (**Figure 3.2 A**). This indicated six genes as potential therapeutic vulnerabilities in SS. Genes were designated as potential dependencies if the first quartile [Q1] of the CRISPR dependency score was ≤ -0.5 . This analysis supported published data from our lab and others that BRD9 is a vulnerability in SS cells (Brien et al., 2018). It also confirmed dependence on the pathognomonic fusion protein SS18-SSX (**Figure 3.2 A**). In SS18-SSX the C-terminal 8 amino acids of the SS18 protein are replaced by 78 amino acids from the C-terminus of 1 of 3 closely related proteins SSX1, SSX2 or SSX4 (Clark et al., 1994; de Leeuw et al., 1995; Skytting et al., 1999). From this approach, the cBAF-specific subunit *DPF2* and the

shared subunits *ACTL6A* and *SMARCA4* (**Figure 3.2 A**) were identified as novel vulnerabilities in SS cells (**Figure 3.2 A**). A heightened dependency was observed on *SMARCA4* (mean score of -0.75) and *ACTL6A* (-1.2), surpassing the published and promising dependency on *BRD9* (mean score of -0.3) and the oncogenic fusion protein itself (-0.6) (**Figure 3.2 A**). This emphasised the importance of further investigating these proteins, marking them as valuable targets for exploration.

To explore the relationship between the identified genetic dependencies and their corresponding gene expression levels, an analysis comparing the expression levels of all SWI/SNF subunits was conducted. This comparison utilized available RNA-seq data from the SS cell lines. The analysis revealed that *ACTL6A* and *SMARCA4* are among the most highly expressed SWI/SNF subunits (**Figure 3.2 B**). The apparent lack of dependency on the *SMARCA4* paralogue, *SMARCA2*, may be attributed to its lower expression in SS cells (**Figure 3.2 B**).

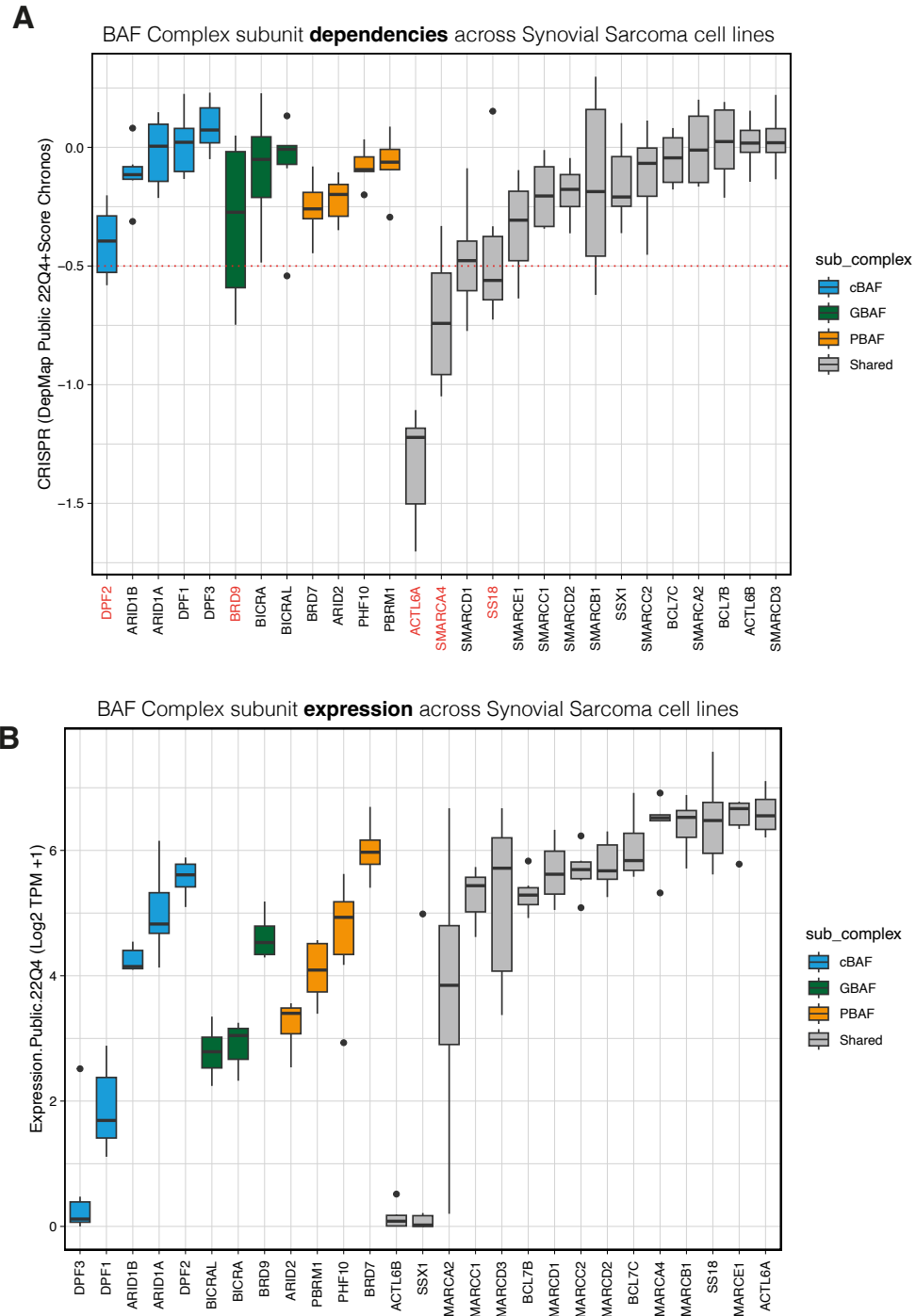


Figure 3.2: Investigating SWI/SNF complex subunit dependencies and expression levels across the synovial sarcoma cell lines in DepMap.

A. SWI/SNF complex subunit dependencies across the synovial sarcoma cell lines in DepMap. Box plots of the median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as solid points. Subunits are colour coded by sub complex incorporation.

B. SWI/SNF complex subunit expression across the synovial sarcoma cell lines in DepMap. Box plots of the median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as solid points. Subunits are colour coded by sub complex incorporation.

The ultimate goal was to identify and pursue impactful vulnerabilities that display a selective effect on SS cells. To this end, all of the cell lines available in DepMap were grouped by lineage subtype and the CRISPR score for each of the identified targets was plotted as a boxplot.

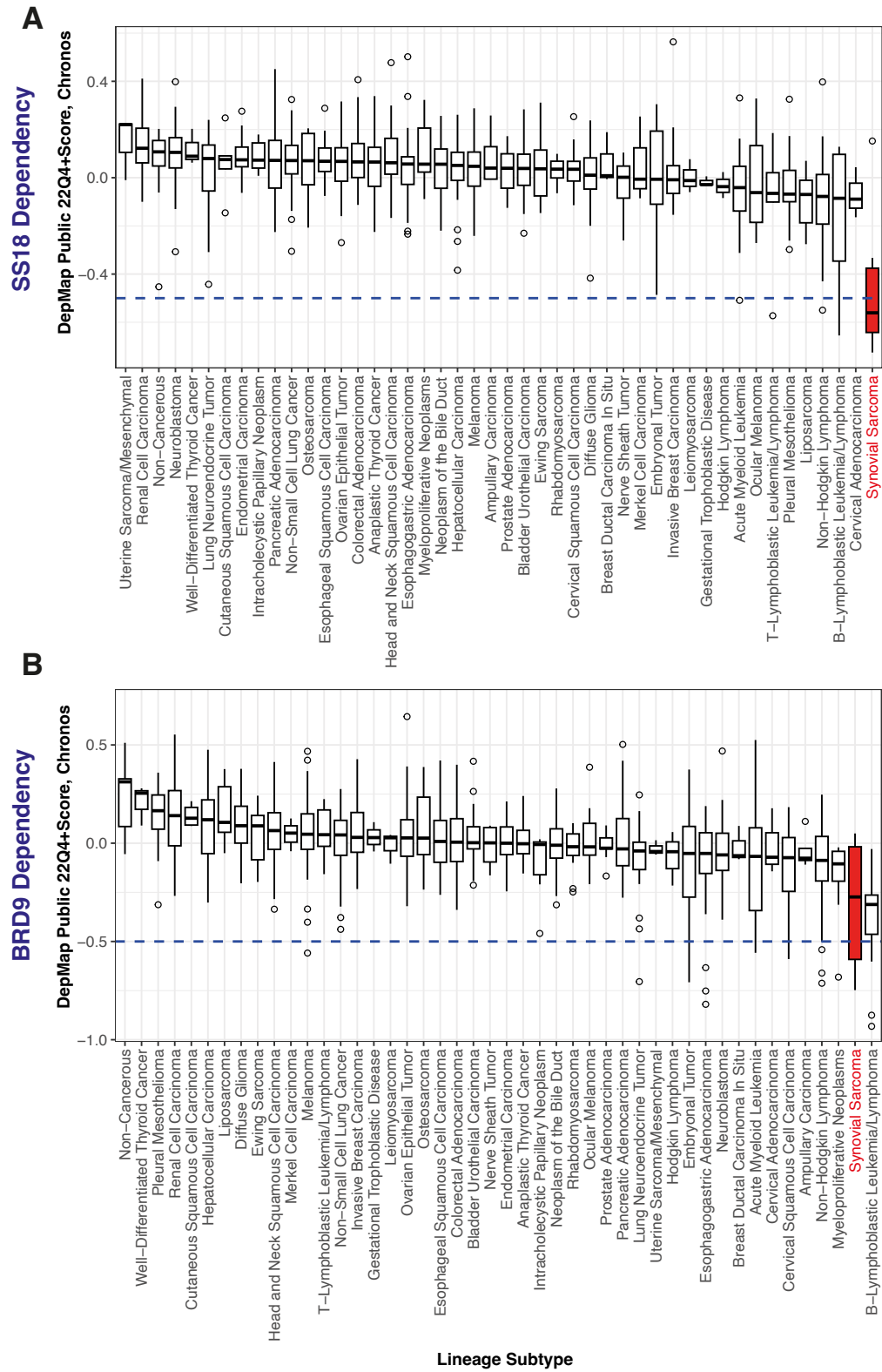


Figure 3.3: The relative dependence of synovial sarcoma cells on SS18 and BRD9 compared to other lineage subtypes.

A. The relative dependence of SS cells on SS18. Box plots of median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as open points.

B. The relative dependence of SS cells on BRD9. Box plots of median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as open points.

SS18 (**Figure 3.3 A**) and *BRD9* (**Figure 3.3 B**) were plotted as positive controls and served as a baseline for the additional targets. As expected, synovial sarcoma was found to be the only context where *SS18* was highlighted as a dependency (**Figure 3.3 A**). In regards to *BRD9*, a strong selectivity on the synovial sarcoma lineage and the B-Lymphoblastic Leukemia/Lymphoma lineages was observed (**Figure 3.3 B**). In stark contrast to the *SS18* and *BRD9* result, *ACTL6A* displayed a strong indiscriminate effect across all cell lineages including those denoted as non-cancerous (**Figure 3.4 A**). Relative to other malignancies *DPF2* was not indicated as a selective dependency in the SS lineage and was therefore not followed up on (**Figure 3.3 B**).

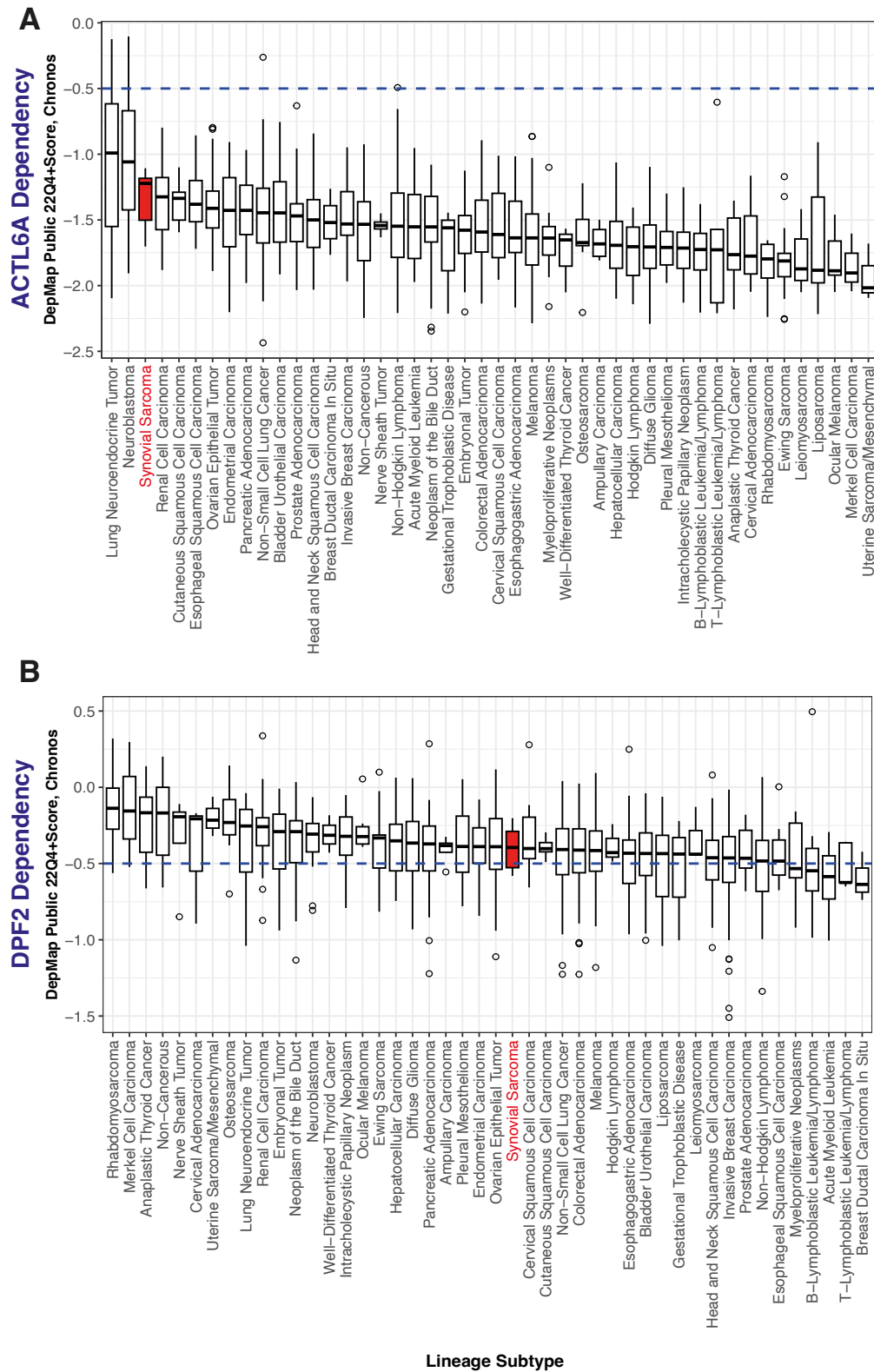


Figure 3.4: The relative dependence of synovial sarcoma cells on ACTL6A and DPF2 compared to other lineage subtypes.

A. The relative dependence of SS cells on ACTL6A. Box plots of median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as open points.

B. The relative dependence of SS cells on DPF2. Box plots of median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as open points.

On the other hand, this analysis highlighted that *SMARCA4* is essential in synovial sarcoma cell lines and that these cells are among the most sensitive to *SMARCA4* loss (**Figure 3.5 A**).

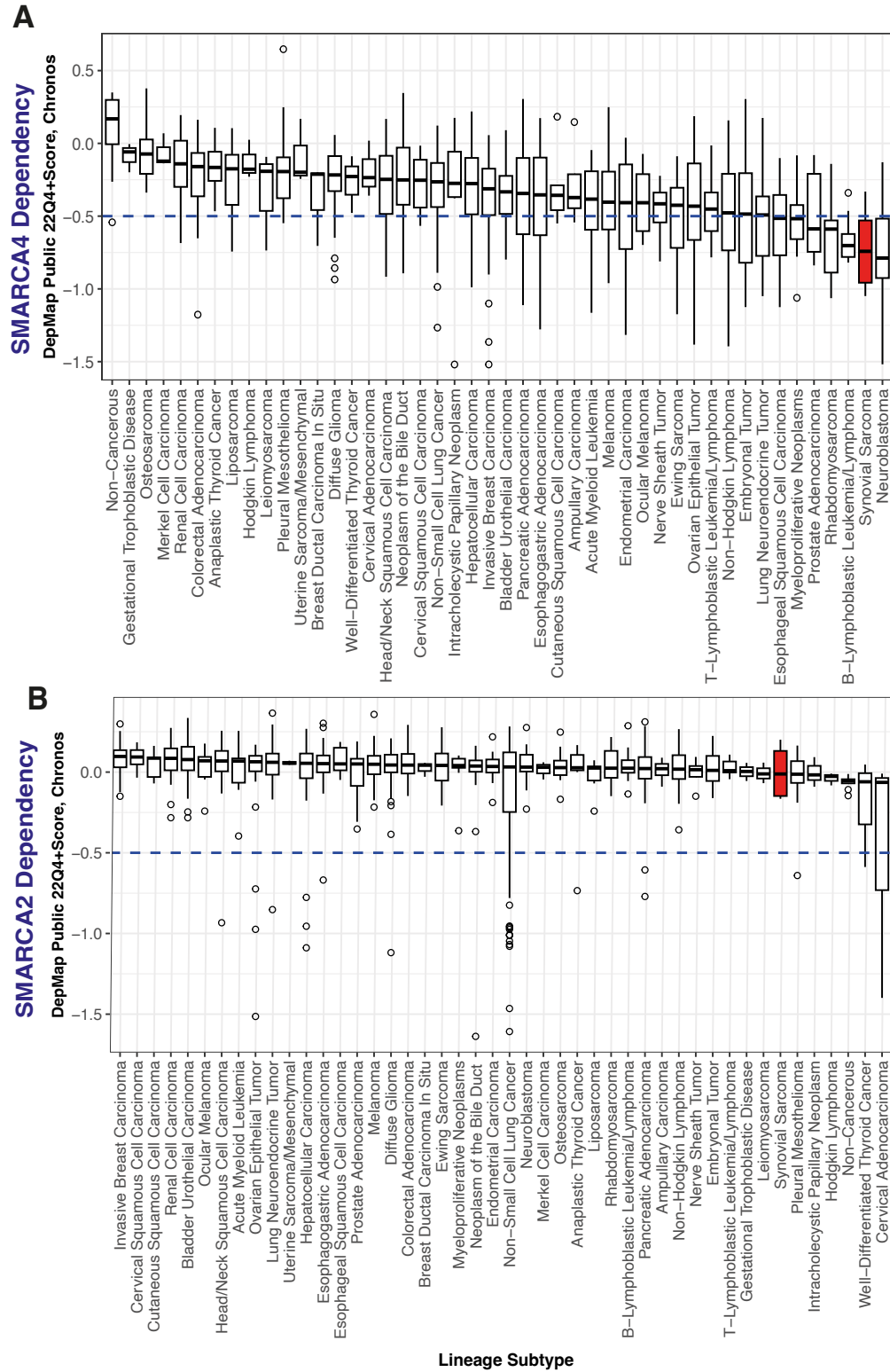


Figure 3.5: The relative dependence of synovial sarcoma cells on SMARCA4 and SMARCA2 compared to other lineage subtypes.

A. The relative dependence of SS cells on SMARCA4. Box plots of median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as open points.

B. The relative dependence of SS cells on SMARCA2. Box plots of median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as open points.

In contrast, the *SMARCA4* paralogue *SMARCA2* (**Figure 3.5 B**) did not show this selective dependency, a fact potentially linked to its reduced expression as indicated in the gene expression boxplot (**Figure 3.2 B**). Finally, a list of chromatin regulatory genes was compiled and filtered for genes listed as common essentials by DepMap. This list was used to explore the ranked gene order of *SMARCA4* as a dependency with *BRD9* and *SS18* as positive controls (**Figure 3.6 A**). From this analysis, it was concluded that SMARCA4 is among the top-scoring chromatin regulatory genes with the CME1, SYO1, and YAMATO cell lines serving as examples (**Figure 3.6 A**).

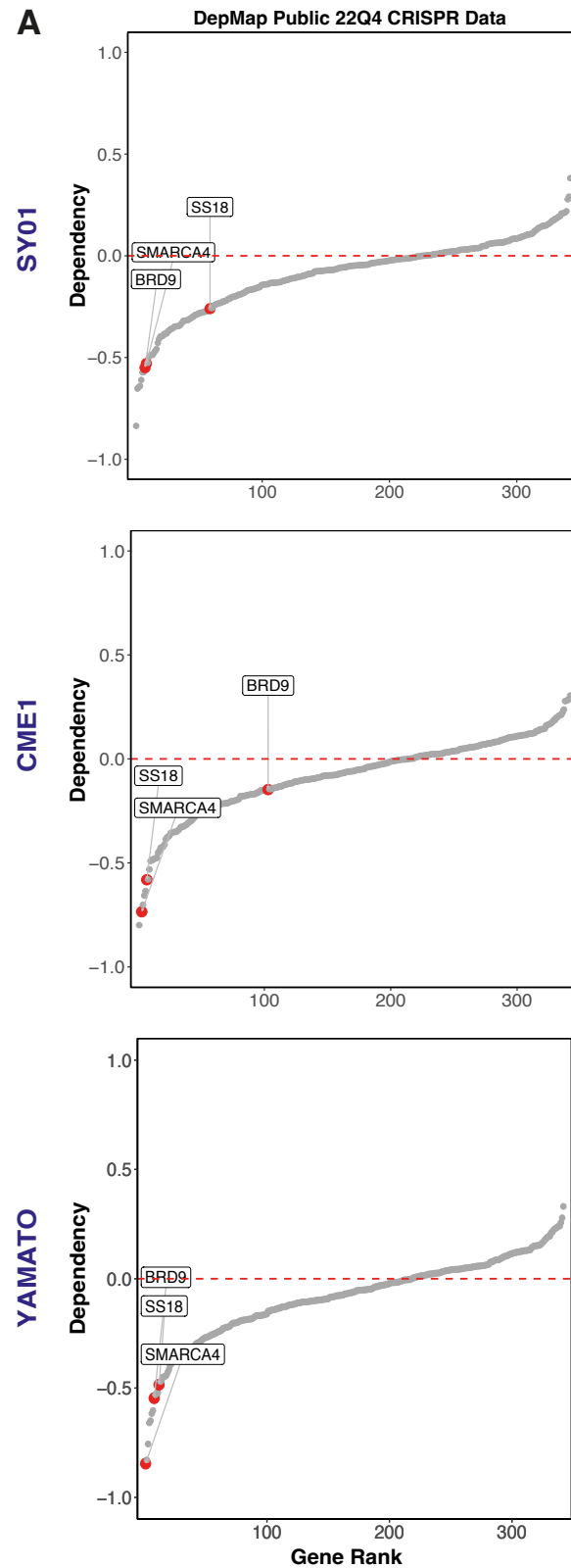


Figure 3.6: SMARCA4 is among the top-scoring chromatin regulatory genes

A. Ranked chronos dependency scores for three SS cell lines for a curated list of 366 chromatin regulatory genes. Common essential genes, as defined by DepMap (Achilles, 19Q3), were removed. SS18, BRD9 and SMARCA4 are highlighted in red.

3.2.2 Establishing a method to acutely degrade *SMARCA4* in synovial sarcoma cells

Following the identification of SMARCA4 as a promising genetic dependency in SS cells, therapeutic opportunities to target SMARCA4 function were explored. To do this a degrader or proteolysis-targeting chimera (PROTAC) based approach was chosen. These therapeutic small-molecules are heterofunctional compounds that engage both a protein of interest and an E3 ubiquitin ligase to induce ubiquitination and subsequent proteasomal degradation (**Figure 3.7 A**) (Lai & Crews, 2016). Such molecules have proven advantageous compared to traditional small-molecule inhibitors since they are capable of more completely abrogating target protein function(s). Importantly, potent SMARCA4 targeting PROTAC molecules have been described and reported to elicit therapeutic responses in SWI/SNF dependent cancer contexts (Farnaby et al., 2019). An important point to note is that this compound targets not only SMARCA4, but also its paralogue SMARCA2, in addition to the PBAF-specific subunit PBRM1 (Farnaby et al., 2019). ACBI1 harnesses the von Hippel Landau (VHL) E3 ligase, recruiting the enzyme to SMARCA2/4/PBRM via engagement of bromodomain structures in each protein (Farnaby et al., 2019).

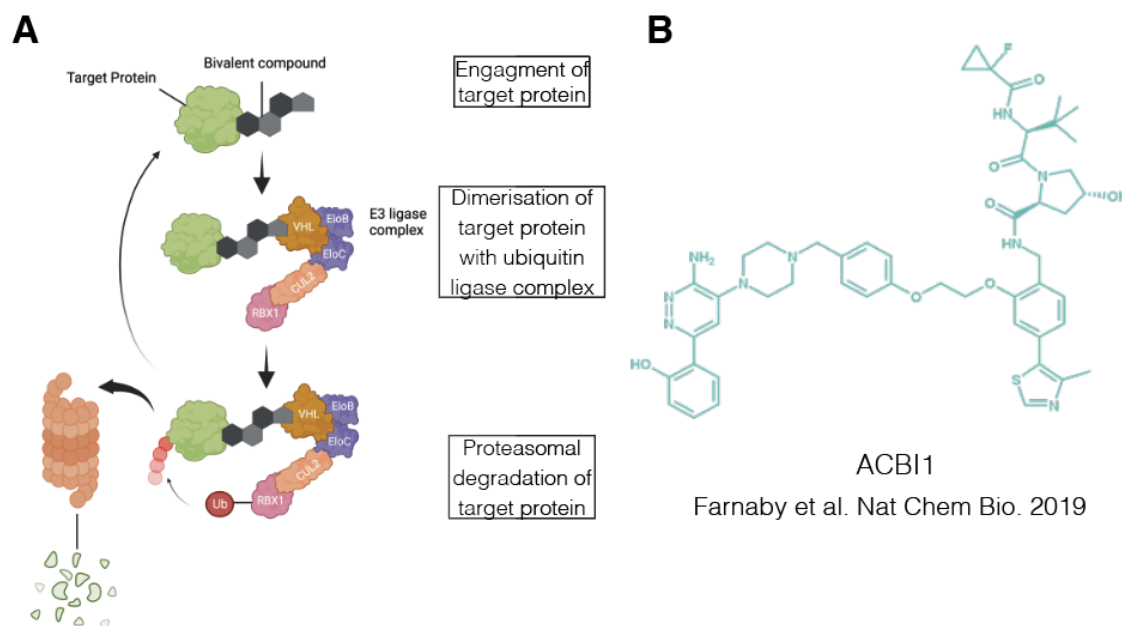


Figure 3.7: Mechanism of protein degradation using degrader compounds.

A. Bivalent degrader compounds work through active recruitment of an E3 ligase complex to a target protein. This leads to ubiquitination of the target protein, tagging it for removal by the proteasome. The compound is subsequently available to elicit additional rounds of degradation through re-engaging the remaining target proteins. **B.** The 2-D structure of ACBI1.

The degradation properties of ACBI1 were evaluated using western blot assays. A 1 μ M concentration was chosen to assess the activity of ACBI1 across a short time course, on the scale of hours. This concentration was previously determined by our group as the minimum concentration required to achieve robust degradation of its targets over 24-hours in SS cells (data not shown). Remarkably, ACBI1 induced extensive degradation of SMARCA4 and SMARCA2 in as little as 4 hours (**Figure 3.8 A**). ACBI1 was found to be less effective at degrading PBRM1 (**Figure 3.8 A**). Accompanying relative western blot quantification for each ACBI1 target was calculated (**Figure 3.8 B**).

As evident in (**Figure 3.1 C**) in section **section 3.1** both SMARCA4 and its paralogue SMARCA2 were found to interact with SS18-SSX1 in the HSSYII cell line via SS18-SSX1 IP-MS. To validate this result, a fusion protein IP was carried out in this cell line (**Figure 3.8 C**). SS18 IP followed by immunoblotting suggested that SS18-SSX1 had been immunoprecipitated and that SMARCA4 and SMARCA2 had been co-immunoprecipitated (**Figure 3.8 C**). The core PRC2 subunit EZH2 would not be expected to co-immunoprecipitate with SS18 and served as a negative control to demonstrate that not all proteins were non-specifically being pulled down with the SS18 antibody. Signal for EZH2 was detected in the input sample and SS18 did not co-immunoprecipitate with EZH2, suggesting that the IP was specific (**Figure 3.8 C**). Furthermore, the IgG IP did not show any signal for SS18, SMARCA2, or SMARCA4, suggesting that the proteins pulled down in the SS18 IP were done so due to specific interactions with the SS18 antibody (**Figure 3.8 C**). The above IP result confirms that both SMARCA4 and SMARCA2 are expressed and interact with the fusion protein in this cell context. Moreover, it highlights the cooperative degradation of SMARCA4 and SMARCA2 with ACBI1 has significant potential benefits, since it would avoid the possibility of compensation for loss of SMARCA4 by SMARCA2; as has been observed in cancer contexts where SMARCA4 function has been lost (Hoffman et al., 2014; McDonald et al., 2017).

Together, these results showcase the excellent degrader properties of ACBI1 in a synovial sarcoma cell line context and highlight this compound as an excellent tool to use to understand the functional contribution of SMARCA4 (and SMARCA2) in SS cells.

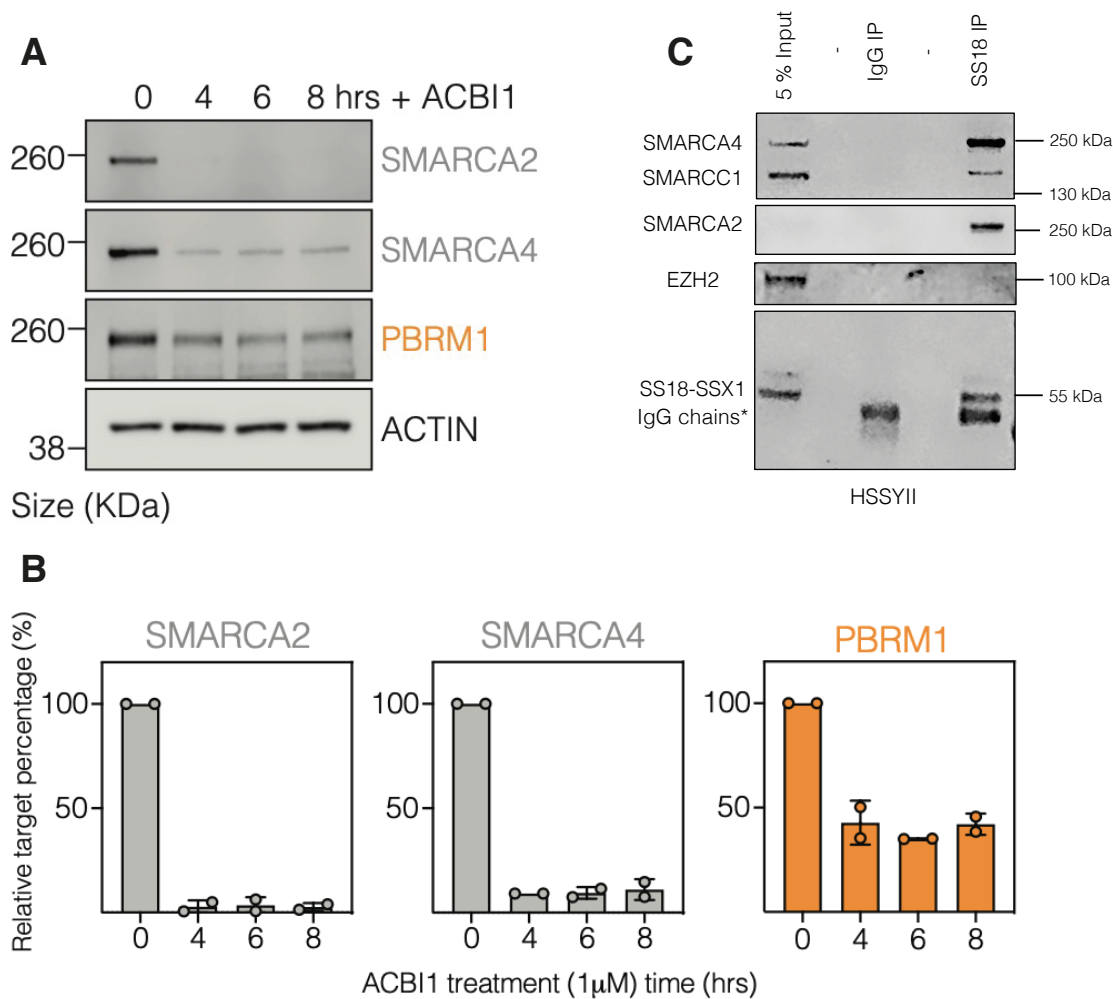


Figure 3.8: Assessing the degradation properties of ACBI1 in synovial sarcoma cells.

A. A representative Western blot for SMARCA4, SMARCA2 and PBRM1 in cells treated with 1 μ M ACBI1 for the indicated time points. Representative data of $n = 3$.

B. Accompanying relative western blot quantification for each ACBI1 target from 2 independent experiments.

C. IP coupled with immunoblotting in HSSYII cells. EZH2 serves as a negative control for the SS18 IP.

3.2.3 Unbiased quantitative chromatin proteomic profiling of synovial sarcoma cells following ACBI1 treatment

To assess the ability of ACBI1 to degrade chromatin-bound SMARCA4 and to understand the broader effects of the compound, unbiased quantitative chromatin mass spectrometry was performed, in triplicate in the HSSYII cell line. Cells were treated with 1 μ M ACBI1 across a time course of 0, 2, and 24-hours (**Figure 3.9 A**). Following the subcellular fractionation protocol, quality control immunoblotting was performed to verify that SMARCA4 had indeed been degraded and that the chromatin-bound fraction had been successfully isolated (**Figure 3.9 B**). Histone H3 signal was found only in the chromatin-bound fraction and GAPDH

was only detected in the cytoplasmic and nuclear soluble fractions as expected (**Figure 3.9 B**). SMARCA4 signal was found to be restricted to the chromatin-bound fraction and completely degraded at the 2-hour time point (**Figure 3.9 B**). SS18-SSX1 fusion protein signal was detected both in the nuclear soluble fraction and chromatin-bound fraction and remained stable across the time course, indicating that the fusion protein does not depend on SMARCA2/4 levels for stability; at least within the time course of these perturbations (**Figure 3.9 B**).

Together, these results indicated successful isolation of the chromatin-bound fraction, demonstrated that ACBI1 can degrade chromatin-bound SMARCA4 in as little as 2 hours and that near complete degradation of SMARCA4 had a minimal effect on the global levels of the fusion protein on chromatin as measured by Western blotting.

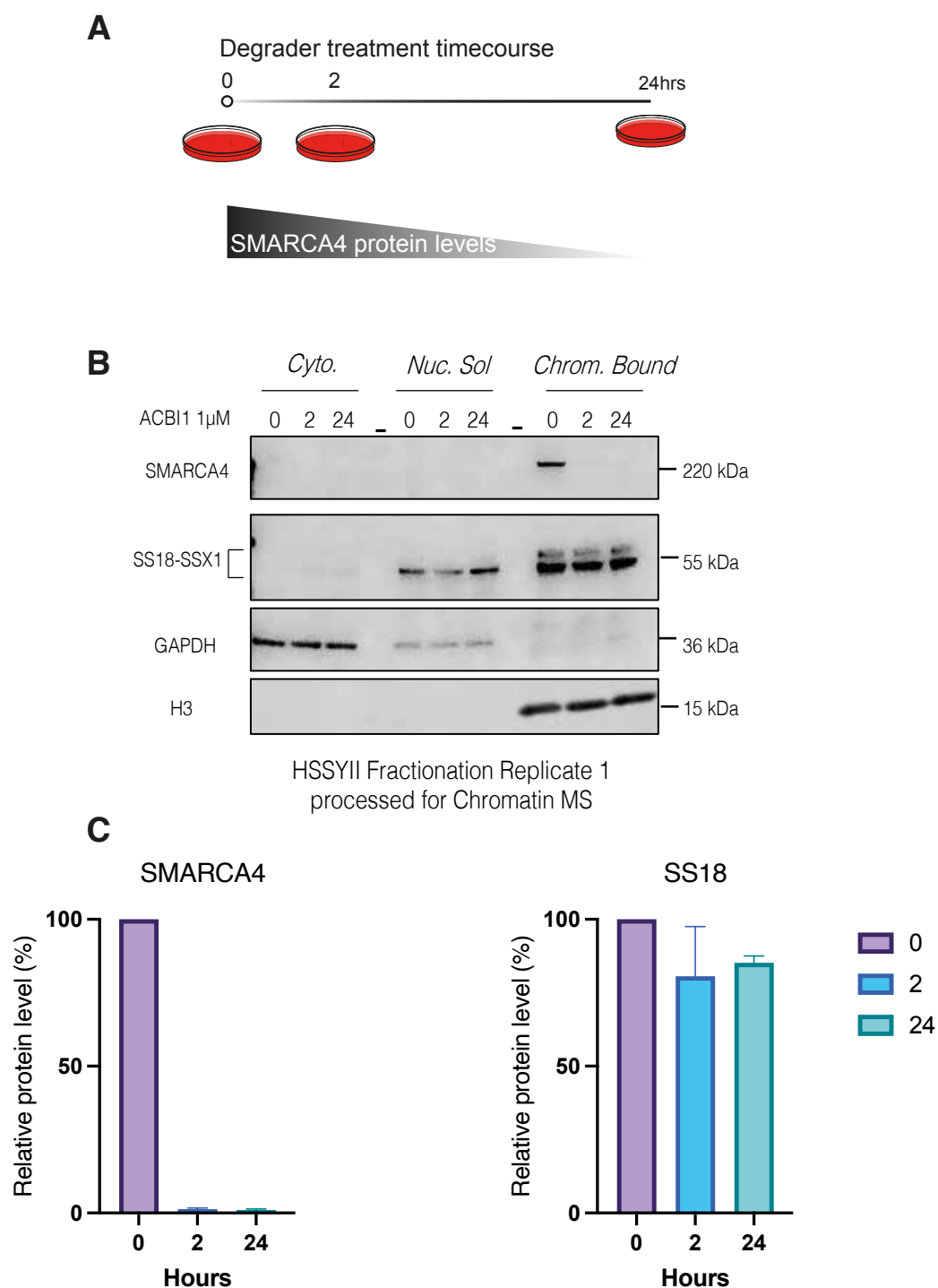


Figure 3.9: Preparing for chromatin mass spectrometry following ACBI1 treatment.

A. Schematic of the time course of ACBI1 in HSSYII cells.

B. Immunoblots performed following subcellular fractionation of ACBI1 treated HSSYII cells. Isolated chromatin fractions subsequently underwent mass spectrometry analyses. Representative data of $n = 3$.

C. Accompanying relative Western blot quantification for chromatin-bound SMARCA4 and SS18 from three independent experiments.

Chromatin-bound fractions were then sent to a collaborator Dr. Giorgio Oliviero (University College Dublin)

who prepared and conducted the mass spectrometry. Analyses of this data demonstrated that as expected, the three direct targets of ACBI1, SMARCA4, SMARCA2 and PBRM1 were significantly depleted from chromatin when compared to DMSO at the 2 and 24-hour time points (**Figure 3.10 B**) (**Figure 3.11 A**). In fact, these 3 proteins were found to be the most significantly depleted proteins at the 24-hour time point (**Figure 3.11 A**). Interestingly, a broader impact on SWI/SNF components was observed (**Figure 3.11 A**). A heatmap visualisation of all the significant SWI/SNF proteins with data centred per protein further highlights this finding (**Figure 3.10 B**). Specifically, at the 2-hour time point, 11 out of 16 significantly depleted proteins ($\approx 68\%$) were SWI/SNF subunits (**Figure 3.11 A**). At the 24-hour time point, 8 out of 17 significantly depleted proteins ($\approx 47\%$) were SWI/SNF subunits (**Figure 3.11 A**). To complement the chromatin mass spectrometry analysis gene expression data from the same cell line was employed to confirm which SWI/SNF subunits are expressed and to what degree. The raw transcriptomic data was processed to generate a Log_2 transcripts per million (TPM) value for each SWI/SNF subunit and plotted as a heatmap (**Figure 3.10 C**). Taken together, these results demonstrate that ACBI1 can remove its direct targets SMARCA2, SMARCA4, and PBRM1 from chromatin. This has consequent implications for additional SWI/SNF components. Considering a total of 1854 proteins were detected by the mass spectrometer, the effect of ACBI1 on SWI/SNF subunits can be considered highly specific.

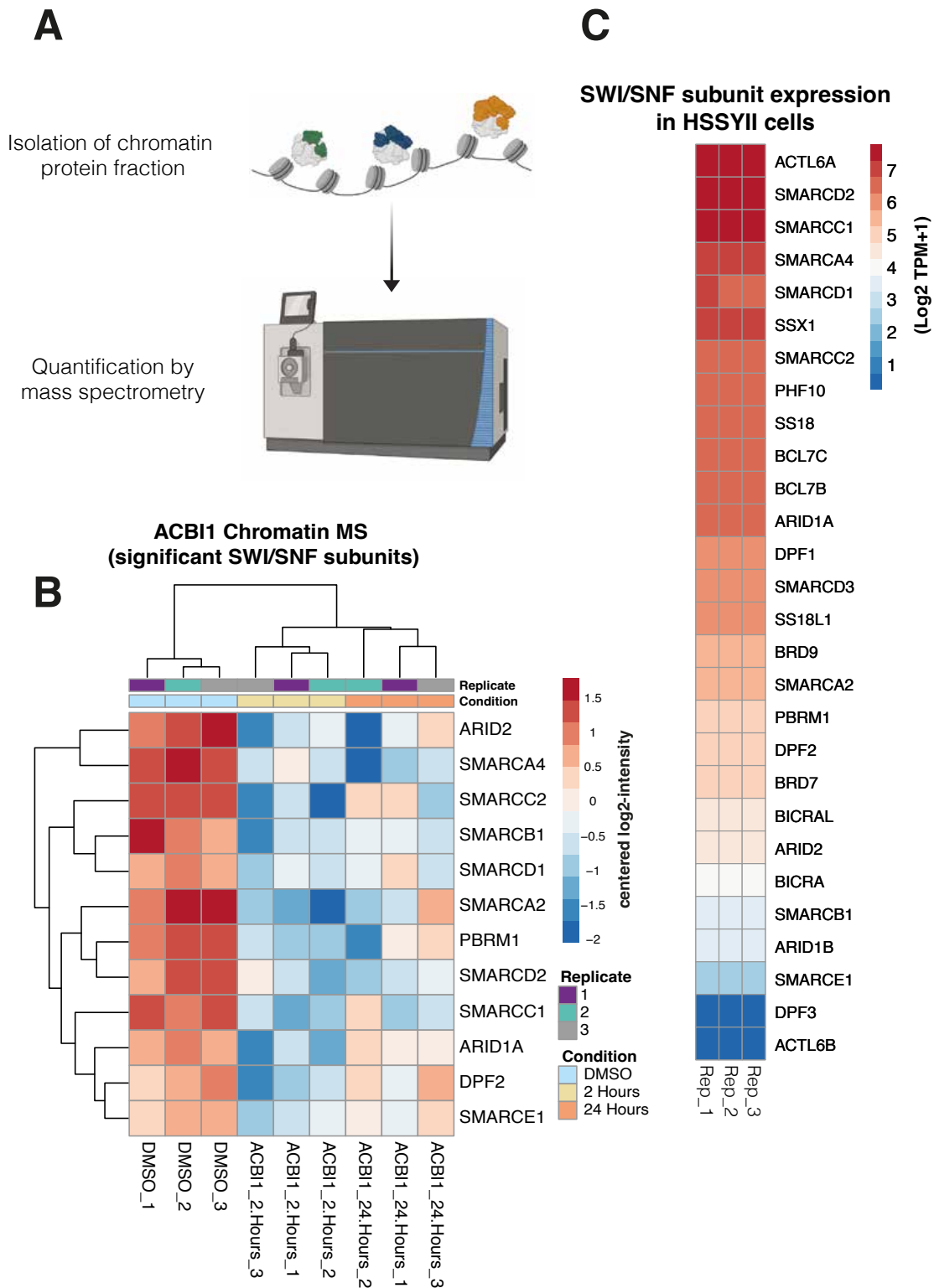


Figure 3.10: Unbiased quantitative chromatin mass spectrometry following ACBI1 treatment. Part I.

A. Schematic of the chromatin mass spectrometry.

B. A heatmap of all significant SWI/SNF proteins across the timecourse with the data centred per protein.

C. mRNA abundance of SWI/SNF components. Displayed as Log₂ transcripts per million (TPM).

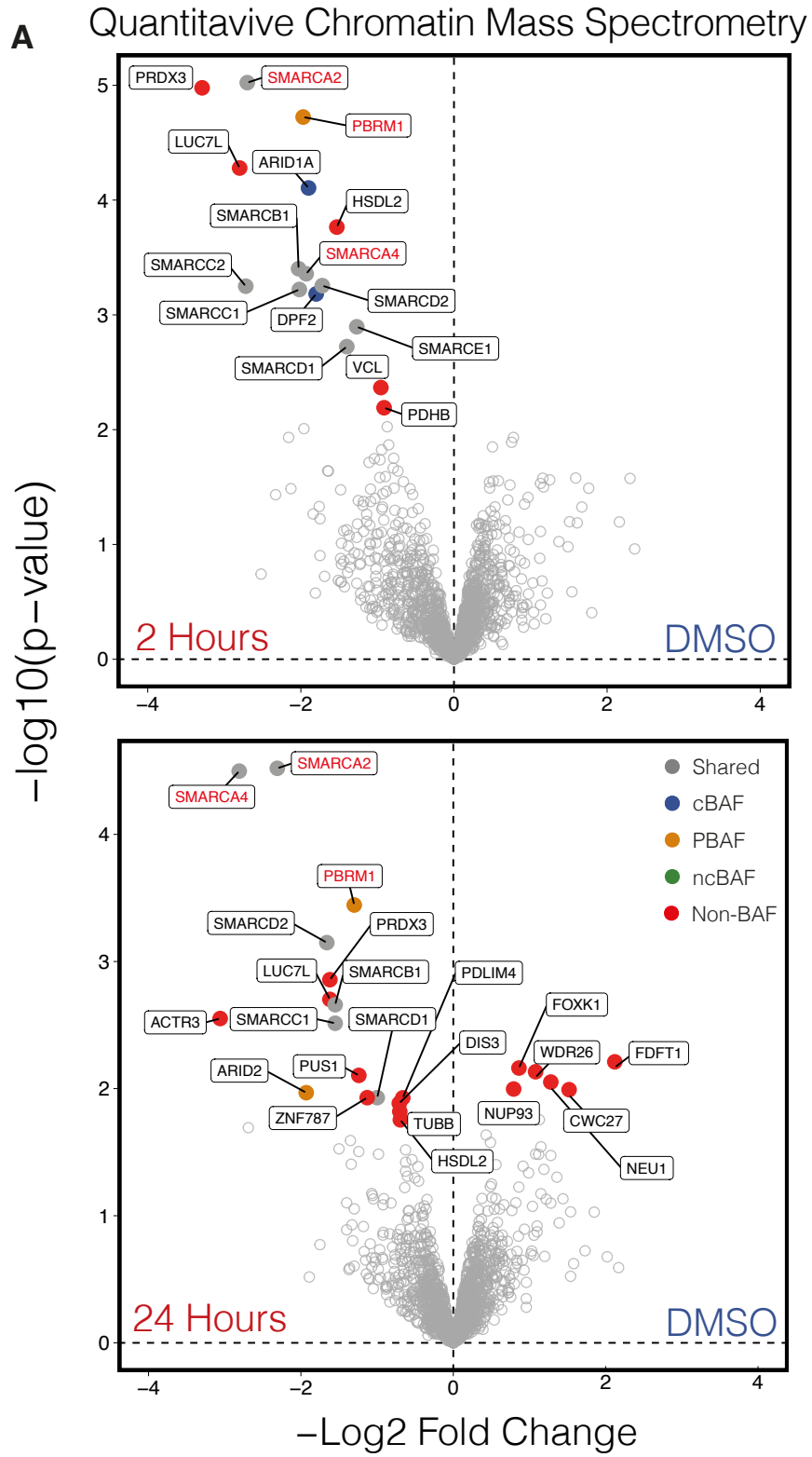


Figure 3.11: Unbiased quantitative chromatin mass spectrometry following ACBI1 treatment. Part II.

A. Volcano plots at the 2-hour timepoint (top) and 24-hour timepoint (bottom) with the label-free quantification intensity in the ACBI1 condition over the DMSO condition is plotted against the $-\log_{10}$ p-value. Statistically significant proteins are made larger and coloured. SWI/SNF components are colour-coded.

3.2.4 SMARCA4/2 degradation induces disassembly of oncogenic SWI/SNF complexes

The logical next question to ask was: Does the degradation of SMARCA4/2 affect the integrity of the SWI/SNF complex in synovial sarcoma cells? To answer this question, large-scale endogenous immunoprecipitations of SMARCC1 followed by mass spectrometry were performed over the same time course as used in **subsection 3.2.3** in triplicate. SMARCC1 was chosen as it is a shared SWI/SNF subunit and therefore can enrich all versions of the SWI/SNF complex (**Figure 3.12 A**). As a quality control measure before mass spectrometry analysis, an aliquot of each sample from each replicate was assessed by immunoblotting (**Figure 3.12 B**). The SMARCC1 IP showed effective performance as evidenced by the substantial presence of SMARCC1 in the pulled-down samples, exceeding 5% (**Figure 3.12 B**). This conclusion is drawn from the comparison of signal strength between the 5% input lane and the IP lanes (**Figure 3.12 B**). This quality control assessment also demonstrated that SMARCC1 can successfully co-immunoprecipitate SMARCA4 (**Figure 3.12 B**). Importantly, SMARCC1 levels remained stable throughout the treatment time course, as evidenced in the input lanes (**Figure 3.12 B**). Moreover, it validated that ACBI1 treatment can remove SMARCA4 from the SWI/SNF complex across a short time frame (**Figure 3.12 B**). The lack of any detectable signal across the IgG replicates gave confidence that the immunoprecipitations were specific. Accompanying relative western blot quantification for SMARCA4 and SMARCC1 were calculated (**Figure 3.12 C**). Together, these results indicated that the experiment was a technical success.

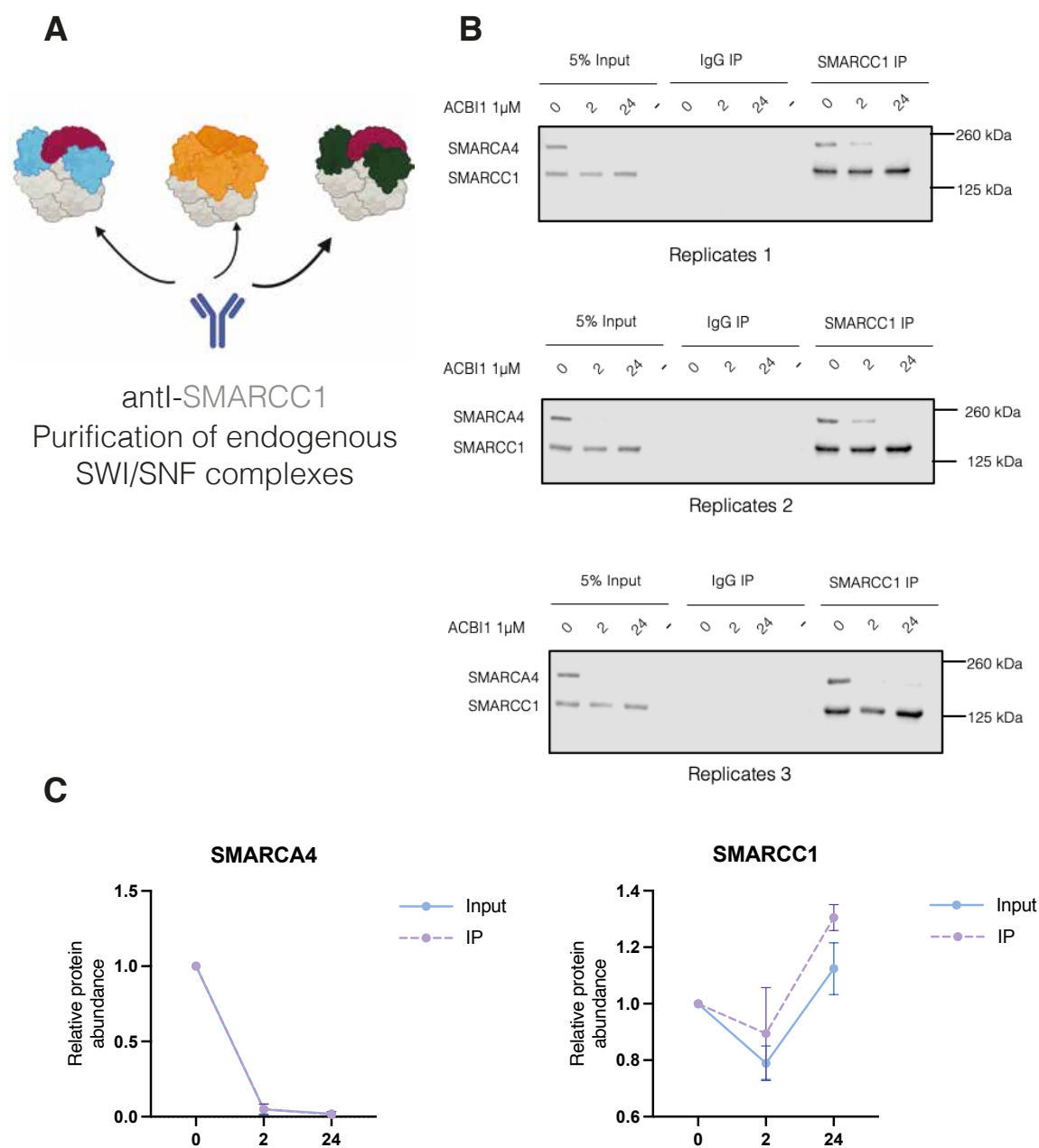


Figure 3.12: Preparing for SMARCC1 immunoprecipitation mass spectrometry.

A. Schematic of the SMARCC1 IP enriching all versions of the SWI/SNF complex.

B. SMARCC1 IP coupled with immunoblotting in HSSYII cells across the indicated ACBI1 time course.

C. Accompanying relative western blot quantification for input and IP signal for SMARCA4 and SS18 from three independent experiments.

As before, the samples were sent to Dr. Giorgio Oliviero (University College Dublin) who prepared and conducted the mass spectrometry. Mass spectrometry analysis demonstrated that SMARCC1 interacts with all three versions of the SWI/SNF complex as evidenced by the capturing of a wide array of cBAF, PBAF

and GBAF-specific subunits (**Figure 3.13 A**). At all time points, the SMARCC1 IP was highly specific as supported by the small proportion of non-SWI/SNF components detected as significantly enriched (**Figure 3.13 A**). Next, the Log₂FC (Log₂ Fold Change) of the relative abundance of all SWI/SNF components across the time course of ACBI1 treatment was calculated using the iBAQ method. iBAQ values are calculated by summing the intensities of all the peptides of a given protein and then dividing by the number of possible observable peptides for that protein, thus normalizing for protein size (Smits, Jansen, Poser, Hyman, & Vermeulen, 2013). As iBAQ values from the IgG IP represent background binding, they were subtracted from the values calculated from SMARCC1 IPs. These values were normalized to the strength of the SMARCC1 signal and then to the DMSO (0-hour) condition and a heatmap visualisation of the data was prepared (**Figure 3.14 B**). From this analysis, a clear loss of the direct targets of ACBI1 was observed across the time course. Interestingly, a broader disassembly of oncogenic SWI/SNF complexes was indicated. Most notably, this included loss of peptides mapping to the fusion protein itself (SS18 and SSX1) and BRD9, in addition to other PBAF and GBAF-specific subunits (**Figure 3.14 A & B**). Of particular note, the cBAF-specific subunits ARID1A and DPF2 remained stable as did the majority of shared SWI/SNF subunits (**Figure 3.12 A & B**). Together, these results demonstrate that ACBI1 can remove its direct targets from their position within SWI/SNF complexes. This in turn induces loss of the fusion protein and BRD9 in addition to other GBAF and PBAF subunits while minimally disrupting cBAF and shared SWI/SNF subunits.

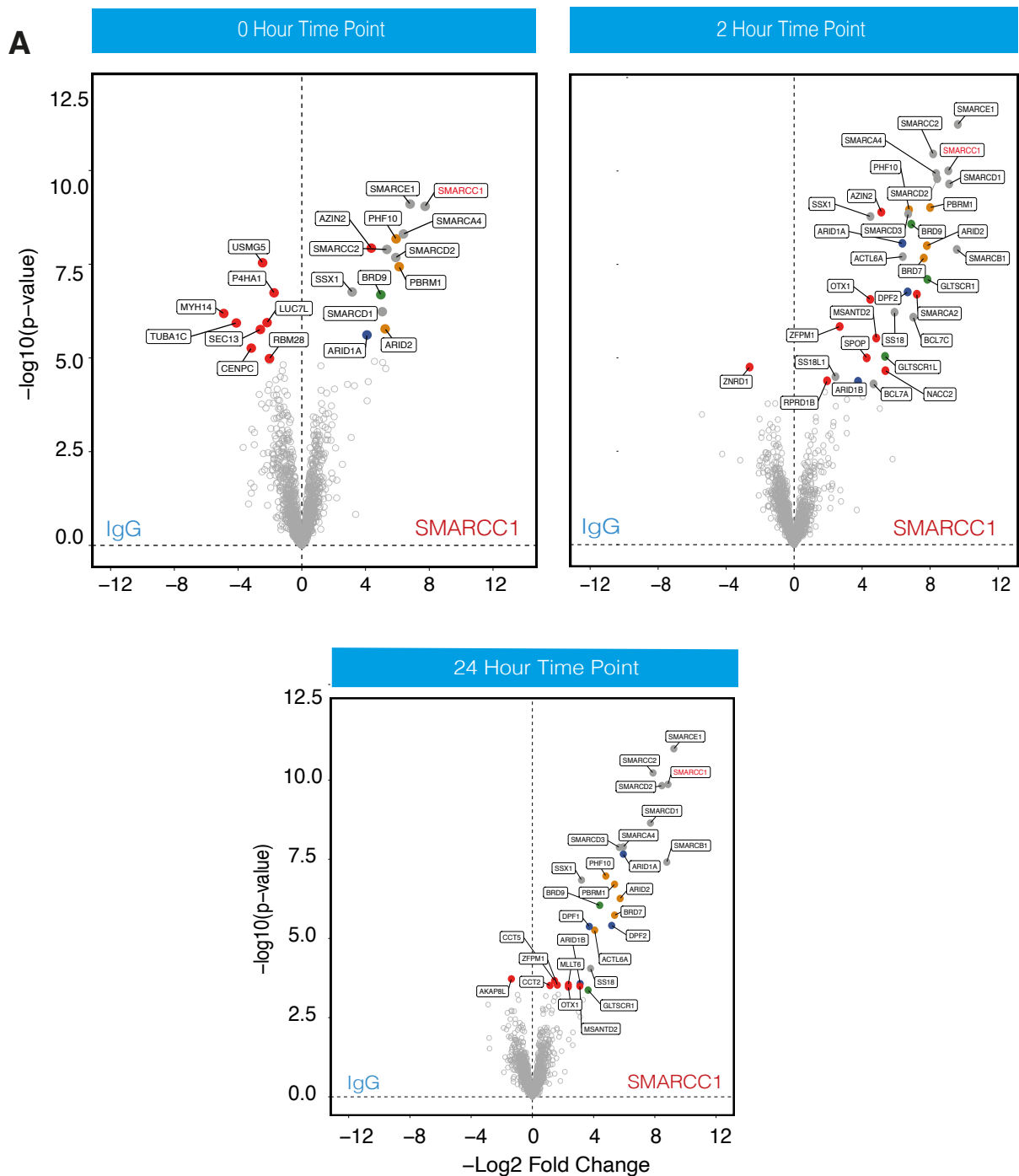


Figure 3.13: SMARCA4/2 degradation induces disassembly of oncogenic SWI/SNF complexes. Part I.

A. SMARCC1 immunoprecipitation (IP) coupled with mass spectrometry analysis in HSSYII cells treated with DMSO or ACBI1 for the indicated time points. The label-free quantification intensity of the SMARCC1 IP over the IgG IP is plotted against the $-\log_{10}$ p-value. Statistically significant proteins are made larger and coloured.

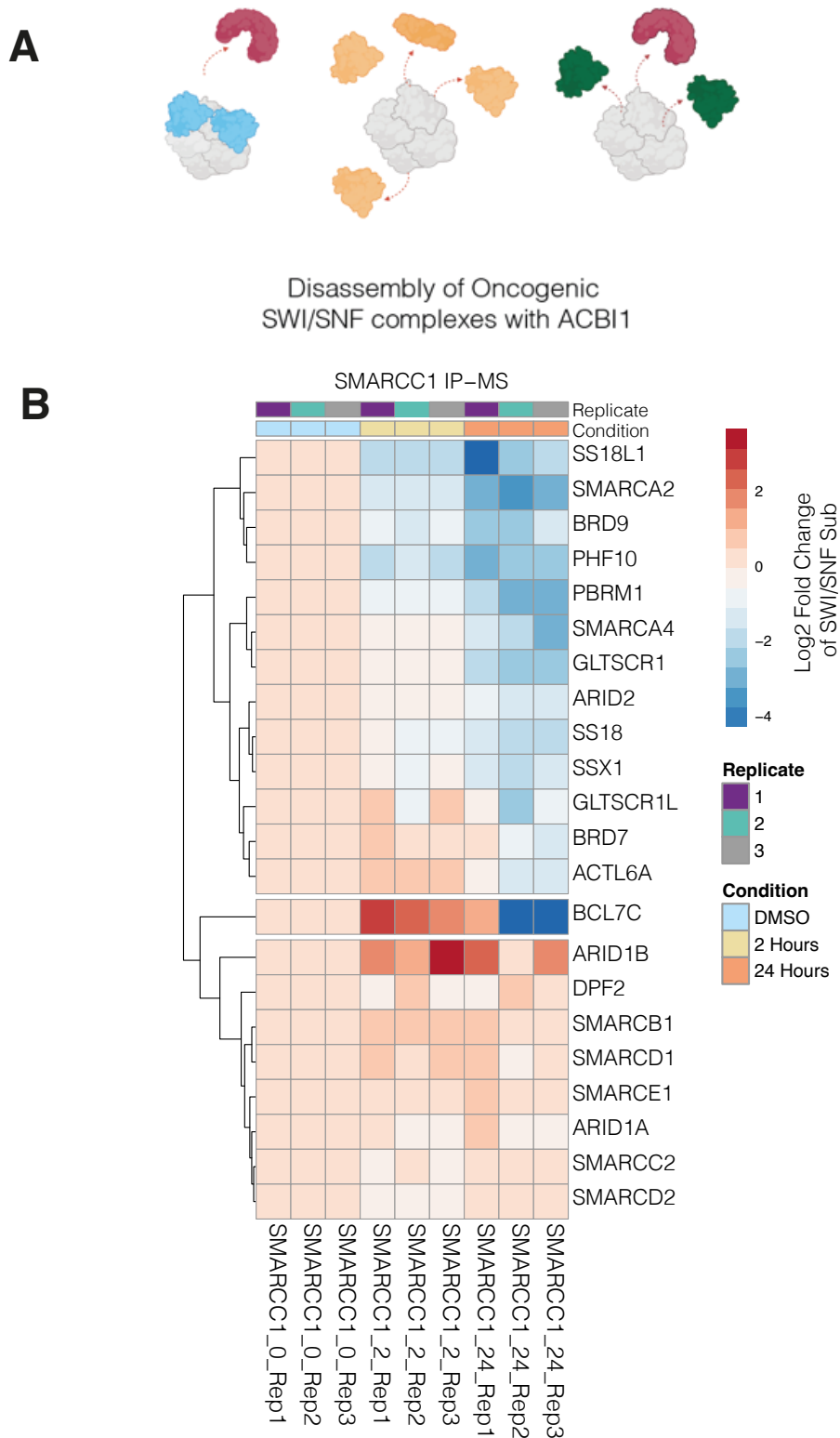


Figure 3.14: SMARCA4/2 degradation induces disassembly of oncogenic SWI/SNF complexes. Part II.

A. Schematic illustrating disassembly of SWI/SNF complexes following ACBI1 treatment.

B. Stoichiometry of SMARCC1 interactors in HSSYII cells. Log₂ Fold Change of the normalised iBAQ values for all SWI/SNF subunits across the ACBI1 time course.

3.3 Discussion

Here, the DepMap cancer dependency mapping resource was used to systematically identify functional dependencies across all SWI/SNF members genes in SS cells. These data sets were integrated with systematic, empirical biochemical characterisations of SS18-SSX containing SWI/SNF complexes in the same setting. Together, these analyses demonstrated that among SS18-SSX containing complexes, SMARCA4 is a key functional dependency. Moreover, SMARCA4 was highly abundant in fusion protein containing complexes; suggesting that targeting SMARCA4 function could provide a means to broadly disrupt molecular mechanisms downstream of SS18-SSX. Following this, a method to perturb SMARCA4 function was established with the aim of using this to gain an understanding of the functional role of SMARCA4 in SS cells. Finally, insights into the biochemical consequences of SMARCA4 loss were illuminated using state-of-the-art quantitative mass spectrometry.

In regards to the DepMap data which compared SWI/SNF subunit dependencies across the SS cell lines in DepMap, it was of interest that of the 23 subunits investigated that displayed robust gene expression levels, only 6 subunits were indicated as important for SS cell survival (**Figure 3.2 A**). A striking observation was the strong specificity of the SS18 subunit as exclusively essential in synovial sarcoma cells (**Figure 3.3 A**). This finding epitomises the concept of a specific functional genomic dependency and represents the perfect therapeutic target in this disease context. Unfortunately, no pharmacological agents currently exist to directly target the fusion protein due to the lack of inherent druggable domains. However, this fact may soon change with the advent of degrader compounds. This is because the identification of binding sites that directly modulate protein function is not necessary. Instead, the binding of a degrader compound at any site on a protein has the potential to induce target degradation. Nonetheless, the success of BRD9 targeting in synovial sarcoma highlights that in the context of a fusion protein-driven malignancy, identifying and targeting indirect vulnerabilities is a viable therapeutic rationale.

It was not unexpected that SMARCA4 was flagged as a genetic dependency in synovial sarcoma cells as it is the catalytic subunit present in all versions of the SWI/SNF complex and abnormal SWI/SNF function is indicated as the primary driver of synovial sarcomagenesis as discussed in **subsection 1.5.3**. As presented in (**Figure 3.1 C**) in section 3.1 SMARCA4 was detected to be one of the most abundant components of SS18-SSX-containing complexes in 2 independent synovial sarcoma cell lines. A fact that may be directly

relevant to its functional importance. A noteworthy aspect of the observed SMARCA4 dependency was its high-ranking position among 366 chromatin regulatory genes, with SMARCA4 consistently scoring among the top genes and clustering with the known dependencies, SS18 and BRD9. SMARCA2 has been shown to be expressed at lower levels in synovial sarcoma cells compared to its paralogue SMARCA4 (**Figure 3.2 B**) and this may explain the fact that synovial sarcoma cells can tolerate its loss (**Figure 3.5 B**). On following up on the ACTL6A result it was discovered that this protein is included in the DepMap curated list of common essential genes (Achilles, 19Q3) and was therefore not followed up on. Genes included in this common essential genes list are indicated to be universally essential for the proliferation of most cell types profiled as part of the DepMap initiative.

A key strength of the tool compound ACBI1 is the efficiency and speed at which SMARCA4 is degraded. This allowed the direct biochemical effects of the degrader to be assessed as opposed to utilising for example a shRNA targeting SMARCA4 that would take longer to take effect and potentially confound data interpretation due to downstream effects. However, one caveat to address is the cooperative degradation of SMARCA2 and PBRM1 in addition to SMARCA4. In the context of these experiments where the HSSYII cell line was used, this is a strength as both SMARCA2 and SMARCA4 are expressed and can interact with the fusion protein (**Figure 3.8 C**). More broadly, SMARCA2 knockout mice have been shown to develop normally as a result of compensatory up-regulation of SMARCA4 expression (Reyes et al., 1998), highlighting the importance of targeting both paralogues to combat functional redundancy. However, the biochemical effects of ACBI1 can not be exclusively attributed to the loss of SMARCA4/2 alone as PBRM1 is also degraded, albeit to a lesser extent (**Figure 3.8 A & B**).

At the outset of the project, it was important to assess the specificity of ACBI1. With the use of unbiased chromatin proteomic profiling, it was demonstrated that this compound exhibited a SW1/SNF-centric effect. As only chromatin-bound proteins were profiled it is unknown how the cytoplasmic and nuclear soluble protein pools are affected. However, whole-cell proteomic profiling of ACBI1 has been carried out in the acute myeloid leukaemia context and showed minimal disruption of other proteins across the proteome (Farnaby et al., 2019). Unfortunately, due to technical limitations of the chromatin mass spectrometry experiment not all expressed SWI/SNF subunits (**Figure 3.10 C**) were detected as binding to chromatin. As a result, this limits the conclusions that can be drawn. It was particularly interesting that global levels of the fusion protein (SS18-SSX1) on chromatin were not altered across the time course (**Figure 3.9 B**).

This suggests that SMARCA4/2 is not essential for the chromatin binding capacity of the fusion protein. Moreover, this finding proves even more intriguing in the context of the dissociation of numerous SWI/SNF components from chromatin and the disassembly of SWI/SNF complexes.

The main finding of the SMARCC1 immunoprecipitation experiment was that ACBI1 treatment induces disassembly of oncogenic SWI/SNF complexes. Considering this result in the context of the modular organisation and assembly of SWI/SNF complexes (Mashtalir et al., 2018) some interesting similarities and differences were observed. The initial step in SWI/SNF complex formation is the dimerisation of SMARCC subunits, this is followed by the formation of the BAF initial core with the incorporation of SMARCD subunits (Mashtalir et al., 2018). Next, the incorporation of SMARCE1 and SMARCB1 components creates the BAF core module. Interestingly, these subunits remain stable across the ACBI1 time course and suggest an intact BAF core module (**Figure 3.14 B**). Interaction with ARID1 leads to the creation of cBAF complexes with the subsequent incorporation of DPF2 (Mashtalir et al., 2018). Indeed, these cBAF-specific subunits remain stable across the time course and suggest that these ARID1/BAF core intermediates remain intact (**Figure 3.14 B**). However, for PBAF complex formation ARID2 has been shown to bind the intact BAF core module and instigate ARID2/PBAF core intermediate complexes via incorporation of BRD7 and PHF10 (Mashtalir et al., 2018). The formation of ARID2/PBAF core intermediates appears to be disrupted across the time course as evidenced by the loss of these subunits (**Figure 3.14 B**). Similarly, the formation of the BRD9/GBAF core is similarly disrupted. Alternatively upon formation of the BAF initial core, GLTSCR1 and GLTSCR1L can be incorporated at the expense of SMARCE1 and SMARCB1, followed by the formation of the ncBAF core module, which binds BRD9 (Mashtalir et al., 2018). Indeed, these GBAF-specific subunits are lost across the time course (**Figure 3.14 B**). These differences may be accounted for by the fact that HEK293T cells were characterised in the previous study, a cellular context where SWI/SNF complexes are not altered by the incorporation of the SS18-SSX fusion oncoprotein. Final form assemblies of the cBAF and GBAF complexes are completed with the incorporation of an intact SS18-containing ATPase module, with an SS18-deficient ATPase module finalising PBAF assembly (Mashtalir et al., 2018). The ATPase subunits SMARCA4/2 have been shown to form extensive cross-links with SS18 in addition to ACTL6A and BCL subunits and have been shown to form a distinct ATPase module (Mashtalir et al., 2018). Consistent with this, across the ACBI1 treatment time course ATPase module subunits are lost, including peptides mapping to the fusion protein.

In summary, a functional dependence on SMARCA4 in SS relative to other cancer contexts was highlighted. The biochemical effects of the novel SMARCA4/2/PBRM1 degrader were evaluated in SS cells and used as a method to disrupt the assembly/stability of fusion protein-containing complexes.

Chapter 4

Characterising the transcriptional & chromatin regulatory role of SMARCA4

4.1 Introduction

Following on from **chapter 3**, which highlighted the importance of SMARCA4 in SS cells and characterized the biochemical consequences of SMARCA4 degradation, this chapter had two key aims:

1. To examine the transcriptional effects of SMARCA4 loss.
2. To examine the chromatin dynamics associated with SMARCA4 degradation.

Addressing these key aims was important because, as described in the main introduction mammalian SWI/SNF complexes are ATP-dependent chromatin remodelers that regulate DNA accessibility and control gene expression dynamics and SMARCA4/2 are the two mutually exclusive catalytic subunits found in all three SWI/SNF subclasses (Centore et al., 2020). Until now, the role of these catalytic subunits in SS disease biology remained unexplored.

Given the functional importance of SMARCA4 in SS it was pertinent to gain an understanding of the transcriptional consequences of SMARCA4 loss in SS. This will facilitate an understanding of the functional

contributions of SMARCA4 in this disease context, which will be important in order to fully exploit the potential of targeting SMARCA4 function as a therapeutic approach. To understand the gene expression program controlled by SMARCA4 in SS cells global transcriptional analysis using QuantSeq 3' mRNA-Seq (Moll, Ante, Seitz, & Reda, 2014) was performed. This novel methodology is a form of targeted RNA-seq that focuses on sequencing only the 3' end of transcripts as opposed to traditional RNA-seq where the entire length of the transcript is sequenced (Moll et al., 2014). As a result, QuantSeq requires a lower sequencing depth and is, therefore, more cost-effective (Moll et al., 2014). However, the targeted nature of QuantSeq restrains its primary use to measuring gene expression as unlike traditional RNA-seq it is not possible to detect novel transcripts, splice variants and non-coding RNAs using this methodology.

SMARCA4 (and the SWI/SNF complex) affects chromatin structure by controlling accessibility to gene enhancers and promoters, facilitating the binding of additional transcriptional regulators at these sites (Alver et al., 2017; Tolstorukov et al., 2013). In this chapter, the genome-wide chromatin accessibility changes induced by SMARCA4 degradation are investigated using Assay for Transposase Accessible Chromatin using sequencing (ATAC-seq). ATAC-seq is the most recent method available for probing genome wide chromatin accessibility. This methodology facilitates the identification of promoter, enhancer and silencer regions, allowing for the regulatory mechanisms of gene expression to be deciphered. The ATAC-seq method devised by the Greenleaf lab (Buenrostro, Wu, Chang, & Greenleaf, 2015) holds numerous advantages over its preceding counterparts namely DNase I hyper-sensitivity (DNase-seq) and Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE-seq) sequencing, including in situ library preparation, reduced assay time, lower starting material requirements and the protocol does not require formaldehyde fixation thus capturing the native chromatin state. The method involves treating the genome with a hyperactive derivative of the prokaryotic Tn5 transposase (**Figure 4.1**). This Tn5 is preloaded *in vitro* with sequencing adapters and inserts these DNA sequences preferentially into open regions of the genome and simultaneously fragments the DNA (**Figure 4.1**). The resulting DNA fragments are then PCR amplified and undergo DNA purification prior to paired-end next generation sequencing (Buenrostro et al., 2015) (**Figure 4.1**).

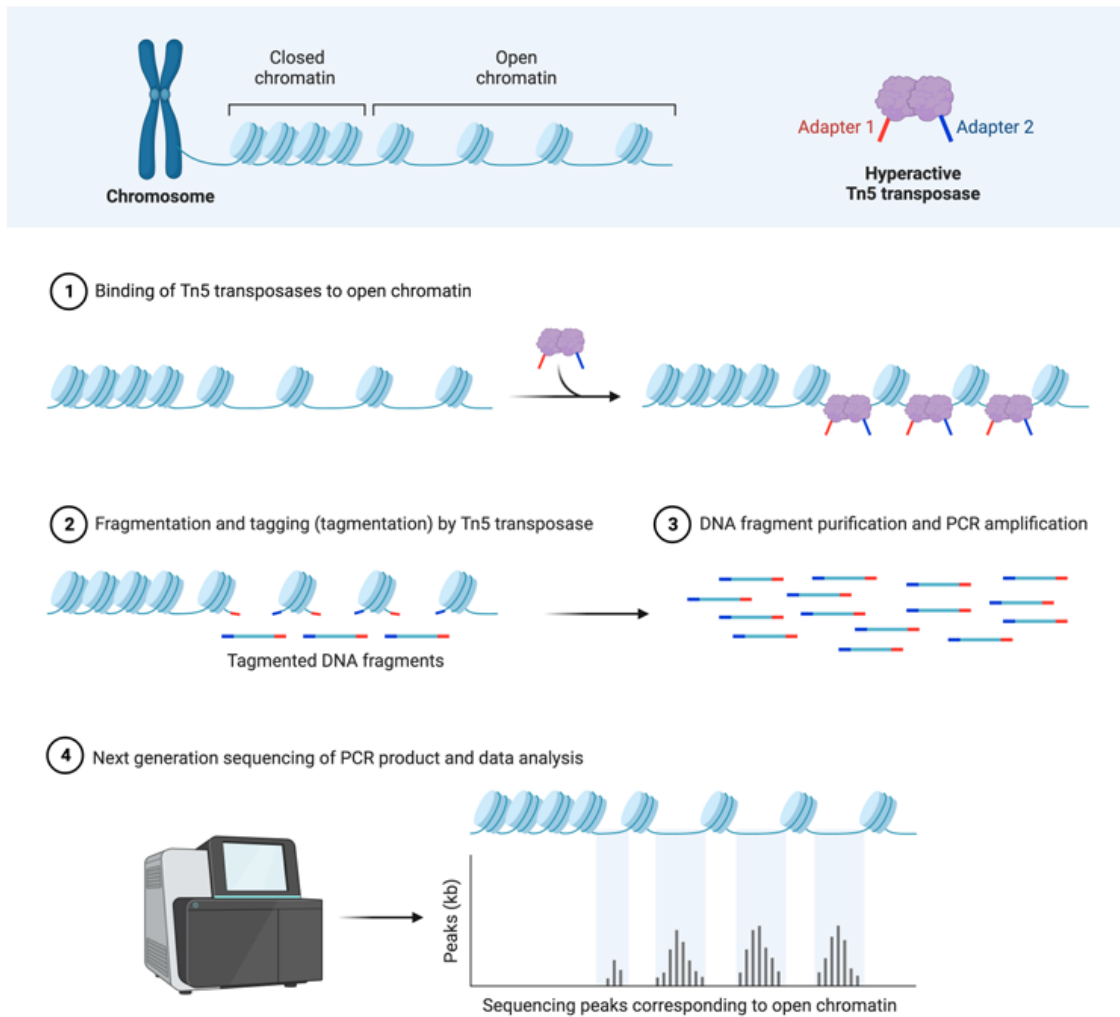


Figure 4.1: Schematic of the ATAC-Seq protocol.

A schematic overview of the ATAC-seq method adapted from biorender.com

To better understand the functional role of SMARCA4 in SS it was essential to establish methods to map its localisation on chromatin. Moreover, it was also key to map the fusion protein to begin delineating the functional interplay between these proteins. To this end, a strong effort was made to establish the Cleavage Under Targets and Release Using Nuclease (CUT&RUN) chromatin profiling methodology in the lab (Skene & Henikoff, 2017; Meers, Bryson, Henikoff, & Henikoff, 2019). CUT&RUN has several advantages over traditional Chromatin immunoprecipitation, coupled with sequencing (ChIP-seq) which has been the primary approach for genome-wide chromatin profiling: 1) The assay is performed *in situ* in non-crosslinked or very lightly cross-linked cells and does not require chromatin fragmentation or solubilisation, as such protein-DNA

interactions are more likely to be maintained in their native state; 2) the method requires fewer numbers of starting cells; and 3) the targeted nature of the method naturally generates very low background signal and as a result high-resolution genome-wide mapping for histone post-translational modification (PTMs) and chromatin-associated proteins can be achieved with only $\approx 1/10$ th the sequencing depth required in conventional ChIP-seq experiments (Skene & Henikoff, 2017). The cost savings of utilising CUT&RUN over ChIP-seq in this research project allowed for a deeper, more rigorous understanding of the targets of interest through the incorporation of multiple replicates. This method developed by the Henikoff lab (Skene, Henikoff, & Henikoff, 2018) involves permeabilising cells or alternatively using nuclei and immobilizing them on lectin-coated magnetic beads. The bead-bound cells/nuclei are successively incubated with an antibody against the PTM or chromatin interacting protein of interest and a fusion of protein A/G to Micrococcal Nuclease (pAG-MNase) which diffuses through the nuclear pores. Ca^{2+} ions are then added to activate the pAG-MNase to cleave the target-DNA complex, which then diffuses out of the nucleus. DNA is extracted from the supernatant and used to prepare libraries for paired-end sequencing. A schematic overview of this protocol is presented (**Figure 4.2**). Having established and optimised the CUT&RUN protocol the genome-wide occupancy of SMARCA4, SMARCA2 and SS18-SSX were mapped before and after SMARCA4 degradation. Importantly integration of these data sets with the gene expression and chromatin accessibility analyses has the capacity to provide comprehensive insights into the role of SMARCA4 in SS.

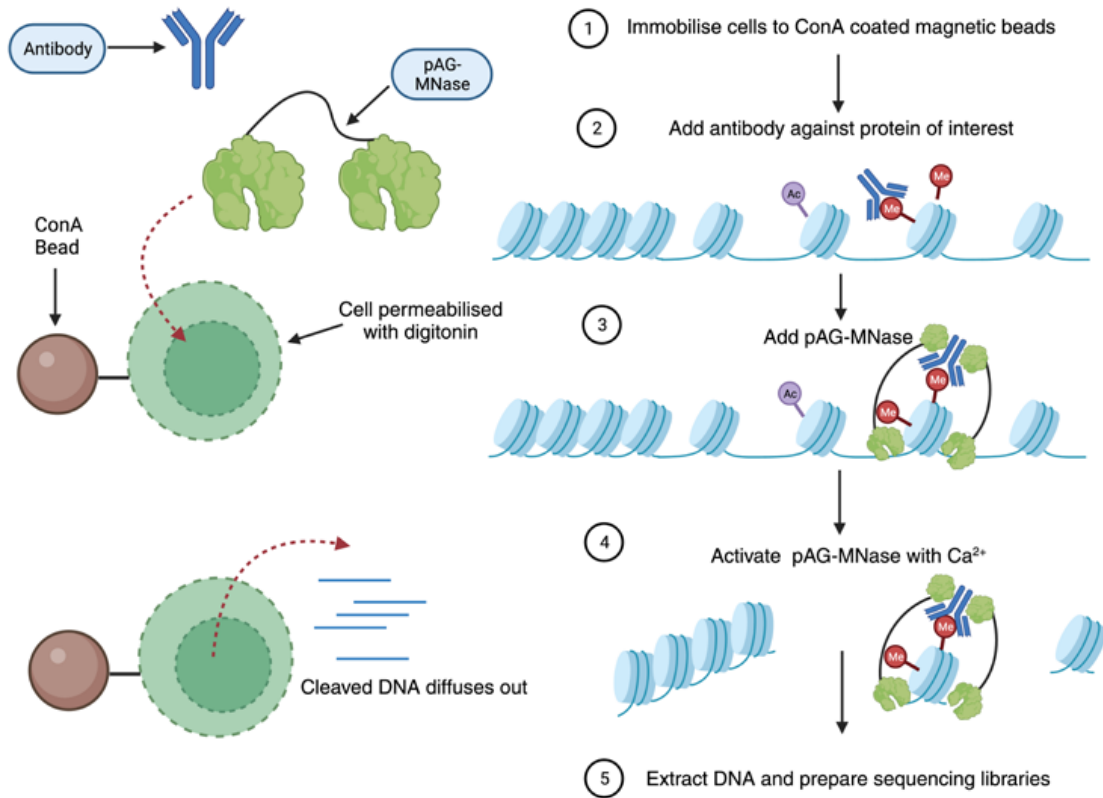


Figure 4.2: Schematic of the CUT&RUN-Seq protocol

A schematic overview of the CUT&RUN protocol. Adapted from (Skene et al., 2018) and made with Biorender.com.

4.2 Results

4.2.1 Comparing traditional whole transcript and 3' RNA-Seq

To compare traditional whole transcript and 3' RNA-seq, HSSVII cells were treated with 100 nM dBRD9-A for 6 and 24 hours and the QuantSeq 3' mRNA-Seq kit (Lexogen, 015.96) was used to prepare Illumina-compatible sequencing libraries. Traditional RNA-seq libraries generated using the NEBNext Ultra RNA Library Prep kit for Illumina (NEB, E7530L) were previously prepared and sequenced by Dr. Gerard Brien. A differential expression analysis was then carried out using the DESeq2 R package (Love et al., 2014) to detect differentially expressed genes (DEGs). Following 6 hours of BRD9 degradation expression of 101 genes showed a ≥ 1.5 -fold decrease while 114 showed an increase ($p \leq 0.05$) for the Quant-seq samples (**Figure 4.3 A**). For the analogous RNA-seq samples 55 genes were significantly downregulated

while 172 genes were upregulated (**Figure 4.3 A**). Although there was a difference in the number of DEGs detected across both methods it was clear that both methods were capturing overlapping robust changes, for example, *SCD*, *INSIG1* and *STARD4* as downregulated and *ZNF692*, *SEMA3C* and *RBM3* as upregulated (**Figure 4.3 A**). A similar result was observed for the 24-hour time point comparison (**Figure 4.3 B**). In order to more broadly visualise this comparison $\log_2(\text{TPM}+1)$ expression values were plotted as a heatmap for all significant genes for both methods and time points (**Figure 4.4 A & B**). From this analysis, it was clear that the DMSO and treatment time points clustered together regardless of the method used and that similar expression trends were observed for both methods (**Figure 4.4 A & B**). This initial comparison of the methods gave confidence to go forward with this novel gene expression profiling method to examine the transcriptional effects of SMARCA4 loss.

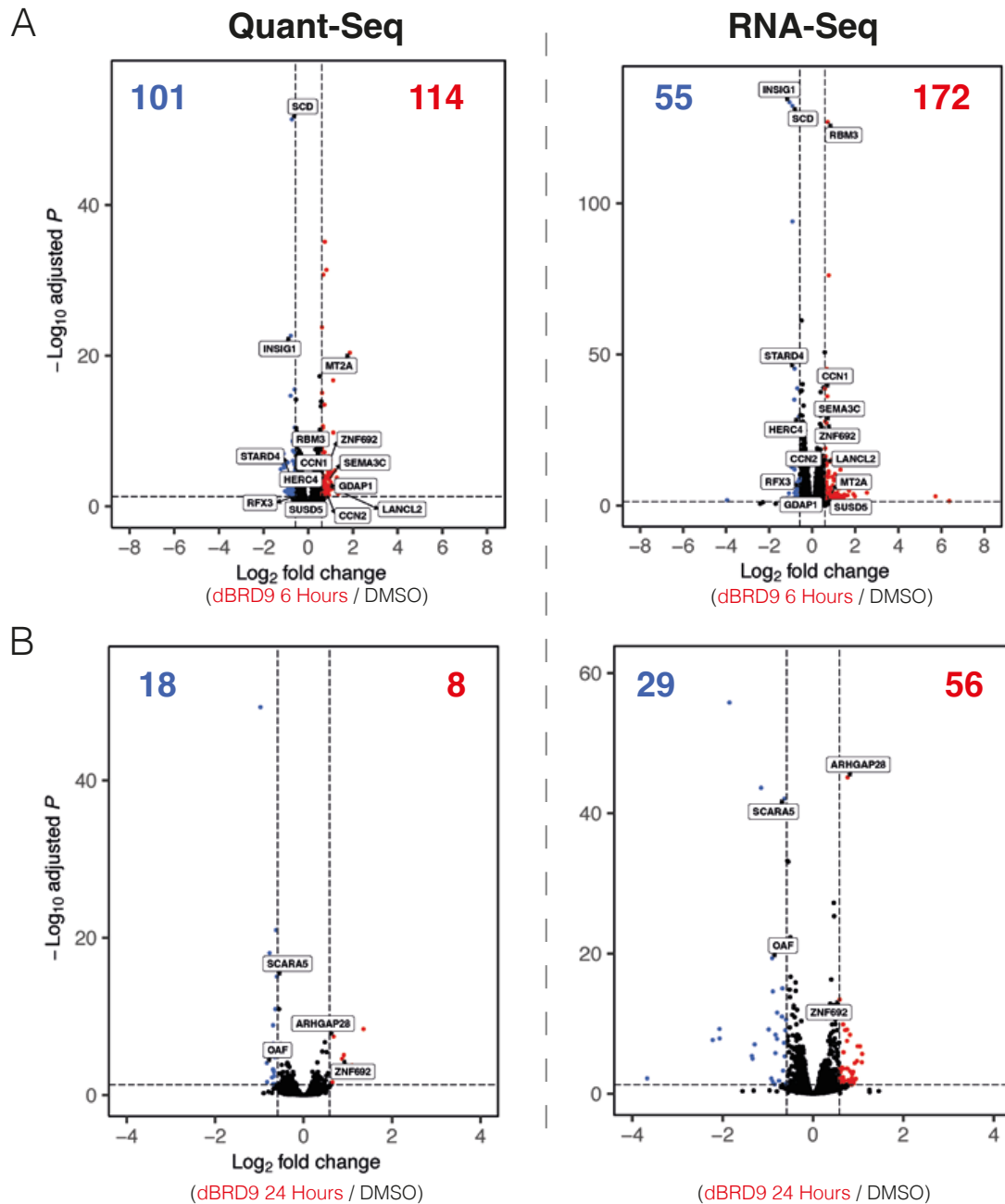


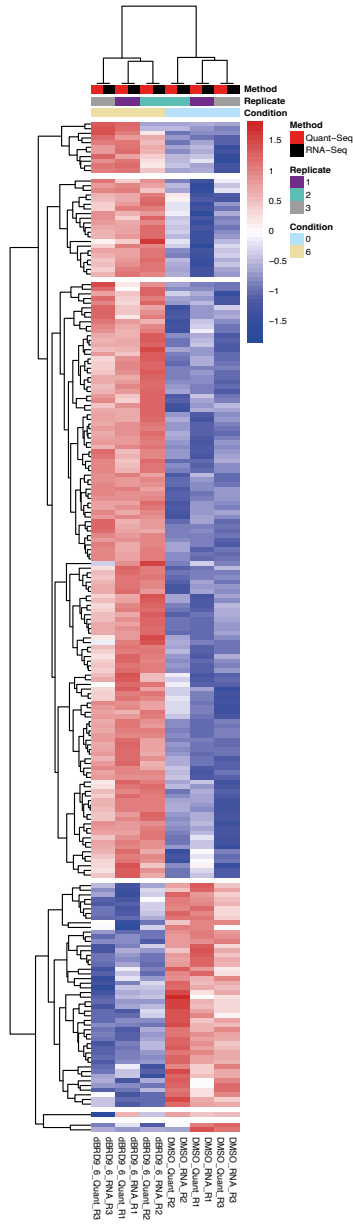
Figure 4.3: Comparing traditional whole transcript and 3' RNA-Seq. Part I

A. Quant-seq (left) and RNA-seq (right) volcano plots presenting gene expression changes observed in the HSSYII cell line treated with 100 nM dBRD9 for 6 hours. Red and blue denote genes significantly up and down-regulated respectively. Selected genes are labelled in boxes.

B. Quant-seq (left) and RNA-seq (right) volcano plots presenting gene expression changes observed in the HSSYII cell line treated with 100 nM dBRD9 for 6 hours. Red and blue denote genes significantly up and down-regulated respectively. Selected genes are labelled in boxes.

A

dBRD9 6 hour significant Genes (log2CPM+1) Quant-Seq vs RNA-Seq



B

dBRD9 24 hour significant Genes (log2CPM+1) Quant-Seq vs RNA-Seq

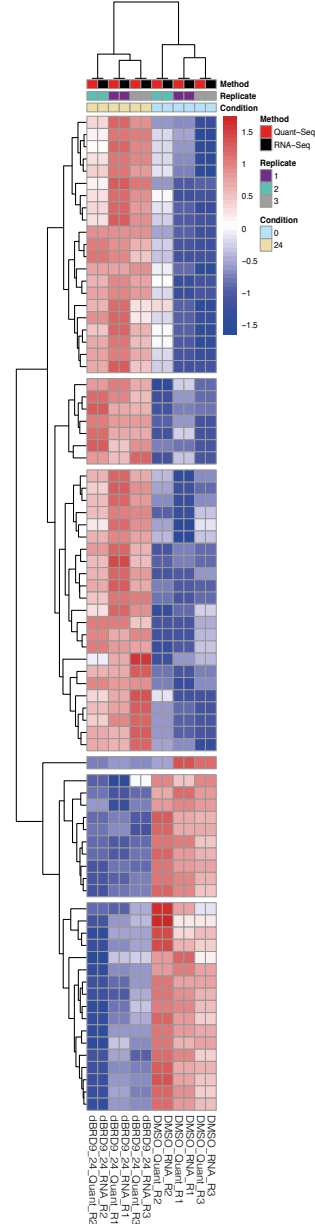


Figure 4.4: Comparing traditional whole transcript and 3' RNA-Seq. Part II

A. Heatmap displaying log₂CPM+1 signal for the significantly DEGs detected by both RNA-seq and Quant-seq upon 6-hour treatment with 100 nM dBRD9.

B. Heatmap displaying log₂CPM+1 signal for the significantly DEGs detected by both RNA-seq and Quant-seq upon 24-hour treatment with 100 nM dBRD9.

4.2.2 Examining the transcriptional effects of SMARCA4 loss

To investigate the transcriptional effects of SMARCA4 loss, global transcriptional analysis was performed using QuantSeq 3' mRNA-Seq across an ACBI1 time course (**Figure 4.5 A**). Importantly, due to the favourable kinetics of the degrader, the very early time points of 2 and 6 hours were included to allow for the characterisation of the primary molecular changes of SMARCA4 loss (**Figure 4.5 A**). Curiously, at the 2-hour time point, a predominant upregulation of gene expression was observed with 205 genes detected as upregulated and 82 genes downregulated (**Figure 4.5 B**). At the 6-hour time point, there was a similar number of up and downregulated genes 258 and 247 respectively (**Figure 4.5 B**). Most unexpectedly, the 24-hour time point was the time point where the least number of DEGs were detected with 71 downregulated and 39 upregulated (**Figure 4.5 B**). Considering the biochemical effects of SMARCA4/2 degradation described in detail in **chapter 3**, and the fact that SS is considered a classic example of a malignancy driven by altered SWI/SNF function, this limited effect on transcription especially at the later time point of 24 hours was surprising.

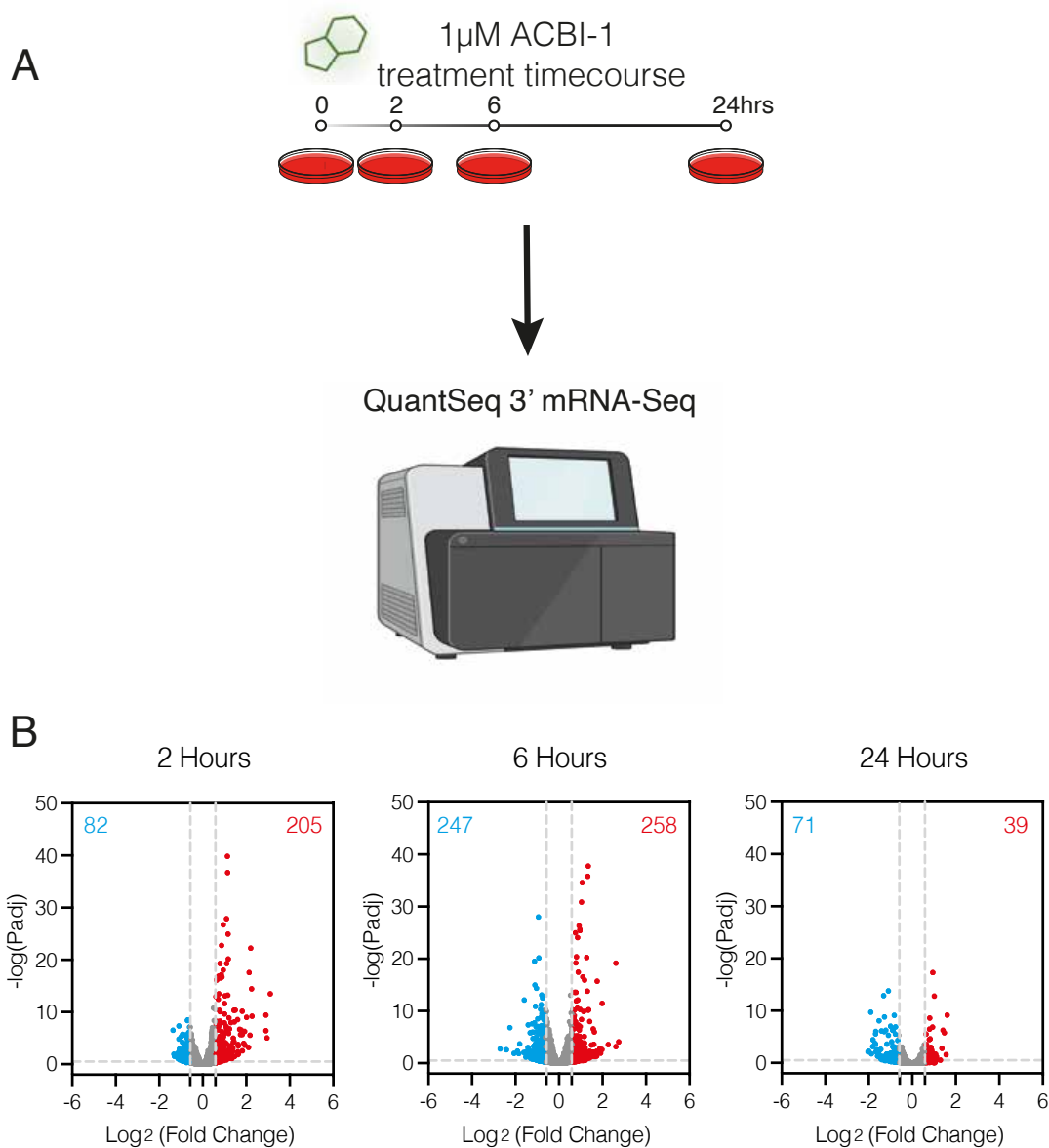


Figure 4.5: Investigating the transcriptional response to SMARCA4/2 degradation

A. Schematic representation of 1 μ M ACBI-1 treatment time course in HSSYII cells.

B. Quant-seq volcano plots presenting gene expression changes observed across the 1 μ M ACBI-1 treatment time course. Red and blue denote genes significantly up and down-regulated, respectively. Differentially expressed genes were detected using a ≥ 1.5 fold change and a < 0.05 Benjamini-Hochberg adjusted P value.

It was important to probe whether the same core genes were consistently downregulated/upregulated across the time course. Surprisingly, only 3 genes were detected as downregulated at all time points (**Figure 4.6 A**). In general, there was minimal consistency indicated across the time points for the downregulated genes (**Figure 4.6 A**). This point is further highlighted by the gene ontology analysis (**Figure 4.6 B**) where

there were no significant gene ontology terms detected for the downregulated genes at the 2-hour time point and there was only a modest overlap between the enriched gene ontology terms at the 6 and 24-hour time points (**Figure 4.6 B**). This scenario was mirrored in the upregulated genes (**Figure 4.6 B**). Notably, there was a switch in the gene ontology terms from developmental-related terms at 2 hours to metabolic and biosynthetic processes at the 6 and 24-hour time points where more overlap was indicated (**Figure 4.6 B**). Overall these findings suggest that across the indicated timecourse SMARCA4/2 degradation results in inconsistent fluctuating changes in gene expression.

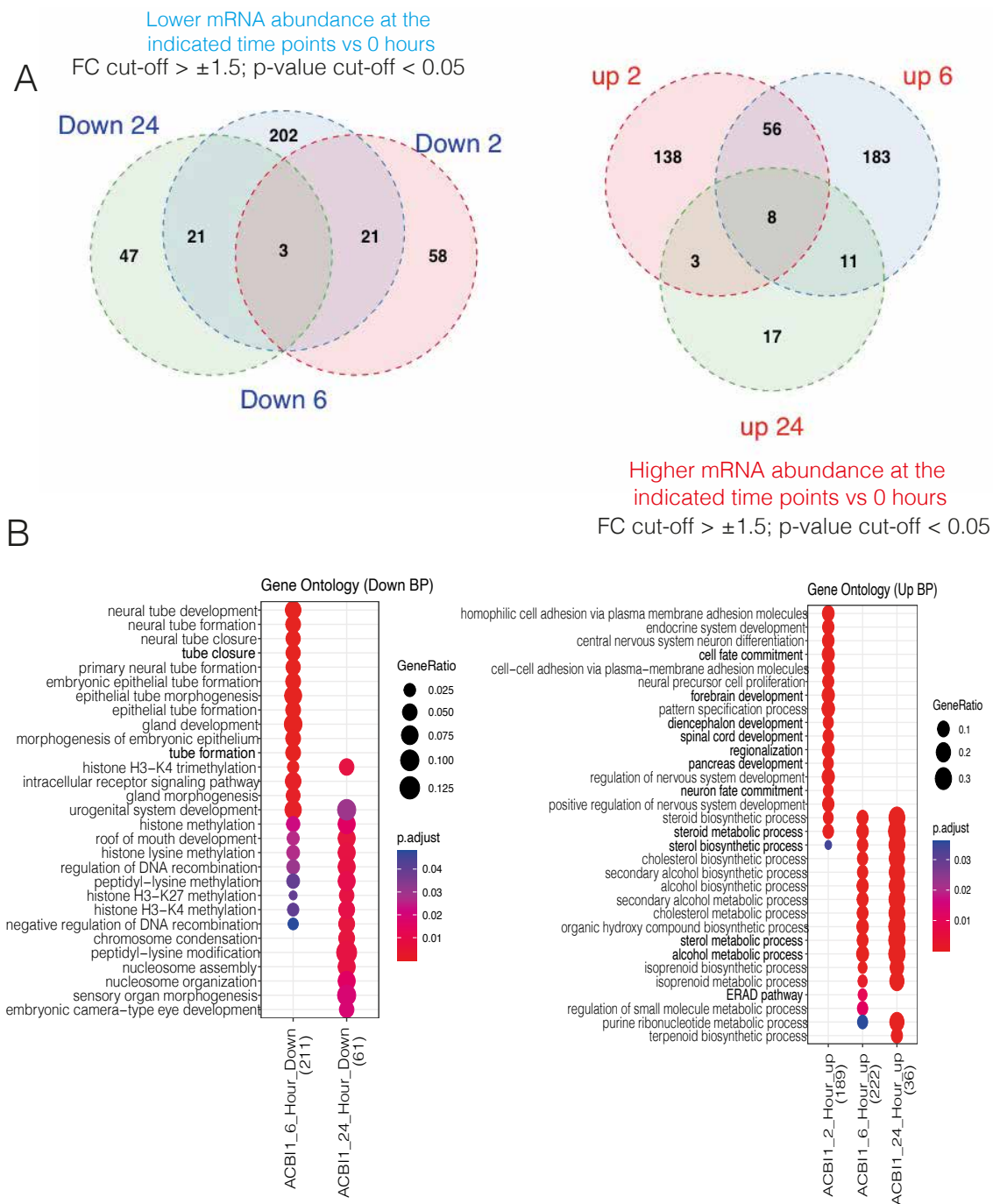


Figure 4.6: Probing the biological impact of SMARCA4/2 degradation

A. Venn diagram of overlapping downregulated (left) and upregulated (right) DEGs in the HSSYII cell line across the time course.

B. Comparative GO analysis of the downregulated (right) and upregulated (left) DEGs across the time-course.

4.2.3 Investigating chromatin accessibility changes induced by SMARCA4 perturbation

In-silico quality control of ATAC-seq data

SMARCA4 (and the SWI/SNF complex) affects chromatin structure by regulating accessibility to DNA regulatory elements (gene enhancers and promoters), facilitating the binding of additional transcriptional regulators at these sites. This is crucial for the proper regulation of gene transcription. As such it was imperative to investigate the chromatin accessibility changes induced by SMARCA4 perturbation. To complement the gene expression analysis the HSSYII cell line was treated with the same dose of ACBI1 across the same time course as used in the Quant-seq experiment **subsection 4.2.2** and ATAC-seq was performed in triplicate (**Figure 4.8 A**).

High-quality ATAC-seq libraries are expected to contain approximately 50% of reads post-filtering as short fragments ($\leq 100\text{bp}$) which denote nucleosome-free regions (NFRs). This is due to the fact that the Tn5 transposase is biased towards inserting sequencing adapters into regions of the highest chromatin accessibility. However, the transposase also inserts sequencing adapters into the linker DNA between neighbouring nucleosomes. As such, when assessed, the fragment size distribution should reveal a clear downward laddering effect, exhibiting the amount and length of DNA fragments from NFRs and those associated with one to numerous nucleosomes. All ATAC libraries generated in this study demonstrated the fragment size distribution pattern indicative of a successful ATAC-seq experiment, with one replicate being used as a representative example (**Figure 4.7 A**). This type of diagnostic plot is particularly important in providing insights into how sample preparation may be improved, as experiments prepared with a too-high ratio of Tn5 concentration to cells can result in a reduced signal-to-noise ratio.

In a successful ATAC-seq experiment, it is expected for the nucleosome-free fragment signal to enrich at the transcription start sites (TSSs) of active genes. Conversely, these regions should be devoid of any nucleosome-bound fragment signal but display periodic peaks of this signal immediately upstream and downstream of those TSSs. Expected signals around TSSs for nucleosome-free fragments and nucleosome-bound fragments are shown as heatmaps (**Figure 4.7 B**) and an average read coverage plot (**Figure 4.7 C**) for a representative replicate, indicating a successful ATAC-seq experiment.

The detection of a clear “footprint” of a well-characterized DNA binding protein such as CTCF is also

indicative of an optimized ATAC-seq experiment. Similar to nucleosomes, DNA-bound transcription factors (TFs) impede the Tn5-mediated cleavage of DNA and result in a defined region of decreased signal strength within a larger region of high signal known as a “footprint”. A clear footprint of the DNA-binding protein, CTCF, for a representative sample, is shown (**Figure 4.7 D**), providing further support for an optimized ATAC-seq assay.

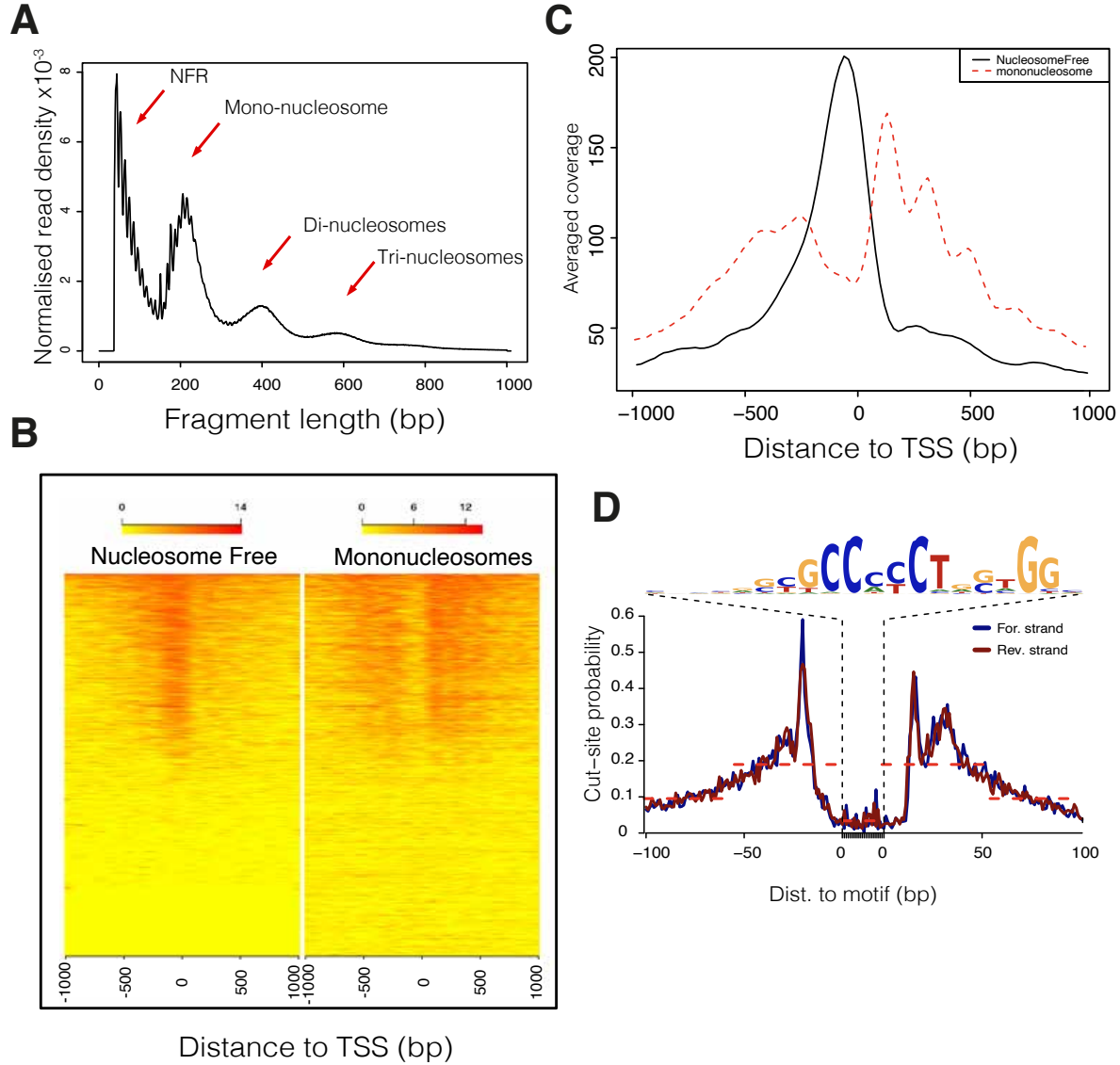


Figure 4.7: Quality control plots for a representative ATAC-seq dataset

A. Size distributions of sequenced fragments with reads passed filtering criteria for a representative replicate. Inferred nucleosome-free fragments and nucleosome-bound (mono-, di- and tri-nucleosome) fragments are labelled.

B. Heatmaps showing the distributions of signal around transcription start sites (TSSs), resulting from inferred nucleosome-free fragments and nucleosome-bound fragments.

C. Smoothed histograms of the signal displayed in B.

D. CTCF footprints inferred for the representative ATAC-seq dataset.

Differential chromatin accessibility analysis

On completion of the quality control process, the chromatin accessibility patterns between control and treated conditions were compared. As expected a predominant decrease in accessibility was observed across the ACBI1 time course (**Figure 4.8 B**). Specifically, at the 2-hour time point, 280 regions were detected to decrease in accessibility while 47 regions increased (**Figure 4.8 B**). At the 6-hour time point, there was a dramatic loss of accessibility with 3014 regions losing accessibility and 517 regions gaining (**Figure 4.8 B**). Finally, at the 24-hour time point, 2776 regions lost accessibility and 1450 regions gained accessibility (**Figure 4.8 B**). Similar to the analysis carried out in **subsection 4.2.2** it was important to investigate whether the same core regions were consistently losing/gaining accessibility across the time course. In contrast to the transcriptomic data, there was more consistency indicated across the genomic regions losing accessibility over time (**Figure 4.9 A**). There was a large degree of overlap between the regions losing accessibility at 6 and 24 hours with $\geq 50\%$ (1929) regions consistently losing accessibility (**Figure 4.9 A**). This point is further emphasised in the GO analysis (**Figure 4.9 B**), where the enriched GO terms were uniform across the time course and almost identical at 6 and 24 hours (**Figure 4.9 B**). Of particular interest was the strong enrichment of GO terms related to development and the switch to the enrichment of terms related to muscle development at 6 and 24 hours (**Figure 4.9 B**). In relation to the regions gaining accessibility, this scenario was not recapitulated, with minimal consistency indicated across the time course ((**Figure 4.9 A**). This was further indicated by the GO analysis which highlighted no overlap among enriched GO terms across the time course (**Figure 4.9 B**). Of note, at the 2-hour time point there was a strong and specific enrichment of GO terms related to metabolic and bio-synthetic processes (**Figure 4.9 B**). These same terms were enriched for the genes upregulated at the 6 and 24-hour time points in the transcriptomic data (**Figure 4.6 B**) suggesting that at 2 hours the regulatory regions of these genes begin to increase in accessibility. These findings show that across the indicated time course SMARCA4/2 degradation results in relatively consistent and stable losses in accessibility, while the gains in accessibility are inconsistent and varying. The large degree of chromatin accessibility losses observed at the later timepoints are interesting considering the modest changes in accessibility observed in (**Figure 4.5 B**).

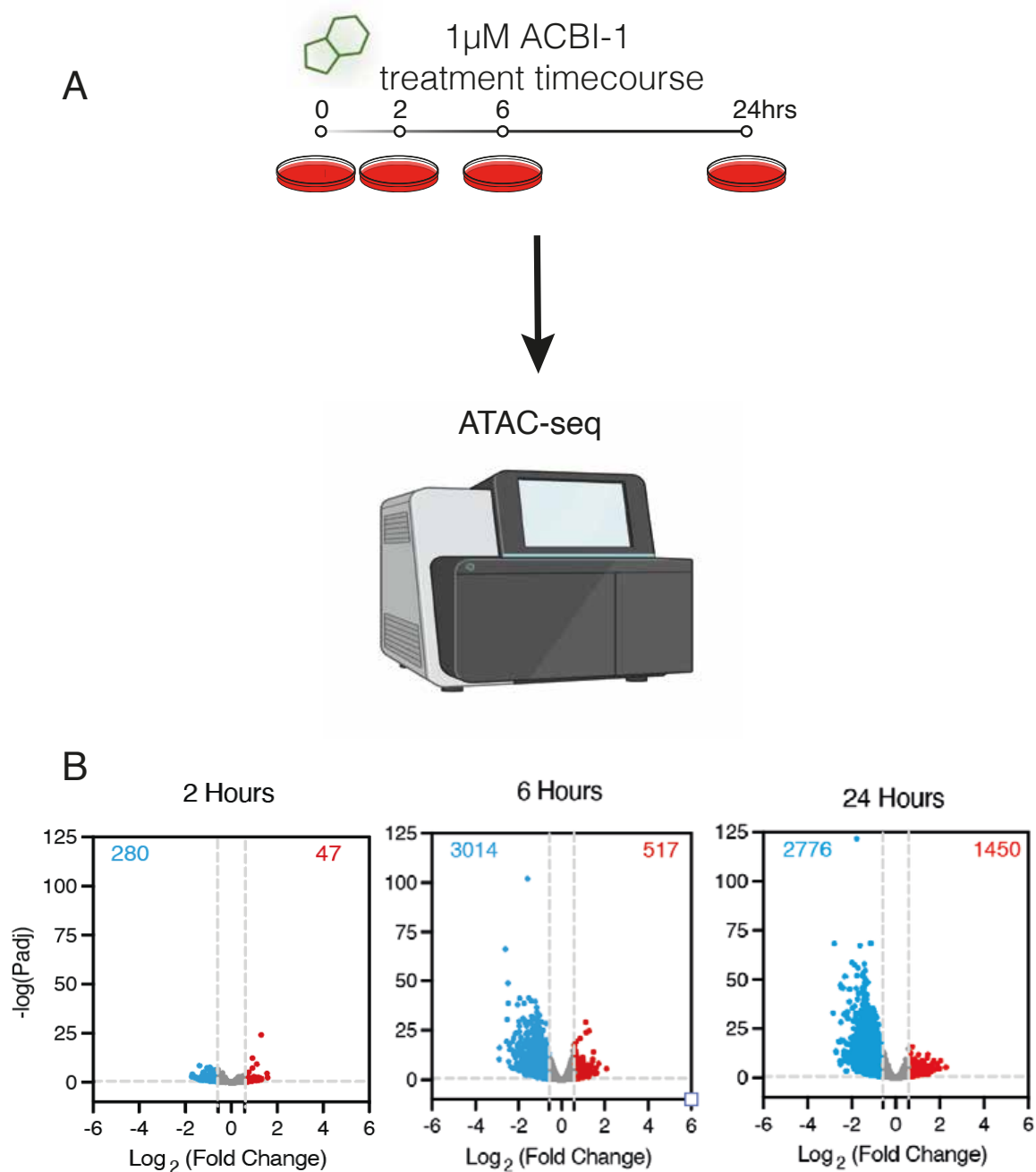


Figure 4.8: Investigating the chromatin accessibility dynamics induced by SMARCA4/2 degradation. Part I.

A. Schematic representation of 1 μ M ACBI-1 treatment time course in HSSYII cells.

B. ATAC-seq volcano plots presenting chromatin accessibility changes observed across the 1 μ M ACBI-1 treatment time course. Red and blue denote regions significantly gaining and losing accessibility, respectively.

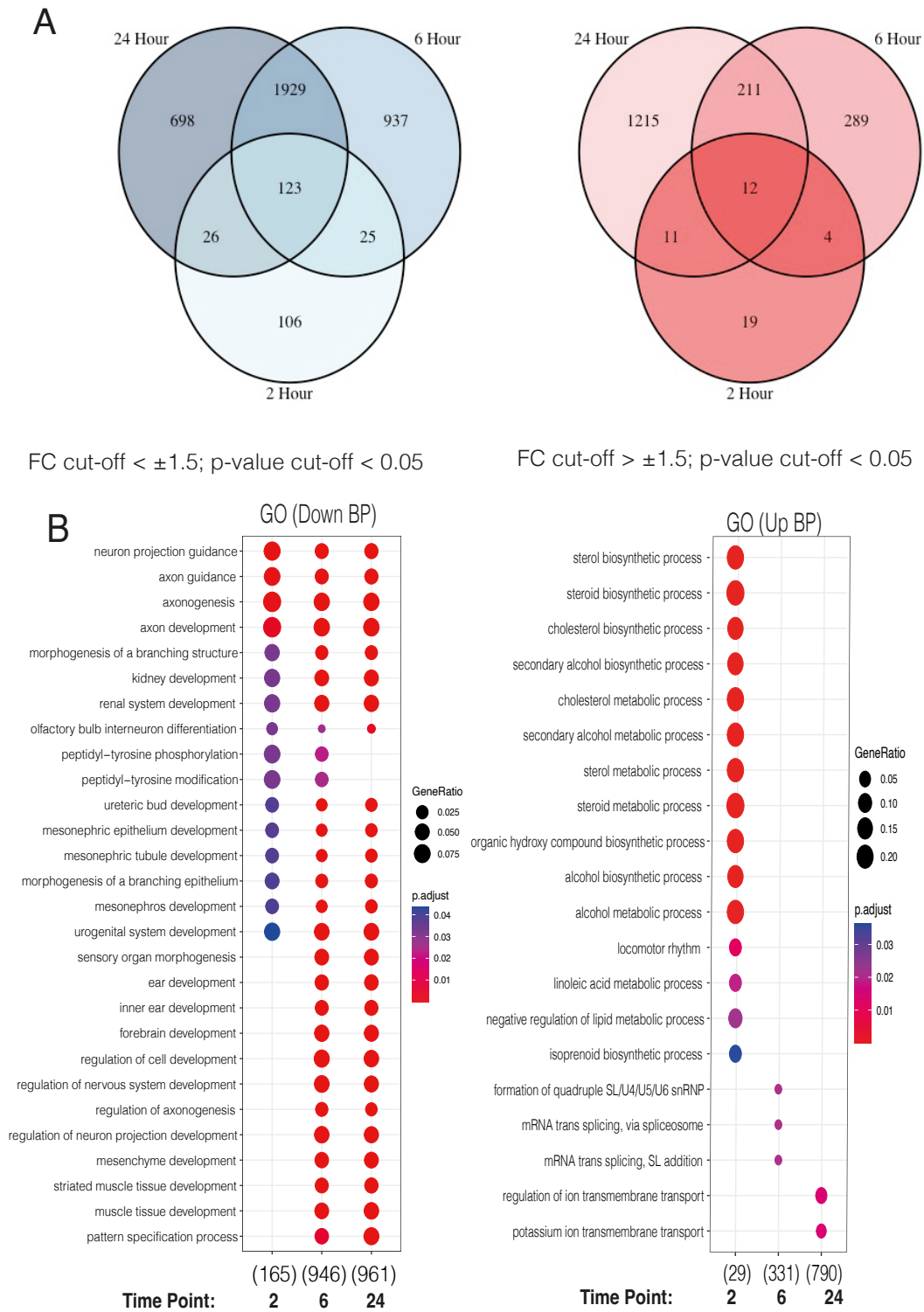


Figure 4.9: Investigating the chromatin accessibility dynamics induced by SMARCA4/2 degradation. Part II.

A. Venn diagram of overlapping closing (left) and opening (right) regions in the HSSYII cell line across the time course.

B. Comparative GO analysis of the downregulated (right) and upregulated (left) DEGs across the time-course.

4.3 Quantitative chromatin mapping with CUT&RUN-Rx

In order to gain deeper insights into the functional significance of SMARCA4/2 within the SS context, it was essential to establish a method to map their location on chromatin. Furthermore, it was equally important to map the fusion protein as this would serve as a crucial step in elucidating the functional relationship between these proteins. With this objective in mind, considerable effort was dedicated to establishing the CUT&RUN genome-wide chromatin profiling methodology in the lab (Skene et al., 2018). In order to ensure that the most accurate quantitative profiling of these proteins was carried out, exogenous reference genome-normalised CUT&RUN-Rx was performed with mouse embryonic stem cells (ESCs) used as spike-in (Orlando et al., 2014). In addition, three biological replicates were sequenced for each condition at each time point, a feat directly attributable to the cost savings facilitated by using CUT&RUN over conventional ChIP-seq (Skene et al., 2018)

To complement the gene expression and accessibility analysis in **subsection 4.2.3** and **subsection 4.2.2** HSSYII cells were treated with 1 μ M ACBI1 for 0, 2 and 24 hours prior to performing the CUT&RUN-Rx protocol (**Figure 4.10 A**). Initial CUT&RUN-qPCR was carried out prior to sequencing to ensure that the experiment was a technical success (**Figure 4.10 A & B**). Strong SMARCA4 enrichment was observed at the positive control site (the LHX2 promoter, an actively transcribed fusion protein target gene) (**Figure 4.10 B I**). As early as the 2-hour time point there was a dramatic reduction in SMARCA4 enrichment levels comparable to that of the 24-hour time point (**Figure 4.10 B I**). As this experiment centred around the use of ACBI1, it was internally controlled for antibody specificity. Additional indication of technical success was the absence of SMARCA4 enrichment at the negative control site (the promoter of the SSX1 gene, expression of which is restricted to testis tissue (**Figure 4.10 B I**) (Chen et al., 2003). In regards to SMARCA2, a stark reduction in enrichment levels was observed at the 2 and 24-hour time point, to levels comparable to the background signal obtained with the non-targeting IgG antibody in this experiment (**Figure 4.10 B II**). On the other hand, SS18-SSX1 enrichment was indicated to stay relatively stable across the time course (**Figure 4.10 B III**).

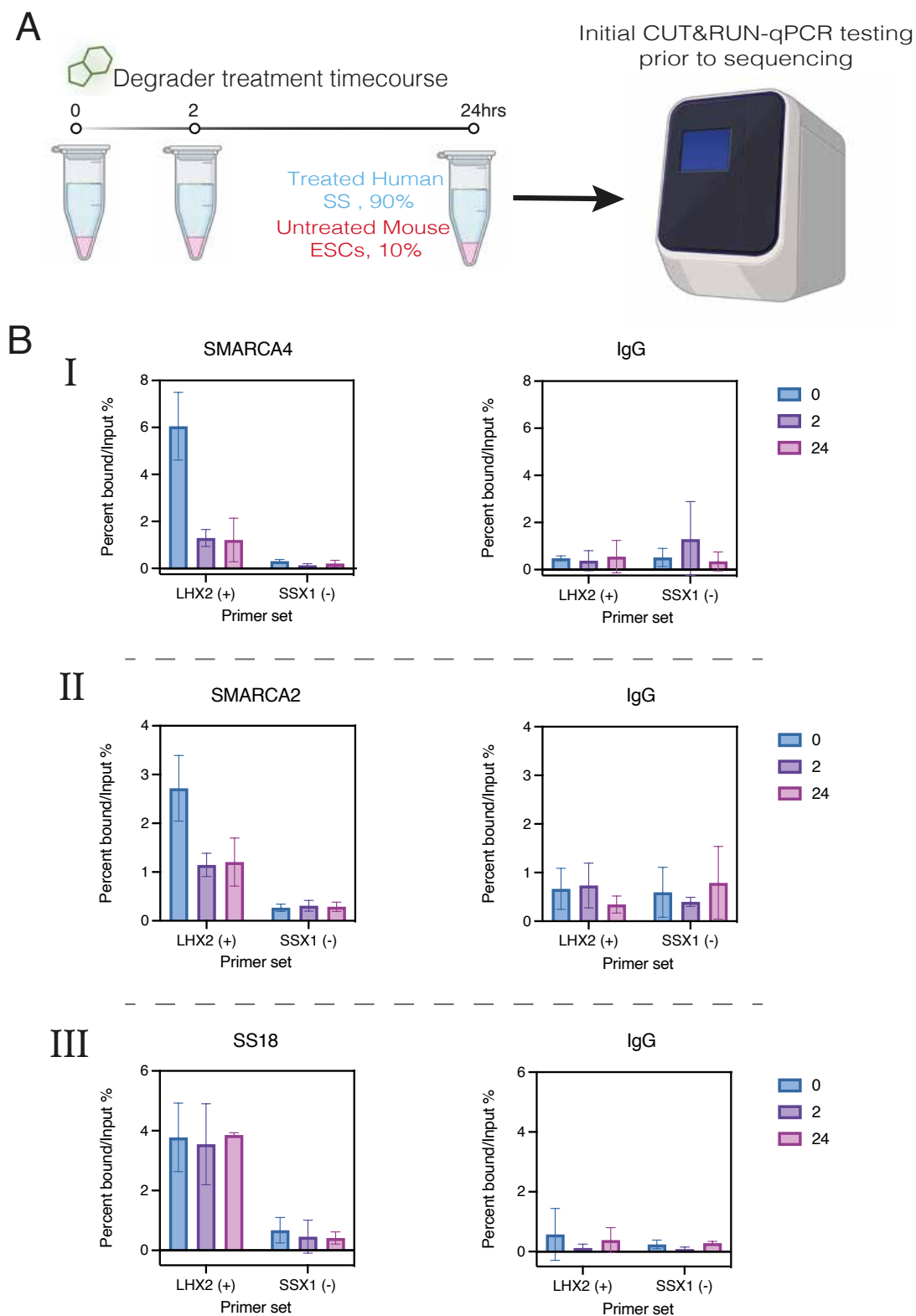


Figure 4.10: Initial quality control CUT&RUN-qPCR.

A. Schematic of the Cut&Run-Rx assay setup.

B. Initial CUT&RUN-qPCR assays were performed in HSSyII cells across a time course of 0, 2 and 24 hours using antibodies against SMARCA4 (I), SMARCA2 (II), SS18 (III) and IgG. q-PCR was performed with primers for the promoter of the active LHX2 gene and the promoter of the inactive SSX1 gene was used as a negative control site. Error bars represent three biological replicates.

After confirming technical success, the three biological replicates at each time point were sequenced and the indicated computational workflow was followed (**Figure 4.11**). Importantly, the final output of step 7 was a BigWig file displaying the average Rx-normalised signal for the three biological replicates (**Figure 4.11**). The final output of step 9 was a list of stringent, high-confidence, consensus peaks common to at least two biological replicates (**Figure 4.11**).

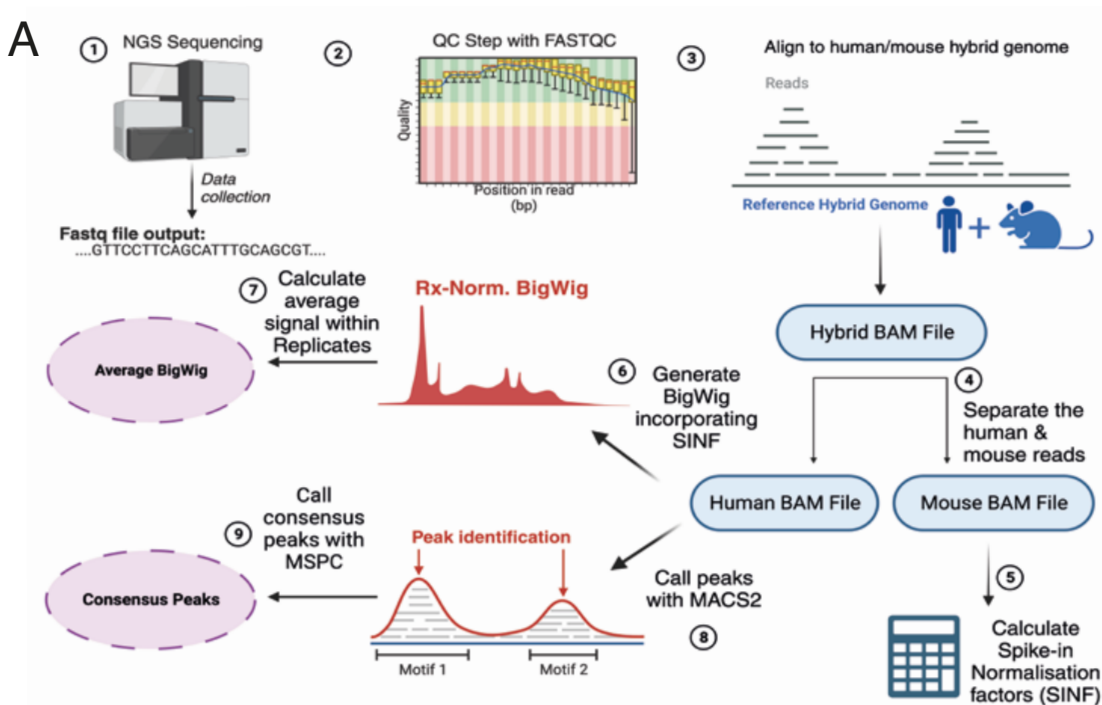


Figure 4.11: CUT&RUN-Rx computational Workflow.

A. Schematic of the CUT&RUN-Rx computational workflow followed. See subsection 2.5.3 for more detail.

In order to investigate the consequences of ACBI1 treatment on SMARCA4, SMARCA2, and SS18-SSX1 levels genome-wide, the average signal for each target at 0, 2, and 24 hours was determined at their respective consensus peak sets (**Figure 4.12 A**). Remarkably, the SMARCA4 signal was almost completely eliminated as early as 2 hours, and this reduction remained comparable to the signal at 24 hours (**Figure 4.12 A**). A similar pattern was observed for SMARCA2 albeit with a slightly less dramatic reduction in signal (**Figure 4.12 A**). In relation to the fusion protein, SS18-SSX1 signal also showed a decrease over the time course, although to a much lesser extent, with an approximate 50% loss of signal at 24 hours post-treatment (**Figure 4.12 A**). A representative gene track of SMARCA4/2 and SS18-SSX average CUT&RUN-Rx

signal across the indicated ACBI1 treatment time course at a key SS18-SSX target gene LHX2 is provided (**Figure 4.12 B**).

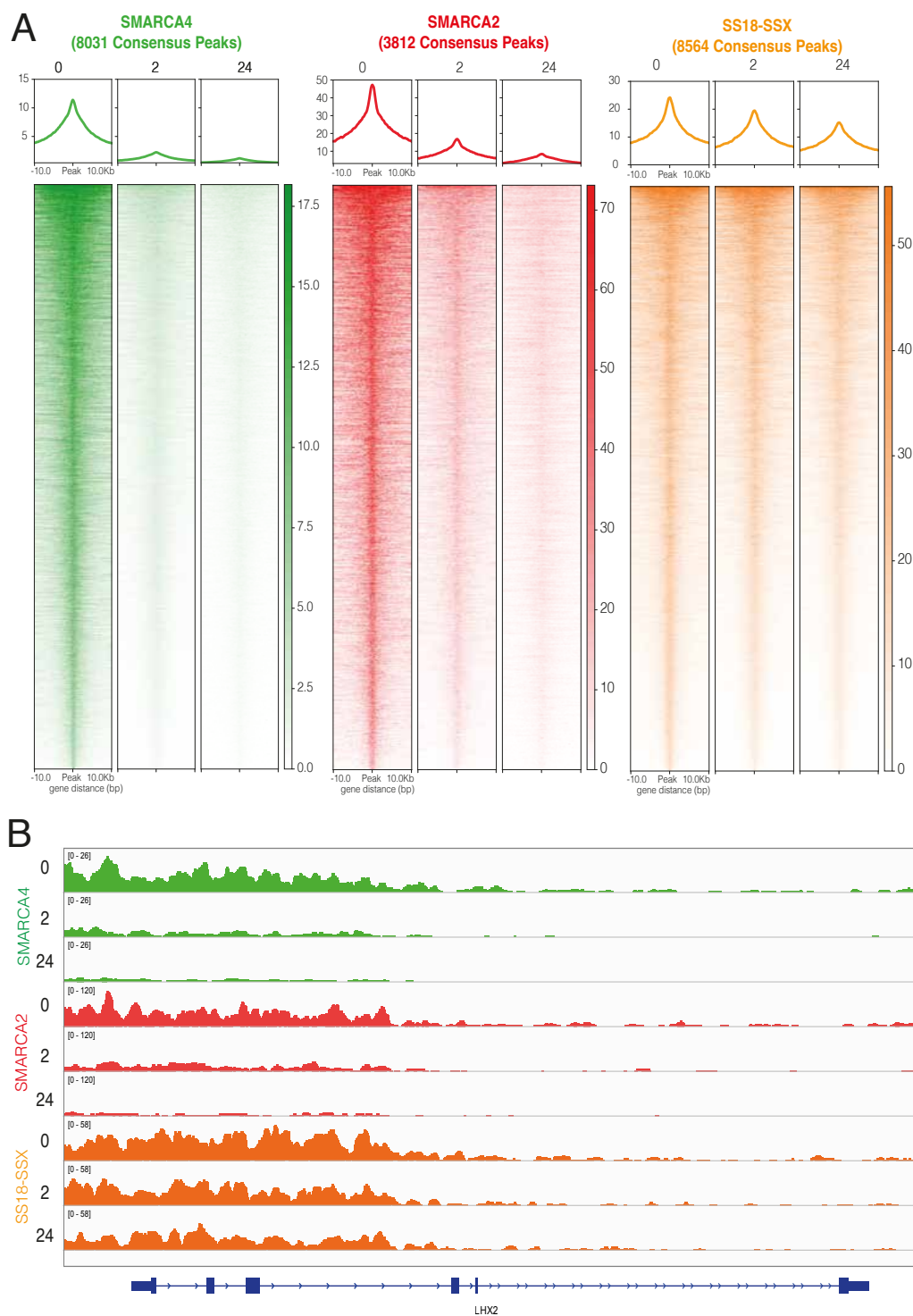


Figure 4.12: ACBI1 treatment induces a dramatic loss of SMARCA4/2 from their target sites. The effect on SS18-SSX localisation is less pronounced. Part I.

A. Heat maps depicting the average CUT&RUN-Rx normalised binding signal of SMARCA4, SMARCA2 and SS18-SSX within -10 and +10 Kb of the peak centre of their respective consensus peak sets across an ACBI1 treatment time course of 0, 2 and 24 hours.

B. A representative gene track of SMARCA4/2 and SS18-SSX average CUT&RUN-Rx signal across the indicated ACBI1 treatment time course at a key SS18-SSX target gene LHX2.

Following on from this, it was important to ask how the SMARCA4, SMARCA2 and SS18- SSX1 consensus peaks relate to each other. To address this, a Venn diagram of overlapping peaks was created. This analysis revealed 3160 regions common to all three proteins (**Figure 4.13 A**). It was also suggested that the majority of SMARCA2 peaks are contained within SMARCA4 peaks (**Figure 4.13 A**). Most interestingly, it suggested that 2916 regions specific to SS18-SSX exist which may represent sites in the genome where the fusion protein functions outside the confines of cBAF or GBAF (**Figure 4.13 A**). To explore this further a similar analysis to that in **Figure 4.12** was carried out for the regions highlighted in the Venn diagram. This analysis indicated that the binding of all three proteins appeared to be strongest where they exhibited co-binding and that SMARCA4, SMARCA2 and SS18-SSX signal was lost to a similar degree at all sites (**Figure 4.13 B**). Moreover, visualising the data in this more non-binary manner suggested that instead of isolated peaks, a spectrum of binding signal was observed (**Figure 4.13 B**).

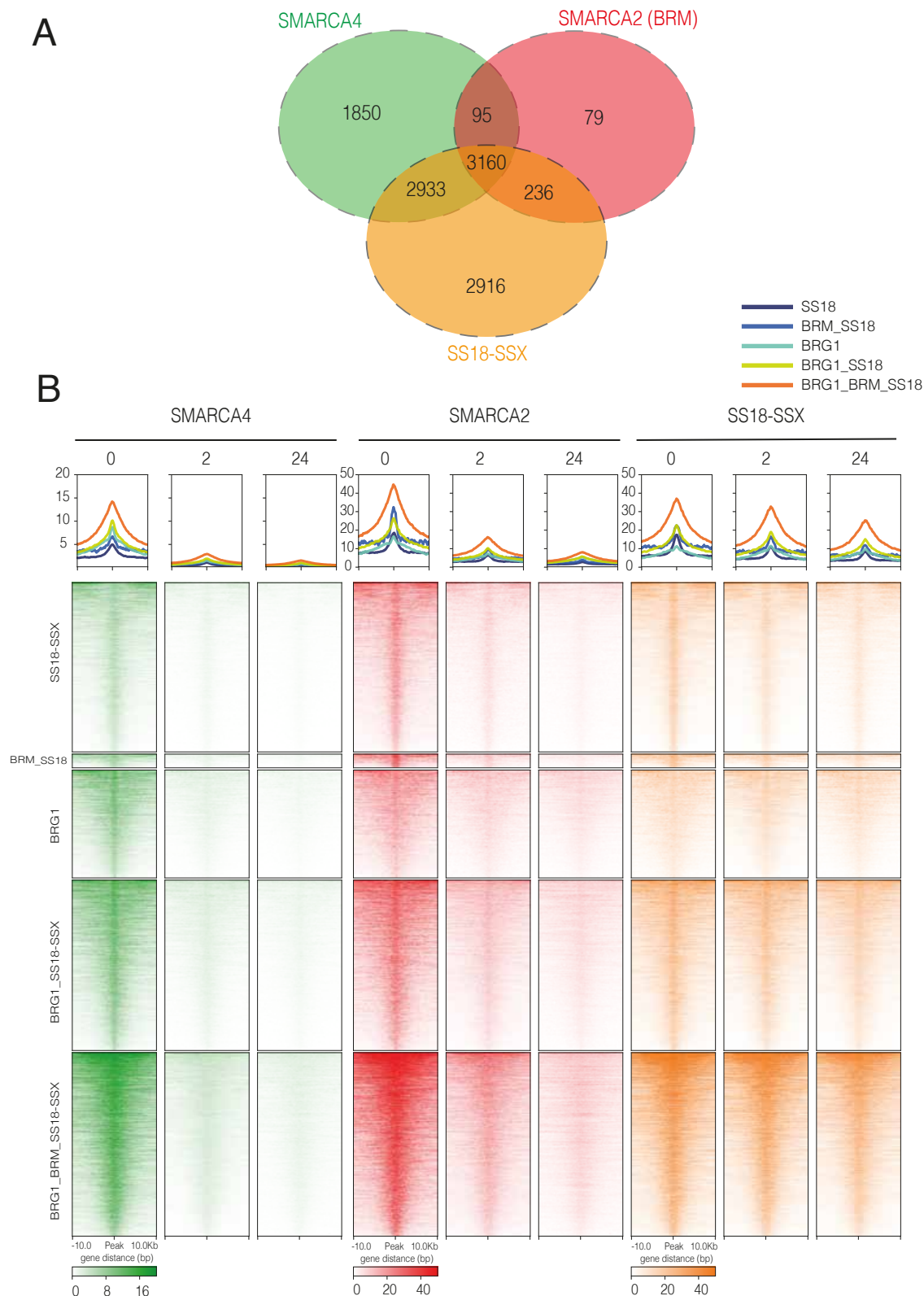


Figure 4.13: ACBI1 treatment induces a dramatic loss of SMARCA4 and SMARCA2 from their target sites. The effect on SS18-SSX localisation is less pronounced. Part II.

A. Venn diagram displaying the extent of overlap between SMARCA4, SMARCA2 and SS18-SSX peaks

B. Heat maps depicting the average CUT&RUN-Rx normalised binding signal of SMARCA4, SMARCA2 (BRM) and SS18-SSX within -10 and +10 Kb of the peak centre at the peak sets derived in panel A across the ACBI1 treatment time course of 0, 2 and 24 hours.

4.4 Short-term SMARCA4 degradation has a minimal effect on synovial sarcoma cell viability

It was important to investigate the effect of SMARCA4/2 degradation on the viability of SS cell. To address this HSSYII and CME1 cell lines were exposed to $1\mu M$ ACBI1 for up to 8 days (**Figure 4.14 A & B**). As described in **section 2.2** cell viability was assessed by two methods, the CTG-assay and manual cell counting. The CTG-assay is a luminescent assay which quantifies viability based on the metabolic activity of cells. Despite the rapid removal of SMARCA4/2 achieved with $1\mu M$ ACBI1 (**Figure 3.8 B**), there was a minimal effect on cell viability at 48 and 72-hours as assayed with CTG (**Figure 4.14 A**) with a maximum decrease in viability of 13% for HSSYII at 72-hours (**Figure 4.14 A**). Manual cell counts showed that by day 5 SS cell viability was impaired with an $\approx 50\%$ reduction in cumulative cell number (**Figure 4.14 B**). Manual cell counting was selected as it is a method more appropriate for longer drug treatments. WB analysis was carried out to investigate if the impaired cell proliferation observed at day 5 was linked to the destabilisation of the fusion protein (**Figure 4.14 C**). Results showed that protein levels of the fusion protein remained stable in both cell lines following ACBI1 treatment across this time frame (**Figure 4.14 C**).

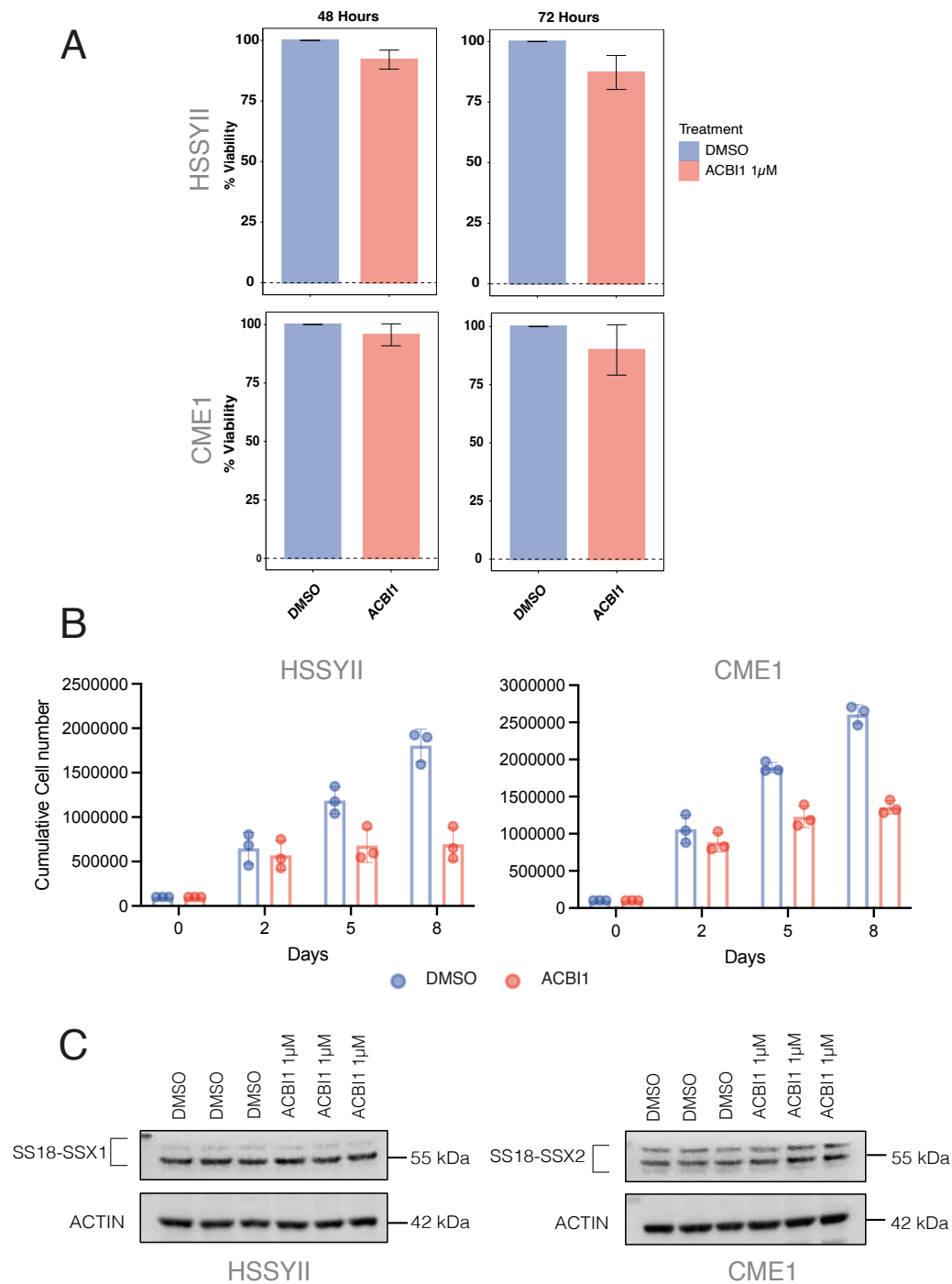


Figure 4.14: Short-term SMARCA4 degradation has a minimal effect on synovial sarcoma cell viability.

A. The response to ACBI1 was assessed as a % reduction in cell viability compared to DMSO at 48 and 72 hours using cell titre glow reagent (CTG). Cell lines are displayed as HSSYII in the top panel and CME1 in the bottom panel. Data represents the average viability and standard deviation of three biological replicates.

B. The response to ACBI1 and DMSO was assessed as cumulative cell number at 2, 5 and 8 days by manual cell counting. Cell lines are displayed as HSSYII in the left panel and CME1 in the right panel. Data represents the average viability and standard deviation of three biological replicates

C. Representative western blot of HSSYII cells (left) and CME1 cells (right) treated with DMSO or $1\mu M$ ACBI1 for 5 days. Actin signal was used as the loading control.

4.5 Discussion

This research chapter employed the SMARCA4/2 degrader ACBI1 to investigate the transcriptional impact and chromatin dynamics linked to SMARCA4/2 degradation in SS cells. Additionally, the repercussions of SMARCA4/2 loss on the viability of two distinct SS cell lines was profiled providing insight into the interplay between SMARCA4/2 function and cellular survival.

Continuous SWI/SNF complex activity has been demonstrated to be required to maintain chromatin accessibility (Schick et al., 2021). In agreement with these findings, SWI/SNF complex perturbation with ACBI1 across a time frame of 2, 6 and 24 hours showed a predominant reduction in chromatin accessibility (**Figure 4.8**). Considering the biochemical effects of SMARCA4/2 degradation characterised in detail in **chapter 3**, the fact that SS is considered a classic example of a malignancy driven by perturbed SWI/SNF function (Kadoch & Crabtree, 2013; Brien et al., 2018; McBride et al., 2018; Banito et al., 2018; Michel et al., 2018) and the result of the chromatin accessibility analysis, the limited effect on transcription following SMARCA4/2 degradation was surprising (**Figure 4.5 A**). However, consistent with this finding, a modest effect on gene expression was observed upon perturbing SMARCA4/2 function across a similar time frame (Schick et al., 2021). This finding also suggests that additional SS18-SSX co-factors may play an important role in synovial sarcomagenesis.

Consistent with the chromatin WB (**Figure 3.9 B**) and chromatin MS analysis (**Figure 3.10 B**) a robust genome-wide depletion of SMARCA4 was observed as early as the 2-hour timepoint (**Figure 4.12**). This was also the case for SMARCA2 (**Figure 4.12 A**). Of particular interest was the fact that although SMARCA4/2 are robustly lost from chromatin as early as 2 hours and disassembly of SWI/SNF complexes was indicated in **chapter 3** considerable SS18-SSX1 signal persisted at its consensus sites across the time course (**Figure 4.12 A**). This suggests that the fusion protein can maintain a relatively high degree of occupancy outside the confines of SWI/SNF complexes. The conserved 34-amino acid C-terminus region of the SSX portion of the fusion protein has been recently identified as a ligand for the nucleosome acidic patch and this imparts a preferential affinity of SS18-SSX bound complexes for nucleosomes marked with H2AK119Ub (McBride et al., 2020). This mechanism could help explain the maintained SS18-SSX signal. Future experiments will address whether longer-term ACBI1 treatments induce a greater loss of SS18-SSX1 from its target sites. Numerous previous studies have demonstrated global changes in various histone PTMs following SWI/SNF

perturbation most notably the active H3K27Ac modification (Schick et al., 2021; Panditharatna et al., 2022; Alver et al., 2017). Future chromatin profiling experiments will address this phenomenon in SS with the aim of shedding light on the observed disconnect between large-scale chromatin accessibility losses and the minimal downregulation of gene expression.

An unexpected finding in this research chapter was that although ACBI1 efficiently induced degradation of SMARCA4/2 within a mere two hours, the impact on cell viability remained remarkably minimal until five days of drug exposure (**Figure 4.14**). This finding may provide insights into why only modest changes in gene expression were detected within the brief time frames of 2, 6, and 24 hours (**Figure 4.5 A**). It is important to remember the context in which SMARCA4 emerged as a genetic dependency in SS cells; the determination was made through harnessing the data generated from genetic dependency screening experiments which were conducted over a time span of several weeks (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.). These findings justify profiling the gene expression changes induced by SMARCA4/2 across a longer time scale in follow up experiments. Given SMARCA's important role as the catalytic subunit of the SWI/SNF complex and its pivotal roles in development, gene regulation and disease pathogenesis it remains crucial to profile the response to ACBI1 in a wider panel of cell lines including additional SS cell lines, normal cells, and cancer cell lines from different lineages.

Chapter 5

Exploring the role of the histone acetyltransferase EP300 in synovial sarcoma

5.1 Introduction

In the initial study from our lab which identified BRD9 as a critical functional dependency in synovial sarcoma (Brien et al., 2018) and laid the foundation for exploring BRD9 degradation in clinical trials (ClinicalTrials.gov identifiers: NCT05355753, NCT05355753) the E1A-binding protein p300 (EP300) but not the highly homologous CREB-binding protein (CREBBP or CBP) was also identified as functionally essential in synovial sarcoma cells (**Figure 5.1 A**). This finding is also important in the context of the results discussed in **chapter 4**, which suggested that additional SS18-SSX co-factors may play an important role in synovial sarcomagenesis. The goal of this research chapter was to follow up on this finding and to investigate the effect of perturbing EP300 function on the transcriptional program of synovial sarcoma cells.

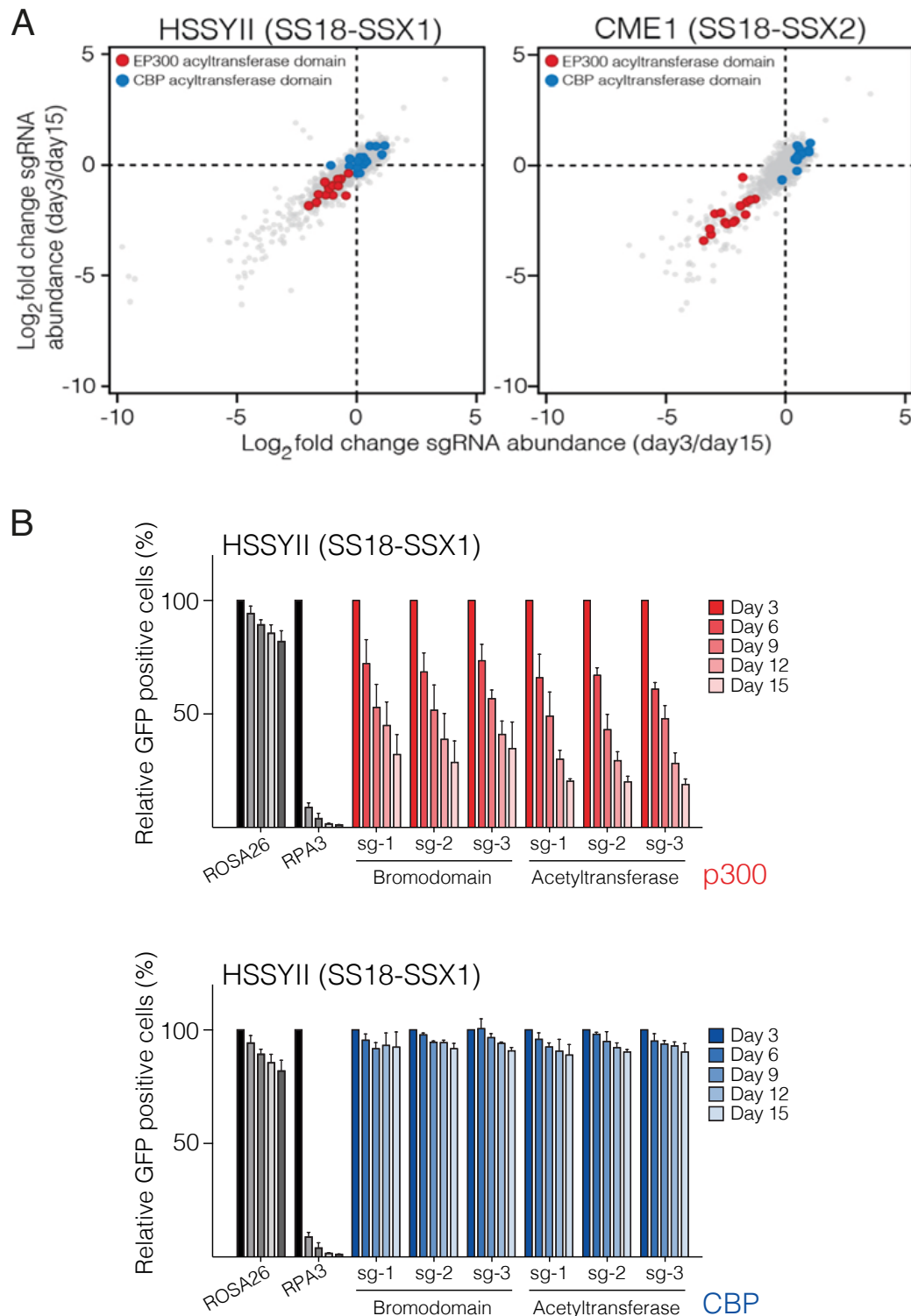


Figure 5.1. EP300 is a functional dependency in synovial sarcoma cell lines.

A. Scatter plot representation of biological duplicate sgRNA screening data in two synovial sarcoma cell lines HSSYII and CME1. Each dot denotes an individual sgRNA and axes represent log₂ fold-change in sgRNA abundance between day-3 and day-15. P300 and CBP acetyltransferase targeting sgRNAs are highlighted in red and blue respectively.

B. Negative selection assay results for sgRNAs targeting both the acetyltransferase and bromodomain of p300 and CBP in HSSYII cells. Graph shows the percentage of the cell population that was GFP-positive at the indicated time point relative to the first time point (Day 3 postinfection). sgRNA targeting ROSA26 is a negative control and sgRNAs targeting RPA3 are positive controls (n = 3, mean ±SD).

Additional motivation to pursue EP300 targeting was the fact that EP300/CBP function has been exploited by cancer cells where they have been demonstrated to support oncogenesis as key secondary regulators of oncoproteins in a number of cancer contexts (see **section 6.3**). However, to date, no published research has investigated the role of EP300 function in synovial sarcomagenesis. Moreover, in the experiment described in **section 5.2** EP300 was also identified in 2 independent SS cell lines as a high confidence fusion protein interactor, signalling functional importance.

In this chapter, the transcriptional response to EP300 targeting was addressed using a chemical biology-based approach, leveraging the availability EP300/CBP targeting compounds. Initially, the transcriptional response to A48-5 a potent and selective small molecule inhibitor of both EP300 and CBP (Lasko et al., 2017) was characterised. As the bromodomain of EP300 had also been shown to be functionally essential in validating work (**Figure 5.1 B**) a recently developed EP300/CREBBP degrader molecule dCBP-1 was also used in complementary experiments (Vannam et al., 2021). This point underscores a significant advantage of degrader compounds, particularly when dealing with proteins that possess multiple crucial functional domains. Achieving complete degradation in such cases is notably more advantageous. One important caveat to address is the dual inhibitory functions of these compounds, a direct consequence of the extensive sequence homology shared by P300 and CBP. As such, the transcriptional response of synovial sarcoma cells to either of these compounds cannot be attributed to the perturbation of P300 alone. However, as our ultimate goal is to provide a pre-clinical rationale and mechanistic insight into the potential clinical utility of targeting P300 in synovial sarcoma patients, utilising A-485/dCBP-1 is relevant and appropriate as it is highly unlikely that clinically useful small molecules can be developed displaying selectivity for either protein.

Unbiased RNA-seq was the method chosen to assess the transcriptional response to A-485 treatment. As EP300 and CBP are important regulators of gene expression special consideration was given to ensuring a reliable gene expression analysis. This involved the use of spiked-in RNA standards permitting normalisation to cell number, the importance of which has been highlighted in the literature (Lovén et al., 2012). Spiked-in standards are especially important to use in contexts where large changes in RNA levels are anticipated. This is because if equal amounts of RNA from two populations of cells are used as input material without permitting normalisation to cell number as in the conventional approach, large differences in RNA yield resulting from the specific experimental manipulation have the potential to be masked and confound interpretations

(Lovén et al., 2012) Indeed, this was the case for the BET bromodomain inhibitor JQ-1 (Filippakopoulos et al., 2010) used as an example in this research chapter to highlight the careful consideration given to conducting a reliable gene expression analysis.

To further explore the relationship between SS18-SSX and EP300 efforts were focused on establishing an isogenic cell model in which the fusion protein could be acutely degraded. To achieve this an approach coined Small Molecule-Assisted Shutoff (SMASh) was adopted (Chung et al., 2015). The cellular context in which to establish this model was carefully considered and the murine C2C12 cell line was chosen. This was because C2C12 cells are myoblasts, precursor cells to muscle fibres (myotubes) that can be differentiated in culture and therefore represent a myogenesis model (Tang et al., 2018). This is important as outlined in the main introduction the cell of origin of SS is believed to be mesenchymal and conditional expression of the fusion protein in muscle progenitor cells of mice leads to the development of tumours that histologically and molecularly resemble the human disease (Haldar et al., 2007).

5.2 EP300 interacts with SS18-SSX and is a strong genetic dependency

To explore additional dependencies in SS use was made of an experiment characterising the composition of endogenous SS18-SSX containing SWI/SNF complexes which combined SS18-SSX IP with MS in two independent SS cell lines previously mentioned in **section 3.1 (Figure 5.2 A)**. As the fusion protein is the primary driver of synovial sarcomagenesis it was anticipated that additional fusion protein interactors may represent promising therapeutic targets. To this end, a list of proteins found to be enriched >4-fold in the SS18-SSX IP in both cell lines was compiled, representing a list of high confidence fusion protein interactors (**Figure 5.2 A & B**). This analysis identified 74 proteins common to both the HSSYII and CME1 cell line which included 22 SWI/SNF members (**Figure 5.2 B**). To access the functional importance of these interactors, the dependency score for each interactor for all SS cell lines in DepMap was plotted as a heatmap (**Figure 5.2 C**). Prior to this analysis any interactor characterised as common essential was removed. Strikingly, apart from SMARCA4 the focus of chapter 3 and 4, SMARCD1, DPF2, SS18 and BRD9 highlighted in chapter 3, EP300 was the only interactor indicated as a dependency in SS (**Figure 5.2 C**). Moreover, EP300 and SS18 itself clustered exclusively (**Figure 5.2 C**). Notably, the SW982 cell line

(highlighted in red in the figure), although believed to have originated from a patient with synovial sarcoma did not contain the pathognomonic SS18-SSX translocation (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.). Remarkably, this cell line was the only SS cell line in DepMap not dependent on SS18 or EP300, suggesting that the dependency on EP300 is directly linked to the presence of SS18-SSX (**Figure 5.2 C**). In agreement with the CRISPR screen result no dependence on CREBBP was evident.

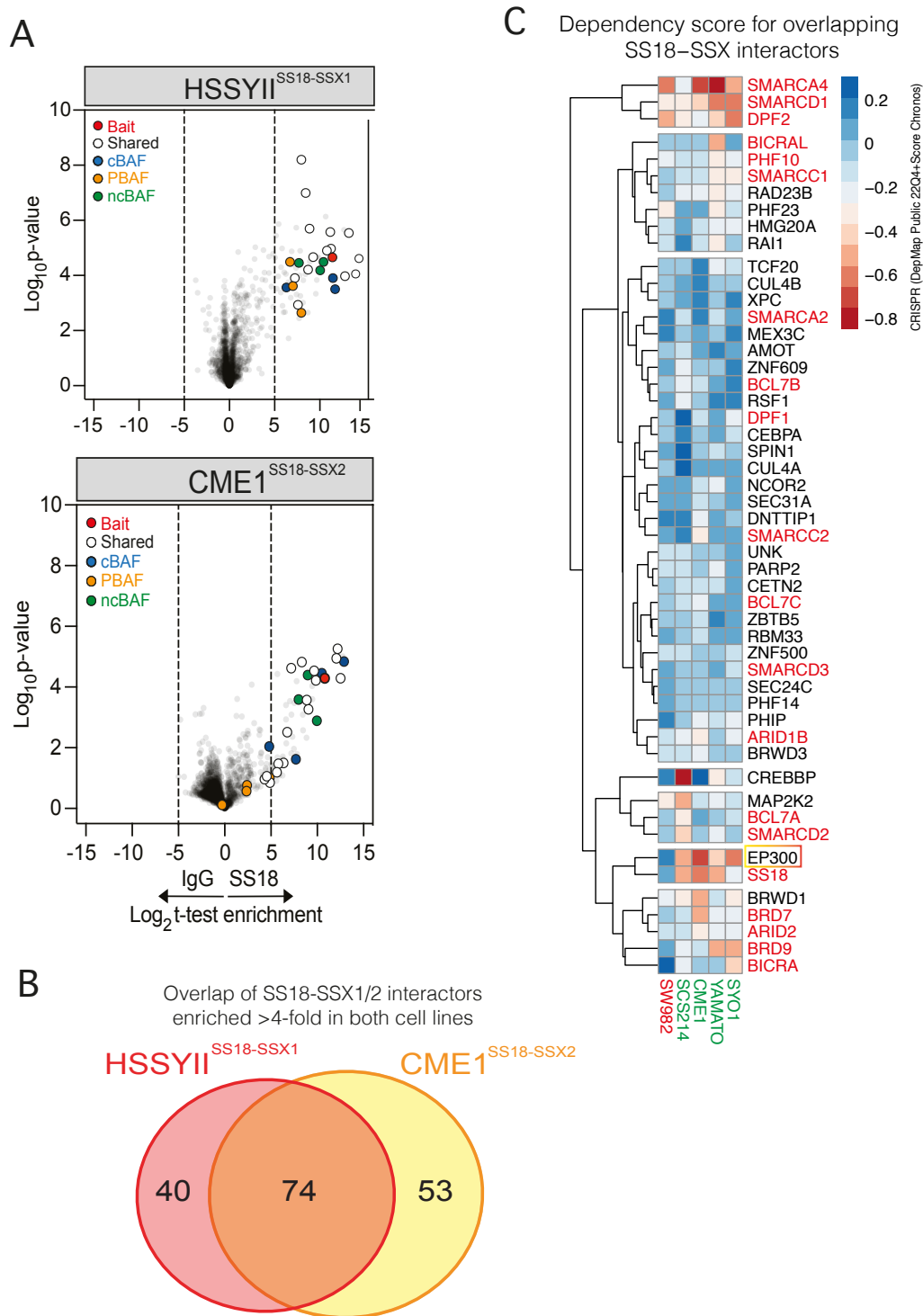


Figure 5.2. EP300 interacts with SS18-SSX and is a strong genetic dependency

A. SS18-SSX1/2 IP coupled with mass spectrometry analysis in HSSYII and CME1 cells. The $\log_2$ t-test enrichment of the SS18 IP over the IgG IP is plotted against the $-\log_{10}$ p-value. SWI/SNF components are colour-coded.

B. Overlap of SS18-SSX1/2 interactors enriched >4-fold in HSSYII and CME1 cell lines.

C. Heatmap displaying the DepMap dependency score for the overlapping SS18-SSX1/2 interactors.

To explore this relationship further the SS18 gene effect and EP300 gene effect were plotted for all cell lines in DepMap (**Figure 5.3 A**). Across all cell lines in DepMap the pearson correlation between SS18 and P300 was 0.135; but remarkably in synovial sarcoma cells this increased to 0.757 (**Figure 5.3 A**). Together, these data indicate that SS18 and P300 are strong co-dependencies in synovial sarcoma; but not across other cancer contexts; suggesting the possibility of some shared mechanistic role specific to synovial sarcoma. The same analysis for CREBBP revealed a Pearson correlation of 0.262 indicating the absence of a co-dependent relationship (**Figure 5.3 A**). Further analysis demonstrated that the dependency score for EP300 was on par of that of SS18 itself and more stark than BRD9 a known genetic dependency under evaluation in clinical trials (**Figure 5.3 B**) (Brien et al., 2018) (ClinicalTrials.gov identifiers: NCT05355753, NCT05355753). As a means to reinforce and verify that CREBBP was not detected as a functional dependency due to the simple fact that it is not expressed, the gene expression levels of these two proteins were directly compared (**Figure 5.3 B**). Across all the SS lines the EP300 and CREBBP genes were observed to be expressed to comparable levels and to a comparable level to BRD9 (**Figure 5.3 B**), supporting the idea that CREBBP is not a functional dependency in synovial sarcoma cells.

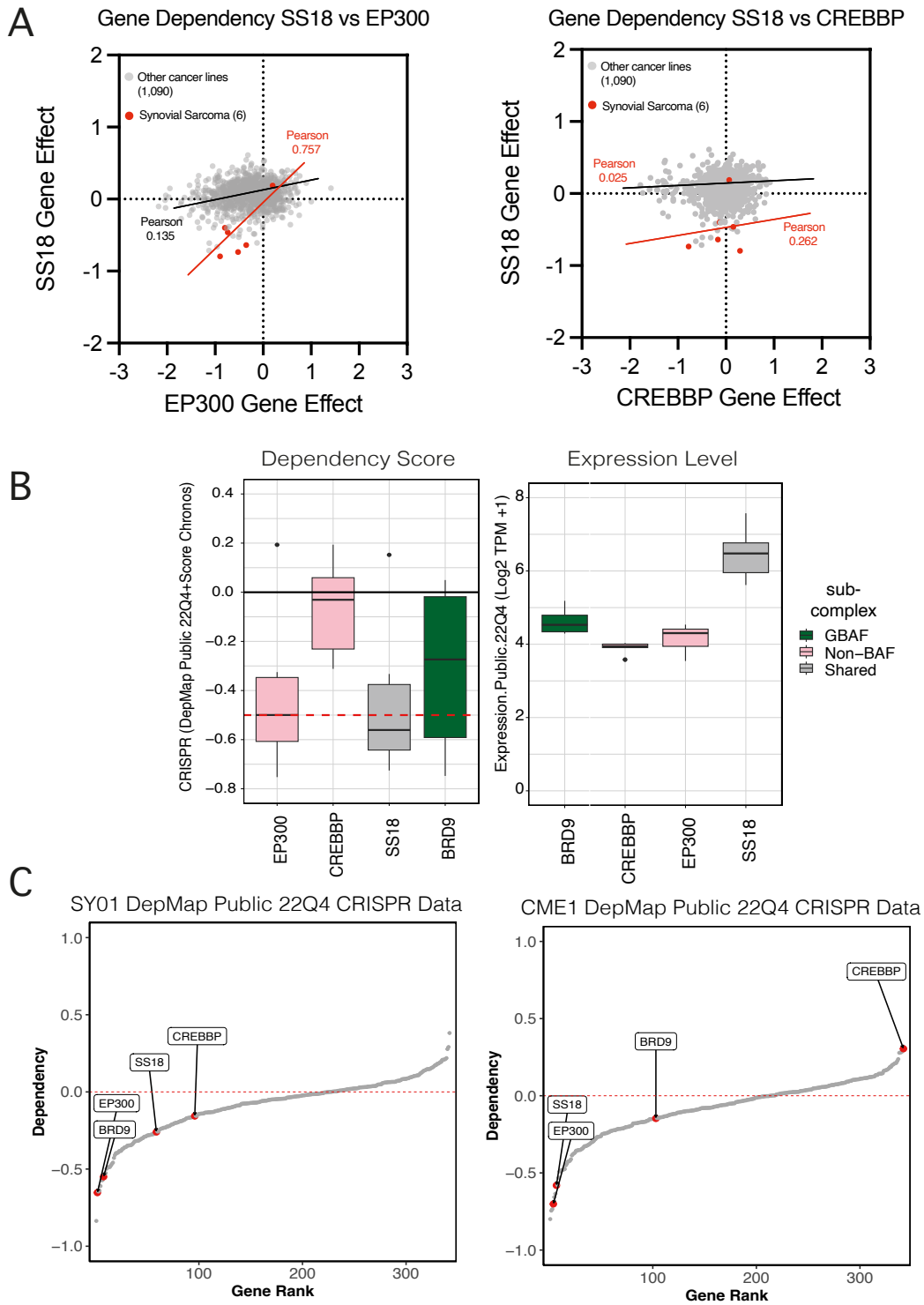


Figure 5.3: EP300 is a strong genetic dependency continued.

A. Scatterplots of the SS18 gene effect vs the EP300 (left) and the CREBBP gene effect (right) with SS cell lines highlighted in red.

B. Boxplots of dependency score and expression level of the indicated genes. Box plots display median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as solid points. Subunits are colour-coded by sub-complex incorporation.

C. Ranked chronos dependency scores for two SS cell lines for a curated list of 366 chromatin regulatory genes. Common essential genes, as defined by DepMap (Achilles, 19Q3), were removed. SS18, BRD9 and EP300 are highlighted in red.

The ultimate goal was to identify and pursue impactful vulnerabilities that display a selective effect on synovial sarcoma cells. To this end, all of the cell lines available in DepMap were grouped by lineage subtype and the CRISPR score for EP300 was plotted as a boxplot (**Figure 5.4 A**). A number of lineages were detected where EP300 was highlighted as a potential genetic dependency (**Figure 5.4 A**). Synovial sarcoma was among the top scoring lineages (**Figure 5.4 A**). CREBBP was observed to be less important for cancer survival generally and as expected synovial sarcoma cells were agnostic to CREBBP targeting (**Figure 5.4 B**).

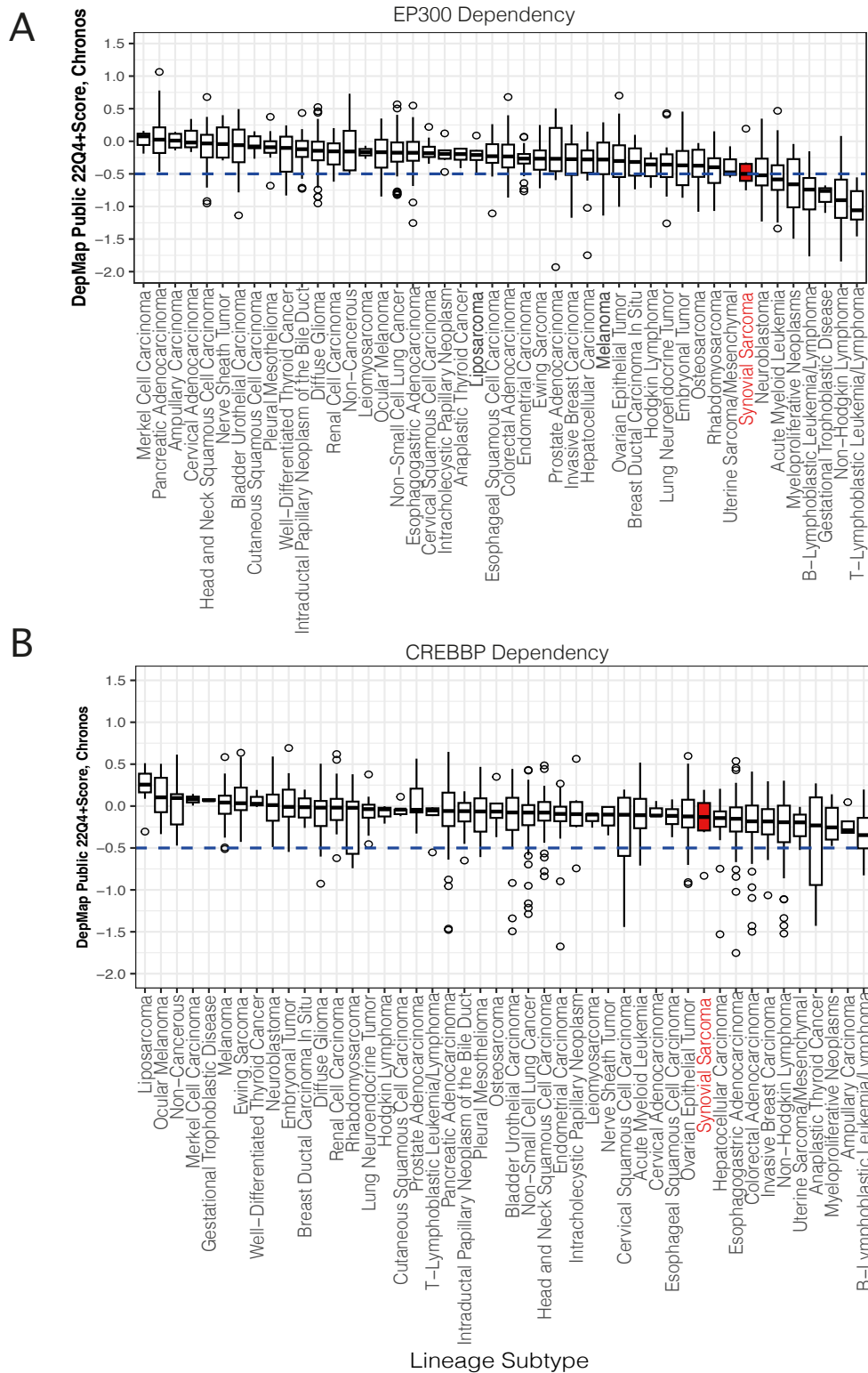


Figure 5.4: Synovial sarcoma is among the top lineages dependent on EP300

A. The relative dependence of SS cells on EP300.

B. The relative dependence of SS cells on CREBBP.

Box plots of median and quartiles, with whiskers showing $1.5 \times$ inter-quartile ranges. Outliers are indicated as open points.

5.3 Investigating the transcriptional response to enzymatic inhibition of EP300

5.3.1 Accessing the cellular activity of A-485

To assess the activity of A-485 in synovial sarcoma cell lines, HSSYII and CME1 cells were treated with a range of concentrations of A-485 or the inactive analogue A-486 for 24 hours (**Figure 5.5 B**). A-485 treatment resulted in a dose-dependent decrease of H3K27Ac and H3K18Ac but not H3K9Ac (**Figure 5.5 B**). This result supported the on-target activity of A485, since previous genetic studies have demonstrated that EP300/CBP selectively acetylates H3K27/H3K18 but not H3K9 residues (Jin et al., 2011b) As anticipated, A-486 was confirmed to be inactive and no inhibition of either modification was observed.

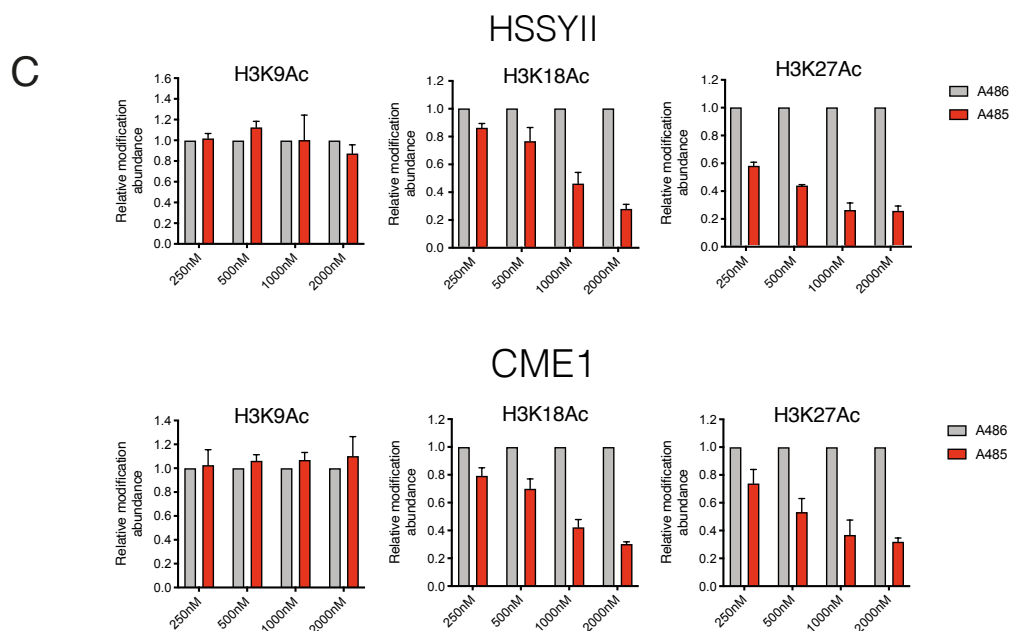
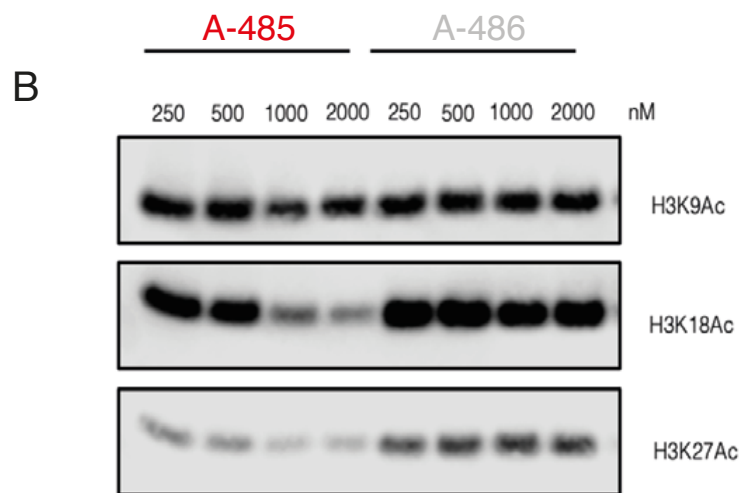


Figure 5.5: Confirming cellular activity of A-485

A. Schematic representation of A48-5 and A48-6 compounds.

B. A representative western blot of HSSYII cells treated with the indicated doses of A-485 or A-486 for 24 hours.

C. Quantification of western blots of H3K27Ac, H3K18Ac and H3K9Ac for 3 independent biological replicates for the HSSYII (top) and CME1 (bottom). Error bars represent standard deviation.

5.3.2 Comparing traditional RNA-seq and spike-in normalised RNA-seq in the context of BET bromodomain inhibition

Following on from establishing a method to perturb EP300 enzymatic activity and prior to investigating the transcriptional consequences, special consideration was given to ensuring a reliable gene expression analysis was conducted. To this end, the importance of including spiked-in RNA standards that permit normalisation to cell number was explored in the context of BET bromodomain inhibition with JQ-1. This data set was generated by Dr. Gerard Brien and was used as it was readily available in the lab and is a context where large changes in RNA levels are anticipated.

HSSYII synovial sarcoma cells were treated with 500 nM JQ-1 for 24 hours (**Figure 5.6 A**). Initial principal component analysis revealed tight clustering of all three replicates and indicated a strong separation between conditions (**Figure 5.6 B**). A stark difference in the number of DEGs was immediately apparent when DESeq2 (Love et al., 2014) was employed to carry out the differential expression analysis including or excluding the spike-in normalisation constants (**Figure 5.6 C**). In the standard normalization context, the expression of 3833 genes showed a > 1.5 -fold decrease ($p < 0.05$), while 3143 showed an increase (**Figure 5.6 C**). When spike-in normalization was used, the expression of 10493 genes showed a > 1.5 -fold decrease ($p < 0.05$) while 363 showed an increase (**Figure 5.6 C**). As the large-scale inhibition of transcription consequent to JQ-1 treatment was only revealed in the spike-in normalisation context it was decided to conduct spike-in normalised RNA-seq when characterising the transcription response to A-485 treatment.

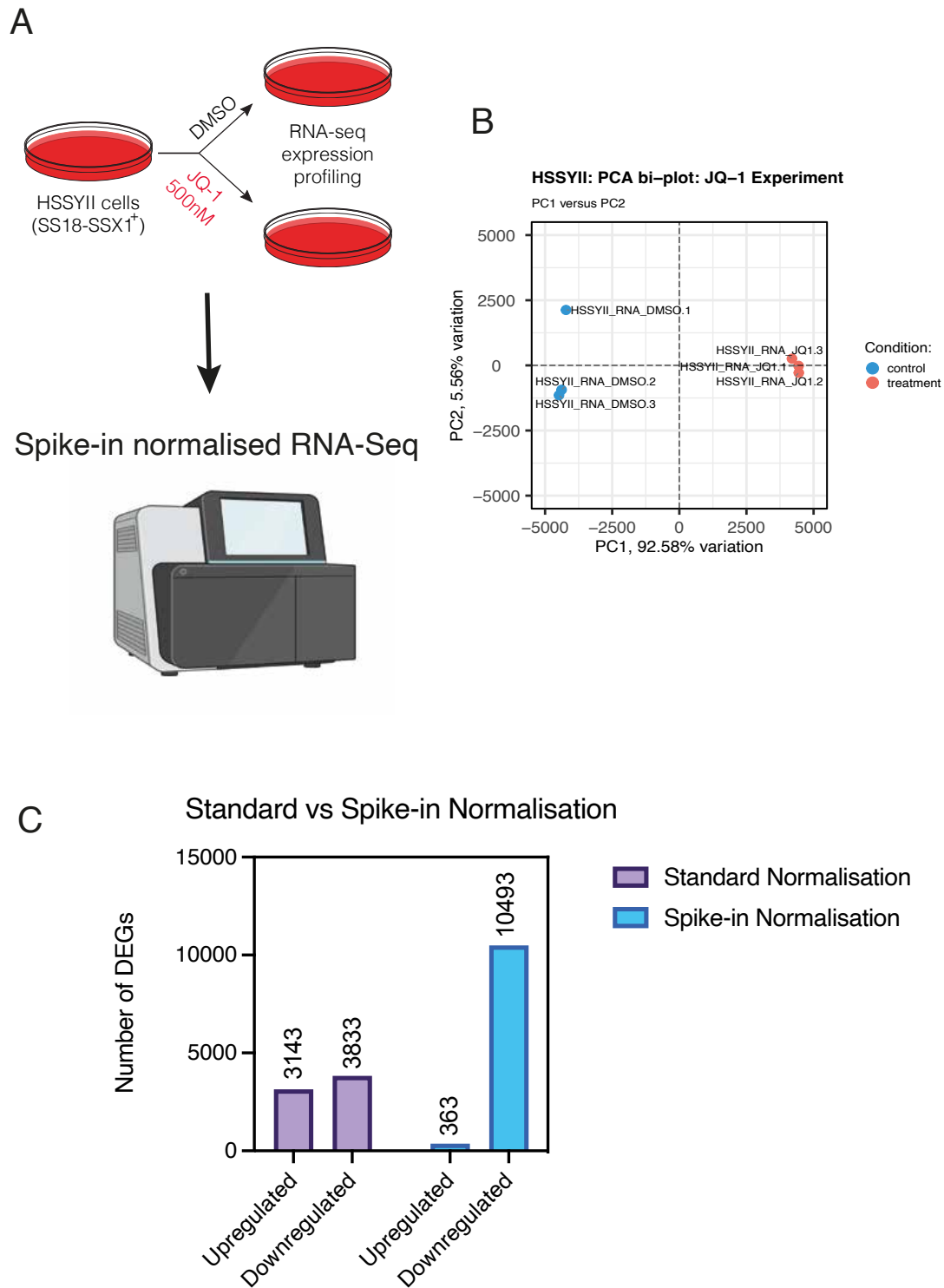


Figure 5.6: Comparing traditional RNA-seq and spike-in normalised RNA-seq

A. Schematic representation of JQ-1 treatment in the HSSYII cell line.

B. Principal component analysis (PCA) of RNA-seq data. Each dot represents a biological replicate. Conditions are colour-coded.

C. Barplots displaying the number of DEGs in either the standard or spike-in normalisation context.

Differential expression analysis following catalytic inhibition of EP300

To assess the gene expression changes induced by EP300/CBP inhibition, HSSYII and CME1 synovial sarcoma cell lines were treated with 2 μ M A48-5 or the inactive analogue A48-6 in triplicate for 24 hours followed by spike-in normalized RNA-seq (**Figure 5.7 A**). In HSSYII cells, the number of DEGs detected when spike-in normalization was included or excluded did not vary to a great extent (**Figure 5.7 B**). This was in stark contrast to the JQ-1 treatment in the same cell line across the same time frame (**Figure 5.6 C**), indicating that A-485 treatment does not have a global effect on transcription. However, cell line-specific effects of A48-5 treatment were suggested, as the number of DEGs did vary for the same analysis conducted for the CME1 cell line (**Figure 5.7 B**). The DEGs detected in the spike-in-normalization context were used for all subsequent analyses. Specifically, in HSSYII cells, the expression of 2574 genes showed a > 1.5 -fold decrease ($p < 0.05$), while 2565 showed an increase (**Figure 5.7 B**). In CME1 cells, 3927 genes showed a > 1.5 -fold decrease ($p < 0.05$), while 468 genes showed an increase in expression (**Figure 5.7 B**).

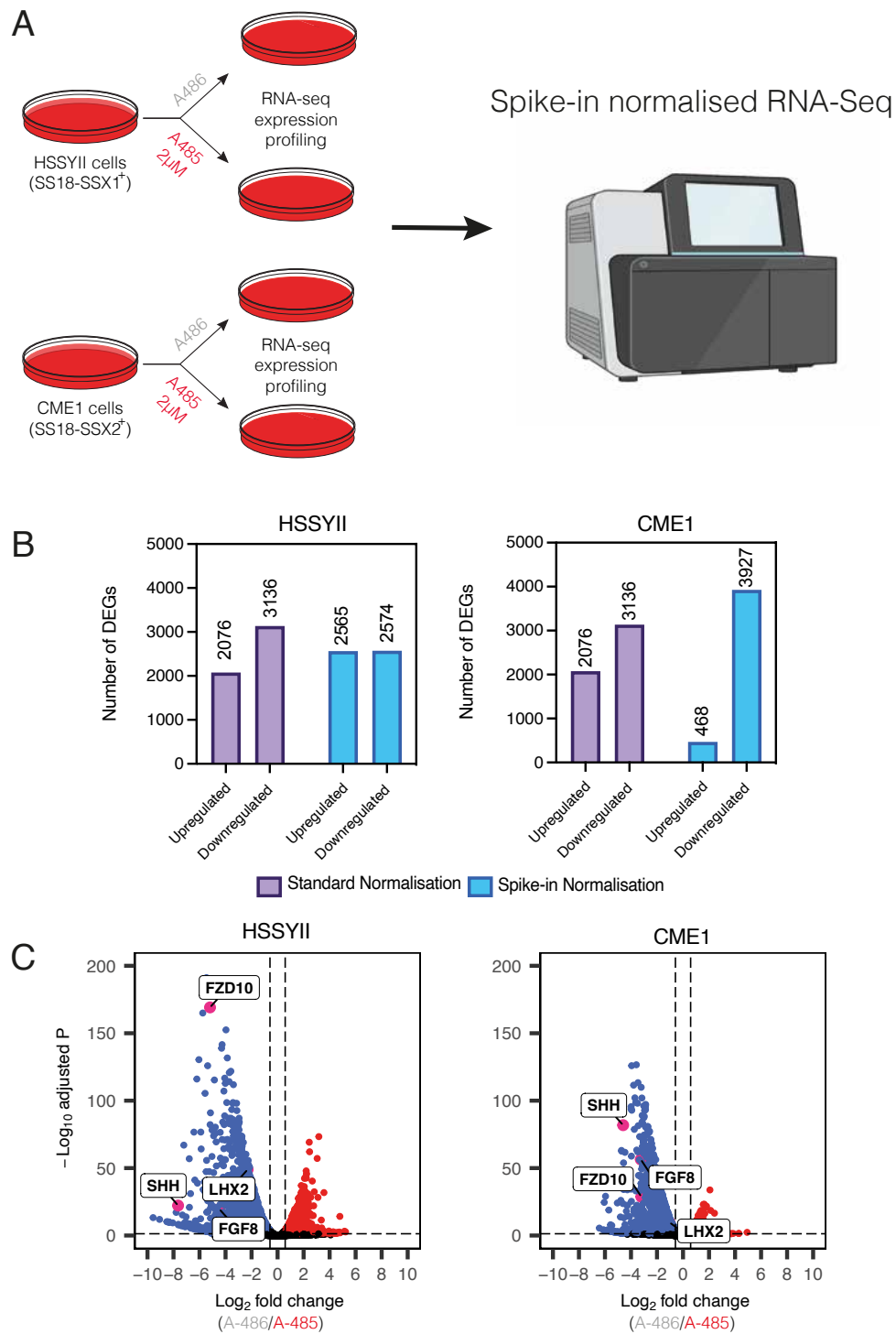


Figure 5.7: Investigating the transcriptional response to P300/CBP inhibition

A. Schematic representation of A48-5/6 treatment.

B. Barplots displaying the number of DEGs in either the standard or spike-in normalisation context with HSSYII (left) and CME1 (right).

C. RNA-seq volcano plots presenting gene expression changes observed in HSSYII (left) and CME1 (right) cell lines treated with 2µM A48-5 or A-486 for 24 hours in the spike-in normalised context. Red and blue denote genes significantly up and down-regulated respectively. Selected SS18-SSX target genes are labelled.

Downstream analysis revealed that there was a large degree of overlap between the downregulated genes in both cell lines, with > 50% of the downregulated genes in the HSSYII cell line also downregulated in the CME1 cell line (**Figure 5.8 A**). In contrast, the upregulated genes in both cell lines did not display this continuity (**Figure 5.8 A**) suggesting a less specific effect for the upregulated genes. As suggested by the high degree of commonality between the downregulated genes, gene ontology (GO) analysis of these genes revealed a large degree of overlap and enriched for GO terms related to development with a specific enrichment for neuro and mesenchyme developmental terms (**Figure 5.8 B**). On the other hand, GO terms enriched for the upregulated genes displayed minimal overlap, with terms associated with autophagy and lysosomal activity being specifically enriched in the HSSYII cell line (**Figure 5.8 B**), while GO enriched terms for CME1 related to synaptic vesicle endocytosis (**Figure 5.8 B**). To complement this analysis Gene Set Enrichment Analysis (GSEA) was carried out using the Hallmark gene set from MSigDB (Liberzon et al., 2015) This is a curated collection of genes that represent specific biological states, processes, or pathways. (Liberzon et al., 2015). GSEA analysis bared strong resemblance in both cell lines and among the top 20 enriched pathways, `HALLMARK_HEDGEHOG_SIGNALING` which has been shown to be important in synovial sarcoma disease biology was dramatically downregulated as were `HALLMARK_MYC_TARGETS` (**Figure 5.8 C**). Moreover, specific downregulation of the `HALLMARK_MYOGENESIS` pathway was observed in the CME1 cell line (**Figure 5.8 C**).

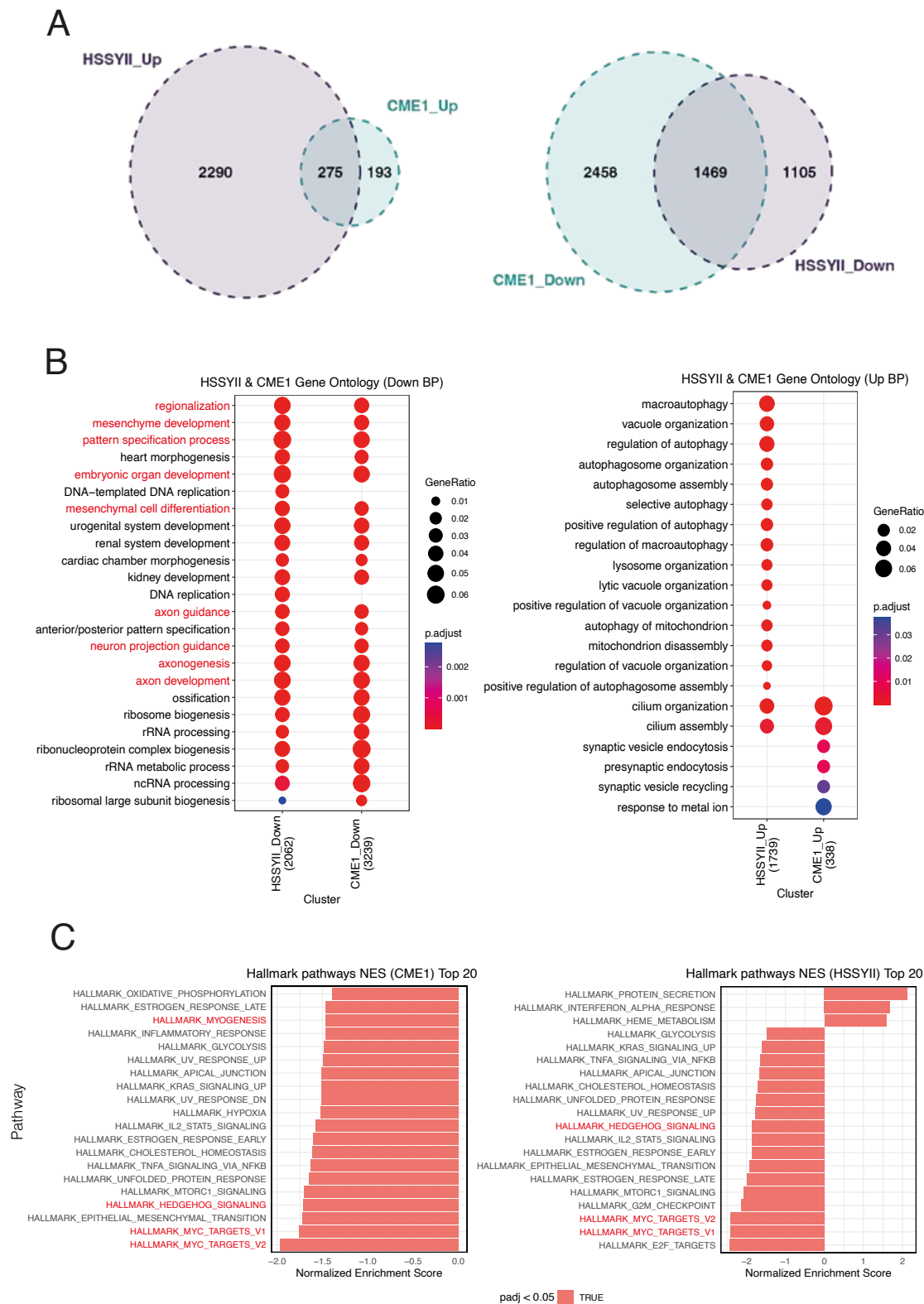


Figure 5.8: Comparing the functional significant of A48-5 treatment

A. Venn diagram of overlapping upregulated (left) and downregulated (right) DEGs in the HSSVII and CME1 cell lines.

B. Comparative GO analysis of the downregulated (right) and upregulated (left) DEGs.

C. GSEA of the top 20 enriched pathways for the CME1 cell line (left) and the HSSVII cell line (right).

5.4 Investigating the transcriptional response to EP300/CBP degradation

5.4.1 Accessing the cellular activity of dCBP-1

To assess the activity of dCBP-1 in synovial sarcoma cells, HSSYII cells were treated with a range of concentrations of dCBP-1 for 6 hours (**Figure 5.9 B**). dCBP-1 treatment resulted in a dose-dependent degradation of EP300 as evident in the representative Western blot and accompanying qualifications (**Figure 5.9 B**). Robust degradation of EP300 was achieved with the 500 nM dose (**Figure 5.9 B**). After evaluating the efficiency of the degrader, the effects of EP300 removal on H3K27Ac levels were addressed via Western blotting (**Figure 5.9 C**). Remarkably, stark reductions in H3K27Ac levels were observed as early as 2 hours and almost complete removal was apparent at 6 hours (**Figure 5.9 C**). Western blotting for cleaved PARP, a marker of apoptosis across the time course revealed no indication of apoptosis (**Figure 5.9 C**).

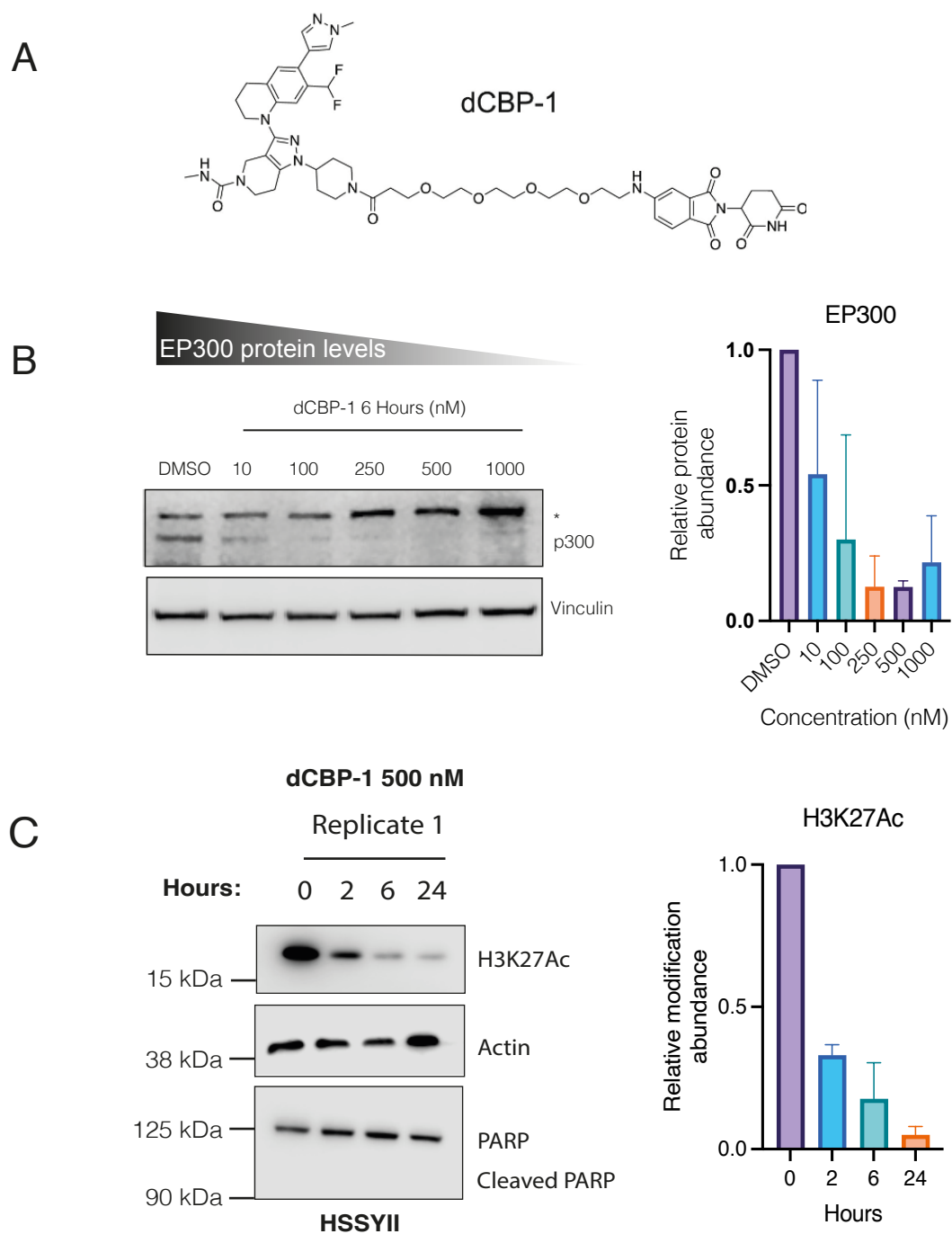


Figure 5.9: dCBP-1 efficiently removes EP300 and H3K27Ac

A. Schematic representation of dCBP-1.

B. A representative western blot of HSSYII cells treated with a range of concentrations of dCBP-1 for 6 hours (left). Quantification of EP300 signal for 2 independent biological replicates (right). Error bars represent standard deviation.

C. A representative western blot of HSSYII cells treated with 500 nM dCBP-1 across the indicated time course (left). Quantification of H3K27Ac signal for 3 independent biological replicates (right). Error bars represent standard deviation.

5.4.2 Differential expression analysis following complete degradation of EP300

To assess the gene expression changes induced by EP300/CBP degradation, HSSYII cells were treated with 500 nM dCBP-1 across the same time course used in the ACBI1 experiments in chapter 4 and global transcriptional analysis was performed using QuantSeq 3'mRNA-Seq (**Figure 5.10 A**). As the spike-in normalisation made little difference for this cell line in the A-485 context (**Figure 5.7 B**), it was decided that spike-in normalisation would not be necessary. Akin to ACBI1, due to the favourable kinetics of the degrader, the very early time points of 2 and 6 hours were included to allow for the characterisation of the primary molecular changes consequent to EP300 loss (**Figure 5.10 A**). In contrast to the analogous experiment carried out with ACBI1, the number of downregulated genes increased steadily across the time course (**Figure 5.10 B**). Specifically at the 2-hour time point, a minimal impact on transcription was observed with only 3 genes being called as downregulated and 12 genes upregulated (**Figure 5.10 B**), at the 6-hour timepoint a predominant downregulation of gene expression was observed with 389 genes downregulated and 43 genes upregulated (**Figure 5.10 B**). The greatest number of transcriptional changes were observed at the 24-hour timepoint with 700 genes downregulated and 300 genes upregulated (**Figure 5.10 B**).

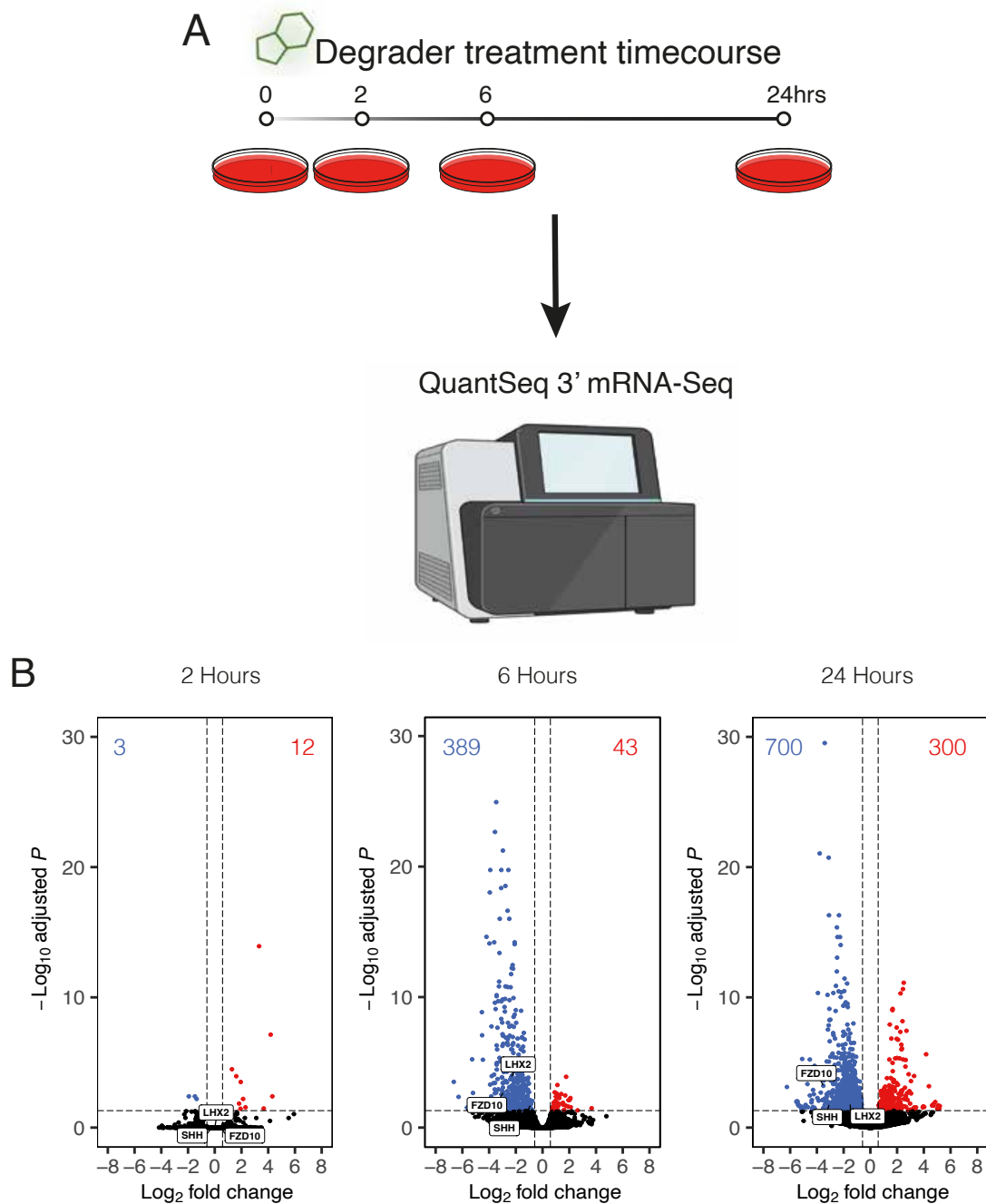


Figure 5.10: Investigating the transcriptional response to P300/CBP degradation

A. Schematic representation of 500 nM dCBP-1 treatment time course in HSSYII cells.

B. Quant-seq volcano plots presenting gene expression changes observed across the 500 nM dCBP-1 treatment time course. Red and blue denote genes significantly up and down-regulated, respectively. Selected SS18-SSX target genes are labelled.

It was important to probe whether the same core genes were consistently downregulated/upregulated across the time course. In contrast to the analogous ACBI1 experiment, all the genes downregulated at 2

hours were downregulated at 6 hours and > 50% of the genes downregulated at 6 hours were also downregulated at 24 hours (**Figure 5.11 A**). There was minimal consistency indicated across the time points for the upregulated genes (**Figure 5.11 A**). This is reminiscent of the A-485 context (**Figure 5.8 A**) suggesting a less specific effect for the upregulated genes. Reflective of these points there was a strong overlap of the gene ontology terms enriched across the downregulated genes that were not observed for the upregulated genes (**Figure 5.11 B**). As observed in the EP300 inhibition context the GO terms enriched for the downregulated genes included a stark enrichment of GO terms related to development with a specific enrichment for mesenchyme/muscle developmental terms (**Figure 5.11 B**). On the other hand, GO terms enriched for the upregulated genes at the 24-hour time point mirrored those obtained in the inhibitor context, including cellular transport and autophagy (**Figure 5.11 B**). To complement this analysis GSEA was carried out in a similar way as conducted in the A-485 context. A strong overlap was observed with the A48-5 experiment with HALLMARK_HEDGEHOG_SIGNALING and HALLMARK_MYC_TARGETS again being observed among the top downregulated gene sets (**Figure 5.11 C**). At both the 6 and 24-hour time points the HALLMARK_WNT_BETA_CATENIN_SIGNALING gene set was also significantly downregulated (**Figure 5.11 C**).

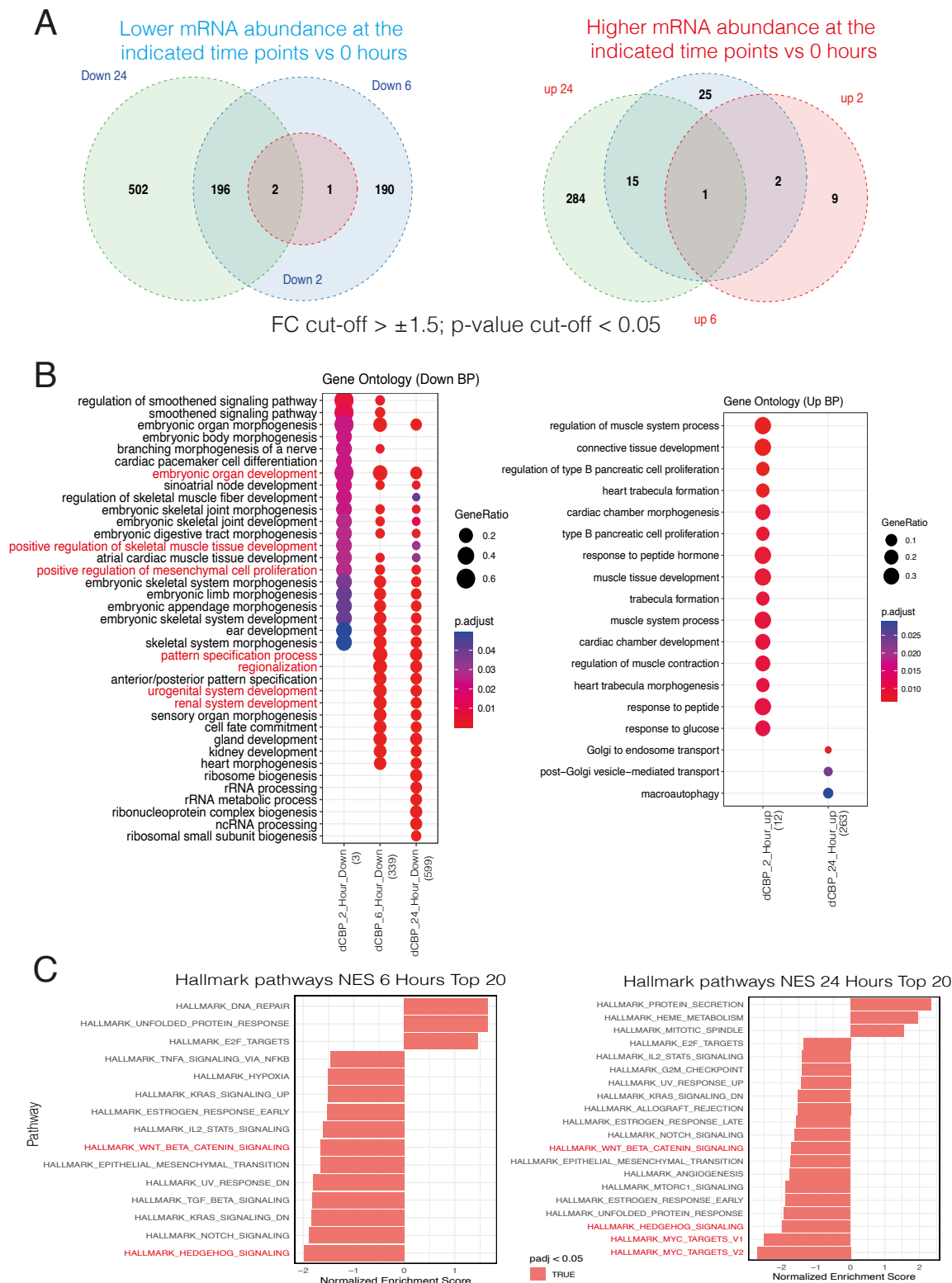


Figure 5.11: Probing the biological impact of dCBP-1 treatment

A. Venn diagram of overlapping downregulated (left) and upregulated (right) DEGs in the HSSYII cell line across the time course.

B. Comparative GO analysis of the downregulated (right) and upregulated (left) DEGs across the time-course.

C. GSEA of the top 20 enriched pathways for the HSSYII cell line at the 6 (left) and 24 (right) hour time points.

It was important to investigate whether the same core genes were consistently downregulated/upregulated across the two methods of EP300/CBP perturbation. Interestingly, $\approx 75\%$ of the genes downregulated after 24 hours of EP300/CBP degradation with dCBP-1 were also downregulated after 24 hours of EP300/CBP enzymatic inhibition with A-485 (**Figure 5.12 A**). A similar percentage $\approx 79\%$ of the genes upregulated after 24 hours of EP300/CBP degradation with dCBP-1 were also upregulated after 24 hours of EP300/CBP enzymatic inhibition with A48-5 (**Figure 5.12 A**). GO analysis of the overlapping downregulated genes were enriched for GO terms associated with development (**Figure 5.12 B**).

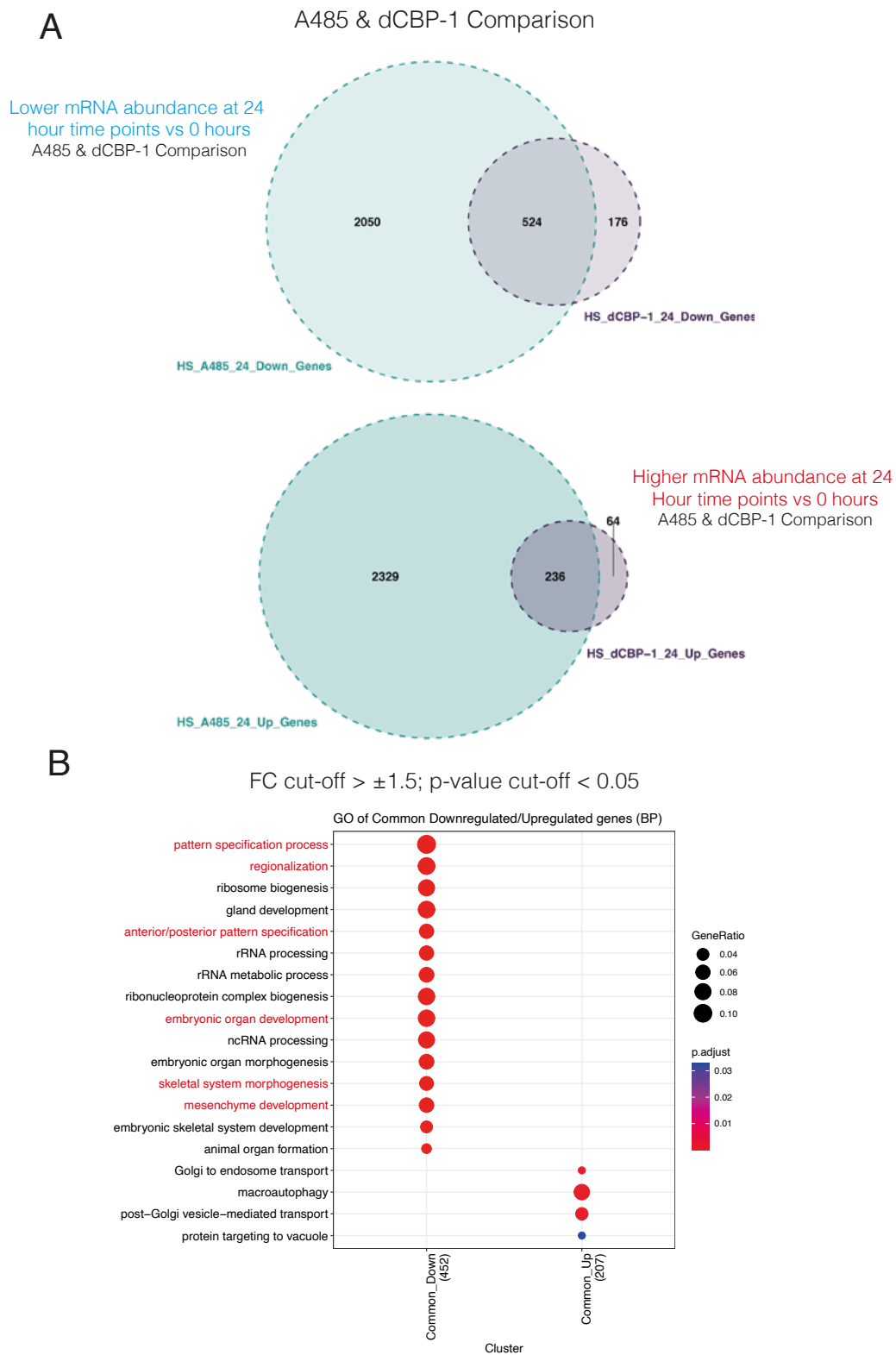


Figure 5.12: Comparing the transcriptional effect of EP300 enzymatic inhibition and degradation

A. Venn diagram of overlapping downregulated (left) and upregulated (right) DEGs in the HSSVII cell line following 24 hours of EP300 enzymatic inhibition (A48-5) or degradation (dCBP-1).

B. GO analysis of the common downregulated and upregulated DEGs across the two methods of EP300 perturbation.

5.5 EP300 supports oncogenic gene expression in synovial sarcoma

Having highlighted EP300 as a SS18-SSX1/2 interactor and genetic dependency in synovial sarcoma and investigating the transcriptional response to EP300 perturbation via enzymatic inhibition and complete protein degradation it was hypothesised that EP300 may interact with SS18-SSX to support oncogenic gene expression in synovial sarcoma (**Figure 5.13 A**). In fact, throughout evaluating the transcriptional response to EP300 perturbation it was noticed that the GO analysis of the downregulated genes bared a strong resemblance to the GO analysis conducted on fusion protein target genes and examining the transcriptional response to fusion protein knockdown (Banito et al., 2018) (**Figure 5.13 B**). In fact, many of the genes that decreased in expression were well defined fusion protein target genes including *SHH*, *FZD10*, *FGF8*, and *LHX2* (Banito et al., 2018; McBride et al., 2018) (**Figure 5.7 C**) (**Figure 5.10 B**). Strikingly, using *SHH* and *FZD10* as examples EP300 enzymatic inhibition with A48-5 completely inhibited the expression of these genes (**Figure 5.13 C**).

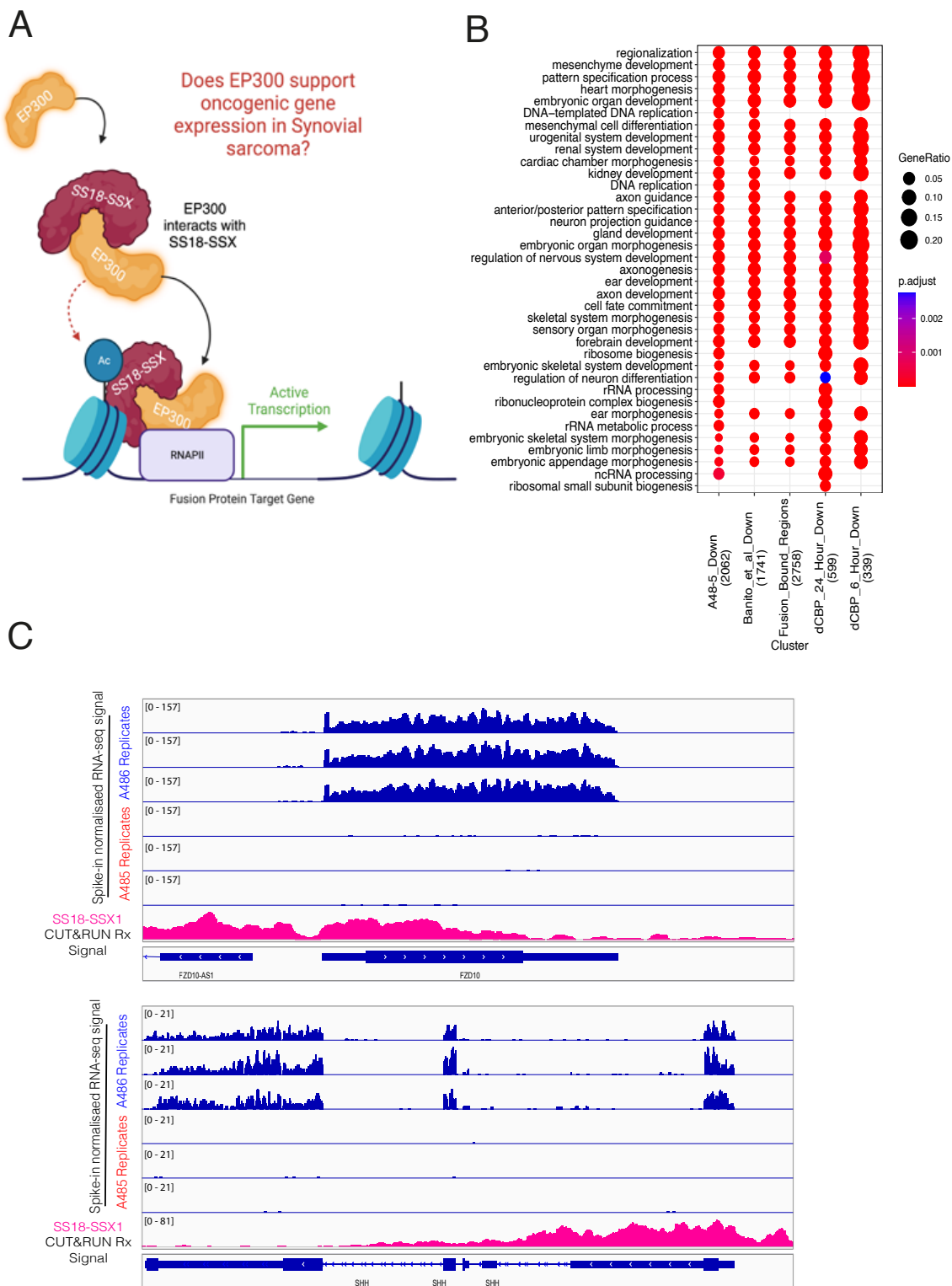


Figure 5.13: EP300 perturbation impacts oncogenic gene expression. Part I.

A. Schematic of EP300 supporting oncogenic gene expression.

B. Comparative GO analysis of genes downregulated following EP300 perturbation with A-485 and dCBP-1, fusion protein target genes and genes downregulated following fusion protein knockdown (Banito et al., 2018)

C. Representative gene tracks of gene expression signal for two key SS18-SSX target genes SHH and FZD10 and SS18-SSX1 CUT&RUN-Rx signal in the HSSVII cell line.

To investigate this further and to determine the extent to which EP300/CBP perturbation may impact oncogenic transcription in synovial sarcoma efforts were made to use the available literature to define the synovial sarcoma transcriptional signature (**Figure 5.14 A**). This involved making use of two key studies (Banito et al., 2018; McBride et al., 2018) characterising the transcriptional response to direct fusion protein knockdown in the HSSYII cell line via RNA-seq, the same cellular context as our EP300 perturbations were carried out. This data was downloaded and analysed and a list of commonly downregulated genes was collected (**Figure 5.14 A**). In addition, use was made of RNA-seq data from the TCGA sarcoma cohort (Abeshouse et al., 2017) which comprised 259 samples covering 8 major sarcoma subtypes. This sarcoma specific dataset was processed by (Banito et al., 2018) to define a synovial sarcoma transcriptional signature by obtaining a list of genes specifically upregulated in synovial sarcoma tumours vs the other sarcoma subtypes (**Figure 5.14 A**).

Initially, to explore how EP300/CBP inhibition impacted the SS transcriptional signature as defined either by genes specifically upregulated in synovial sarcoma patient tumours vs other sarcoma subtypes (Layer 1) (**Figure 5.14 A**) or by the high confidence HSSYII specific list (Layer 2) (**Figure 5.14 A**). The raw transcriptomic data was processed to generate a Log_2+1 transcripts per million (TPM) value for each gene and plotted as a heatmap (**Figure 5.14 B**). This analysis revealed a clear and dominant downregulation of these gene-sets in the A48-5 condition (**Figure 5.14 B**).

To investigate this further custom GSEA analysis was employed. This analysis demonstrated in a more statistically rigorous approach, a stark downregulation of these gene sets (**Figure 5.15 A**), in addition to a list of consensus fusion protein target genes defined in chapter 4 (**Figure 4.12 A**). Separate enrichment plots are included in addition to the bar chart to further highlight this point (**Figure 5.15 B**). As studies have yet to characterise the gene expression changes induced by fusion protein knockdown in the CME1 cell line it was decided only to include the genes specifically upregulated in synovial sarcoma patient tumours vs other sarcoma subtypes and again these were shown to be downregulated (**Figure 5.15 C**).

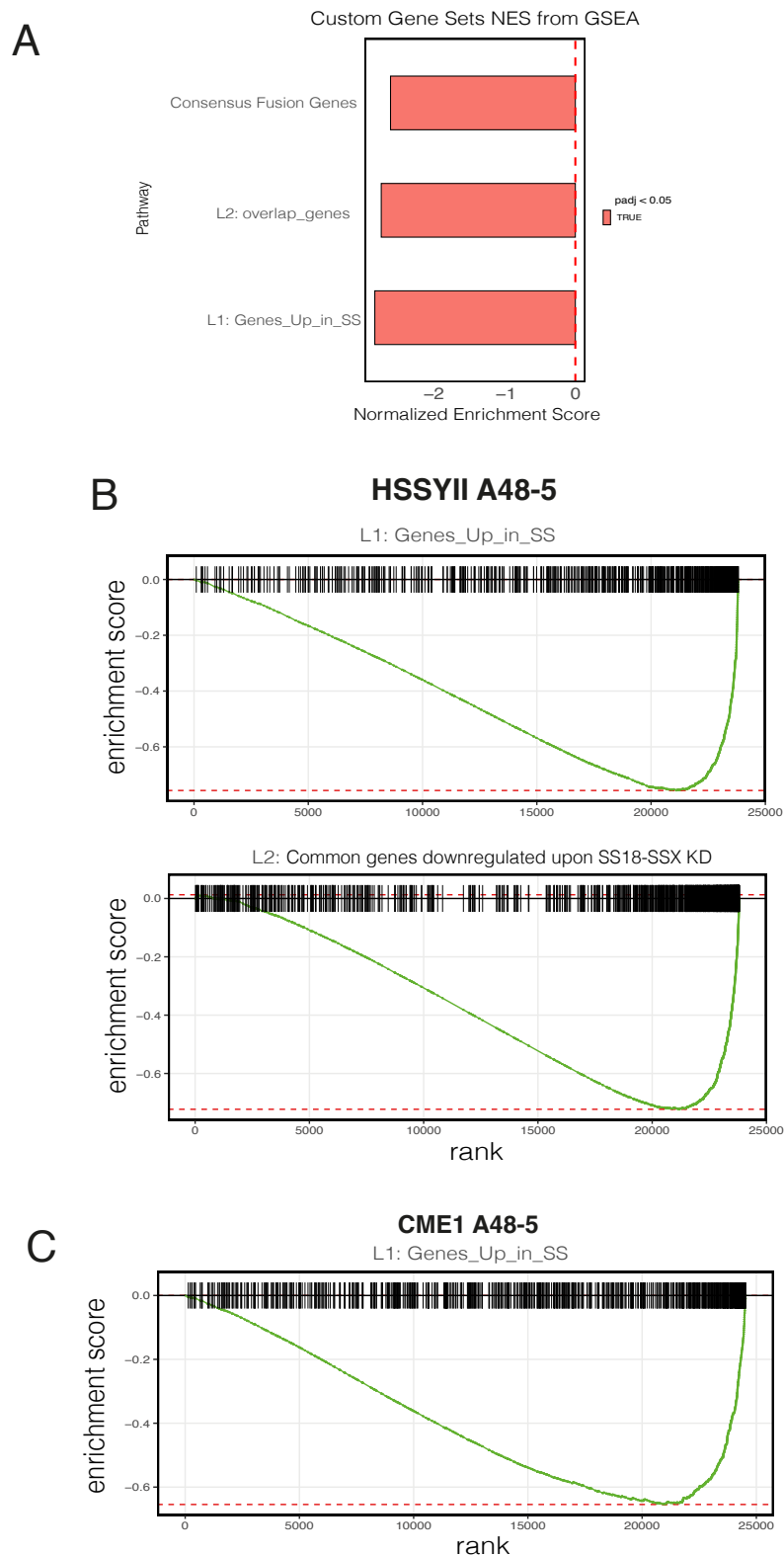


Figure 5.15: GSEA confirms enzymatic inhibition of EP300 suppresses the SS transcriptional signature

A. Barplots displaying the normalised enrichment score (NES) for the three gene sets of interest.

B. Enrichment plots of gene expression change in the HSSYII cell line treated with A-485 compared to the gene sets of interest.

C. Enrichment plots of gene expression change in the CME1 cell line treated with A-485 compared to the gene set of interest.

An analogous approach was taken with the dCBP-1 data and these gene sets were observed to be dramatically downregulated at as early as the 6-hour timepoint (**Figure 5.16 A & B**).

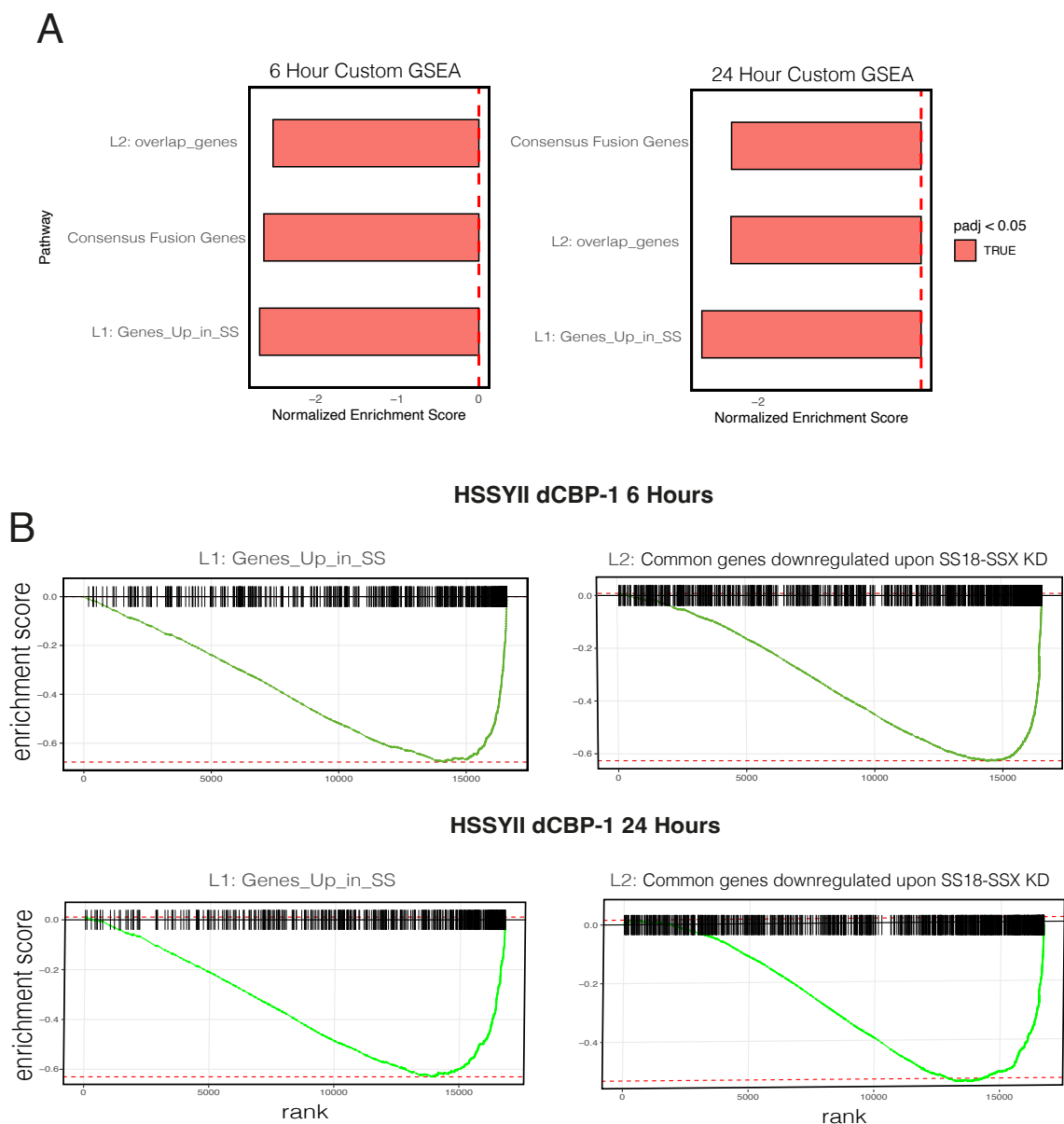


Figure 5.16: GSEA confirms degradation of EP300 suppresses the SS transcriptional signature
A. Barplots displaying the normalised enrichment score (NES) for the three gene sets of interest for the 6 (left) and 24 hour (right) dCBP-1 data.
B. Enrichment plots of gene expression change in the HSSYII cell line treated with dCBP-1 for 6 (top) and 24 (bottom) hours compared to the gene sets of interest

5.6 Establishing an isogenic cell model in which SS18-SSX1 can be acutely degraded

In order to generate an isogenic system whereby the relationship between SS18-SSX and EP300 could be examined the Small Molecule-Assisted Shutoff (SMASh) methodology (Chung et al., 2015) was adopted. Using a gateway cloning compatible SMASh-tag vector which contained a puromycin-resistant cassette, a C2C12 cell line stably expressing a degradable HA-tagged SS18-SSX1 was established (**Figure 5.17 A**).

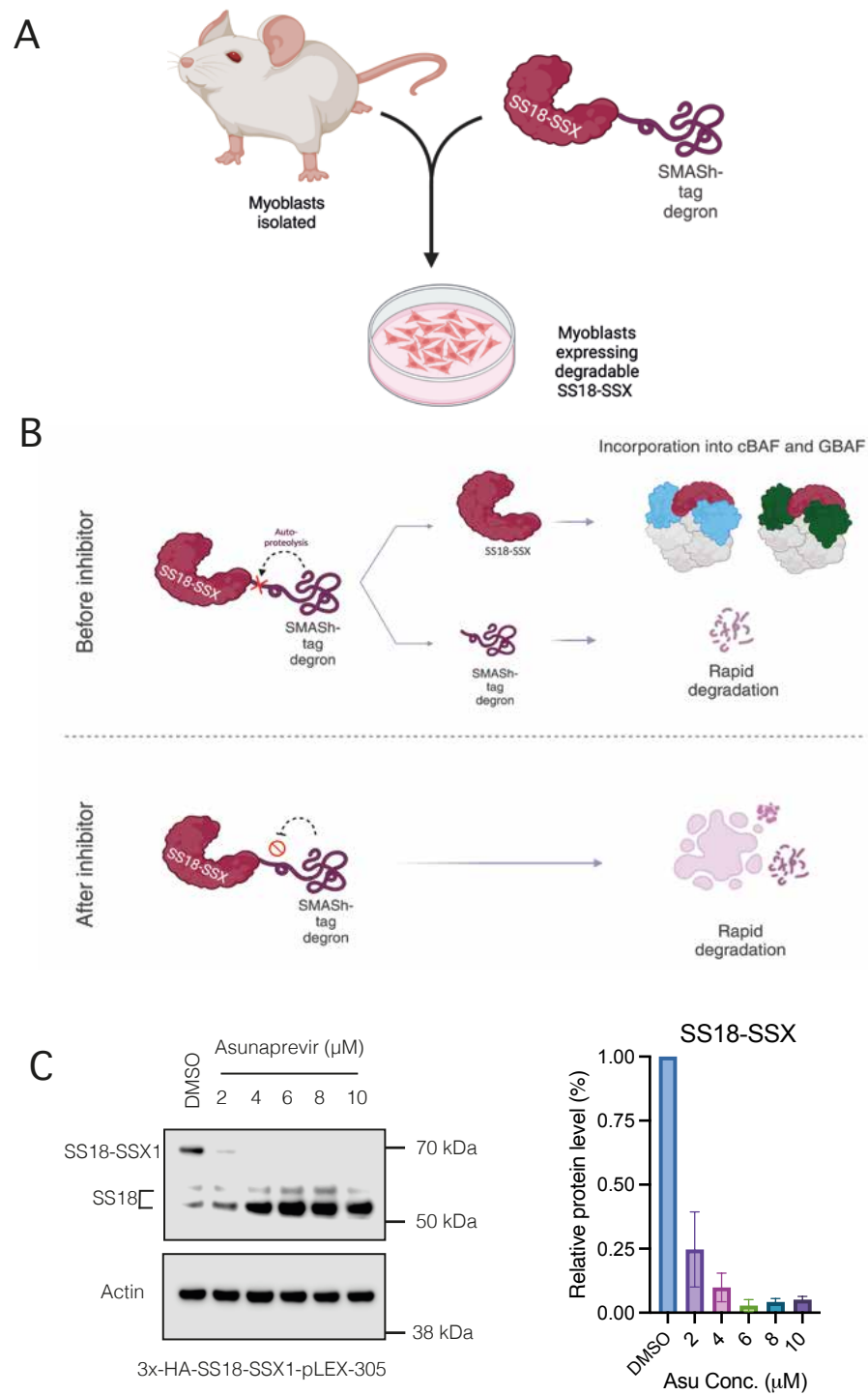


Figure 5.17: SS18-SSX can be acutely regulated by the SMASH methodology

A. Schematic of generating a degradable SS18-SSX cell model.

B. Schematic of the SMASH system. Made with Biorender.com.

C. SS18-SSX1 regulation by SMASH-tagging is dose-dependent. Immunoblot of SS18-SSX1, WT SS18 and Actin levels. C2C12 expressing degradable SS18-SSX1 for 24 hours across the indicated dose range (Left). Quantification of SS18-SSX1 levels by immunoblot. SS18-SSX1 signal was normalized to Actin signal, and then plotted as a percent of the signal in the untreated condition (Right) ($n = 3$, error bars represent standard deviations).

How does the SMASh methodology work? The protein of interest (POI) is tagged with both NS3 (a viral protease) and a degron (Chung et al., 2015). After translation, the tagged protease cleaves off the degron and itself via intramolecular cleavage, releasing the POI in its native form. NS3 and the degron are subsequently removed by intracellular machinery (Chung et al., 2015) (**Figure 5.17 B**). In the presence of the protease inhibitor asunaprevir, this intramolecular cleavage is inhibited, the degron is retained and subsequent proteasomal degradation of the POI ensues (Chung et al., 2015) (**Figure 5.17 B**).

To assess SS18-SSX1 protein regulation by the SMASh system C2C12 stably expressing 3x-HA-tagged SS18-SSX1 were cultured in DMSO or a dose range of asunaprevir (2-10 μ M) for 24 hours and SS18-SSX1 signal was detected via Western blotting, with actin serving as a loading control (**Figure 5.17 C**). As evident in the representative Western blot and accompanying quantification, SS18-SSX1 was robustly expressed and degraded in a dose-dependent manner (**Figure 5.17 C**). This result demonstrates that SS18-SSX1 can be acutely degraded via the SMASh method. Interestingly, expression of SS18-SSX1 was observed to destabilise endogenously expressed wild-type SS18 and SS18-SSX1 degradation was observed to rescue this effect (**Figure 5.17 C**). Although, additional replicates are needed to confirm this result.

Proliferating C2C12 myoblasts can be induced to exit the cell cycle and differentiate into myotubes by following a simple differentiation protocol (see subsection 2.1.3) (**Figure 5.18 A**). To investigate how SS18-SSX1 expression/degradation influences myogenic differentiation C2C12 cells expressing degradable SS18-SSX1 and control parental C2C12 cells were treated with DMSO or 2 μ M asunaprevir for 24 hours prior to being induced to differentiate for 4 and 6 days. A non-induced population was used as a control. A representative Western blot of SS18-SSX1 levels across the experiment is shown (**Figure 5.18 B**). In the parental control context, C2C12 differentiation progressed as expected with dramatic upregulation of the myogenic regulatory factors (MRF), including MyoD and MyoG (myogenin) (Tang et al., 2018) (**Figure 5.18 C**). Remarkably, expression of SS18-SSX1 starkly inhibited differentiation (**Figure 5.18 C**). Importantly degradation of SS18-SSX1 with asunaprevir treatment partially rescued this effect (**Figure 5.18 C**). Together, these results show that the expression of SS18-SSX1 in C2C12 myoblasts inhibits myogenic differentiation and that this can be partially rescued by SS18-SSX degradation via the SMASh system. Importantly robust but not complete degradation of SS18-SSX1 was achieved with the 2 μ M dose used (**Figure 5.18 C**).

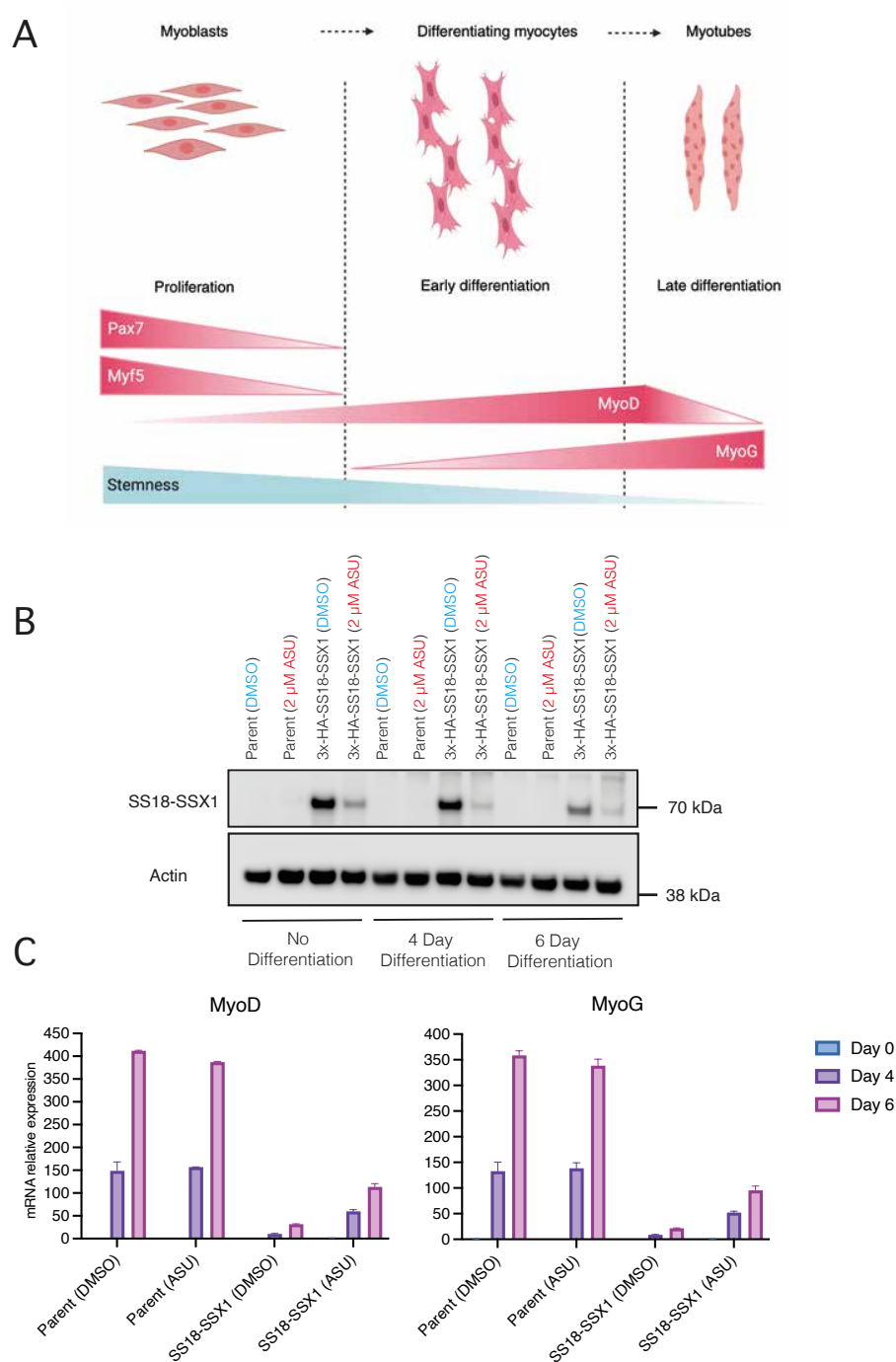


Figure 5.18: SS18-SSX1 impairs myogenic differentiation of C2C12 cells

A. Schematic summary of the differentiation of C2C12 cells. Myoblasts proliferate, differentiate, and fuse to form multinucleated myotubes. Transcription factors and their representative levels at each stage are indicated.

B. A representative Western blot of SS18-SSX1 levels across the indicated differentiation experiment. Actin served as the loading control.

C. Effect of SS18-SSX1 on relative mRNA profiles of MyoD and MyoG in C2C12 cells. (n = 2, error bars represent standard deviations)

5.7 Discussion

In this chapter, the finding that EP300 is a genetic dependency in SS was investigated. A high-confidence list of fusion protein interactors was compiled and EP300 was identified as a SS18-SSX1/2 interactor (**Figure 5.2 A & B**). Following on chemical biology-based approaches were established to perturb EP300 function and utilised to gain insight into why in the context of transcriptional regulation EP300 is a critical functional dependency (**Figure 5.5 B**) (**Figure 5.9 B**). Finally, a relevant cellular model in which to further explore the relationship between SS18-SSX and EP300 was established (**Figure 5.17**).

In the SS18-SSX1/2 IP-MS results there was a high degree of overlap evident among the fusion protein interactors detected in both cell lines and these common proteins were taken as high-confidence fusion protein interactors, potentially functionally relevant in SS cell biology (**Figure 5.2 A & B**). However, cell line-specific interactors were also identified and may represent SSX1/2-specific interactors (**Figure 5.2 A & B**), the existence and functional importance of which has not been explored to date in the literature. As expected a large proportion of the common interactors were SWI/SNF components (**Figure 5.2 C**). Strikingly, EP300 was the only non-SWI/SNF component indicated to be a dependency (**Figure 5.2 C**). Moreover, EP300 clustered exclusively with the fusion protein itself and was not indicated as a genetic dependency in the SW982 cell context characterised as a synovial sarcoma line but lacking the characteristic fusion protein (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.)(**Figure 5.2 C**). This finding is strongly suggestive of a co-dependent/functional relationship between SS18-SSX1/2 and EP300.

Consistent with its role as a positive regulator of gene expression perturbation of EP300 function either by inhibiting its catalytic activity with A-485 (**Figure 5.7 B**) or achieving complete protein degradation with dCBP-1 resulted in a predominant downregulation of DEGs (**Figure 5.10 B**). This effect was more limited than that achieved with the BET bromodomain inhibitor JQ-1 (**Figure 5.6 C**) alluding to a more nuanced impact on transcription. Contrary to what was expected enzymatic inhibition of EP300 (**Figure 5.7 B**) had a greater impact on transcription than complete protein degradation with dCBP-1 (**Figure 5.10 B**). A number of factors may be at play here. For one the starting point for the drug design of dCBP-1 is the EP300/CBP bromodomain inhibitor GNE-781 and not A-485 (Vannam et al., 2021). Moreover, an A-485 concentration of 2 μ M was used, while a lower 500 nM concentration of dCBP-1 was used. As a result, perhaps a more selective perturbation of EP300 was achieved with dCBP-1. Secondly, both experiments are

not directly comparable as two different gene expression profiling methodologies were used, RNA-seq and Quant-seq respectively.

Systematic GO analysis of genes downregulated following EP300 perturbation identified terms related to mesenchymal development/differentiation and neurogenesis (**Figure 5.13 B**). This result gave the first indication that P300 activity may somehow play a role in sustaining the synovial sarcoma oncogenic transcriptional program. This is because fusion protein containing BAF complexes have been demonstrated to bind to and activate the aberrant expression of developmentally important transcription factors to maintain the proliferative undifferentiated state of synovial sarcoma cells (Banito et al., 2019; McBride et al., 2018). In relation to the neurodifferentiation terms, SS tumours are characterised by an unusual transcriptional signature, with a clear upregulation of neurogenic factors (Ishibe, Nakayama, Aoyama, Nakamura, & Toguchida, 2008) and mouse models of SS display a strong upregulation of genes related to neuro-development/function (Garcia et al., 2012) **HALLMARK_WNT_BETA_CATENIN_SIGNALING**, **HALLMARK_HEDGEHOG_SIGNALING**, **HALLMARK_MYOGENESIS** and **HALLMARK_MYC_TARGETS** were among the top downregulated gene sets (**Figure 5.8 C**) (**Figure 5.11 C**). WNT signalling has been shown to be abnormally upregulated by the fusion protein (Garcia et al., 2012) and genetic disruption of this signalling pathway has been shown to prevent tumour formation *in vivo* (Barham et al., 2013). Expression of the signalling protein SHH has been shown to be upregulated in SS tumours compared to other sarcoma subtypes (Banito et al., 2018). Moreover, the MYC proto-oncogene is associated with the development of a wide range of human tumour types where abnormal activation drives many hallmarks of cancer including aberrant proliferation, altered cellular metabolism, angiogenesis and can inhibit cellular senescence and differentiation (Gabay, Li, & Felsher, 2014).

Strengthening the idea that perturbing P300 impinges on the synovial sarcoma transcriptional programme, custom GSEA capitalizing on the availability of RNA-seq data from the TCGA sarcoma cohort (Abeshouse et al., 2017) was performed (**Figure 5.14 A**) (**Figure 5.16 A & B**). This analysis revealed that A48-5 and dCBP-1 treatment significantly reduced the expression of genes upregulated in synovial sarcoma patient tumours vs other sarcoma subtypes (**Figure 5.14 A**) (**Figure 5.16 A & B**). Probing further, this impingement became apparent again when these compounds were demonstrated to reduce the expression of genes that are downregulated when the fusion protein is knocked down (**Figure 5.14 A & B**) (**Figure 5.16 A & B**) and a list of stringently defined fusion protein target genes (**Figure 5.14 A**) (**Figure 5.16 A**). Collectively, these findings begin to illuminate why transcriptionally synovial sarcoma cells rely on EP300

function for survival. One important limitation to address is the fact that due to their extensive sequence homology, the compounds used to perturb EP300 function also perturb CBP (Lasko et al., 2017; Vannam et al., 2021). As a result, the above findings cannot be attributed to EP300 perturbation alone. However, as synovial sarcoma cells were discovered to be selectivity-dependent on EP300 function and not CBP via genetic screening (**Figure 5.1 A**), it is fair to infer that the negative effect these compounds have on the synovial sarcoma transcriptional program is a function of EP300 perturbation. However, future work will directly address this using additional genomic tools to specifically perturb P300 or CBP function.

Consistent with previous work demonstrating that expression of the fusion protein in mouse muscle progenitors *in vitro* (Garcia et al., 2012) and *in vivo* (Haldar et al., 2007) expression of degradable HA-tagged SS18-SSX1 in mouse myoblasts impaired myogenic differentiation (**Figure 5.18 C**). The strength of this model lies in the utilisation of the SMASh system, permitting robust expression and acute degradation of the fusion protein (**Figure 5.18 B**). Initial differentiation experiments suggest that the myogenic block induced by fusion protein expression is at least partially reversible (**Figure 5.18 C**). These experiments will be completed with a higher dose of asunaprevir to achieve more complete SS18-SSX1 degradation to potentially achieve a stronger reversal of the myogenic block. This cell model will be utilised in the context of EP300 degradation in future experiments, to investigate the functional interplay between EP300 and the fusion protein.

In summary, the findings presented here illuminate the significance of EP300 as a key genetic dependency in synovial sarcoma, providing valuable insights into its role in sustaining the fusion gene-driven transcriptional program crucial for the survival of SS cells.

Chapter 6

General Discussion

6.1 Summary of findings

In **chapter 3**, the DepMap cancer dependency mapping resource (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.) was employed to systematically uncover novel functional dependencies across all SWI/SNF member genes in SS cells (**Figure 3.2 A**) with SMARCA4 being highlighted as a novel vulnerability (**Figure 3.5 A**). The integration of these findings with the fact that SMARCA4 was found to be one of the most abundant components of complexes containing SS18-SSX (**Figure 3.1**), strongly suggested functional importance. The observation of SMARCA4's high-ranking position among 366 chromatin regulatory genes, consistently clustering with known dependencies SS18 and BRD9, further strengthens its significance (**Figure 3.6 A**). These results suggested that targeting SMARCA4 could broadly disrupt molecular mechanisms downstream of the oncogenic fusion protein SS18-SSX. The SMARCA4/2 PROTAC ACBI1 (Farnaby et al., 2019) was used to explore the biochemical consequences of SMARCA4 degradation in SS cells. Time-resolved quantitative IP-MS demonstrated that ACBI1 treatment induced the acute disassembly of oncogenic SWI/SNF complexes (**Figure 3.14**). This SWI/SNF disassembly was further accompanied by a highly specific loss of oncogenic complexes from the chromatin fibre (**Figure 3.10 B**) (**Figure 3.11 A**).

In **chapter 4**, ACBI1 was employed to explore the transcriptional impact (**Figure 4.5**) (**Figure 4.6**) and chromatin dynamics associated with SMARCA4/2 degradation in SS cells (**Figure 4.8**) (**Figure 4.12**). The chapter also examined the effects of SMARCA4/2 loss on the viability of SS cell lines, shedding light on the interplay between SMARCA4/2 function and cellular survival (**Figure 4.14**). Despite a predominant

reduction in chromatin accessibility (**Figure 4.8**), a modest effect on gene expression was observed (**Figure 4.5**). Quantitative chromatin mapping using CUT&RUN-Rx revealed a robust genome-wide depletion of SMARCA4 and SMARCA2 from chromatin as early as the 2-hour timepoint (**Figure 4.12**). Despite the early loss of SMARCA4/2 and SWI/SNF complex disassembly, notable SS18-SSX signal persisted at its consensus sites throughout the time course (**Figure 4.12**). A minimal impact on cell viability was found despite efficient SMARCA4/2 degradation within two hours, with effects observed only after five days of drug exposure (**Figure 4.14**). This finding prompts further exploration into the temporal dynamics of SMARCA4/2-induced changes in gene expression and highlights the need for longer-term profiling experiments. It also suggested that additional SS18-SSX co-factors may play an important role in synovial sarcomagenesis.

chapter 5 focused on the identification and functional exploration of EP300 as a genetic dependency in SS. SS18-SSX IP-MS results in 2 independent SS cell lines revealed a set of common fusion protein interactors, largely composed of SWI/SNF components (**Figure 5.2 A & B**). Strikingly, EP300 was the sole non-SWI/SNF component identified as a genetic dependency, suggesting a unique functional relationship with the fusion protein (**Figure 5.2 C**). This relationship was further underscored by EP300's exclusive clustering with the fusion protein based on DepMap dependency data and its absence as a genetic dependency in the SW982 cell line. SW982 cells share histological features with SS cells but lack the characteristic fusion protein (*DepMap: The Cancer Dependency Map Project at Broad Institute*, n.d.) (**Figure 5.2 C**). Perturbation of EP300 function, either through enzymatic inhibition or complete protein degradation, predominantly resulted in the downregulation of differentially expressed genes (**Figure 5.7 B**) (**Figure 5.10 B**). Notably, the impact on transcription was nuanced, with enzymatic inhibition exhibiting a greater effect than protein degradation. Gene ontology analysis implicated EP300 in processes related to mesenchymal development, differentiation, and neurogenesis, hinting at its role in sustaining the oncogenic transcriptional program in SS (**Figure 5.13 B**). Custom gene set enrichment analysis using TCGA sarcoma cohort data reinforced the notion that A-485 and dCBP-1 treatments significantly reduced the expression of genes associated with synovial sarcoma patient tumours (**Figure 5.14**) (**Figure 5.15**) (**Figure 5.16**). The findings further extended to the downregulation of genes affected by fusion protein knockdown, emphasising the intricate interplay between EP300 and the SS transcriptional program (**Figure 5.14**) (**Figure 5.16**). Establishing a relevant cellular model expressing degradable SS18-SSX in mouse myoblasts highlighted the impairment

of myogenic differentiation induced by the fusion protein (**Figure 5.18 C**). The reversible nature of this myogenic block (**Figure 5.18 C**) opens avenues for future experiments exploring the functional interplay between EP300 and the fusion protein in this model.

6.2 The current state of the synovial sarcoma research landscape

The overarching goal of this research was the identification of novel therapeutic targets for the treatment of SS. This research is of clinical relevance as there are currently no approved rational therapeutic approaches to treat SS. The primary treatment for SS involves surgical resection, often combined with radiotherapy or traditional chemotherapy. However, combining radiation and chemotherapy can be significantly toxic, with a risk of treatment-related adverse effects (Fiore et al., 2021; McBride et al., 2018). The low overall long-term survival rate among SS patients (*approx* 40%) highlights the limited efficacy of current interventions, emphasising the need for more effective approaches (Fiore et al., 2021). A shift toward more targeted therapies is crucial for advancing the treatment of SS.

SS tumours exhibit a pathognomonic chromosomal translocation known as t(X;18), resulting in the formation of the characteristic fusion gene SS18-SSX (Clark et al., 1994; de Leeuw et al., 1995; Skytting et al., 1999). Cancer genomics investigations have shown that SS tumours typically lack significant additional genetic abnormalities, strongly implying that the SS18-SSX fusion protein is the primary driver of the disease's pathogenesis (Nielsen et al., 2015; Michel et al., 2018). In line with this, findings from a genetically engineered mouse model indicate that the sole expression of SS18-SSX is enough to induce a penetrant disease resembling SS in mice (Halдар et al., 2007). SS development is widely attributed to abnormal SWI/SNF function (Kadoch & Crabtree, 2013; Brien et al., 2018; McBride et al., 2018; Banito et al., 2018; Michel et al., 2018). Recent insights have clarified key mechanisms underlying the disease's biology. The SS18-SSX fusion protein integrates into cBAF complexes, displacing the wild-type SS18 protein and leading to the degradation of the tumour suppressor SMARCB1 (McBride et al., 2018; Kadoch & Crabtree, 2013). Notably, SS18-SSX also associates with GBAF complexes (Brien et al., 2018; McBride et al., 2018). Genome-wide analyses showed these aberrant complex formations globally retarget SWI/SNF complexes to repressed polycomb domains, activating associated gene expression (McBride et al., 2018). Suppressing SS18-SSX, thereby restoring the WT SWI/SNF complex composition, promotes PRC2-mediated H3K27me3

gain, reduces chromatin accessibility, and induces mesenchymal differentiation (McBride et al., 2018). These findings underscore the potential therapeutic opportunity in targeting mechanisms related to fusion protein recruitment and SWI/SNF complex function in SS.

In line with this, work in our lab through the use of CRISPR/Cas9-based functional genomics has highlighted BRD9 (a dedicated GBAF specific subunit) as a crucial SS18-SSX co-factor in SS cells (Brien et al., 2018). This finding has also been supported by others (Michel et al., 2018). BRD9 was characterised to functionally associate with SS18-SSX-containing SWI/SNF complexes and support oncogenic gene expression (Brien et al., 2018). Furthermore, the loss of BRD9 in SS cells resulted in a suppression of tumour progression *in vivo* (Brien et al., 2018). This pre-clinical data is promising and optimistically has led to the initiation of two independent clinical trials for the treatment of SS (ClinicalTrials.gov Identifiers: NCT05355753, NCT05355753). This finding highlights that in the context of fusion protein-driven malignancies which have proven intractable to direct therapeutic targeting, targeting downstream vulnerabilities is a viable therapeutic rationale. However, these clinical trials remain in their early stages and the efficacy of BRD9 targeting in SS patients remains undetermined. As such it is important to continue to identify and explore additional opportunities to target SS18-SSX function in this malignancy. This holds particular significance due to the common occurrence of partial clinical responses in patients, especially when drugs are administered as single agents (Garraway & Jänne, 2012). Such responses are frequently attributed to the development of drug resistance within a population of tumour cells which poses a substantial clinical challenge (Garraway & Jänne, 2012). Circumventing these issues requires the availability of additional therapeutic opportunities that can be exploited.

6.3 Therapeutic relevance of targeting SMARCA4 and EP300 in synovial sarcoma

This research project identified two novel therapeutic targets in SS, namely SMARCA4 and EP300. Given the fact that SS development is widely attributed to abnormal SWI/SNF function, targeting the key catalytic subunit in this cellular context may provide promising therapeutic outcomes. Importantly, in the context of this research project, SMARCA4 has emerged as a potential therapeutic target in additional cancer contexts. A recent CRISPR screen in patient-derived H3K27M-glioma neurospheres demonstrated

a dependence on SMARCA4 (Panditharatna et al., 2022). The authors showed that SWI/SNF complex activity maintained glioma stem cells in a cycling, oligodendrocyte precursor cell like state. Genetic and pharmacological perturbation of SMARCA4 was shown to inhibit proliferation, promote the progression of differentiation along the astrocytic lineage, and improve survival in patient-derived xenograft models of H3K27M-glioma (Panditharatna et al., 2022). In this study, the mouse models underwent extended systemic treatment with Compound 14 a SMARCA4/2 ATPase inhibitor. This compound was well tolerated, with no adverse effects observed (Panditharatna et al., 2022). In advanced stages of neuroblastoma (NB), elevated expression of SMARCA4 has been observed to correlate with poorer outcomes (Jubierre et al., 2016). Moreover, perturbation experiments *in vitro* and *in vivo* demonstrated an essential role for SMARCA4 in NB cell proliferation and as a key regulator of oncogenic pathways (Jubierre et al., 2016). Consistent with this finding neuroblastoma was one of the additional lineage subtypes identified in my analysis which demonstrated a selective dependence on SMARCA4 (**Figure 3.5 A**). An additional lineage subtype displaying a strong dependence on SMARCA4 identified in my analysis was rhabdomyosarcoma (**Figure 3.5 A**). A recent publication has identified SMARCA4 as being overexpressed in the aggressive alveolar subtype of this malignancy and that SMARCA4 function is essential for oncogenic transcription (Laubscher et al., 2021). SMARCA4 also scored in a shRNA screen as a dependency in a mouse model of AML (Zuber et al., 2011). Follow-up studies demonstrated that SMARCA4 was critical to sustain transcription factor occupancy at a set of lineage-specific enhancers of the *MYC* gene (Shi et al., 2013). This finding has led to the initiation of clinical trials in patients with AML (ClinicalTrials.gov Identifier: NCT04891757) using the compound FHD-286, an inhibitor of SMARCA4/2 ATPase activity.

In regard to the observation in **chapter 5** that EP300 function supports oncogenic transcription in SS cells, EP300 function has been shown to be exploited in additional cancer contexts where it has been demonstrated to support oncogenesis as a key secondary regulator of oncoproteins. For example, the fusion protein AML-ETO the oncogenic driver in some subtypes of acute myeloid leukemia (AML) is itself acetylated by EP300 and this acetylation has been demonstrated to be essential for leukemogenicity in mouse models (Wang et al., 2011). An additional example relates to invasive endometriosis, where the HAT activity of EP300 has recently been shown to be essential for driving invasive phenotypes in mouse models of ARID1A mutant invasive endometriosis (Wilson et al., 2020). Moreover, a 2021 study has also demonstrated a role for EP300 catalytic activity in sustaining oncogenic gene expression in multiple myeloma and chronic

lymphocytic leukaemia (CLL), two additional cancer contexts where EP300 has been identified as a functional dependency (Hogg et al., 2021).

A potent and selective inhibitor of the EP300/CBP bromodomain known as CCS1477 (inobrodib), has exhibited promising pre- and early-phase clinical efficacy in both AML and multiple myeloma patients. In a number of relapsed or refractory AML cases, CCS1477 monotherapy has induced differentiation responses (Nicosia et al., 2023). In addition, synergy has been observed when combined with standard-of-care cytotoxic agents in pre-clinical studies. Importantly, CCS1477 has been proven to be well tolerated (Nicosia et al., 2023).

6.4 Concluding remarks on directly targeting the fusion protein

A striking observation worth revisiting was the strong specificity of the SS18 subunit as exclusively essential in SS cells compared to other lineage subtypes (**Figure 3.3 A**). This underscores the concept of a specific functional genomic dependency, presenting an ideal therapeutic target within this disease context. Presently, there are no available pharmacological agents to directly target the fusion protein due to the absence of inherently druggable domains. Developing methods to directly target the fusion protein is a prime focus in the field of SS disease biology. Directly targeting oncogenic drivers in other cancer contexts has proven highly successful with the most notable example of this being in the context of chronic myeloid leukemia (CML). Like SS this malignancy is characterised by a pathogniomic fusion protein, the Philadelphia chromosome, which results from the t(9;22)(q34;q11) balanced reciprocal translocation that generates the BCR-ABL oncogenic fusion protein with aberrant kinase activity (Quintás-Cardama & Cortes, 2009). The compound imatinib binds to the BCR-ABL kinase domain preventing the fusion protein from exerting its role in the oncogenic pathway resulting in leukemic cell apoptosis (Sacha, 2014). Imatinib was approved by the FDA as a first-line therapeutic option for newly diagnosed CML in 2002 (Sacha, 2014). With the advent of degrader compounds, targeting oncogenic drivers which have proven undruggable with traditional pharmacological approaches may soon be possible. This is because the identification of binding sites that directly modulate protein function is not necessary. Instead, the binding of a degrader compound at any site on a protein has the potential to induce target degradation. The concept of directly targeting the oncogenic fusion protein in SS is still in its infancy and even with the advent of PROTACs may prove unsuccessful. With this in mind

the continued pursuit and identification of essential co-factors of the fusion such as BRD9, SMARCA4 and EP300 is essential.

6.5 Future directions

In **chapter 3** of this thesis, SMARCA4 was highlighted as a novel genetic dependency in SS cells (**Figure 3.2 A**) (**Figure 3.5**). Despite near complete loss of SMARCA4/2 in as early as two hours (**Figure 4.12**), a minimal impact on cell viability until day five of drug exposure was observed (**Figure 4.14**). These findings coupled with only a modest effect on gene expression at 24-hours (**Figure 4.5**) warrants follow-up experiments profiling the gene expression changes induced by SMARCA4/2 degradation to be conducted across a longer time frame. It is important to note that the time frame of the genetic dependency screening experiments conducted by the DepMap initiative which were harnessed to highlight the dependency on SMARCA4 spanned several weeks. It will be important to comprehensively assess the response to ACBI1 across a broader range of cell lines. This expanded analysis should encompass additional SS cell lines, normal cells and cancer cells from a range of different lineages given SMARCA4's vital role as the catalytic subunit of the SWI/SNF complex and its significant contributions to development, gene regulation, and disease pathogenesis. Several studies have shown widespread alterations in histone PTMs following SWI/SNF perturbation (Schick et al., 2021; Panditharatna et al., 2022; Alver et al., 2017). Future chromatin profiling experiments in SS will specifically investigate this phenomenon to better understand the disconnect between the substantial chromatin accessibility losses and the minimal downregulation of gene expression. Given the crucial role of the histone acetyltransferase EP300 as revealed in **chapter 5**, special attention will be given to mapping this protein and its established histone marks in the context of SMARCA4/2 degradation. Additional SWI/SNF components found to be destabilised following SMARCA4/2 degradation will also be included in future chromatin mapping studies. A current focus involves more integration of the transcriptomic, chromatin accessibility, and chromatin mapping data sets.

In **chapter 5** EP300 was the only non-SWI/SNF component identified as both a fusion protein interactor and as a genetic dependency in SS cells, suggesting a unique functional relationship with the fusion protein (**Figure 5.2 C**). Transcriptomic analysis following EP300 perturbations revealed that EP300 activity supported oncogenic gene expression in SS cells. Future work will focus on carrying out time-resolved

quantitative chromatin mapping using CUT&RUN-Rx of both EP300 and the fusion protein in the context of EP300 perturbation. Integration of these data sets with the gene expression and chromatin accessibility analyses will provide further insights into the role of EP300 in SS cells. Akin to the experiments carried out in response to ACBI1 (**Figure 4.8**), the genome-wide chromatin accessibility changes induced by EP300 degradation will be investigated using ATAC-seq. It will be of interest to see if these accessibility changes correlate more strongly with the gene expression changes than those observed for the SMARCA4/2 perturbation experiments.

Quantitative IP-MS data has demonstrated that SWI/SNF complexes containing the SS18-SSX fusion oncoprotein associate with P300 in two SS cell lines (**Figure 5.2**). To determine the biochemical composition of EP300 associated SWI/SNF complexes future studies will conduct EP300 IP-MS. To ensure a stringent characterisation, identified EP300-SWI/SNF protein interactions will be validated using two independent P300-specific antibodies in endogenous co-immunoprecipitations (co-IP) coupled with western blotting. Furthermore, these analyses will be complemented by reciprocal co-IPs using antibodies specific for identified EP300 interactors. Together these experiments will characterize the biochemical composition of P300/SS18-SSX containing SWI/SNF complexes. Given the established diversity that exists amongst SWI/SNF complex assemblies the insights derived here will be essential to gaining a clear understanding of the role of EP300 associated complexes in this disease. As outlined for the SMARCA4/2 degrader ACBI1 it will be important to comprehensively assess the phenotypic response to EP300 perturbation across a broad range of cell lines including SS cells, normal cells and cancer cells from a range of different lineages. In **chapter 5** substantial effort was invested into establishing a relevant cellular model in which the fusion protein could be acutely degraded. This cell model will be used in the context of EP300 degradation in future experiments, to investigate the functional interplay between EP300 and SS18-SSX. As this model permits direct degradation of SS18-SSX it can be leveraged to gain further insight into the effects of directly versus indirectly targeting the fusion protein.

6.6 Conclusions

The data presented in this thesis support a number of conclusions:

1. SMARCA4 is a genetic vulnerability in synovial sarcoma cells.
2. SMARCA4/2 degradation induces the disassembly and concomitant loss of oncogenic SWI/SNF complexes from chromatin in synovial sarcoma cells.
3. EP300 interacts with the oncogenic fusion protein SS18-SSX and supports oncogenic gene expression in synovial sarcoma.

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