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An epigenetic approach for Ewing sarcoma patients

María Sánchez Jiménez

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Facultat de Medicina
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An epigenetic approach for Ewing sarcoma patients

Dissertation submitted by:

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“Cause love's such an old-fashioned word
and love dares you to care for
the people on the edge of the night
and love dares you to change our way of
caring about ourselves”

Under Pressure – Queen & David Bowie

A mamá, papá y Carmenchu,

A mi hermana,

A Ignasi.

I.	INTRODUCTION	1
1.	A GENERAL OVERVIEW OF PEDIATRIC CANCER	2
2.	INTRODUCTION TO EWING SARCOMA.....	4
2.1.	<i>Soft tissue sarcomas.....</i>	4
2.2.	<i>Definition of Ewing sarcoma.....</i>	5
2.3.	<i>Epidemiology</i>	6
2.4.	<i>Clinical features.....</i>	8
2.5.	<i>Diagnosis and risk stratification.....</i>	8
2.6.	<i>Treatment.....</i>	11
2.7.	<i>Genetic profile of Ewing sarcoma.....</i>	13
2.7.1.	<i>The fusion gene.....</i>	13
2.7.2.	<i>Genetic landscape in Ewing sarcoma.....</i>	15
2.8.	<i>Cell of origin.....</i>	17
3.	EPIGENETICS.....	18
3.1.	<i>A brief definition.....</i>	18
3.2.	<i>Chromatin structure</i>	19
3.3.	<i>Mechanisms of chromatin modification.....</i>	20
3.3.1.	<i>Histone modifications and the histone code.....</i>	21
3.3.2.	<i>Chromatin states.....</i>	23
3.4.	<i>Chromatin remodeling in EwS.....</i>	25
3.5.	<i>Polycomb group: Role of RING1B in EwS.....</i>	27
3.6.	<i>PRC1 canonical and non-canonical complexes.....</i>	28
3.7.	<i>BET proteins.....</i>	34
3.7.1.	<i>Definition: Histone readers.....</i>	34
3.7.2.	<i>Role in cancer: EwS.....</i>	36
3.7.3.	<i>BET inhibitors.....</i>	37
4.	AURORA KINASES.....	38
4.1.	<i>Definition</i>	38
4.2.	<i>Overview of AURKs in cancer.....</i>	43
4.3.	<i>Aurora kinase inhibitors and clinical trials.....</i>	44
4.4.	<i>AURK inhibition in Ewing sarcoma.....</i>	49
II.	HYPOTHESIS AND AIMS	51
III.	MATERIALS AND METHODS.....	56
1.	MATERIALS.....	57
1.1.	<i>Antibodies</i>	57
1.2.	<i>Interference RNA oligonucleotides.....</i>	58

1.3.	<i>Oligonucleotides sequences</i>	58
2.	CELL CULTURE.....	60
2.1.	<i>Cell lines</i>	60
2.2.	<i>Isolation and characterization of hpMSCs</i>	61
2.3.	<i>Pharmacological treatment of cell cultures</i>	62
2.4.	<i>Cell viability assays for synergy studies</i>	63
2.5.	<i>Small interfering RNA transfection</i>	63
2.6.	<i>Genome editing by CRISPR-Cas9: AURKB knock-out</i>	64
3.	FLOW CYTOMETRY ANALYSIS.....	64
3.1.	<i>Characterization of hpMSCs</i>	64
3.2.	<i>Apoptosis analysis with annexin V staining</i>	65
4.	MOLECULAR BIOLOGY TECHNIQUES	66
4.1.	<i>RNA extraction</i>	66
4.2.	<i>Reverse transcription quantitative polymerase chain reaction (RT-qPCR)</i>	66
4.3.	<i>DNA extraction and amplification by PCR</i>	67
4.4.	<i>Protein extraction</i>	68
4.5.	<i>Western blotting</i>	68
4.6.	<i>Protein immunoprecipitation</i>	69
4.7.	<i>Chromatin immunoprecipitation followed by qPCR</i>	70
4.8.	<i>Chromatin immunoprecipitation followed by sequencing</i>	72
4.9.	<i>RNA extraction followed by sequencing</i>	73
4.10.	<i>Proteomics</i>	73
4.11.	<i>Immunohistochemistry</i>	74
5.	IN VIVO EXPERIMENTS	75
IV.	RESULTS.....	77
	CHAPTER 1	78
1.	EWS CELL LINES ARE SENSITIVE TO AURKB INHIBITION	78
1.1.	<i>AURKB is widely expressed in EwS</i>	78
1.2.	<i>AURKB is a direct target of EWSR1-FLI1</i>	79
1.3.	<i>AZD-1152 is specifically effective on EwS cell lines causing cell death and apoptosis</i>	82
2.	STUDY OF AURORA KINASE B DISTRIBUTION GENOME-WIDE.....	83
2.1.	<i>AURKB is enriched at active enhancers in A673 EwS cell line</i>	83
2.2.	<i>AURKB co-localizes genome-wide with EWSR1-FLI1 and RING1B at active enhancers</i>	88
2.3.	<i>AURKB distribution genome-wide in HeLa Tet-On cell line model</i>	93
3.	EWSR1-FLI1/RING1B COMMON TARGET GENES ARE REGULATED BY AURKB MODULATION	95
3.1.	<i>AURKB is an essential gene in EwS</i>	95

3.2.	<i>EWSR1-FLI1/RING1B targets are regulated by AURKB knockdown with small interference RNA and short hairpin RNA</i>	98
3.3.	<i>EWSR1-FLI1/RING1B targets are regulated by AURKB inhibition with AZD-1152</i>	99
4.	UNDERLYING THE MECHANISTIC RELATION BETWEEN AURKB, EWSR1-FLI1 AND RING1B	104
4.1.	<i>AURKB modulation of RING1B E3 ubiquitin ligase activity</i>	104
4.2.	<i>AURKB modulation of RING1B phosphorylation</i>	105
5.	EVALUATION OF THE SYNERGISTIC COMBINATION OF AZD-1152 AND SUPERENHANCERS DISRUPTORS <i>IN VITRO</i> AND <i>IN VIVO</i>	110
5.1.	<i>Combination of AURKB inhibition and BET inhibition is potentially synergistic in EwS cell lines</i>	110
5.2.	<i>Combination of AZD-1152 and BMS-986158 is potentially synergistic in EwS in vivo models</i>	118
	CHAPTER 2	119
6.	ESTABLISHMENT AND CHARACTERIZATION OF HPMSCs	119
7.	GENOME-WIDE CHARACTERIZATION OF HPMSCs	122
V.	DISCUSSION	125
1.	TARGETING AURKB IN EWS CELL LINES	127
2.	AURKB IS ENRICHED AT ACTIVE ENHANCERS IN A673 CELL LINE	129
3.	AURKB RECRUITMENT BY EWSR1-FLI1	132
4.	AURKB DOWNREGULATION AND INHIBITION IN EWING SARCOMA	133
5.	UNDERLYING AURKB'S ROLE IN EWING SARCOMA	134
6.	SYNERGISTIC COMBINATION OF AZD-1152 AND BET INHIBITORS	137
7.	EPIGENETIC BACKGROUND OF HPMSC LINES	139
VI.	CONCLUSIONS	141
VII.	BIBLIOGRAPHY	144

I. INTRODUCTION

1. A general overview of pediatric cancer

The classical biological definition of a pediatric tumor referred to a heterogeneous group of solid and hematological neoplasms occurring predominantly during childhood, the period that extends from birth to adolescence. Pediatric cancer relates to the complex process of growth and development, differing to a large extent from adult cancer; instead of being a disease associated to the phenomenon of aging (Mora, 2012). In contrast to most adult tumor types, pediatric cancers are often driven by a limited number of genetic alterations.

One of the defining biological characteristics of pediatric tumors is their resemblance to progenitor tissues. Gene expression studies comparing different stages of normal tissue development have consistently shown a strong similarity between these cancer cells and the immature cells of the developing organs from which they arise (De Preter et al., 2006; Rivera & Haber, 2005; Scotting et al., 2005; Xia et al., 2002). It is not only the tissue or cell of origin that matters, but also the specific developmental stage during which these tumors arise. Evidence from genetically engineered mouse models suggests that pediatric tumors often originate from stem or progenitor cells that are blocked at a particular point in their differentiation (Funato et al., 2014; Mora, 2012; Nakagawara & Ohira, 2004; Scotting et al., 2005).

Pediatric tumors can be classified into infant, childhood or puberty tumors, according to a mathematical model describing the normal growth and development (ICP) (Karlberg, 1989). This model characterizes growth as the sum of three biological phases: infancy, childhood, and puberty. During infancy, growth is rapid but slows quickly, which is primarily influenced by nutrition, occurs the highest growth rate shortly after birth and velocity decreases rapidly. Childhood growth is depicted by a polynomial regression curve, largely driven by the initial impact of growth hormone (GH). Puberty marks a final, accelerated growth phase, fueled by the interplay between GH and sex hormones.

Pediatric cancer encompass a large variety of entities, including leukemia, lymphomas, central nervous system tumors, sarcomas of bone and soft tissue, neuroblastoma,

retinoblastoma, rhabdoid, liver, renal and germ cell tumors among other rare cancers. Given that each tissue develops within specific growth periods, pediatric cancers tend to correspond with these developmental stages (**Figure I. 1**). Sarcomas are relevant developmental puberty tumors arising in the fast growing zones of bones. We have focused on Ewing sarcoma (EwS), a puberty related tumor from an unclear lineage of undifferentiated cells that grow preferentially in the GH-insulin-like growth factor (IGF) and sexual steroids environment which characterizes puberty growth (Mora et al., 2009).

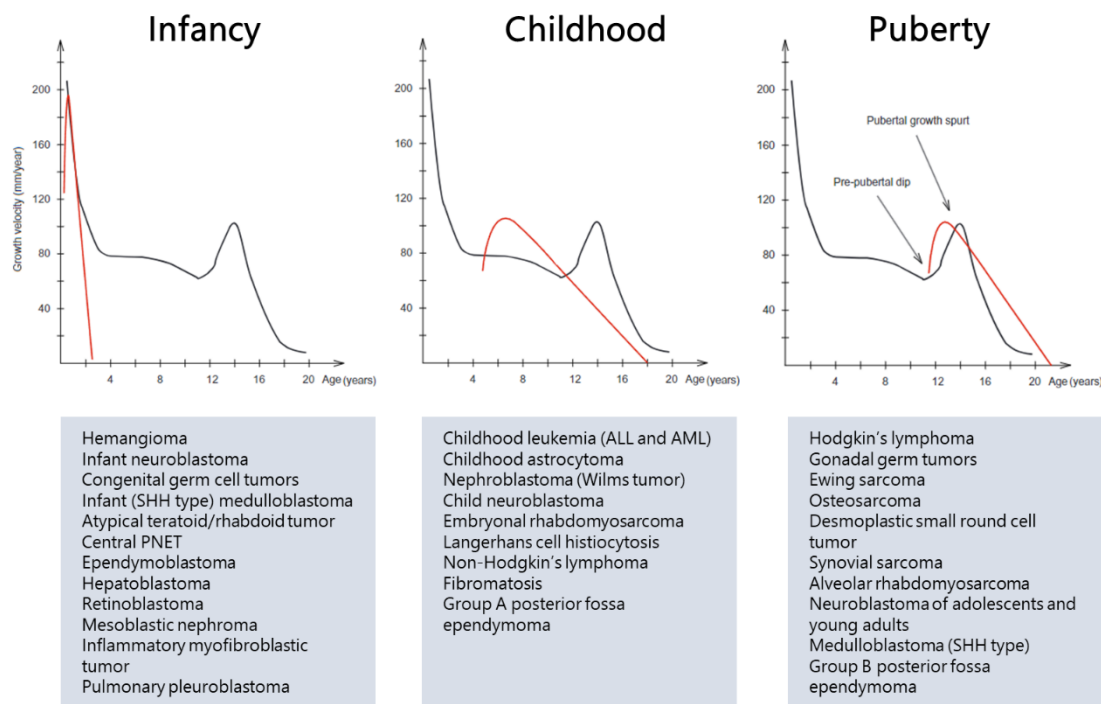


Figure I 1. Growth velocity curve of normal human growth overlaps with the age curve presentation of pediatric tumors. Each graph shows the overlap between the age curve presentation (red) and the growth velocity curve during 0–20 years of postnatal life. In the mathematical model described by Karlberg (1989), called ICP model, three additive and partly superimposed components characterized by different types of growth can be distinguished: infancy (left), childhood (middle) and puberty (right). In this regard, the first 2 years of postnatal life are characterized by a deceleration growth phase, followed by a stable and slow growth phase from 4–11 years of age. Finally, there is a growth spurt from 12–18 years of age, coinciding with puberty. These growth stages overlap with age presentation of paediatric tumours, as indicated in the yellow boxes below each graph. ALL, acute lymphoblastic leukaemia; AML, acute myeloblastic leukaemia; PNET, primitive neuroectodermal tumour; SHH, sonic hedgehog homolog. Adapted from (Mora, 2012)

2. Introduction to Ewing sarcoma

2.1. Soft tissue sarcomas

The advances in classifying and understanding the pathogenesis based on the correlation of histologic and genetic findings have been particularly significant in the field of soft tissue and bone oncopathology (Doyle, 2014). Most recent classifications have integrated morphology, immunohistochemistry and molecular genetics with different health disciplines (medical, surgical and radiation oncologists), to obtain more precise definitions (Sbaraglia et al., 2021). Sarcomas, tumors of putative mesenchymal origin, account for nearly 21% of all pediatric solid malignant cancers and less than 1% of all adult solid malignant cancers (Burningham et al., 2012). They are diverse types of malignancies that arise from bone, cartilage, or connective tissues, such as muscle, fat, peripheral nerves, and fibrous or related tissues (Taylor et al., 2011). In line with the diverse nature of mesenchymal tissues, more than 50 histologic sarcoma subtypes have been described, such as osteosarcoma (OS), Ewing sarcoma (EwS), rhabdomyosarcoma (RMS), liposarcoma, synovial sarcoma (SS), and chondrosarcoma. (Figure I. 2)

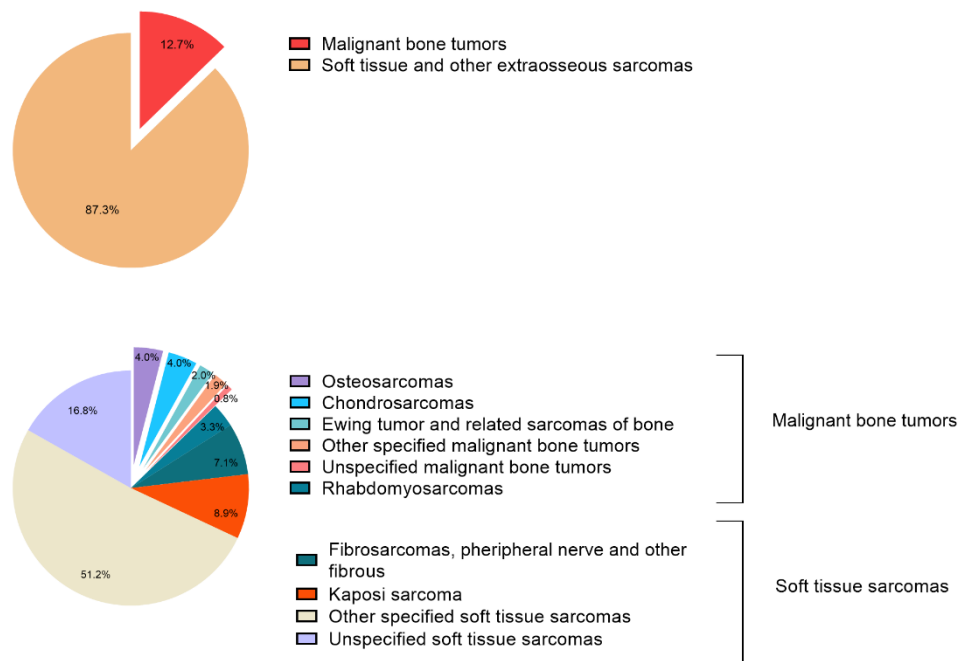


Figure I. 2. Distribution of new sarcoma cases by histology (2008). Upper image showing percentages of malignant bone and soft tumors. Lower image showing percentages of every subtype of malignant bone tumors and soft tissue sarcomas. Adapted from (Burningham et al., 2012)

Current understanding of the key genomic aberrations in soft-tissue sarcomas is limited to the most recurrent alterations or translocations (Barretina et al., 2010). Most sarcomas with simple genetic alterations are translocation-associated sarcomas (which account for approximately one-third of all sarcomas). These tumors tend to arise *de novo* and, in some cases, harbor only a single defining cytogenetic abnormality that is present at initiation and is retained throughout their clonal evolution (Taylor et al., 2011). Sarcomas span a wide range of complexity among human malignancies in terms of their copy-number alterations (Beroukhi et al., 2010). They vary from translocation-associated sarcomas with generally few copy-number alterations, to karyotypically complex subtypes that are heterogeneous, unstable and profoundly altered in genomic copy number.

More specifically, according to the most recent WHO Classification of Soft Tissue and Bone Tumors, we can find four categories of undifferentiated small round cell sarcomas: Ewing sarcoma, round cell sarcoma with EWSR1-non-ETS fusions, CIC-rearranged sarcoma, and sarcoma with BCOR genetic alterations. The WHO currently defines EwS as harboring EWSR1/FUS::ETS fusions. Detection of these fusions alone, however, is not diagnostic of Ewing sarcoma, and it is likely there are rare mesenchymal tumors, in addition to adamantinoma-like Ewing sarcoma, with the same fusions that are not properly classified as Ewing sarcomas, but this is an area of ongoing investigation (Dehner et al., 2024).

2.2. Definition of Ewing sarcoma

In 1921, James Ewing defined for the first time a “diffuse endothelioma of the bone”, a painful condition that, contrary to osteosarcoma, was radiosensitive and microscopically characterized by well-defined round cells with pale cytoplasm and big nuclei (Ewing, 1921). Nowadays, Ewing sarcoma (EwS) is described as an aggressive neoplasm of bone

and/or soft tissue with a peak incidence during adolescence and young adulthood (Gaspar et al., 2015). It is the second most common bone cancer that affects children and adolescents; however, it is the leading neoplasm in bone and soft tissue in Spain.

As it was pointed out before, EwS is the archetype and most commonly occurring member of the undifferentiated small round cell sarcoma group. Historically, chest wall tumors were designated “Askin tumor,” and cases with morphologic features of neuronal differentiation were designated “(peripheral) primitive neuroectodermal tumor,” however the use of these terms is discouraged. Further, current evidence supports the assertion that *EWSR1::NFATc2* sarcoma, *CIC*-rearranged sarcoma and *BCOR* alteration sarcoma, previously known as “Ewing-like” sarcomas, are distinct entities biologically and now conceptionally in the most recent WHO Soft Tissue and Bone Tumour Classification (Dehner et al., 2024).

2.3. Epidemiology

Ewing sarcoma predominantly affects children, adolescents, and young adults (AYAs), with a peak incidence at age 15, and is slightly more common in boys and men (sex ratio 3:2) (Jawad et al., 2009). It is primarily observed in populations of European descent, with an incidence of approximately 1.5 cases per million children and AYAs (Grünewald et al., 2018). In Spain, the annual incidence rate is 5.2 per million between 0 and 14 years, almost twice as high as osteosarcoma (3.5 per million), according to the national Spanish pediatric tumor registry (RNTI) (Mora et al., 2017). By contrast, the incidence is much lower in Asian and African populations, with rates of around 0.8 and 0.2 cases per million children, respectively (Fraumeni & Glass, 1970; Jawad et al., 2009; Jensen & Drake, 1970). The low incidence among African Americans implies a genetic rather than environmental basis for the disease (Fraumeni & Glass, 1970; Worch et al., 2011). Indeed, Caucasians have a nine-fold higher incidence of Ewing sarcoma compared to African Americans, and this incidence has increased over time among Caucasians but not in other racial groups (Jawad et al., 2009). In this sense, the complex relationship between race and *EWSR1* somatic translocation underscores the importance of considering racial and ethnic

differences in the diagnosis, treatment, and research of EwS (Worch et al., 2011). Survival rates are also influenced by factors such as local disease stage, primary tumor location in the appendicular skeleton, and tumor size (≤ 8 cm). Female sex is associated with improved survival among Caucasian patients as an independent predictor, but this benefit does not extend to other racial groups. Further research is necessary to understand the racial disparities in incidence and the sex differences in survival outcomes.

Metastasis at diagnosis is considered one of the main important prognostic factor with lungs, bone, and bone marrow being the common metastatic sites. The 5-year overall survival (OS) for patients with localized EwS is approximately 70-80%. In the case of patients with metastasis the OS decreases to less than 30%, with the exception of patients with isolated pulmonary metastasis, where the OS stands around 50%. In the absence of metastasis, the tumor site is the most significant prognostic factor, with patients having proximal primary tumors (such as those in the pelvis and sacrum) experiencing poorer outcomes compared to those with distal tumors. (Riggi et al., 2021). Other prognostic factors of bad outcome include age over 14 years, tumor volume over 200 mL, presence and number of bone lesions, additional pulmonary metastases, and bone marrow involvement.

Several different clinical trials in EwS have consistently identified age as prognostic marker, although the age cutoff has varied among studies. Indeed, results from our center confirmed in the GEIS21 study the importance of age (≥ 18 years cutoff) as an independent predictor of worse outcome, superior to the detection of metastasis. In this clinical trial the mP6 protocol, consisted of chemotherapeutic cycles 1, 2 and 4 with cyclophosphamide, doxorubicin and vincristine; and 3 and 5 cycles with ifosfamide and etoposide, was completed including adults and children within the GEIS consortium (Mora et al., 2017). Furthermore, the most recent report from the Children's Oncology Group (COG) trial of localized EwS and the retrospective analysis of all COG trials indicate that age ≥ 18 years is an important predictor of outcome (Marina et al., 2015). According to these results, patients older than 18 years with metastasis or pelvic primary tumors

should be prioritized to treatments using new agents since conventional drugs are unlikely to provide much benefit at a high toxicity cost. Nevertheless, currently there is no robust tool to estimate the life expectancy of EwS patients that survive (Gaspar et al., 2015; Grünewald et al., 2018).

2.4. Clinical features

EwS tumors most commonly arise in bone. The ribs, pelvis, or the diaphysis or meta-diaphysis of long bones are most frequently involved, though no part of the skeleton is exempt. EwS often presents with nonspecific clinical features. Patients typically experience localized pain, swelling is common and can be mistaken for a minor injury, while in some cases, the only symptom may be the detection of a firm mass upon palpation. A pathological fracture occurs in 10 to 15% of cases. In advanced disease, nonspecific symptoms such as fever, night sweats, fatigue, and weight loss may arise. Blood tests might reveal elevated markers like alkaline phosphatase, which are generally not diagnostic, although an increased serum lactate dehydrogenase level is associated with tumor burden and has both diagnostic and prognostic value (Biswas et al., 2014; Widhe & Widhe, 2000).

On X-rays, Ewing sarcoma of the bone typically appears as a poorly defined, permeative lesion within the bone marrow. It often extends into the surrounding soft tissues, sometimes significantly, and may show an onion-skin or, less commonly, a sunburst-like periosteal reaction. Magnetic resonance imaging (MRI) is valuable for assessing the full extent of the tumor, as X-rays often underestimate its size. In cases of extraskkeletal tumors, the tumor may erode the underlying cortical bone, forming a concave indentation known as saucerization (Dehner et al., 2024; Zöllner et al., 2021).

2.5. Diagnosis and risk stratification

Diagnosis relies on histologic and molecular analysis of biopsy specimens or surgically resected tumor tissue. This diagnosis should be confirmed at a sarcoma reference center using biopsy material sufficient for conventional histology, immunohistochemistry,

molecular pathology, and biobanking (ESMO/European Sarcoma Network Working Group, 2014; Grünewald et al., 2018). Histologically, EwS is classified as a small round blue cell tumor and has common features with several cancers including neuroblastoma, lymphoblastic lymphoma, alveolar rhabdomyosarcoma, peripheral neuroectodermal tumor (PNET), poorly differentiated synovial sarcoma, and rare old-called “Ewing-like” tumors. The cells have scant, weakly eosinophilic cytoplasm with glycogen granules in the form of periodic-acid-Schiff-positive. Cells show round nuclei with evenly distributed chromatin and minimal mitotic activity; the chromatin is finely stippled, and nucleoli are usually not observable (Grünewald et al., 2018; Riggi et al., 2021; Riggi & Stamenkovic, 2007). Since EwS lacks matrix among tumor cells, reticulin stains are negative. Occasionally, larger cells with irregular nuclei and larger nucleoli are seen, particularly in the atypical (large cell variant) cases. The main differences in these cells compared to conventional Ewing sarcoma are larger nuclei with irregular contours, conspicuous nucleoli, and typically negative periodic acid–Schiff staining (Grünewald et al., 2018). Some cases show rosette formation (oriented towards a central space), showing more neural differentiation, and a “light cell-dark cell” pattern due to viable cells juxtaposed to pyknotic (condensed and damaged) cells (Folpe et al., 2005) (**Figure I. 3.**).

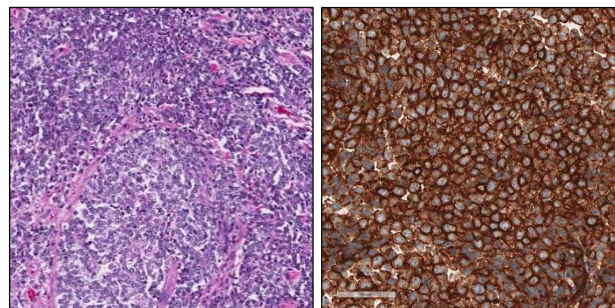


Figure I. 3. Histologic and immunohistochemical features of EwS. In the left, hematoxylin and eosin section showing sheets of a small blue cell tumor with closely packed cells with indistinct cell borders and inconspicuous nucleoli. Note the cytoplasmic clearing. In the right, CD99 stain showing strong and diffuse membranous staining of tumor cells. Adapted from <https://atlasgeneticsoncology.org/solid-tumor/5010/bone-and-soft-tissue-ewing-sarcoma>.

Immunohistochemically, strong CD99 diffuse membrane expression, although not specific, is positive in approximately 95% of cases, and its absence nearly rules out EwS (Ambros et al., 1991; Perlman et al., 1994; Toomey et al., 2010). FLI1 is more specific but limited by expression in other cancers; its sensitivity is reduced by variant translocations not involving *FLI1* (Hornick, 2014). Caveolin 1 and homeobox protein NKX-2.2 expressions are also useful, especially in CD99-negative cases (Llombart-Bosch et al., 2009; Machado et al., 2018). PAX7 is expressed in the large majority of EwS in addition to rhabdomyosarcomas, BCOR::CCNB3 sarcoma and synovial sarcoma (Toki et al., 2018). Characterization of the EwS histopathology is essential for diagnostics as well as for the assessment of tumor necrosis during treatment, as it correlates with patient OS (Gaspar et al., 2015).

Molecular pathology is essential for confirming EwS, especially with unusual clinical and pathological features. A definitive diagnosis requires identifying signature chromosomal translocations via in situ hybridization or quantitative polymerase chain reaction (ESMO/European Sarcoma Network Working Group, 2014; Machado et al., 2009; Riggi et al., 2021). Assays using EWSR1 break-apart probes detect EWSR1 gene rearrangements, important for differentiating from other sarcomas with EWSR1 fusions with non-ETS genes. Next-generation sequencing (NGS) is recommended when FISH and/or RT-PCR results are inconclusive. Laboratories relying on FISH, RT-PCR and NGS prevent misdiagnosis of EwS with EWSR1–ERG fusions, which may be FISH-negative for EWSR1 rearrangements (S. Chen et al., 2016; Sorensen et al., 1993).

Prognostic factors, such as the presence of metastases, histological response to chemotherapy, tumor volume, and age at diagnosis, are used as stratification criteria, allowing for subsequent adjustments in treatment intensity (Widhe & Widhe, 2000). As an overview, patients with local disease and small tumors, together with good histological response, are classified as standard risk. Patients with adverse histological response and large tumors are considered as localized high-risk. Primary disseminated tumors and relapses are associated with poor outcome as very high-risk metastatic (Grünewald et al., 2018). Finally, recent studies demonstrated that the presence of

circulating tumor DNA (ctDNA) is linked to a higher risk of disease recurrence and reduced overall survival. This finding suggests that ctDNA could serve as a valuable prognostic biomarker in EwS, providing insights into the disease's progression and potentially guiding more tailored therapeutic approaches (Shulman et al., 2018).

2.6. Treatment

Treatment for Ewing sarcoma typically involves a multidisciplinary approach combining chemotherapy, surgery, and radiotherapy (RT). Patients generally receive neoadjuvant chemotherapy to shrink the tumor, followed by local control measures such as surgery and/or radiation, and then adjuvant chemotherapy. Primary amputation is rarely necessary, as tumor shrinkage from neoadjuvant chemotherapy and the option of definitive radiotherapy make limb-sparing treatments more feasible. Neoadjuvant chemotherapy, with or without preoperative radiotherapy, significantly reduces tumor size and helps prevent metastatic progression. Combined strategies of surgery and RT yield to more favorable outcomes in local control than either definitive RT or surgery alone (DuBois et al., 2015). The choice between surgery and radiotherapy is determined by numerous factors, e.g., patient age, tumor site, size, and local extension, requiring a careful and multidisciplinary balance of both approaches (Zöllner et al., 2021). The duration of the initial period of neoadjuvant chemotherapy mainly determines the timing of surgical local therapy (Lin et al., 2019).

Treatment is completed with postoperatively multiagent cytotoxic chemotherapy. Current treatment protocols involve intensive multi-agent chemotherapy with drugs like vincristine, ifosfamide, doxorubicin, etoposide (VIDE) and cyclophosphamide (Juergens et al., 2006) combined with the surgery and/or radiation just mentioned to manage both primary and metastatic disease. For metastatic patients, whole lung irradiation or high-dose busulfan and melphalan chemotherapy together with autologous stem cell transplantation (SCT) have been studied to improve outcomes (Grünewald et al., 2018; Luksch et al., 2012). The combination of gemcitabine and docetaxel described by our

institution obtained a 66% of response rates when high doses applied (Mora et al., 2009), but not superior to other regimens evaluated.

The US Children's Oncology Group (COG) compared the 3-weekly (so-called uncompressed regimen) versus 2-weekly (compressed) regimens of vincristine, doxorubicin and cyclophosphamide alternating with ifosfamide and etoposide. As a result of sequential COG clinical trials, outcomes for patients with localized disease were improved (five-year event-free survival (EFS) is 87%) through chemotherapy intensification with this compressed regimen (Reed et al., 2023; Womer et al., 2012). In contrast, patients with newly diagnosed metastatic or recurrent disease have not seen improvements in outcomes for several decades. For example, on the most recently completed COG trial AEWS1221, the three-year EFS rate was 38.3%. Importantly, a range of rational strategies to intensify therapy for these patients has not achieved improved outcomes, highlighting a degree of intrinsic chemotherapy resistance for these patients. To improve the outcome of these patients, many treatments have been proposed, such as the addition of a gemcitabine and docetaxel regimen (Mora et al., 2009, 2017), whole lung irradiation or high-dose besulfan and melphalan chemotherapy with autologous stem cell transplantation (Luksch et al., 2012; Oberlin et al., 2006). However, some relapsed patients may benefit from the combination of specific agents. One combination includes the use of vincristine, irinotecan, and temozolomide agents (Raciborska et al., 2013) while another combination involves temsirolimus in combination with irinotecan and temozolomide (Bagatell et al., 2014).

Nonetheless, most patients with recurrent EwS will not survive. Relapse in patients with EwS can occur due to the presence of highly resistant clone of tumor cells, which may have either pre-existed in the primary tumor or developed during anticancer treatment (Castillo-Ecija et al., 2020). A critical focus for the field, therefore, is to develop novel strategies for these patients with unsatisfactory survival (Reed et al., 2023). Many randomized clinical trials are currently investigating the efficacy of new agents. Nevertheless, genomic characterization of bone sarcomas has not yet identified opportunities for existing molecularly targeted therapies. Similarly, immunotherapy has

yet to make a significant impact on these tumors. Some ongoing trials include the addition of monoclonal antibodies against the receptor of insulin-like growth factor 1 (IGF1) (trials with both R1507 and robatumumab) to dose-compressed chemotherapy (Anderson et al., 2016; Pappo & Dirksen, 2017). Therapies that inhibit the mTOR pathway (temsirolimus), topoisomerase (irinotecan) or the DNA repair mechanism (temozolamide) are also under research (Bagatell et al., 2014). Adjuvant immunotherapy with autologous lymphocytes, tumor lysate, or keyhole limpet hemocyanin-pulsed dendritic cell vaccination with or without recombinant human interleukin 7 (IL-7) has been shown to improve survival in patients with metastatic pediatric sarcomas including EwS (Merchant et al., 2016).

2.7. Genetic profile of Ewing sarcoma

2.7.1. The fusion gene

Non-random chromosomal translocations are often characteristic features of a number of bone and soft-tissue sarcomas. At the pathophysiologic level, many of these translocations behave as aberrant oncogenes that play crucial roles in tumor development by deregulating target gene expression (Sankar & Lessnick, 2011).

The defining molecular feature of EwS is the FET-ETS fusion protein. In approximately 85% of the cases, the balanced translocation involves chromosomes 11 and 22, t(11;22)(q24;q12) (Delattre et al., 1992). It fuses the EWSR1 RNA-binding protein 1 (EWSR1) gene of the FET (FUS/TLS, EWSR1, TAF15) family (previously called TET) of RNA-binding proteins with a member of the erythroblast transformation-specific (ETS) family, i.e. the Fli-1 proto-oncogene gene (*FLI1*), leading to the EWSR1-FLI1 fusion gene. Exon 7 of EWSR1 joins to either exon 6 (fusion type I; 60% of cases) or exon 5 (type II; 25% of cases) of FLI1. In the remaining 15% of cases, chromosomal rearrangements have been observed involving other members of the same gene families. For instance, variants have been identified where EWSR1 is fused with a different ETS transcription factor, such as ERG (Zucman et al., 1993). Other, less common translocations, each found in about 1% of cases, involve EWSR1 rearrangements with other ETS family members such as ETS

variant 1 (ETV1), ETS variant 4 (ETV4), or the FEV transcription factor gene (FEV) (Jeon et al., 1995; Peter et al., 1997; Urano et al., 1998). Additionally, alternative variants have been identified where ETS genes fuse with EWSR1 paralogs from the FET gene family, like the FUS RNA-binding protein gene (FUS) or the TATA-box binding protein associated factor 15 gene (TAF15) (Ng et al., 2007; Watson et al., 2018). All the types of possible translocations are summarized in **Figure I. 4**.

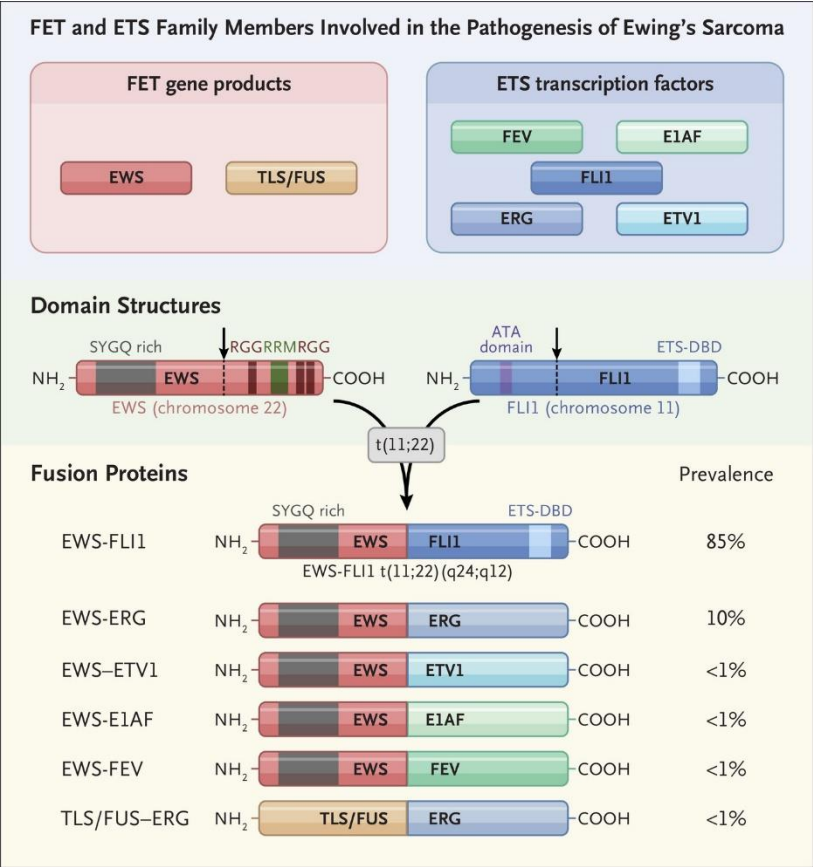


Figure I. 4. Fusion Proteins in Ewing sarcoma. Oncogenic translocations in EwS involve a member of the FET gene family (FUS, EWSR1, or TAF15) paired with various members of the ETS transcription factor family (such as FLI1, ERG, ETV4, and FEV).

EWSR1–FLI1 plays a crucial role in cellular transformation as a powerful transcriptional regulator (Kinsey et al., 2006; R. Smith et al., 2006). The N-terminus that contains the transactivation domain of EWSR1 is fused to the C-terminal DNA-binding domain of FLI1, with both elements being essential for the transformation process (Jaishankar et al., 1999; Lessnick et al., 1995). The oncogenic effects of EWSR1–FLI1 are achieved through direct or indirect regulation of genes necessary for transformation. Although EWSR1–FLI1 is

indispensable for this process, its ectopic expression in most human cell lines fails to activate similar genetic programs or induce transformation. This suggests that cell-specific factors significantly influence EWSR1–FLI1 activity (Lessnick et al., 2002; Miyagawa et al., 2008; Riggi et al., 2008).

2.7.2. Genetic landscape in Ewing sarcoma

Identifying secondary genetic alterations with a significant prevalence could contribute to understand the molecular mechanisms underlying the most aggressive forms of EwS. In the case of EwS, like other translocation-positive sarcomas, its genetic background is characterized by one of the lower mutational rates of all cancers with 0.15-0.45 mutations per megabase (Brohl et al., 2014; Crompton et al., 2014). Copy number alterations (CNAs) are the most common feature with gains of chromosome 8 (50% of cases), chromosome 2 (25% of cases), 1q (25% of cases), and loss of 16q (30%) being the most common (Kullendorff et al., 1999). Chromosome 1q gain is frequently associated with 16q loss as the result of an unbalanced t(1;16) rearrangement, which is associated with poor clinical outcome (Kullendorff et al., 1999; Mugneret et al., 1988; Stark et al., 1997). The most frequent chromosomal deletions involve the small arm of the chromosome 9 (9p), producing the loss of the tumor suppressor cyclin-dependent kinase inhibitor 2A (CDKN2A), which has been associated with poor prognosis (Brohl et al., 2014; Crompton et al., 2014).

According to Crompton et al., next-generation sequencing (NGS) studies of EwS cohorts demonstrated that pathogenic TP53 mutations were detected in 5-20% of cases and frequent loss of function mutations in the cohesion complex family member stromal antigen 2 gene (STAG2) (20%) (mutational landscape of EwS is summarized in **Figure I. 5**) (Brohl et al., 2014; Crompton et al., 2014; Tirode et al., 2014). RPTN, a member of the epidermal differentiation complex, and PRB2, a member of the human salivary proline-rich protein family, were also significantly mutated on the basis of this analysis (Crompton et al., 2014). The prognostic significance of STAG2 mutation appears to be strongly dependent on the coexistence of a TP53 mutation. STAG2 and TP53 mutation were

described to cooperate to increase genetic instability in an aggressive subtype of EwS. However, mutations of STAG2 or TP53 are not required for tumor initiation or maintenance, while loss of STAG2 increases migration and invasion of MSC without modifying their proliferation rate. It has been described that STAG2 inhibition, together with TP53 mutation and the expression of the oncogene, are synergistic in the process of sarcomagenesis, although they are not sufficient to fully transform primary murine MSCs (El Beaino et al., 2020). The functional significance of these mutations as EwS modifiers remains to be elucidated. Recent studies showed that the loss of STAG2 expression in EwS modifies the transcriptional activity of EWSR1-FLI1, leading to a more invasive cellular phenotype (Adane et al., 2021; Surdez et al., 2021). CDKN2A was found deleted in around 14% of EwS cases throughout deletion of chromosome 9p. Furthermore, STAG2 and CDKN2A mutations were found as mutually exclusive in EwS tumor samples and cell lines (Brohl et al., 2014; Crompton et al., 2014; Tirode et al., 2014).

Considering the low mutational burden of these tumors and the fact that the mutations occur primarily in genes regulating epigenetic processes, epigenetics is believed to play a key role in the initiation and progression of EwS.

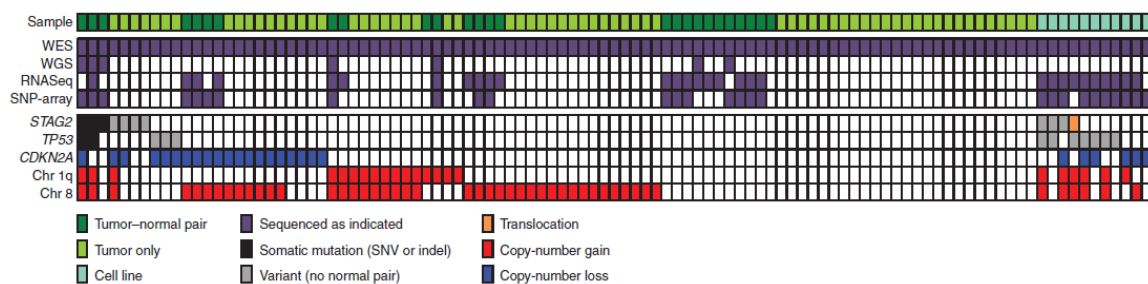


Figure I. 5. Mutational landscape of Ewing sarcoma tumors. Sample data, sequencing method, and recurrent aberrancies detected by massively parallel sequencing are indicated for 92 Ewing sarcoma tumors and 11 Ewing sarcoma cell lines. Box colors for each panel are indicated in the key. SNV, single-nucleotide variant. Retrieved from (Crompton et al., 2014).

2.8. Cell of origin

Although in his first description in 1921 James Ewing postulated endothelium as the cell of origin, this issue still remains unclear it has been hypothesized to be diverse including neural crest or mesenchymal stem cells (MSC). The lack of a known cell of origin contributes to the difficulty in understanding exactly the tumorigenic process of EwS and adds difficulty to the design of experimental models.

Initial studies suggested a neural crest origin due to the immature neural phenotype of many tumors and the capability of EwS-derived cell lines grown *in vitro* to undergo neural differentiation (Noguera et al., 1994). This evidence was supported in the study by von Levetzow et al., where whole genome expression profiling revealed that the molecular signature of EwS was more similar to human neural crest stem cells (hNCSC) than any other normal tissue, including hMSC (von Levetzow et al., 2011). Furthermore, ectopic expression of EWSR1-FLI1 in undifferentiated hNCSC resulted well-tolerated and led to altered expression of both well established as well as novel EWSR1-FLI1 target genes, initiating transition to an EwS-like state. Other researchers in the field have observed that knockdown of EWSR1-FLI1 in EwS cell lines leads to an expression profile similar to bone marrow -derived mesenchymal progenitor cells (Tirode et al., 2007).

Human MSCs (hMSCs) are multipotent precursors that can be differentiated *in vitro* into various cell types, typically osteoblasts, chondrocytes, and adipocytes, as well as into endothelial lineage (Oswald et al., 2004). EWSR1-FLI1 overexpression in adult hMSCs blocks their differentiation, and generates a transcriptome profile reminiscent of EwS (Riggi et al., 2008). Many of these EF-regulated genes are even more induced when the oncogene is expressed in pediatric hMSCs (hpMSCs), while numerous genes that are among the most prominent EwS markers are induced in hpMSCs but not in their adult counterparts (Riggi et al., 2010). Notably, although hpMSCs provide a far more permissive environment, hpMSCs that express EF (EF-hpMSCs) are unable to form tumors *in vivo*, which has led to the suggestion that a cooperating genetic event or a unique cellular phenotype is additionally required. To address these two issues, Gordon and colleagues

developed an approach to model EwS that exploits the in vitro potential of human embryonic stem cells (hESCs) to differentiate into cells from the three germ layers when forming embryoid bodies (EBs). EWSR1-FLI1 expression in EBs derived from p53-deficient hESCs leads to in vitro transformation, yet EB-derived cells lack tumor formation capacities *in vivo* (Gordon et al., 2016).

If there is no significant recurrent mutation in EwS tumor cells besides the fusion oncogene, why the fusion gene is permissible only in certain cells driving transformation? Most recent experiments suggest developmental epigenome as the hidden factor behind the activity of the fusion oncogene. A notable effort has been done studying the epigenetic changes set by the fusion protein in different tumor cell models. Those models include multiplicity of embryonic cells, such as NCSCs, MSCs, and allographs of embryonic osteochondrogenic progenitors, giving rise to tumors that display most of the characteristics of EwS (Castillero-Trejo et al., 2005; Riggi et al., 2005, 2010; Tanaka et al., 2014; von Levetzow et al., 2011). In sum, EWSR1-FLI1 oncogene only recapitulates the characteristics of EwS tumors in some specific backgrounds.

3. Epigenetics

3.1. A brief definition

In 1942, the term “epigenetics” was used for the first time by the embryologist Conrad Waddington, defining it as the “whole complex of developmental processes that lie between genotype and phenotype” (Waddington, 1942). Currently, epigenetics refers to the heritable and reversible mechanisms that modify DNA and chromatin without altering the genetic sequence, thereby regulating the timing, location, and level of gene expression, and maintaining cellular identity by controlling transcriptional states (Nichol et al., 2016; Sánchez-Molina et al., 2022). In cancer, epigenetics is crucial in oncogenesis and tumor progression, particularly in pediatric tumors, like EwS, where the mutational burden remains very low.

3.2. Chromatin structure

Chromatin is a macromolecular complex made up of DNA, non-histone and histone proteins that serves as the structural framework for packaging the entire genome (Dawson & Kouzarides, 2012). The minimal structural unit of the chromatin is the nucleosome, which is composed of ~147 base pairs of DNA wrapped around an octamer formed by two of each of the histones H3, H2A, H2B, and H4 (Aranda et al., 2015). The nucleosome cores are linked together by segments of linker DNA, creating a "beads-on-a-string" nucleosomal arrangement with a diameter of 11 nm. These nucleosomal arrays are then further compacted into a more condensed 30-nm chromatin fiber, which is commonly considered the secondary structure of chromatin. The maximum level of chromatin condensation is achieved when the chromatin fiber measures from 700 nm to 1.4 μm during mitosis, leading to the easily recognized X-shaped chromosomes (Woodcock & Dimitrov, 2001) (**Figure I. 6**). In contrast, when cells are in interphase and non-dividing, chromosomes are relaxed and compartmentalized into nuclear domains that are known as chromosome territories, constituting a basic feature of nuclear architecture (Cremer & Cremer, 2001). Two major chromatin structures can be classified according to their level of condensation: heterochromatin and euchromatin. Heterochromatin resembles a condensed structure that is characterized by lower levels of expression, whereas euchromatin is transcriptionally permissive and displays a relaxed organization (G. Li & Reinberg, 2011; V. W. Zhou et al., 2011).

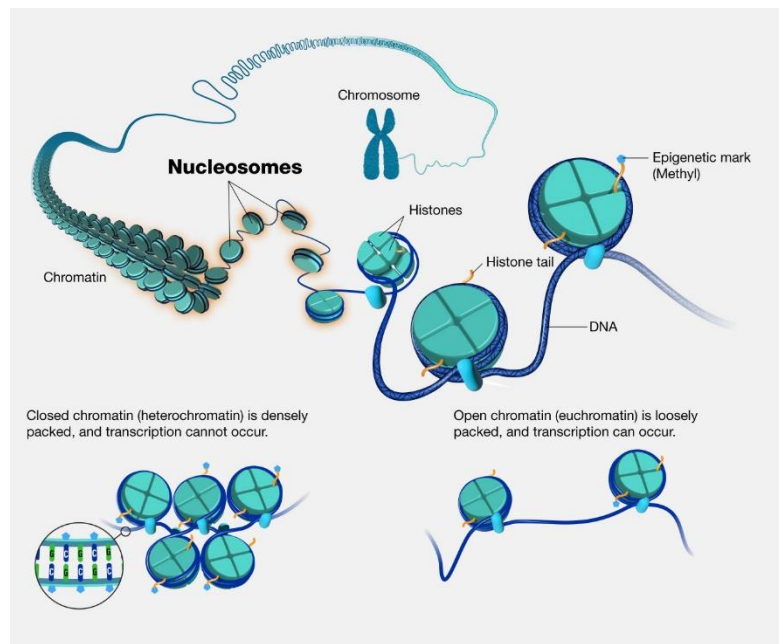


Figure I. 6. Levels of chromatin organization. Diagram showing the different compaction degree between heterochromatin and euchromatin, as well as nucleosome composition. Retrieved from <https://www.genome.gov/genetics-glossary/Nucleosome>.

3.3. Mechanisms of chromatin modification

Chromatin exhibits a highly structured and dynamic nature. The precise control of chromatin packaging can influence the accessibility of various protein complexes that interact with DNA, including the transcriptional machinery complex (Kouzarides, 2007). Several epigenetic mechanisms regulate DNA access in response to upstream or external signals. These mechanisms include DNA methylation, nucleosome remodeling, post-translational modifications (PTM) of histones, incorporation or substitution of histone variants, and non-coding RNA (ncRNA). Notably, these mechanisms do not function independently but rather collaborate to form a complex, interconnected, epigenetic network that governs gene expression. Given the crucial role of these mechanisms in transcription, it is unsurprising that epigenetic dysregulation has emerged as a significant and recurring theme in cancer research over the past decade (Berdasco & Esteller, 2013). In this work, we focus on histone modifications and the enzymatic activities responsible for them.

3.3.1. Histone modifications and the histone code

Histones are conserved small basic proteins with a highly positive charged N-terminal tail that enables their tight binding to the negatively charged DNA from the chromatin. Histones are primarily classified into two categories: core histones and linker histones. The core histones consist of four types: H2A, H2B, H3, and H4. Linker histones, such as H1 or its homologue H5, form the second class. In eukaryotes, core histones are typically produced during DNA replication, with their expression peaking in the S phase of the cell cycle (Arents & Moudrianakis, 1995; Lawrence et al., 2016).

Histone variants are alternative forms of histone proteins, especially core histones, that exhibit structural similarities but possess unique properties and functions. These variants influence the amount of DNA wrapped around nucleosomes and alter nucleosome stability. By doing so, histone variants play a key role in enhancing the complexity and flexibility of chromatin structure and in regulating epigenetic mechanisms in eukaryotic cells. Histone variants have roles in a range of processes including DNA repair, meiotic recombination, transcription initiation and termination, sex chromosome condensation, and chromatin packaging in sperm cells (Talbert & Henikoff, 2010).

Histones and histone variants undergo various post-translational modifications (PTMs) in the N-terminal tails that extend from the nucleosome. These modifications are covalent, dynamic, and reversible, including processes like acetylation, methylation, phosphorylation, ubiquitylation, neddylation, sumoylation, ADP-ribosylation, deimination, and proline isomerization (Bannister & Kouzarides, 2011; Kouzarides, 2007). Histone modifications are key to regulating DNA access for processes like gene transcription, replication, DNA repair, chromosome compaction and localization. Epigenetic changes in these modifications can disrupt gene expression and contribute to cancer development (Chi et al., 2010).

Histone modifications form a complex code in which synergistic or antagonistic interactions regulate chromatin's accessibility to transcription factors, ultimately influencing gene activation or repression. These combinations of post-translational

modifications (PTMs) on histone proteins, particularly on their tails, create a "histone code" that is interpreted by other proteins to regulate gene expression. Epigenetic regulators responsible for histone modifications contain specialized domains that enable them to write, read or erase chromatin modifications. Writers, such as acetyltransferases and methyltransferases are responsible for adding methyl and acetyl groups. Erasers, including demethylases and deacetylases, deplete the histone tails from the acetyl and methyl groups. Finally, readers, such as PHD domains or bromodomains interact with the acetylated lysine in the histone NH₂-terminal tail, stimulating transcription and nucleosome remodeling (Chan & Morey, 2019; Dawson, 2017; Jenuwein & David Allis, 2001). All these proteins, as well as the modifications they regulate, play a crucial role in regulating chromatin structure and accessibility by creating binding sites for chromatin remodelers, histone chaperones, DNA/histone-modifying enzymes, and general transcription factors (Bannister & Kouzarides, 2011). Mutations in epigenetic writers, readers, erasers, and components of chromatin-remodeling complexes are widespread in cancer. Nearly all cancers harbor mutations in at least one of these key chromatin-regulating proteins. Epigenetic modifications are their therapeutic opportunities in cancer are summarized in **Figure I. 7** (Dawson, 2017).

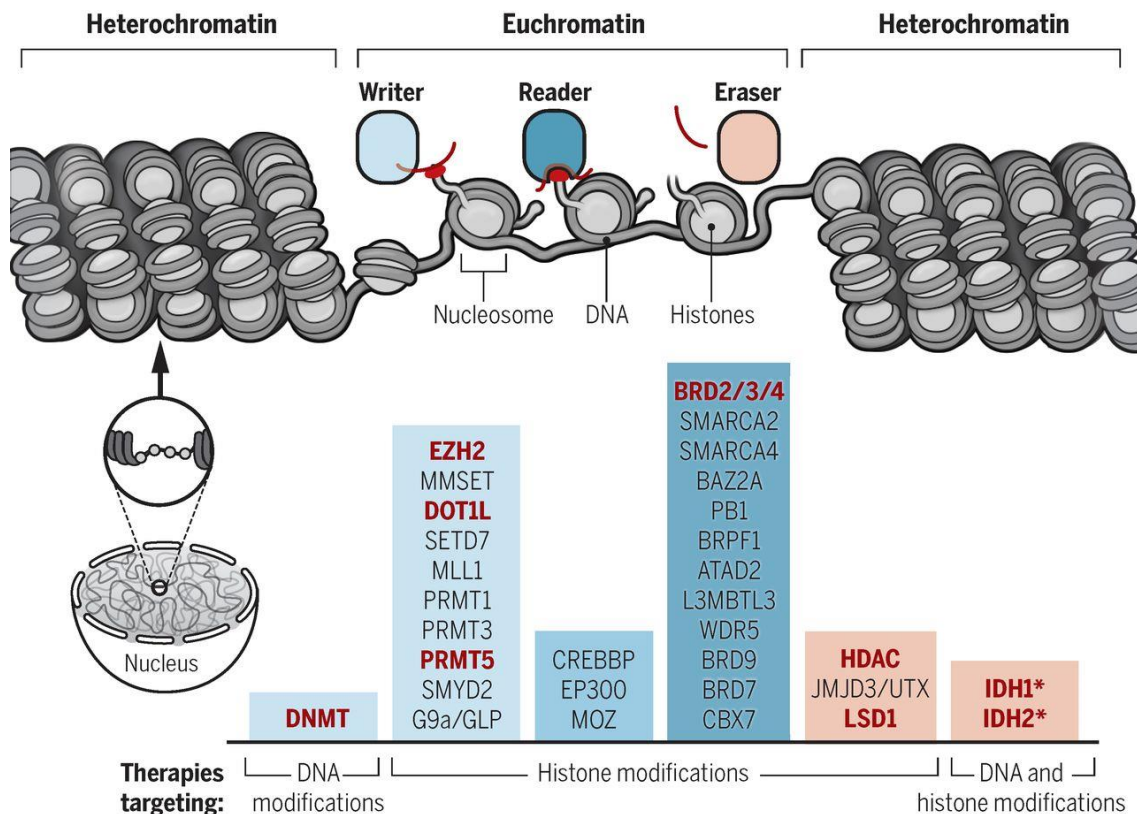


Figure I. 7. Current and emerging epigenetic therapies. Chromatin exists in two major states: an open relaxed conformation called euchromatin, within which most transcriptionally active genes reside, and a more condensed compact state called heterochromatin, which is largely transcriptionally silent. The dynamic transition between these states is mediated by chromatin modifications such as methylation and acetylation, which are laid down by epigenetic writers, bound by epigenetic readers, and removed by epigenetic erasers. Many epigenetic proteins have more than one functional domain, allowing them to function as epigenetic readers and writers or epigenetic readers and erasers. A growing number of small molecule drugs are being developed to target these epigenetic regulators. Highlighted in red are the targets for epigenetic therapies that are either in routine clinical use or currently being evaluated in clinical trials. IDH1 and IDH2 are marked with asterisks because although they are not epigenetic proteins, mutations in these proteins profoundly affect epigenetic erasers of DNA methylation (TET proteins) and histone methylation (Jumonji-C domain proteins). Retrieved from (Dawson, 2017).

3.3.2. Chromatin states

The association between these chromatin marks have been systematically analyzed genome-wide to define 'chromatin states'. In 2015, the NIH Roadmap Epigenomics Consortium generated the largest collection of human epigenomes for primary cells and tissue: this project established global maps of regulatory elements and defined 15 different chromatin states (Kundaje et al., 2015).

As an example, an active chromatin state is associated with acetylated histones that, through the neutralization of their tailed positive charge, decrease the interaction with the negatively charged phosphate groups of DNA. This process of acetylation thus relaxes chromatin, making it accessible to transcriptional machinery. Biologically, the genomic regions linked to a particular chromatin state, together with TFs and RNA polymerase II, can be associated with specific functional elements, such as transcription start sites (TSS), enhancers, active or repressed genes, exons, or heterochromatin. These associations can be deduced from the unique combinations of chromatin marks present in their spatial context and must be decoded for the regulation of gene expression (Ernst & Kellis, 2010; French, 2016). For instance, H3K4me1 alone identifies primed enhancers, while the combination of H3K4me1 and H3K27ac indicates active enhancers. Promoters are distinguished by detectable levels of H3K4me3 along with a high H3K4me3-to-H3K4me1 ratio. Additionally, H3K36me3 modifications and RNA polymerase II ChIP signals are linked to transcribed regions, whereas the presence of H3K27me3 or H3K9me3 is associated with repressive chromatin states (Jiang & Mortazavi, 2018; V. W. Zhou et al., 2011). Poised or bivalent promoters, that have characteristics of both activating and repressive chromatin, are marked by H3K4me3 together with H3K27me3 (Figure I. 8).

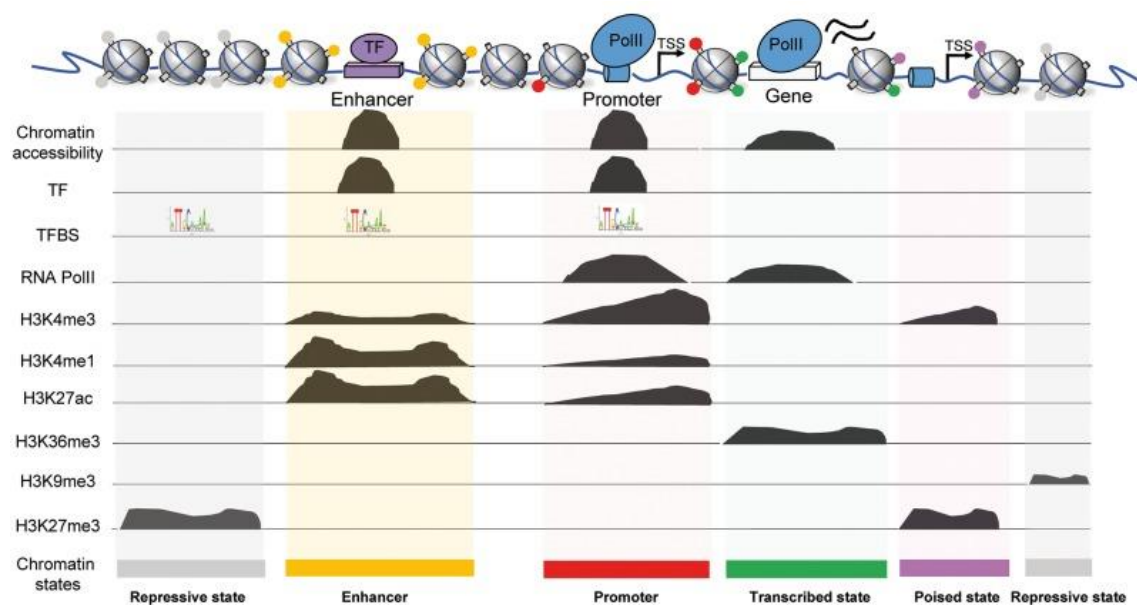


Figure I. 8 Chromatin states are defined by different combinations of histone modifications, TFs and RNA Pol II binding. In this example, a typical repressive state (gray) is characterized by high H3K27me3 heterochromatin facultative signal or H3K9me3 heterochromatin constitutive signal, an enhancer state (yellow) would show a high occupancy ratio of H3K4me1 to H3K4me3 as well as high H3K27ac and the promoter state (red) would show a high occupancy ratio of H3K4me3 to H3K4me1 as well as RNA Pol II binding at the promoter, whereas poised promoter state (magenta) would show the occupancy of H3K4me3 and H3K27me3 bivalent modifications. Actively transcribed region (green) is characterized by enrichment of H3K36me3 with some RNA Pol II binding along the gene body. Retrieved from (Jiang & Mortazavi, 2018).

3.4. Chromatin remodeling in EwS

Considering EwS genetic background, the role of the epigenome is crucial for understanding the molecular mechanisms driving tumor development and for identifying novel targets for new and combinational therapies (Riggi et al., 2021). Epigenetics is essential for creating the right environment for EWSR1-FLI1, in a process similar to cellular reprogramming, before cell fate decisions are made (Suvà et al., 2013). Chromatin regulators (CRs) can make cells more prone to the epigenetic changes needed for transformation, leading to resistance to differentiation, arrest, or apoptosis. Thus, the specific chromatin regulators from the developmental window where the translocation occurs could interact with CRs to modulate DNA accessibility, influencing gene expression and EwS progression.

EWS-ETS proteins localize to the nucleus and bind DNA at multimeric GGAA repeats through the FLI1 C-terminal DNA binding domain. The EWSR1 N-terminal domain functions as a highly potent transcriptional activator significantly stronger than the native FLI1 N-terminal domain. It has been shown that both FLI1 and EWSR1-FLI1 exhibit similar DNA binding affinity and specificity for GGAA motif (Mao et al., 1994); however, EWSR1-FLI1 is a stronger transactivator compared to the wild-type EWSR1 due to the lack of the C-terminal inhibitory domain (Gangwal et al., 2008; K. K. C. Li & Lee, 2000). EWSR1-FLI1's binding may not be restricted to authentic ETS binding sites, as it also binds with high affinity to multimeric repeats of GGAA.

EWS-ETS fusion proteins functions as a transcriptional activator, and repressor, with both functions being critical for oncogenic transformation, of specific sets of target genes and generate a widespread epigenomic reprogramming that, with the right timing and cellular

context, gives rise to the transformed phenotype of Ewing sarcoma (**Figure I. 9**) (Sheffield et al., 2017; Tomazou et al., 2015).

When binding to single GGGA motifs, the displacement of more active wild-type ETS factors by EWSR1-FLI1 is particularly relevant for understanding gene repression. EWSR1-FLI1's ability to block differentiation into mesenchymal lineages may result from the direct repression of enhancers involved in normal development, such as *LOX* and *TGFBR2*, through direct interaction and recruitment of the nucleosome remodeling and deacetylase repressor complex (NuRD), which includes histone deacetylases and histone demethylase LSD1 (Sankar et al., 2013).

As a pioneer factor, the fusion gene binds to DNA acting as a scaffold that through protein-protein interactions, reshapes chromatin structure and rewires the epigenome (Perry et al., 2019). EWSR1-FLI1 multimers bind at GGAA microsatellite repeats and induce chromatin opening. Through the recruitment of the nucleosome remodeling BRG1/BRM-associated factor (BAF) complex, the oncogene establishes de novo superenhancers that interact with promoters to drive expression of key genes for tumorigenesis as *NKX2-2*, *IGF1* or *CCND1* (Boulay et al., 2017; Riggi et al., 2014). These structures, which are the basis of epigenetic deregulation in EwS and other pediatric tumors, are also considered their Achilles heel. De novo super-enhancers function in long-range regulation by the formation of extensive chromatin loops with open chromatin areas depleted of nucleosomes, reduced DNA methylation, and enrichment of activating histone marks such as H3K27ac and H3K4me1 (Boulay et al., 2017; Riggi et al., 2014; Sheffield et al., 2017; Tomazou et al., 2015). 78% of EWSR1-FLI1 binding sites display H3K27ac in EwS cell lines and primary tumors, both harboring highly concordant chromatin states (Riggi et al., 2014). Understanding the epigenetic mechanisms that enable cancer cells to rapidly adapt and exhibit reversibility presents a significant opportunity for developing new cancer treatment strategies (Dawson, 2017).

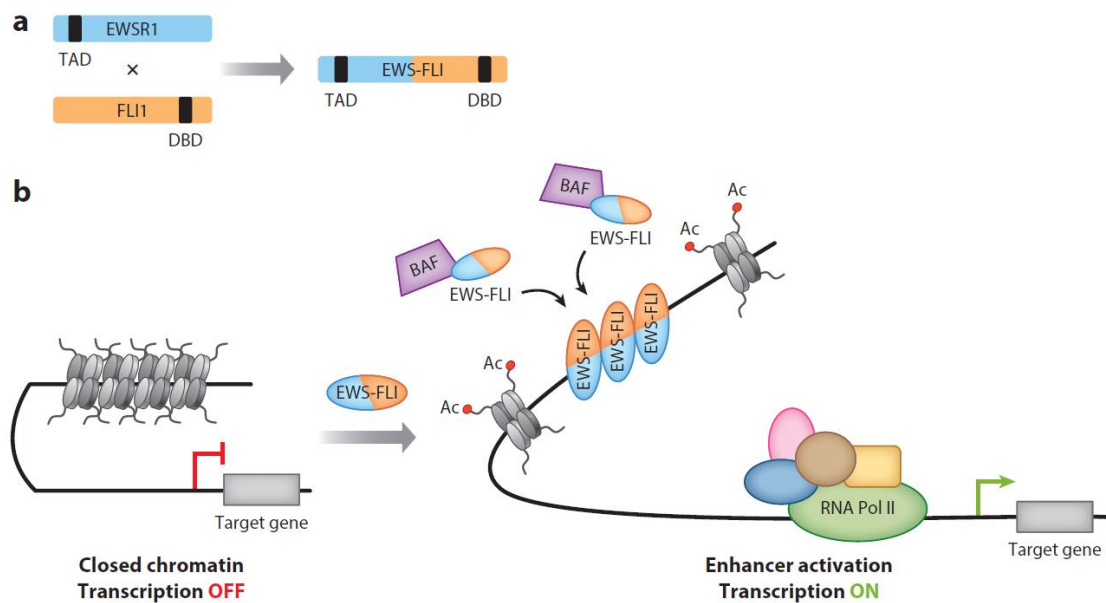


Figure I. 9. The oncogenic function of EWSR1-FLI1 fusion transcription factor in Ewing sarcoma. (A) Generation of the EWSR1-FLI1 fusion transcription factor. The N-terminal portion of EWSR1 containing the transactivation domain (TAD) is fused with the C-terminal portion of FLI1 containing the ETS DNA-binding domain (DBD). (B) The role of EWSR1-FLI1 in transcriptional activation in Ewing sarcoma. In the absence of EWSR1-FLI1, chromatin is in a closed conformation and transcription is turned off at GGAA microsatellites. Once the translocation occurs, EWSR1-FLI1 binds to target genes and recruits the BRG1/BRM-associated factor (BAF) complex to enhancers of its target genes. This EWSR1-FLI1 recruitment leads to chromatin opening, acetylation of H3K27, and enhancer activation, which in turn, results in transcriptional reprogramming responsible of tumorigenesis. Retrieved from (Perry et al., 2019).

3.5. Polycomb group: Role of RING1B in EwS

Polycomb group (PcG) proteins are epigenetic regulators of transcription with crucial roles in stem-cell identity, differentiation and disease. They were identified in *Drosophila melanogaster* more than 40 years ago as repressors of homeotic (Hox) genes during early embryonic development, being a paradigmatic model of epigenetic regulation of gene silencing. The function of PcG is an example of how histone PTMs, discussed in the previous section, are involved in transcriptional regulation.

As previously mentioned, PcG proteins associate into two functional transcriptional repressive complexes (PRC), known as PRC1 and PRC2. These two main complexes are grouped according to their composition and their enzymatic activity on specific histone

residues. PRC1 complexes have the core subunit RING-finger protein 1 (RING1A) or RING1B, which displays an E3 ligase activity that monoubiquitinates histone H2A at lysine 119 (H2Aub). The core of the second complex PRC2 is formed by the proteins Eed, Suz12, and Ezh1 and Ezh2 subunits. EZH2 is overexpressed in EwS and its knockdown inhibits tumor growth and metastasis in vivo (Richter et al., 2009; Riggi et al., 2008). PRC2 deposits the histone H3 lysine 27 di- or trimethyl repressive mark (H3K27me3) through the Ezh1/2 histone methyltransferase enzymes, which directly block gene transcription. PRC1 and PRC2 are structured as collections of varied assemblies, crucial for the complexes' recruitment to chromatin, stabilization, and potential modulation of their activity (Hanot et al., 2023; Morey et al., 2013).

The mechanism by which PRC complexes are recruited to chromatin is intriguing. In *Drosophila*, Polycomb group (PcG) binding regions, known as Polycomb response elements (PREs), were identified as DNA motifs recognized by sequence-specific DNA-binding proteins, such as transcription factors (TFs) (Müller and Kassis, 2006). This led to the proposal of a classical, hierarchical recruitment model in which (i) PRC2 is recruited to these PREs via interactions with TFs, (ii) this recruitment promotes H3K27 methylation, and (iii) the methylation provides a binding site for the PRC1 complex. However, this model does not account for why PRC1-bound regions often lack H3K27me3. An alternative model suggests that context-dependent interactions among TFs, PRC1, and PRC2 are cooperative rather than strictly hierarchical (Aranda et al., 2015; Schuettengruber et al., 2017).

3.6. PRC1 canonical and non-canonical complexes

The core of the PRC1 complex consists of the E3 ubiquitin ligase RNF2 (RING1B) (or its paralog RING1), which catalyzes H2AK119ub1 and forms a dimer with one of six Polycomb group RING finger (PCGF) proteins. Ubiquitination is a post-translational modification of proteins that is required for many cellular processes, including protein degradation by the proteasome, cell cycle progression, transcriptional regulation, DNA repair and signal transduction. It consists on the activation and transfer of ubiquitin to

target proteins in a coordinated three-step enzymatic cascade that involves E1, E2 and E3 enzymes. This PTM occurs at non-histone proteins and all four histones, but H2A and H2B are the best characterized. The PCGF proteins not only enhance the E3 ligase activity of RING1/RNF2 but also determine the selection of auxiliary proteins that are incorporated into a specific PRC1 assembly. PRC1 complexes are generally categorized as either canonical PRC1 (cPRC1) or non-canonical PRC1 (ncPRC1) (**Figure I. 10**). In cPRC1 complexes, alongside RING1/RNF2, PCGF2 or BMI1 (PCGF4) assembles with one of the chromobox proteins (CBX2, CBX4, CBX6, CBX7, or CBX8) that bind H3K27me3, one of the polyhomeotic proteins (PHC1/2/3) crucial for Polycomb-mediated repression, and potentially an SCM protein (SCMH1 or SCML1/2/3). In contrast, ncPRC1 complexes can be assembled around any of the six PCGF proteins and include RYBP or its paralog YAF2, along with various auxiliary proteins, depending on the PCGF subunit, leading to distinct ncPRC1 assemblies such as ncPRC1.1, ncPRC1.2/4, ncPRC1.3/5, and ncPRC1.6 (Figure). A key distinction between cPRC1 and ncPRC1 lies in their chromatin recruitment mechanisms. cPRC1 complexes are recruited to H3K27me3-marked chromatin via recognition by CBX2, 4, 6–7 proteins, while ncPRC1 complexes are recruited independently of H3K27me3, guided instead by their auxiliary subunits (Aranda et al., 2015; Hanot et al., 2023; Morey et al., 2013).

At the molecular level, cPRC1 and ncPRC1 complexes share some target genes but also occupy distinct subsets. cPRC1-CBX is associated with higher levels of H3K27me3 binding compared to ncPRC1-RYBP. Additionally, genes targeted by ncPRC1-RYBP are more highly transcribed, whereas those bound by cPRC1-CBX complexes typically undergo stronger gene silencing (Morey et al., 2013).

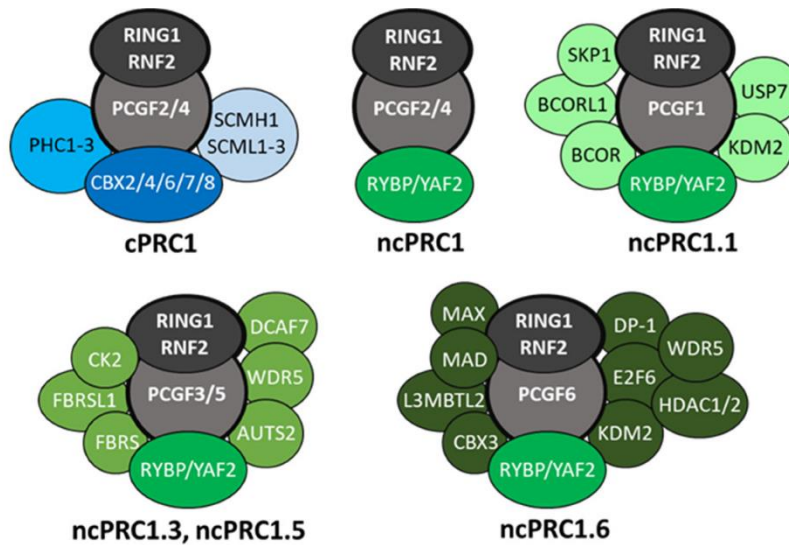


Figure I. 10. PRC1 complexes. The core PRC1 complex is composed of RING1A or RING1B associated with one of the six PCGF proteins. Canonical PRC1 (cPRC1) includes PCGF2 or BMI1 (PCGF4), one of the chromobox proteins (CBX2, CBX4, CBX6-8), a polyhomeotic protein (PHC1/2/3) and eventually an SCM protein (SCMH1 or SCML1/2/3). In contrast, non-canonical PRC1 (ncPRC1) can include all PCGF proteins (PCGF1/2/3/4/5/6) associated with RYBP or YAF2. The identity of the PCGF subunit imposes the nature of the auxiliary proteins present in the assembly, thus defining diverse distinct ncPRC1 complexes. Adapted from (Hanot et al., 2023).

Despite Polycomb activity is primarily linked to gene repression, Polycomb complexes can also associate with active promoters and enhancers, where they play a role in positively regulating gene expression (Chan & Morey, 2019) (**Figure I.11**). Genes bound by RYBP, which boosts PRC1 activity, exhibit lower levels of Ring1B and H2AK119ub, leading to higher transcription compared to those associated with Cbx7 (Morey et al., 2013). Pcf2, also known as Mel18, enhances the expression of crucial mesoderm transcription factors during cardiac differentiation (Morey et al., 2015). The switch from PRC1's role from repressor to activator has been proposed to be related to a change in the subunits conforming the complex (Creppe et al., 2014). For instance, during neuronal differentiation, CBX8-PRC1 complex aids in the activation of genes during differentiation into neuronal cells replacing CBX7-PRC1 repressive transcriptional program in the pluripotent state (Creppe et al., 2014). Additionally, in the central nervous system-derived ML-DmBG3-c2 cells from flies, PRC1 was found to play a dual role in gene regulation through its interaction with the Cohesin complex (Schaaf et al., 2013). In these cells, PRC1

is recruited to both active and repressed genes, with the Cohesin complex aiding in PRC1 recruitment to actively transcribed genes. Notably, depletion of PRC1 led to reduced occupancy and phosphorylation of RNA polymerase II, resulting in diminished transcriptional activity (Pherson et al., 2017).

It was very relevant to our research the role of PRC1 mediating transcriptional activation described by Frangini et al. (Frangini et al., 2013), where authors proposed that RING1B and AURKB bind to several active promoters in quiescent lymphocytes, promoting recruitment of RNAPII and thus activating gene expression. AURKB phosphorylates the USP16 deubiquitinase and the UBE2D3 ligase, which in turn enhance the deubiquitination activity of USP16 and block the E2 ligase activity of UBE2D3. In this way, inhibition of PRC1-mediated H2A ubiquitination allows the PRC1 complex to function as a coactivator.

It is also described that PRC1 facilitates the interaction between the Meis2 promoter and a tissue-specific enhancer, thereby promoting gene activation (Kondo et al., 2014). Last, CK2 colocalizes with RING1B and phosphorylates it at S41 and S168. Due to the phosphorylation of the catalytic subunit of PRC1, RING1B reduces its ubiquitin ligase activity, triggering the recruitment of p300 acetyltransferase and other machinery and leading to gene activation (Gao et al., 2014).

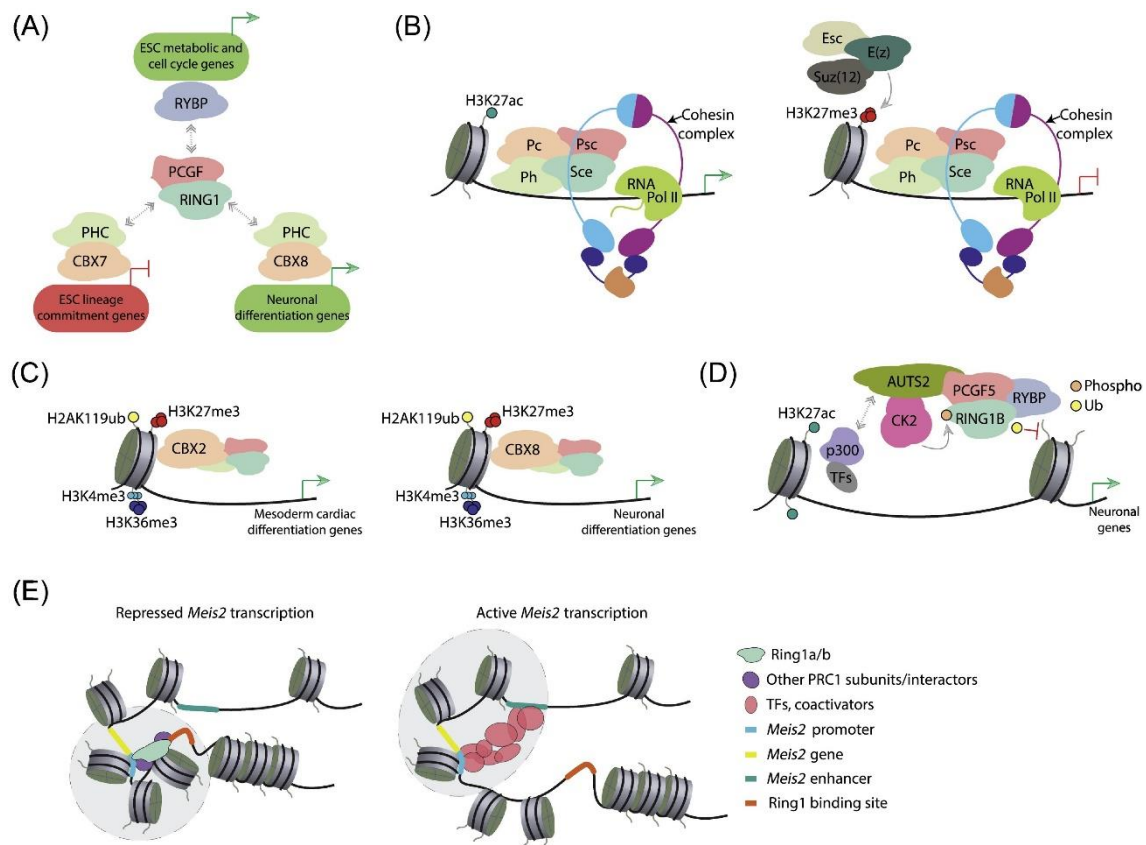


Figure I. 11. Non-classical Functions of PRC1 in Embryonic and Adult Stem Cells. (A) The CBX/RYPB subunit determines the function of PRC1 in ESCs. While CBX7-PRC1 mediates classic repression of lineage commitment genes in the pluripotent state, a CBX8-PRC1 complex activates genes upon neuronal differentiation. Similarly, RYBP-PRC1 activates metabolic and cell cycle genes. (B) In *Drosophila* nerve cells, PRC1 associates with the Cohesin complex and RNA polymerase II both at actively transcribed regions (left) and repressed regions (right). *Drosophila* core PRC1 is composed of four subunits: Polycomb (Pc), Posterior sex combs (Psc), Polyhomeotic (Ph), and Sex combs extra (Sce). *Drosophila* PRC2 contains three components: enhancer of zeste [E(Z)], suppressor of zeste 12 (Suz12), and extra sex combs (Esc). (C) CBX2-PRC1 and CBX8-PRC1 are involved in the activation of cardiac and neuronal differentiation genes, respectively, through its recruitment to genomic regions marked by both activating (H3K4me3, H3K36me3) and repressing histone marks (H3K27me3, H2AK119ub). (D) A PRC1-AUTS2 complex is recruited to neuronal genes in adult mouse brain cells where CK2 phosphorylates RING1B at serine 168 (S168), which inhibits mono-ubiquitination of H2AK119, leading to transcriptional activation. (E) PRC1 activates and represses *Meis2* expression during early midbrain development by modulating chromatin structure. In the repressed state, Ring1a/b binding at the *Meis2* promoter prevents *Meis2* enhancer promoter interaction (left). During midbrain development, Ring1a/b dissociates from the *Meis2* promoter, allowing interaction with the *Meis2* enhancer (right). Abbreviations: AUTS2, Autism susceptibility candidate 2; CK2, casein kinase 2; cPRC, canonical PRC; ESC, embryonic stem cells; ncPRC, non-canonical PRC; PcG, Polycomb-group; PCGF, PcG RING finger domain paralog; PHC, polyhomeotic homologous protein; PRC, Polycomb repressive complex; RYBP, RING1A/B and YY1-binding protein; TF, transcription factor; Ub, ubiquitin. Retrieved from (Chan & Morey, 2019).

RING1B in cancer

RING1B was described to be overexpressed in several cancers (Yan et al., 2021), such as gastrointestinal tumors, colonic tumors and germinal center-derived lymphomas (Sánchez-Beato et al., 2006), hepatocellular carcinoma (Qu & Qu, 2017), breast cancer (Bosch et al., 2014; Chan et al., 2018), melanoma (Rai et al., 2015), cutaneous squamous cell carcinoma (Hernández-Ruiz et al., 2018) or pancreatic cancer (Benitz et al., 2019), mostly associated with tumorigenic process.

According to new data, RING1B activating function supports tumorigenesis in breast cancer and melanoma. In melanoma, RING1B exerts an oncogenic and prometastatic role that is dependent on both transcriptional activation and repressive functions. Mechanistically, E3 ligase activity of RING1B is necessary for the invasive behavior of the tumor through repression of the latent transforming growth factor beta binding protein 2 (LTBP2) promoter. On the other hand, RING1B drives proliferation of melanoma cells through direct transcriptional upregulation of the cell cycle regulator cyclin D2 (CCND2) (Rai et al., 2015). In these cells, MEK-1-mediated RING1B phosphorylation enables recruitment of KDM6A and p300 to activated target genes (Black & Whetstine, 2015). In breast cancer, RING1B is also recruited to active promoters and enhancers at ER α and FOXA1/2 consensus binding sequences independently of H3K27me₃, regulating gene transcription. RING1B depletion has a strong dual role in chromatin accessibility, both closing and opening chromatin at enhancers (Chan et al., 2018; Chan & Morey, 2019). Furthermore, high levels of RING1B in these studies correlate with lower survival of breast cancer patients.

It has been described that EwS tumors have a unique PcG target gene profile, overexpressing several HOX genes, compared to nonmalignant tissues. This is driven by the fusion protein, leading to epigenetic changes that sustain active HOX gene expression, contributing to tumorigenesis (Svoboda et al., 2014). In EwS, our group has shown that RING1B is highly expressed in primary EwS tumors, contributing to EwS development through sodium channel, voltage gated, type VIII, alpha subunit (Nav1.6), and NF- κ B-mediated repression (Hernandez-Muñoz et al., 2016). Further evidence for RING1B being involved in tumorigenesis came also from our group, which recently found

that RING1B co-localizes genome-wide with EWSR1-FLI1 at active enhancers having a key role in EwS tumorigenesis *in vivo*. While retaining its repressive canonical activity, we found RING1B to be necessary for the recruitment of the fusion oncogene to key activated targets including NK2 Homeobox 2 (NKX2-2), SRY-Box 2 (SOX2) or IGF1 (Sánchez-Molina et al., 2020).

3.7. BET proteins

3.7.1. Definition: Histone readers

BET proteins are defined as a family of proteins containing two bromodomains (BRDs) and an extra-terminal (ET) domain. Besides BET proteins, there are several proteins that also contain bromodomain modules such as histone acetyl transferases (HATs), methyltransferases, helicases, ATP-dependent chromatin remodeling complexes, transcriptional coactivators and mediators (Filippakopoulos et al., 2012). Bromodomain containing proteins are classified as histone readers since they have a hydrophobic pocket that recognizes acetylated lysine tails of histones H3 and H4.

The BET protein family consists of BRD2, BRD3, BRD4, and BRDT, this last one limited to the testis. They were first discovered in *Drosophila* and the first mammalian BET protein functionally characterized was BRD2, which has an 80% identical amino acid sequence to BRD4. Both BRD2 and BRD4 were described to be biologically crucial since their deletion of either is embryonic lethal (Houzelstein et al., 2002). BRD4 is characterized by remain bound to chromosomes during mitosis throughout the cell cycle, being essential for transcription of the genes expressed at the end of the mitosis and the beginning of G1. BRD4 binds to active acetylated chromatin localized at promoters and especially at enhancers and superenhancers of active genes, and is brought close to RNAPII, being included in the Mediator complex, that links TFs to RNAPII activation machinery. Therefore, BRD4 acts as a scaffold to recruit other proteins, like the Positive transcriptional elongation factor complex (P-TEFb) and is required for transcriptional elongation (Bhagwat et al., 2016; Moon et al., 2005) (**Figure I. 12**). Besides acetylated

lysines of histones, it is described in AML that BRD4 can be recruited to enhancer and promoter regions and regulate hematopoietic TFs gene transcription (Roe et al., 2015).

The ET domain of BET proteins serves as a protein interaction module that binds a range of chromatin-modifying proteins, including the transcriptional activating methyltransferase, NSD3, as well as chromatin remodeling enzymes such as the member of the SWI/SNF complex, SMARCA4 (French, 2016). BET proteins, that act as scaffolds to recruit other proteins, are participating with the Mediator complex, as important transcription elongation factors (Stathis & Berton, 2018).

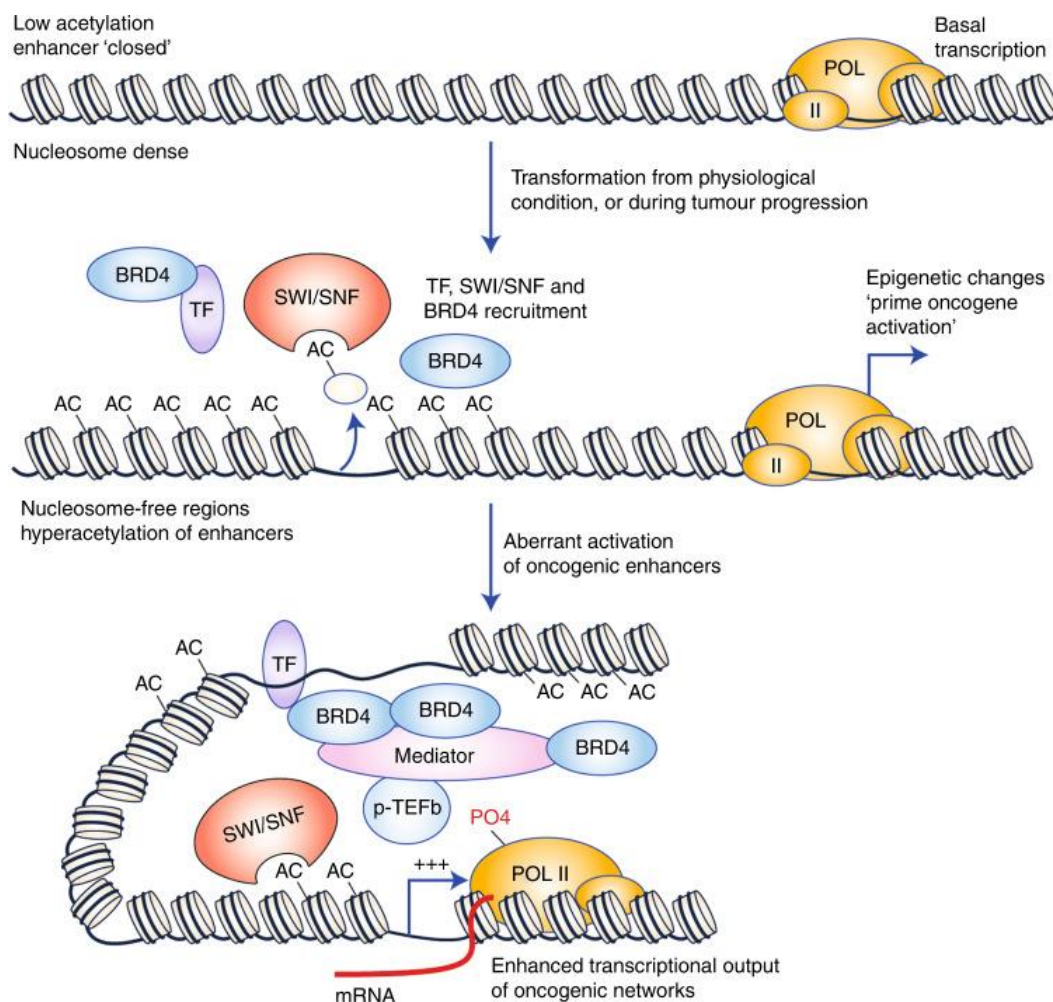


Figure I. 12. Transcriptional activation of oncogenes by BRD4. Under physiological conditions, proliferation and survival genes are transcribed at a basal rate to maintain homeostasis. During the transformation of normal epithelium to neoplasia or, similarly, during the progression of a primary tumor to a more invasive

stage, chromatin surrounding proto-oncogenes becomes enriched for histone acetylation, especially at enhancer regions. This change in chromatin programming allows nucleosome decompaction, which facilitates the recruitment of bromodomain chromatin remodelers, such as the SWI/SNF complex, that further open chromatin to allow the binding of transcription factors (TF). Acetylation also facilitates the recruitment of bromodomain-carrying coactivators, such as BRD4. BRD4 potently activates transcription through the recruitment of the Mediator complex, which connects enhancer elements with the RNA POLII complex at the promoter region of proto-oncogenes. Mediator, through association with CDK9, a component of the p-TEFb elongation complex, phosphorylates RNA POL II on serine 2 of its C-terminal domain, thereby stimulating transcriptional elongation. Retrieved from (Shorstova et al., 2021).

3.7.2. Role in cancer: EwS

Given that histone lysine acetylation has been associated with transcriptional activation, misregulation of histone acetylation could result in aberrant expression of oncogenes and, consequently, initiation and/or maintenance of cancer (Fu et al., 2015). BET-family members influence cell cycle progression by activating oncogenes such as MYC, JUNB, CCND1 and CCNA1. BET proteins may directly activate oncogene transcription through recruitment to hyper-acetylated regulatory regions, as observed in the regions surrounding MYC and CCNA1 genes (Shorstova et al., 2021). BRD4 is the most relevant BET protein in cancer and is involved in multiple tumors like lymphoma, acute leukemia, renal carcinoma, multiple myeloma, prostate, breast and ovarian cancer, neuroblastoma, medulloblastoma and Ewing sarcoma (Fu et al., 2015).

One of the most significant roles of BET proteins is observed in NUT-midline carcinoma, a tumor characterized by rearrangements involving the NUTM1 gene and BRD4. The fusion oncogene BRD4-NUT, driven by the BRD4 promoter, is a key factor in driving cellular transformation. Treatment of tumor cells with BET inhibitors, discussed further in the next section, leads to a genome-wide decrease in BRD4 binding at both enhancers and promoters, resulting in significant downregulation of gene expression. Notably, this downregulation includes key oncogenes associated with super-enhancers, such as MYC, which likely contributes to the antitumor effects of BET inhibitors. Evidence suggests that the reduction in BRD4 binding is more pronounced at super-enhancers, leading to a faster and stronger suppression of their target genes compared to those regulated by standard enhancers (Stathis & Bertoni, 2018).

There are several studies describing the role of BET proteins in Ewing sarcoma. It has been demonstrated that the oncogene EWSR1-FLI1 and EWSR1-ERG depend on BET proteins as mediators of their function. These fusion oncoproteins form a functional complex together with BRD4, MED1 and RNAPII. Disruption of this complex by silencing expression of BRD4 or BET inhibitors, like JQ1, impairs EWSR1-ETS transcription program, as well as EwS cell viability and tumor growth *in vivo* (Gollavilli et al., 2018; Hensel et al., 2016). A separate study on JQ1 in EwS xenografts demonstrated both improved survival and reduced tumor vascularization (Jacques et al., 2016). This anti-angiogenic effect was further supported by additional research showing that JQ1 suppressed the expression of tumor-derived angiogenic factors and directly inhibited endothelial cell function and tube formation (Bid et al., 2016). Another study confirmed that JQ1 treatment reversed the EWSR1-FLI1 transcriptional signature and slowed the growth of Ewing sarcoma xenografts. Similar to the action of HDAC inhibitors, BET inhibitors, like JQ1, was also used to influence key pathways, such as IGF1 (Loganathan et al., 2016).

3.7.3. BET inhibitors

Given the crucial role of BET proteins, particularly BRD4, in cancer, pharmacologic BET inhibition has clear preclinical antitumor activity in a variety of solid tumors and hematologic cancers. BET inhibitors mainly work by displacing BRD4 out of super-enhancer regions that, in cancer, are mainly found in oncogenes and genes related to tumorigenesis (Z. Q. Wang et al., 2023). BET inhibitors are essentially mimetics that specifically bind the acetyl-lysine binding pocket of both bromodomains of all BET proteins with high affinity. In this work, we focus on three inhibitors that encapsulate and represent the evolution of these drugs over the past 10 to 15 years: JQ1, I-BET 151 and BMS-986158.

JQ1: As it has been mentioned in the previous section, one of the first pan-BET inhibitors described was JQ1, which has the highest affinity to BRD4. This molecule interacts with the region that binds BRD4 to the acetylated lysines and displaces this BET protein from nuclear chromatin, inducing differentiation and suppressing tumor development

(Filippakopoulos et al., 2010; Shorstova et al., 2021; Z. Q. Wang et al., 2023). Anti-cancer properties of JQ1 have been widely described in a large number of tumors such as NMC, AML, medulloblastoma, breast and lung cancer and, particularly relevant, Ewing sarcoma. However, next-generation compounds have been developed due to the poor pharmacokinetic profile and low oral bioavailability of JQ1.

I-BET151: I-BET151 induces cell cycle arrest and inhibits the proliferation of tumor cells in various hematological malignancies and solid tumors, including breast cancer, glioma, melanoma, neuroblastoma, and ovarian cancer. Its anticancer effects are associated with its impact on the NF- κ B, Notch, and Hedgehog signaling pathways, as well as its influence on the tumor microenvironment (TME) and telomere elongation (2021 frontiers ibet151). It represented an improvement in the bioavailability and the terminal half-life, compared to other BET inhibitors such as JQ1 (Dawson et al., 2011). Importantly, combination of I-BET151 and the AURKA inhibitor Alisertib improved antitumor efficacy against EwS tumor xenografts (Tomino et al., 2019).

BMS-986158: Due to its novel structure, that implies a higher potency, it has demonstrated potent inhibition of BRD4 and C-MYC expression. This drug was identified and selected for investigation due to its potent preclinical cytotoxic effects against hematologic malignancies and solid tumors, including ovarian cancer, lung squamous cell carcinoma, lung adenocarcinoma, colorectal cancer, and triple-negative breast cancer (Gavai et al., 2021). It is in phase I/II clinical trial in advanced solid tumors and, very remarkably, in 2019 opened the first-in-child study with this kind of inhibitors (Pearson et al., 2021).

4. Aurora Kinases

4.1. Definition

Aurora Kinases (AURKs) are highly conserved serine/threonine protein kinases that coordinate cell cycle progression through G2/M, playing multiple roles in cell division processes as chromosome condensation, kinetochore-microtubule interactions, chromosome orientation, metaphase and correct cytokinesis, as well as maintenance of

genomic stability (Carmena & Earnshaw, 2003). This family of proteins contains three members: Aurora A (AURKA), Aurora B (AURKB), and Aurora C (AURKC). The Aurora gene was identified in *Drosophila melanogaster* in a search for genes that regulate the structure and function of the mitotic spindle (Adams et al., 2001). AURKs are composed of three domains, a N-terminal domain (39-139aa), a kinase domain (250 – 300aa) and a C-terminal domain (15–20aa). The C-terminal catalytic domain is highly conserved among the three kinases and the N-terminal domain (NTD) is variable (**Figure I. 13**). The NTD has been hypothesized to interact with other substrate proteins (Carmena & Earnshaw, 2003). Despite AURKs share a high degree of homology between them in their catalytic domain their functions do not overlap. Because of their implication and aberrant overexpression in many tumor types, causing chromosomal instability and aneuploidy, they have become dedicated targets by pharmaceutical companies. In this work we will focus on AURKB's role in Ewing sarcoma.

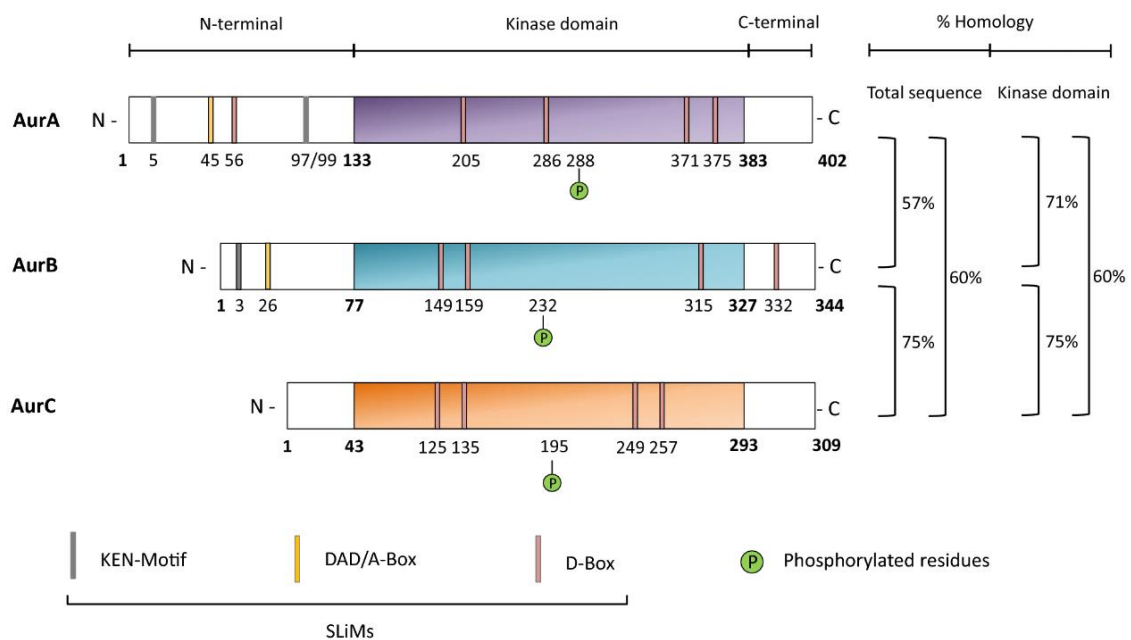


Figure I. 13. Structure of the Aurora kinase domains. Aurora kinases are constituted by (i) an N-terminal domain, believed to control protein localization, (ii) a large and conserved catalytic domain containing the activation T-loop and (iii) a short C-term domain with a D-box (Destruction Box). AURKA and AURKB also contain a KEN motif and an A-box that contributes to APC/C-dependent proteolysis. Numbers represent the residue position. Percentages of homology of the total amino acid sequence are evaluated (i) 57% between AURKA and AURKB, (ii) 75% between AURKB and AURKC and (iii) 60% between AURKA and AURKC. Percentages of homology of the catalytic domain are evaluated at (i) 71% between AURKA and AURKB, (ii)

75% between AURKB and AURKC and (iii) 60% between AURKA and AURKC. Retrieved from (Willems et al., 2018).

Aurora kinase A (AURKA): The *Aurora A* gene maps to chromosomal region 20q13.2, which is frequently amplified in diverse human cancers. AURKA localizes to the duplicated centrosomes that separate during late S/early G2 in mitosis, nearby the spindle poles. It has been implicated in several processes, including centrosome maturation and separation, spindle assembly and metaphase I spindle orientation (Bavetsias & Linardopoulos, 2015; Carmena & Earnshaw, 2003).

Aurora kinase B (AURKB): The *Aurora B* gene maps to human chromosomal region 17q13.1. AURKB initially localizes to the centromeres during the early stages of mitosis. As mitosis progresses and the spindle elongates, AURKB shifts from centromeres to the spindle midzone and the cell cortex. By the final stages of cytokinesis, it concentrates at the midbody (Keen & Taylor, 2004). AURKB was identified in a screening for kinases that were overexpressed in tumors being linked for the first time to carcinogenesis (Bischoff et al., 1998). AURKB expression and activity in proliferating tissues is tightly regulated by the cell cycle, with expression peaking during the G2–M phase transition (Terada et al., 1998). High levels of expression of AURKB in model systems has been linked to chromosome instability (Ota et al., 2002).

AURKB plays a crucial role in managing kinetochore–microtubule interactions in higher eukaryotes. Disruption of its function through RNA interference (RNAi), microinjection of antibodies that blocked its activity, or treatment with small-molecule inhibitors led to chromosome congression (the correct alignment of the chromosomes on the midzone of the mitotic spindle) defects (Ditchfield et al., 2003; Hauf et al., 2003). AURKB is also essential for chromosome condensation, cytokinesis and the activation of the spindle-assembly checkpoint, a circuit that delays mitotic progression until all chromosomes have achieved a proper bipolar orientation on the mitotic spindle (Carmena & Earnshaw, 2003). At the later stages of mitosis, AURKB localizes at the midbody and plays a critical role in the final separation of the two daughter cells.

AURKB are responsible for one of the classic modifications of chromatin in mitosis, the phosphorylation of histone H3 on S10. AURKB is also responsible for histone H3 phosphorylation on S28 from prophase to metaphase. Both H3 S10 and S28 phosphorylation occurred specifically during early mitosis and are required for proper chromosome condensation and segregation in mammals (Goto et al., 1999, 2002).

The chromosomal passenger complex CPC orchestrates critical mitotic events, including chromosome condensation, segregation, and cytokinesis. Aurora kinase B is the catalytic subunit within the CPC, among three additional proteins: the inner centromere protein (INCENP), survivin, and borealin. Disruption in the localization or function of INCENP, survivin, or borealin leads to the mislocalization of CPC components, decreased AURKB activity and impaired cell division. INCENP, survivin, and borealin subunits of CPC are essential for AURKB activation, localization, and function during mitosis (Adams et al., 2001). INCENP plays a dual role by binding to AURKB, activating it, and regulating its spatial localization, while survivin and borealin enhance AURKB activity through phosphorylation (Carmena, Wheelock, et al., 2012). INCENP and survivin are crucial for targeting AURKB to various substrates as the CPC transitions from centromeres to the central spindle. Phosphorylation of INCENP by AURKB further boosts AURKB activity, and survivin is essential for proper localization of both AURKB and INCENP, also contributing to apoptosis regulation.

AURKB activation is a complex process that can also be regulated by PTMs, such as ubiquitylation and sumoylation, that further influence AURKB localization and activity. Checkpoint kinase 1 (Chk1), which monitors DNA damage, and Plk1, which phosphorylates survivin, also influence AURKB activation. This highlights the interconnected roles of these proteins in ensuring proper mitotic progression.

During interphase, AURKB localizes in the nucleus and has another role in cell fate decisions through modulating the epigenetic states of histone H3 and the maintenance of telomeres (Ma & Poon, 2020). Localization of AURKA and AURKB is crucial to understand their functions along the cell cycle (**Figure I. 14**).

Aurora kinase C (AURKC): AURKC expression seems to be restricted to normal testicular tissue and is a chromosome segregation mediator protein with a very similar role to AURKB. It could further promote cancer cell survival and cause centrosome amplification and chromosome segregation defects (Quartuccio & Schindler, 2015). However, AURKC role in meiotic cells and its expression in cancer is an area of ongoing research.

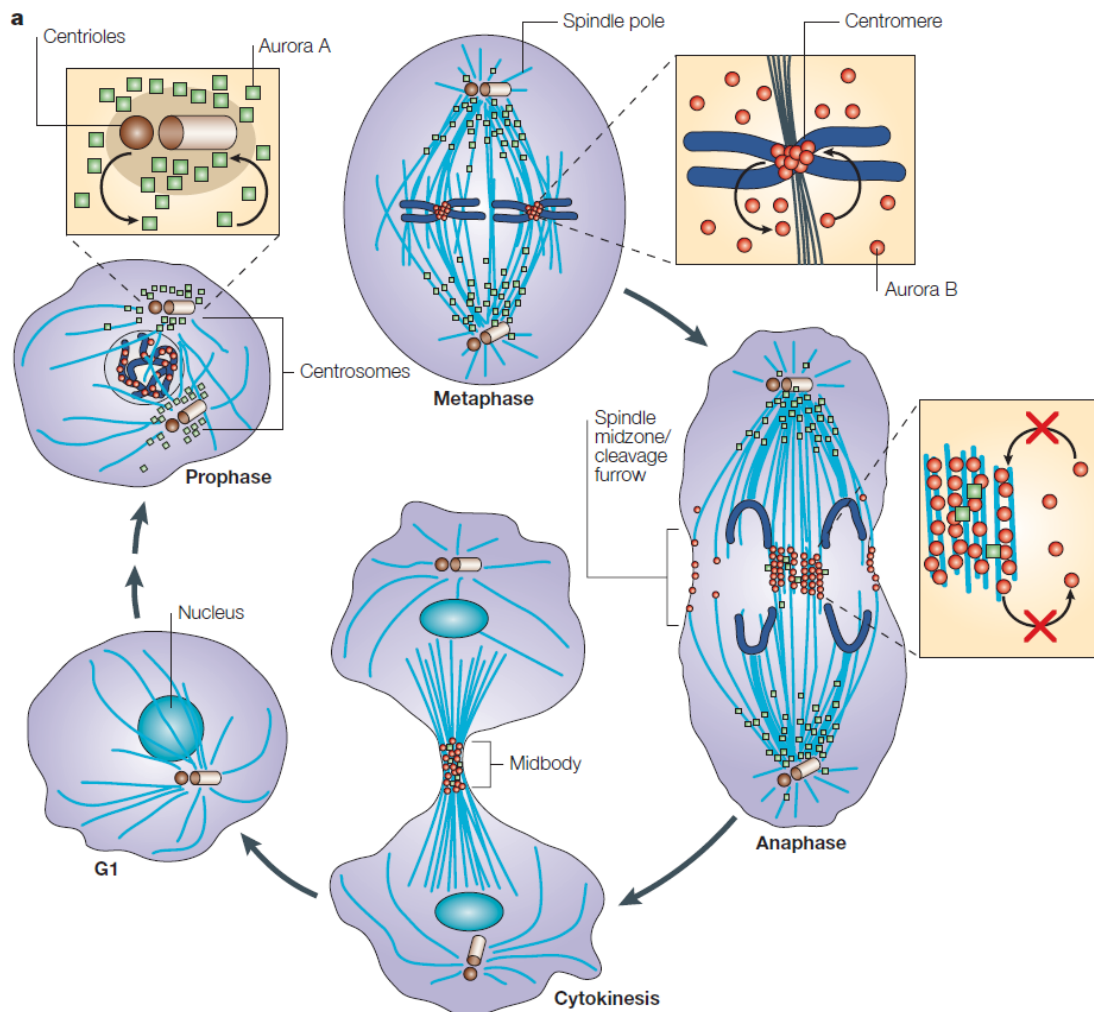


Figure I. 14. Localization of Aurora kinases. The relative localization of AURKA and AURKB in mitotic cells is shown. The level of both kinases is substantially reduced in G1 cells. By prophase, AURKA (green boxes) is concentrated around the centrosomes, whereas AURKB (red circles) is nuclear. In metaphase, AURKA is on the microtubules near the spindle poles, whereas AURKB is located in the inner centromere. In anaphase, most AURKA is on the polar microtubules, but some might also be located in the spindle midzone. AURKB is concentrated in the spindle midzone and at the cell cortex at the site of cleavage-furrow ingression. In cytokinesis, both kinases are concentrated in the midbody. The inset boxes show the results of fluorescence recovery after photobleaching (FRAP) studies, and indicate that AURKA and AURKB are dynamic at centrosomes and centromeres, respectively, but that Aurora B becomes immobile when targeted to the spindle midzone. Retrieved from (Carmena & Earnshaw, 2003).

4.2. Overview of AURKs in cancer

The genes encoding the three human AURKs map to regions that are affected by chromosomal abnormalities in different cancer types, and overexpression of each of the three human Auroras has been detected in several tumor cell lines (Bischoff et al., 1998; Sen et al., 1997; Tatsuka et al., 1998). Considering that AURKs are cell cycle-associated genes, their differential expression in tumor samples, that are highly proliferative, was not a strong indicator of their implication in tumorigenesis. However, alterations to the balance of expression of these genes related them to cancer development through the generation of genomic instability (Gautschi et al., 2008; S. L. Smith et al., 2005). Although AURKs are not directly involved in the tumor initiation, they cooperate with other oncogenes in the tumor maintenance, progression, survival and stemness (Willems et al., 2018).

As a relevant tumorigenic mechanism, AURKA and AURKB are regulators of MYC stability in MYC-driven cancers, where inhibition of either of them led to tumor suppression. Furthermore, they engaged in a positive feedback loop with MYC, in which they stabilize it and MYC oncogenes promote the expression of these proteins. Depletion of these proteins disrupts this circuit, preventing tumor growth and maintenance (Thng et al., 2021).

Aurora kinases are associated with aneuploidy, cell cycle promotion, genomic instability, inhibition of apoptosis, AURKA is amplified or overexpressed in several tumor types including breast, ovarian, pancreatic cancer and gastric tumors (Gritsko et al., 2003; Katayama et al., 1999; Sakakura et al., 2001; Sen et al., 1997; H. Zhou et al., 1998). Moreover, AURKA is a prognostic factor in some of these tumors including breast, colorectal and gastric cancers (Aradottir et al., 2015; Koh et al., 2017; J. Wang et al., 2011). For example, in breast cancer, AURKA binds and phosphorylates the breast cancer-associated gene product, BRCA1, *in vitro* and *in vivo* and regulates its function (Ouchi et al., 2004). There is also a positive association between AURKA overexpression/ gain-of-function and p53 lose-of-function mutations, resulting in centrosome amplification and

setting the stage for chromosomal instability and the propagation of aneuploid cells (Meraldi et al., 2002).

AURKB high levels have been reported in primary non-small cell lung carcinoma (NSCLC), hepatocellular carcinoma, glioma, acute myeloid leukemia, ovarian, cervical, gastric, prostate, oral and breast cancer (Bavetsias & Linardopoulos, 2015; Gupta et al., 2024). High expression of this protein is associated with poor prognosis of NSCLC and hepatocellular carcinoma (Gupta et al., 2024; H. Zhao et al., 2022) and with increased levels of H3S10 phospho in colorectal cancer. Regarding pediatric tumors, AURKB is highly expressed in neuroblastoma, retinoblastoma, osteosarcoma and Ewing sarcoma. AURKB plays a critical role in ensuring that mitotic events initiated by AURKA are properly completed. Essentially, AURKB functions as a checkpoint, arresting cell cycle if AURKA's function is compromised. This prevents errors during mitosis. However, if both AURKBs are inactive, the cell bypasses this checkpoint and progresses through mitosis, indicating that AURKB is responsible for mitotic arrest in the absence of AURKA (H. Yang et al., 2005). This leads to a failure in cytokinesis, resulting in polyploidy, where the cell duplicates its DNA through a process known as DNA endoreduplication.

4.3. Aurora kinase inhibitors and clinical trials

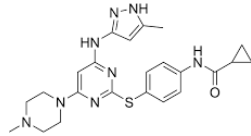
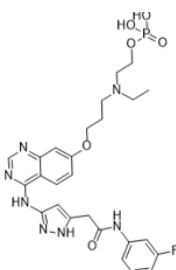
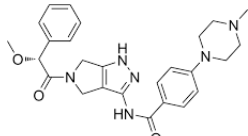
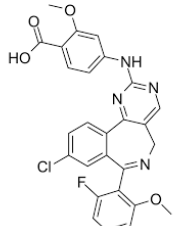
It was not clear from the preclinical data whether aurora kinase inhibitors, if clinically active in humans, could function primarily as cytotoxic (associated with tumor cell death and tumor response) or cytostatic (associated with growth inhibition and disease stabilization) agents (Gautschi et al., 2008). Targeting protein–protein interactions with small molecule inhibitors has been proved to be a great challenge. Selectively targeting the enzymatic activity of kinases with small molecules that can occupy the catalytic ATP (and sometimes the substrate) binding site have much better precedents.

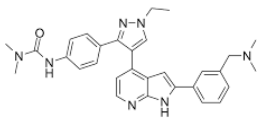
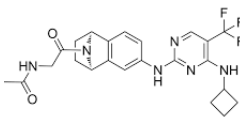
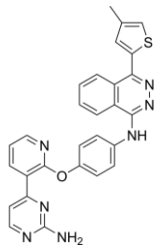
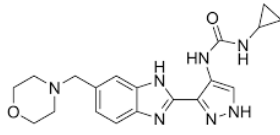
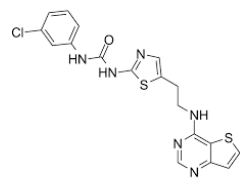
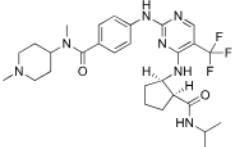
Aurora kinases inhibitors (AKIs) are small molecules that show potential efficacy in cancer treatment due to their cytotoxic activity via induction of apoptosis, polyploidy or cell cycle arrest. AKIs bind to AURKBs through hydrogen bond in the core region and noncovalent interactions (Kollareddy et al., 2012).

Three small molecules —Hesperadin, ZM447439 and VX-680—were the first generation of AKIs identified and evaluated (Ditchfield et al., 2003; Harrington et al., 2004; Hauf et al., 2003). Notably, all of them yield similar cellular effects in assays, particularly by blocking histone H3S10 phospho and disrupting cell division. Interestingly, these compounds did not interfere with the progression of the cell cycle itself. Instead, treated tumor cells proceeded through mitosis with normal timing but subsequently underwent genome re-replication, leading to tetraploidy. This effect distinguished Hesperadin, ZM447439 and VX-680 from traditional antimitotic agents, which typically halt cells in mitosis. Effects caused by AKIs could be directly dependent on the status of p53. In this way, p53 might act as a 'back-up' to the spindle-checkpoint system to prevent the propagation of cells with an abnormal genome. Consistently, p53 is required to prevent endoreduplication in osteosarcoma cells after treatment with the AKI ZM447439 (Ditchfield et al., 2003). These three inhibitors, although effective against both Aurora kinase A and B, mainly caused their effect through AURKB inhibition.

Currently there are about 20 AKIs in different stages of preclinical and clinical development (Bavetsias & Linardopoulos, 2015; Gupta et al., 2024; Jing & Chen, 2021; Pradhan et al., 2021). Nearly 70 clinical trials ranging from Phase I to Phase III have been conducted or are ongoing to evaluate these inhibitors in various cancers. In Table 1 are summarized the most relevant AKIs in clinical trials based on classic and most recent literature. These inhibitors are classified into two types: pan inhibitors, which target all three isoforms (AURKA, AURKB, and AURKC) due to their structural similarities, and selective inhibitors, which specifically inhibit either AURKA or AURKB. Pan inhibitors include compounds such as Tozasertib, Danusertib, AMG 900, SNS-314 and AT9283, while such as MLN8237 for AURKA, and AZD-1152 and BI-811283 for AURKB stand among selective inhibitors. While selective inhibitors may be associated with fewer toxic side effects, they can also be more prone to developing drug resistance. In contrast, pan inhibitors, though potentially more toxic, might be more effective against resistant cancer cells. The most promising agents include Alisertib (AURKA-specific) and AZD1152 (AURKB-specific), both of which have progressed to Phase III trials for advanced solid

tumors. Another potential candidate is PHA-739358, which has shown encouraging results in preclinical studies for multiple myeloma, leukemia and advanced solid malignancies.

Compound	Structure	Aurora inhibition	Activity
VX-680/ MK-0457/ (Tozasertib)		<u>AURKA</u> - 0.6 nM AURKB - 18 nM AURKC - 4.6 nM	Phase I trial - Advanced solid malignancies (NCT02532868) Phase I and phase II trial - Leukemia (NCT00111683)
AZD-1152/ AZD-1152-hQPA/AZD2811 (Barasertib)		AURKB - 3-40 nM	Phase I and II – leukemia (NCT00497991) Phase I, II – leukemia NCT00952588 Phase III – leukemia (NCT00952588)
PHA-739358 (Danusertib)		<u>AURKA</u> - 13 nM AURKB - 79 nM AURKC - 61 nM	Phase II – Multiple myeloma (NCT00872300), leukemia (NCT00335868) and hormone refractory prostate cancer (NCT00766324)
MLN8237 (Alisertib)		<u>AURKA</u> - 1.2 nM AURKB - 396.5 nM	Phase I - Advanced solid malignancies (NCT00249301 and NCT00652158) Phase III trial - Phase III trial - Relapsed/refractory peripheral T-cell lymphoma(NCT01482962)

GSK1070916		AURKA - 490 nM <u>AURKB</u> - 0.38 nM AURKC - 1.5 nM	Phase I trial - Advanced solid malignancies (NCT01118611)
PF-03814735		AURKA - 5 nM <u>AURKB</u> - 0.8 nM	Phase I - advanced solid tumors (NCT00424632)
AMG 900		AURKA - 5 nM AURKB - 4 nM AURKC - 1 nM	Phase I – Acute myeloid leukemia
AT9283		AURKA – 3 nM AURKB – 3 nM AURKC - <10 nM	Phase I – Advanced solid Malignancies and non – Hodgkin's lymphoma (NCT00443976), Leukemia (NCT01431664), refractory solid malignancies (NCT00985868), phase II – multiple myeloma (NCT01145989)
SNS-314		<u>AURKA</u> - 9 nM AURKB - 31 nM AURKC - 3.4 nM	Phase I trials - Advanced solid malignancies (NCT00519662)
BI 811283		AURKB - 9 nM	Phase I - Advance solid malignancies (NCT00701324)

			Phase II trial – Leukemia (NCT00632749)
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Table I.1. Aurora kinase inhibitors in clinical trials.

Inhibition of AURKB activity disrupts proper resolution of kinetochore-microtubule attachments, which prevents correct chromosome alignment and segregation, ultimately halting cell division. This inhibition likely has a direct impact on cytokinesis as well. By avoiding the spindle assembly checkpoint, cells are able to exit mitosis with normal timing, resulting in the formation of tetraploid cells. Since AURKB inhibitors do not block cell cycle progression, these abnormal tetraploid cells continue to proliferate despite the genomic instability, which eventually leads to cell death.

AZD-1152 or **Barasertib** is an AstraZeneca quinazoline prodrug, which is converted in plasma into its active metabolite AZD-1152-HQPA, which in turn has high affinity for AURKB and AURKC. Initially, in preclinical models of melanoma, colon cancer and solid tumors, AZD-1152 significantly inhibited the growth of human tumor xenografts. In the phase I, dose limiting toxicities was grade 3 neutropenia, with few non hematologic toxicities. Pharmacokinetic studies confirmed rapid conversion of AZD-1152 into AZD-1152-HQPA. Prolonged drug administration significantly increased the proapoptotic effect of AZD-1152-HQPA (Gautschi et al., 2008). Then AZD-1152 moved to phase II in solid tumors and leukemia. In preclinical studies *in vitro*, leukemia cells treatment with this drug resulted in accumulation of 4n/82 DNA cells, which underwent apoptotic cell death (J. Yang et al., 2007). In colon cancer, AZD-1152 caused dose-dependent growth inhibition in xenograft models as well as decreased levels of phospho-H3 (ser10), the direct substrate of AURKB (Wilkinson et al., 2007). This mechanism of action was found to be similar for both *in vitro* and *in vivo* conditions. In the phase II studies developed at that time in acute myeloid leukemia, B-cell lymphoma and solid malignancies, these tumors mainly achieved a 25% response rates and stable disease, respectively, with

neutropenia and leukopenia as the most common side effects (Boss et al., 2011; Löwenberg et al., 2011; Schellens et al., 2006).

BI 811283 is an AURKB inhibitor that is currently in a Phase II in the clinics. Treatment of cancer cell lines with BI 811283 resulted in polyploidy and senescence, with a very low apoptotic effect. In NSCLC and colon cancer xenografts tumor growth was inhibited with tumor regression reported in a subset of animals (Gürtler et al., 2010). It was also tested in clinical phase I in advanced and metastatic solid tumors with stable disease achieved for ~30% of patients and side effects of hematotoxicity and neutropenia observed (Mross et al., 2010; Scheulen et al., 2010).

4.4. AURK inhibition in Ewing sarcoma

Wakahara et al. studied for the first time the regulatory mechanisms that control Aurora kinases in Ewing sarcoma. Authors described that protein levels of both AURKA and AURKB decreased upon EWSR1-FLI1 knockdown by small interfering RNA, indicating a regulatory role of the fusion gene on these proteins. In addition, they demonstrated that EWSR1-FLI1 up-regulated the transcription of AURKA and AURKB and that their promoters contain positive regulatory elements required for the oncogene binding and activation. Finally, they define by ChIP assay that EWSR1-FLI1 bound the AURKB and AURKA promoter regions at -71 and -84 bp, respectively, upstream of their TSS. These results confirmed AURKB and AURKA as direct targets of the oncogene concomitant with the high expression of these two kinases in EwS (Wakahara et al., 2008).

In the context of mitosis regulation, EWSR1 wild type is described to recruit AURKB to the midzone during cytokinesis. The disruption of this process by EWSR1-FLI1 then contribute to chromosomal instability and aneuploidy related to EwS tumorigenesis (Park et al., 2014, 2021).

Then, in an integrated kinase inhibitors screening in EwS cell lines, Winter et al. identified tozasertib and danusertib (currently described as pan-AKIs) with specific efficacy at nanomolar concentrations for EwS. Among the target kinases found, they detected AURKA and AURKB as the targets responsible for these drugs sensitivity. They also

in EwS cells. Finally, we hypothesized AURKB as a modulator of RING1B activity in the chromatin remodeling of EwS and so established the basis to develop this project (**Figure I. 16**) (Sánchez-Molina et al., 2020).

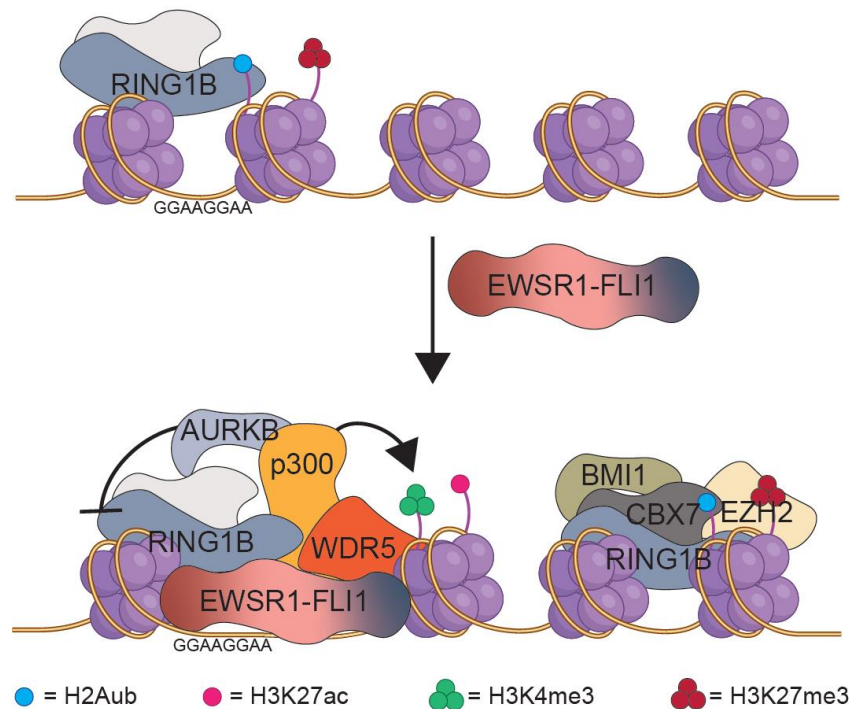


Figure I. 16. Schematic representation illustrating the EWSR1-FLI1 recruitment by RING1B to repressed regions containing GGAA repeats. Once EWSR1-FLI1 has been recruited, additional cooperating factors such as AURKB might inhibit RING1B ubiquitin ligase activity, which, in turn, is able to participate in transcription activation. Adapted from (Sánchez-Molina et al., 2020).

II. HYPOTHESIS AND AIMS

CHAPTER 1

Ewing sarcoma (EwS) is the most common malignant neoplasm in bone and soft tissues among adolescents and young adults in Spain, characterized by a chromosomal translocation between an ETS factor and a member of the FET family of genes. In the 85% of cases, it gives rise to EWSR1-FLI1 fusion protein, which acts as an aberrant transcription factor affecting gene expression (Delattre et al., 1992; Sankar et al., 2013). Although EWSR1-FLI1 is essential for tumorigenesis, it is not solely sufficient for malignant transformation, highlighting the importance of cell specificity and developmental epigenome in its activity (Crompton et al., 2014). EWSR1-FLI1 is capable to induce chromatin opening and create de novo enhancers that interact with its target gene promoters, creating specific enhancer and super-enhancer signatures that are dependent on the oncogene expression (Riggi et al., 2014; Tomazou et al., 2015). Enhancers are *cis*-regulatory elements that drive cell-type-specific gene expression, activating transcription of their target genes at great distances in the genome (Calo & Wysocka, 2013).

Although traditionally repressive, RING1B collaborates with transcription activation at super-enhancers in cancers such as melanoma and breast cancer. Results from our lab demonstrate that RING1B co-localizes with EWSR1-FLI1 at active enhancers, regulating genes critical for EwS tumorigenesis like *NKX2-2*, *SOX-2*, and *IGF-1* (Sánchez-Molina et al., 2020).

Despite the important role of RING1B in the activation of oncogenic enhancers, specific inhibitors have been only described for its repressive E3 ubiquitin ligase activity. Thus, we hypothesize that inhibition of AURKB may represent a novel and specific targeted therapy for RING1B modulation, whose activating role at enhancers we have already demonstrated as necessary for EwS tumorigenesis. As single-agent targeted therapies are rarely curative, and AZD-1152 showed toxicity in previous clinical trials, we propose that combining **AURKB inhibitors** with drugs already proven effective in Ewing sarcoma, such as enhancer disruptors, could enable the use of lower, more effective doses while minimizing side effects. To elaborate on this hypothesis, we will address three aims:

- **Aim 1:** To describe genome wide the distribution of AURKB and its colocalization with RING1B and EWSR1-FLI1 at active enhancers.
- **Aim 2:** To elucidate the mechanisms behind the regulation of RING1B and EWSR1-FLI1 at enhancers by AURKB.
- **Aim 3:** To evaluate the synergistic combination of AZD-1152 and different enhancer disruptors *in vitro* and *in vivo*.

CHAPTER 2

The GEIS-21 conducted in our institution confirmed in a multivariate analysis the importance of age (≥ 18 years cutoff) as an independent predictor of worse outcome, superior to the detection of metastasis (Mora et al., 2017). In the epigenetic context of EwS, characterizing chromatin modifications is crucial to further elucidate the tumorigenesis process of the disease. Using as putative cell of origin mesenchymal stem cells isolated from bone marrow of different age donors (grouped below or above 18 years) we aim to understand how the epigenomic background affects EwS development. We hypothesize that the epigenetic changes produced in mesenchymal stem cells coming from different age donors (adult or pediatric) will be different enough to help us understand the pathogenetic mechanisms underlying age (18 years as cut-off) as prognostic biomarker. However, there is no reported comparison between chromatin states generated by the oncogene in different types of mesenchymal cells. Furthermore, age has not been correlated with the epigenetic background to explain its prognostic significance.

- **Aim 1:** To establish different hMSC models from younger (pediatric) or older (adult) than 18 years olds including healthy donors (hpMSC-HD or hMSC-HD) and EwS patients (hpMSC-P or hMSC-P).
- **Aim 2:** Using ChIP-seq technology, analyze chromatin marks for active enhancers in samples generated from different bone marrow derived hMSC (HD or P).

III. MATERIALS AND METHODS

1. Materials

1.1. Antibodies

Primary antibodies are detailed in **Table III. 1** and were used according to manufacturer's datasheet. Western Blot antibodies were diluted in PBS-Tween 20 0.1% and 0.01% bovine serum albumin (BSA) (Sigma Aldrich) and conserved at -20°C.

Antibody	Application	Work dilution	Reference	Company
RING1B	WB; ChIP; IP	1:500; 1-5 µg	D139-3	MBL
FLI1	WB ChIP	1:1000 1-5 µg	MBS373012 554266	MyBiosource BD Pharmigen
Tubulin	WB	1:30000	T6199	Sigma Aldrich
H2Aub	WB; ChIP	1:2000; 1-5 µg	D27C4	Cell Signaling
Histone H3	WB	1:1000	ab1791	Abcam
Cleaved PARP	WB	1:1000	#5625	Cell Signaling
AURKB	WB; ChIP	1:1000; 1-5 µg	ab2254	Abcam
FLAG	WB; ChIP	1:1000; 1-5 µg	F1804	Sigma Aldrich
H3K4me1	ChIP	1-5 µg	ab8895	Abcam
H3K27ac	ChIP	1-5 µg	ab4729	Abcam
H3K4me3	ChIP	1-5 µg	ab8580	Abcam
H3K27me3	ChIP	1-5 µg	07-449	Milipore
AURKA	WB; ChIP	1:1000; 1-5 µg	A300-071A	Bionova
Vinculin	WB	1:1000	ab18058	Abcam
IgG	ChIP	1-5 µg	C15410206	Diagenode
Phospho-Histone H3 (Ser10)	WB	1:1000	#9701	Cell Signaling
Phospho-RING1B (Ser168)	WB; IP	1:1000; 1-5 µg	ab234421	Abcam
Phospho-Histone H3 (Ser28)	WB; ChIP	1:1000; 1-5 µg	ab5169	Abcam

Table III. 1. Primary antibodies used for Western blot (WB) and antibodies used for chromatin immunoprecipitation (ChIP) and immunoprecipitation (IP) experiments. Table provides detailed information relative to the dilution, use, source and the reference of each antibody.

Fluorescent Western blots were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences). Secondary antibodies used were IRDye 680LT donkey anti-rabbit (925-

68023), IRDye 800CW donkey anti-mouse (926-32212), IRDye 800 CW goat anti-rabbit IgG (926-32211), and IRDye 680RD goat antimouse IgG (926-68070).

1.2. Interference RNA oligonucleotides

Small interfering RNA (siRNA) and short hairpin RNA (shRNA) sequences were used to perform knockdown experiments, detailed in **Table III. 2**.

Primer	Type	Sequence	Company
AURKB seq#1	siRNA	TTGATGACTTTGAGATTGG	Sigma Aldrich
AURKB seq#2	siRNA	GGAGGAGGATCTACTTGATT	Sigma Aldrich
AURKB sh9	shRNA	GAAGAGCTGCACATTTGACGA	Dharmacon
AURKB sh10	shRNA	TGATGGAGAATAGCAGTGGGA	Dharmacon

Table III. 2. Interference RNA sequences. Information about the sequence, source and type of interference RNA used in this work.

1.3. Oligonucleotides sequences

All of the oligonucleotides (primers) were provided by Sigma Aldrich. For gene expression analysis, oligonucleotides were developed by KiCqStart SYBR Green from Sigma Aldrich. Oligonucleotides that were used for ChIP experiments (genomic primers) targeting the promoter or enhancer region of a gene, were designed using the UCSC Primer3 online platform (Rozen & Skaletsky, 1999) using the DNA sequence provided from the Eukaryotic Promoter Database (EPD) (Praz et al., 2002) or selecting the region of interest in the Genome Browser. All oligonucleotides are detailed in **Table II. 3** and validated for their specific roles in molecular assays.

Primer	Forward (5'-3')	Reverse (5'-3')	Type
AURKB	TATGCATGTAGAGGCTCAACAAT	TCTGGAAGTGAGGGAAGCAT	Genomic
CACNB2	GCAAGACTGTAAGCCCCATGG	CTAGGACACATTCAGAATCTGGC	Genomic
CAV1	GGTTCAAGAGTACATGTGCAGG	GGGAGTAGGCTTTGTAGCTGG	Genomic

CAV2	TTTTGCTGACCCATGCTTCTC	GCTCTATACCCCAACATTTTCTGTG	Genomic
CCND1	GAGACAAGGTCCCACTCTGTCTG	GAGACAAGGTCCCACTCTGTCTG	Genomic
COL2A1	CAATGCCTACGCCTCATGT	GCCAATAGATGGCAACACAG	Genomic
DICER	TCTTTTCCCCGATCTGTTGC	TTGCTTCAGCCCAGGATTTTC	Genomic
ENC1	GCTGACACTGGTCCCCAAAG	AGCAGATGCCTTGGGTTTGA	Genomic
FCGRT	GAAAGCAGATGAATGGTTGCCAGG	CTGTCACCTCTATCCGAGTTCC	Genomic
FEZF1	GTGCGGCTAACCTGTTCTACG	GGTCTGGCTCCTGTGTGC	Genomic
HNF1B	GCCTTTCTCTTCCAGGAGGT	CACCGTGTTCCCTTAAGACG	Genomic
IGF1	TCTATTTTCAGCATTGCAGGGG	GGATCCCTGCTGTTTTGGC	Genomic
IGFBP3	CCTGCCTGGATTCCACAG	CTGCATGACGCCTGCAAC	Genomic
JARID2	TGGTTTACATGGGGCCTGTT	TGGATGGCCAGATTTATGTGC	Genomic
LOX	CTGTGTGTAGGTAATTGAGAAATGGG	CCGGCATTTTGAAAAAGAGACAGG	Genomic
NKX2-2	AAGGATGGAACTTGGCCTCA	AAGAGTAGTGGTCTTCCCATCCA	Genomic
NR0B1	GATTCTGTATCAGCTGGTATATACC	GCATCAGGAAGCCTGGATCC	Genomic
SOX2	GAAGTGCACCCTATGCCAGT	CAAGTCCTCTGTGGGGGTTA	Genomic
TAL1	GGACACAACGAAATCAGTCAAAC	GTTCTCAGCAGGGATCTGATTCT	Genomic
VRK1	TGGCTCAGGCCTTTGTCATC	TGAAGACCATTGCCCCATCT	Genomic
AURKB	ATTGGAGTGCTTTGCTATG	TTAGGTCCACCTTGACG	cDNA
BMI-1	CCAGCGGTAACCACCAATCT	CTTAACAGTCTCAGGTATCAACCAGAA	cDNA
CACNB2	TACTCCAGTAAATCAGGAGG	TGATGGAGGTGTTGATTTTC	cDNA
CAV1	CAGGGACATCTCTACACC	TCAAAGTCAATCTTGACCAC	cDNA
CCND1	GCCTCTAAGATGAAGGAGAC	CCATTTGCAGCAGCTC	cDNA
EWSR1- FLI1	CCAAGTCAATATAGCCAACAG	GGCCGTTGCTCTGTATTCTTA	cDNA
EZH2	AAGAAATCTGAGAAGGGACC	CTCTTACTTCATCAGCTCG	cDNA
FCGRT	TATTGGGAGAAAGAGACCAC	GAAATTCATGAACTCCTCGC	cDNA

GAPDH	CTTCAACAGCGACACCCACT	GTGGTCCAGGGTCTTACTC	cDNA
IGF1	CCCAGAAGGAAGTACATTTG	GTTTAACAGGTAACCTCGTGC	cDNA
JARID2	CTCAGGAAAAAGAAGTGGTC	CTCCCTTATAGATGCACTTG	cDNA
KIT	ACAAAACCAGAAATCCTGAC	CAGTTCCTGGACAAAAATACC	cDNA
NKX22	CTTCAGTACTCCCTGCAC	CTTGGAGAAAAGCACTCG	cDNA
NR0B1	CAGTCAGCATGGATGATATG	CACAGCTCTTTATTCTTCCC	cDNA
SOX2	ATAATAACAATCATCGGCGG	AAAAAGAGAGAGGCAAACTG	cDNA
TBP	TTCGGAGAGTTCTGGGATTGTA	TGGACTGTTCTTCACTCTTGGC	cDNA
RPL27	CGTCAATAAGGATGTCTTCAG	GTTCTTGCCTGTCTTGATC	cDNA
TBP	TTCGGAGAGTTCTGGGATTGTA	TGGACTGTTCTTCACTCTTGGC	cDNA
VRK1	AGGTGTACTTGGTAGATTATGG	ATCTTTTGGGGTCTTCTTG	cDNA

Table III. 3. Genomic and cDNA primers used for PCR. A list of the type and direction (forward or reverse) of the sequences of each primer is shown. Type indicates if the primer sequence targets a region of the cDNA or a genomic region, for gene expression or for ChIP techniques, respectively.

2. Cell culture

2.1. Cell lines

The EwS commercial cell lines (A673, TC71, SK-ES-1) and HEK293 cell line immortalized with the SV40 Large T-antigen (293T) were acquired from the American Type Culture Collection (ATCC). Stably transfected HeLa Tet-On cells encoding the EWSR1-FLI1 (HeLa Tet-On EF) were kindly provided by Dr. Daniel J. García-Domínguez (Institute of Biomedicine of Seville (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla/CIBERONC, Sevilla) (García-Domínguez et al., 2020). Stable knockdown of EWSR1-FLI1 in the A673 EwS cell line was kindly provided by Dr. Óscar Martínez Tirado (Institut Català d'Oncologia (ICO), L'Hospitalet de Llobregat, Barcelona) (Tirado et al., 2006). Stable knockdown of AURKB in the A673 EwS cell line was kindly provided by Dr. Brian

D. Crompton (Dana-Farber Cancer Institute, Boston) during the predoctoral stay at his lab.

All cell lines were cultured in RPMI 1640 medium (Gibco®) supplemented with 10% fetal bovine serum (FBS) (HyClone, Lonza), 5 mM of L-glutamine (Gibco®) and 10,000 U/mL of penicillin-streptomycin (Gibco®). They were maintained at 37°C with 5% CO₂ in humidified conditions. When required, cells were split and diluted with Trypsin-EDTA solution (Gibco®). For long term storage, cells cryopreserved in FBS with 10% DMSO (Sigma Aldrich). **Table III. 4** summarizes the molecular characterization of each EwS cell lines that were used.

Cell line	Type of fusion	Origin	Gender	p53 Status
A673	EWSR1-FLI1 (Type I)	Bone	Female	Mutant
TC-71	EWSR1-FLI1 (Type I)	Bone	Male	Mutant
SK-ES-1	EWSR1-FLI1 (Type II)	Bone	Male	Wild-type

Table III. 4. EwS cell lines used. Information about the molecular characteristics of the EwS cell lines used in this work.

2.2. Isolation and characterization of hpMSCs

hpMSCs were extracted from the bone marrow of pediatric patients provided by Hospital Sant Joan de Déu Barcelona following the guidelines and approval of the hospital's Ethics Committee for Clinical Investigation. The isolation and characterization experiments were performed according to previously described protocols (Riggi et al., 2008; Suvà et al., 2009) that were established by Dr. Cinzia Lavarino's group and Dr. Elisabet Figuerola in our institution.

hpMSCs were cultured at low confluence in alpha-MEM medium (Gibco®), 10% NBGS (Fetal newborn calf serum, Gibco®) 100 µg/mL penicillin/streptomycin and 10 ng/mL of recombinant human platelet derived growth factor BB (PDGF-BB) (Stemcell). They were maintained at 37°C with 5% CO₂ in humidified conditions. When required, cells were split

and diluted with Trypsin-EDTA solution (Gibco®). For long-term storage, cells were cryopreserved in FBS with 10% DMSO (Sigma Aldrich).

hpMSCs were characterized based on their capacity to multilineage differentiation into adipocytes, chondrocytes, and osteoblasts (Riggi et al. 2008), according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). To this end, cells were cultured in three differentiation media, including the StemMACS ChondroDiff Media, the StemMACs AdipoDiff Media and the StemMACS OsteoDiff Media (Miltenyi Biotec), respectively. To evaluate the chondrogenic potential, 2.5×10^5 cells were grown in suspension with the differentiation media. After 21 days, a cellular nodule was formed and fixed in formalin, embedded in paraffin and processed according to standard protocols. Finally, Alcian blue with nuclear fast red (BHD Chemicals) was used to stain the nodule and detect positive chondrogenic cells. For the adipogenic culture, cells were fixed at day 21 in 4% PFA and stained with 0.5% Oil Red O solution in isopropanol (Sigma Aldrich). To evaluate the osteogenic capacity cells cultured 10 days in osteogenic media were fixed with a solution of 4% paraformaldehyde (PFA) (Sigma Aldrich) and subsequently stained for 10 minutes at room temperature. The staining solution comprised one tablet of a buffered solution containing 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro blue tetrazolium (NBT), pH 9.5 (Sigma Aldrich). This solution serves as a substrate for alkaline phosphatase, producing a visually observable blue-purple product.

2.3. Pharmacological treatment of cell cultures

Drugs used *in vitro* along this thesis are recapitulated in **Table III. 5**. AZD-1152-HQPA (Astrazeneca) was provided by Sigma-Aldrich, JQ1, I-BET151 and BMS-986158 were purchased from Selleck Chemicals. Depending on the experiment, treatments were evaluated at different times. Protein analysis, IC_{50} determinations and some viability assays were performed after 72 hours of drug incubation. For synergy studies, the treatment duration was 48 hours. Finally, RNA expression analysis, ChIP experiments and phosphorylation changes were evaluated after 24 hours.

Inhibitor	Range of concentration	Target	Vehicle	Company
AZD-1152	0.008-0.4 $\mu\text{mol/L}$	AURKB	DMSO	Sigma Aldrich
JQ1	0.3-30 $\mu\text{mol/L}$	BET proteins	DMSO	Selleck Chemicals
I-BET151	0.3-30 $\mu\text{mol/L}$	BET proteins	DMSO	Selleck Chemicals
BMS-986158	0.3-30 $\mu\text{mol/L}$	BET proteins	DMSO	MedChemExpress

Table III. 5. In vitro inhibitors. Table provides information regarding to the inhibitors used in this thesis with information about the concentration, vehicle, and source.

2.4. Cell viability assays for synergy studies

Cells were seeded in 384-well white flat bottom plates (Corning) at a final density of 1,000 cells in 50 μL of media per well using a robotic dispenser (BioTek). Immediately after plating, media with AZD1152-HQPA at a 7-point, twofold dilution was added robotically by a Bravo Liquid Handler (Agilent) to the plate in combination with media containing BMS-986158 at an 11-point, twofold dilution. This methodology was also followed to perform the subsequent combinations of AZD-1152 with JQ1 and I-BET151. Cells were treated with four replicates of each concentration of each combination. After 48 and 72 hours, 10 μL of Cell-TiterGlo reagent (Promega) were added to each well. Luminescence was read using the FLUOstar Omega microplate reader (BMG Labtech). Dose–response curves for each individual compound were generated from wells in microtiter plates treated with only one compound.

These experiments were performed during a short-term stay at Crompton's Lab in Dana-Farber Cancer Institute (Boston, MA, USA), following the methodology previously established by the group (S. Wang et al., 2019).

2.5. Small interfering RNA transfection

Transient transfection of a specific siRNA (refer to Table II) was carried out in 6-well plates, with each well containing 3.5×10^4 cells. The siRNA-lipid complex was prepared by mixing 25 pmol of siRNA with 10 μL of Lipofectamine RNAiMAX Reagent (Invitrogen) in OPTIMEM media, following the manufacturer's protocol guidelines. Subsequently, the

transfection mixture was added dropwise to the culture media in each well and incubated for 72 hours. After incubation, the cells were harvested for further experimentation. All measurements are per well.

2.6. Genome editing by CRISPR-Cas9: AURKB knock-out

Knockout (KO) of AURKB in A673 cells was achieved following the protocol Gene Knockout Kit v2 (Synthego), using a ribonucleoprotein (RNP) complex comprising purified Cas9 nuclease duplexed with chemically modified synthetic single guide RNA (sgRNA). A multi-sgRNA strategy was employed, where three sgRNA were provided by Bionova (Synthego) to knockout AURKB gene. Sequences were CGAGCAGCGAACAGCCA, TAGAGACGCAGGATGTT and AGAGTATGCCCCCGCG, respectively. Cas9 complexed served as the negative control, and a human scramble (SCR) was used as the positive control. Mock conditions with parental (untransfected) cells were routinely included as an additional negative control.

The sgRNA/Cas9 ratio used for RNP formation was 1.3:1, consisting of 3.9 pmoles sgRNA and 3 pmoles of recombinant Cas9. LipofectamineTM Cas9 Plus reagent was added to the reaction and incubated for 10 minutes to generate Cas9/sgRNA RNP, following manufacturer's protocol. For RNP delivery into cells, LipofectamineTM CRISPRMAX was employed as the transfection reagent at a ratio of 1.5 per reaction. The RNP-transfection solution was mixed with 1×10^5 cells prior to plating and distributed into 24-well plates. After 72 hours, CRISPR-edited cell pools were isolated by cell sorting in. Isolated clones were expanded and cells were collected for subsequent validation by Sanger sequencing of the PCR product. The Inference of CRISPR Edits (ICE) analysis software (Conant et al., 2022) was used to analyze the obtained sequences of edited cells. Knockout clones were then checked by Western blot following standard protocols.

3. Flow cytometry analysis

3.1. Characterization of hpMSCs

In order to fully characterize and verify the phenotype of the potential hpMSCs, they were analyzed by flow cytometry using the Human Mesenchymal Stem Cell Verification Kit (n.

FMC020 R&D Systems). 1×10^5 cells for condition were processed and stained following the manufacturer's protocol. They were added the following positive markers and fluorochromes: CD73-FITC, CD105-PerCP and CD90-APC; and a cocktail of the following negative markers and fluorochrome: CD45-PE, CD34-PE, CD11b-PE, CD79A-PE, and HLA-DR-PE. Markers corresponding to positive and negative isotypes were also added as a control to the samples, as well as DAPI as a marker of cell viability. Cells were acquired in the cytometer FACSCanto II (Becton Dickinson) using the acquisition software FACASDiva, and data obtained were analyzed using FloWJo software v10.7.2. Cytometer facility was kindly provided by Sandra Pont Martí and Dra. Montserrat Torreadell from Hospital Sant Joan de Déu Barcelona. Dra. Alexandra Avgustinova (IRB Barcelona), guided and supervised all the analysis.

3.2. Apoptosis analysis with annexin V staining

Annexin V staining was used to determine the apoptotic population of cells. It is a human anticoagulant with high affinity for phosphatidylserine (PS), an anionic phospholipid. In healthy cells, PS is typically found on the inner surface of the plasma membrane. However, during apoptosis, PS relocates from the inner to the outer layer (extracellular side) of the plasma membrane, a structural change observed in apoptotic cells. This externalization of PS serves as a signal for recognition and subsequent phagocytosis by macrophages. Annexin V and propidium iodide (PI) staining of cells allows to distinguish early apoptotic fraction of the cells (only positive for annexin V) versus late apoptotic fraction (positive for Annexin V and PI). Live cells will be negative for both markers and the necrotic population will show a positive PI staining.

In our experiments, A673, TC71 and 293T cells were treated with 20nM of AZD1152 and 72 h later cell death was measured using the Alexa Fluor 488 fluorophore kit (V13241, Invitrogen™), according to manufacturer's instructions. Cells were collected and, after centrifugation, 100 μ L containing 1×10^6 cells/mL were resuspended in Annexin-binding buffer. These cells were labelled with 5 μ L of Alexa Fluor 488 Annexin V and 1 μ L of PI working solution (100 μ g/mL) and incubated in dark conditions at room temperature. After 15 min, 400 μ L of the buffer was added to the cells and samples were run in Gallios

multicolor flow cytometer (Beckman Coulter) set up with the 3-lasers, 10 colors standard configuration. Fluorescence was measured with an excitation/emission wavelength of 499/521 for annexin V and 535/617 nm for PI. Histograms and cytograms were further analyzed with FlowJo v10.7.2. This service was provided by the Cytometry Unit from Centres Científics i Tecnològics (CCITUB), which also supervised the results.

4. Molecular biology techniques

4.1. RNA extraction

Total RNA was isolated from collected cells using the Rneasy Mini Kit (Qiagen®) and then purified according to manufacturer's protocol. For mice samples coming from the *in vivo* experiment, RNA was extracted starting from 20-30 mg of tumor tissue previously frozen at -80°C. During the lysis, 2-mercaptoethanol was added and the material was homogenized using a tissue grinder. Then, the same kit as for cell samples was employed to continue with the extraction. RNA samples were quantified in the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

4.2. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

A 1 µg aliquot of each purified RNA sample was reverse-transcribed to cDNA in a reaction catalysed by a retrotranscriptase enzyme (M-MLV Reverse Transcriptase M1705 - Promega®) (200 U/µL). Random Primers (C1181 - Promega®) and RNase inhibitor (RNasin(R) Plus RNase Inhibitor N2615 – Promega®) (2500 U/µL) were also added to the reaction. The process includes two steps, a mix containing the RNA, random primers and water was first heated at 85°C during 3 minutes to induce the annealing with the template. Then dNTPs at 10mM, the enzyme and the RNase inhibitors were added to a final volume of 20 µL. The retrotranscriptase reaction was performed in GeneAmp PCR System 9700 Fast Thermal Cycler (Applied Biosystems) following a protocol of 1 hour at 44°C followed by 10 minutes at 92°C to inactivate the enzyme. The cDNA obtained was diluted 1:5 with RNase free water and stored at -80°C until sent to RNAseq or analyzed by qPCR. Expression changes were evaluated by qPCR. Amplification of cDNA with

specific forward and reverse oligonucleotides (**Table III. 3**) was performed using SYBR Green™ PCR Master Mix (4309155 – Applied Biosystems). Each cDNA sample was run in triplicate and its levels were analyzed using QuantStudio 6 Flex (Applied Biosystems™). Relative quantification of qPCR data was analyzed using the comparative 2- $\Delta\Delta$ CT method with an endogenous control as housekeeping gene.

4.3. DNA extraction and amplification by PCR

To proceed with the sequencing of the CRISPR-edited clones, DNA was extracted from the collected cell pellets. Cell lysis buffer (Cat. N° 158908, Qiagen) was added to cell pellets, which were incubated at room temperature for 72 h, and then Protein Precipitation Solution (Cat. N° 158912 Qiagen) was added following manufacturer's instructions. After centrifugation, the supernatant was transferred to a new tube containing isopropanol, and precipitated DNA was obtained by centrifugation and washed with 70% ethanol. After removing ethanol, the DNA pellet was rehydrated with DNA hydration solution (Qiagen). DNA was quantified using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

Sequencing primers of our gene of interest were purchased from Sigma. Purified DNA was amplified starting from a concentration of 10 ng/ μ L. A mix containing MgCl₂ 25mM (Promega), dNTPs 10mM (Applied Biosystems), 3' and 5' primers of interest at 10 μ M and GoTaq DNA Polymerase (Promega) (5U/ μ L) and H₂O was added to 5 μ L of DNA. Reaction was performed following a preheating of 5' at 95°C and then 35 cycles of [15'' 95°C, 15'' annealing temperature (58°C, 60°C or 62°C in standard conditions), 30'' 72°C and 7' 72°C]. Samples can then be run in an agarose gel following standard protocols and/or be sent to sequencing. Subsequently, amplified material was run in 2% agarose gels with SYBR Safe Gel DNA Stain (Thermo Fisher Scientific) at 120V for 20-30 min. Sample (3 μ L) and the 1 kb DNA ladder (Promega) were loaded into the gel with 4 μ L of 5 $\times$ GoTaq green loading dye (Promega). Visualization of the DNA bands in the gel was performed by UV light exposure by using the iBright™ CL1500 Imaging System (Invitrogen).

4.4. Protein extraction

Cultured cells were harvested using either a cell scraper or trypsin-EDTA solution (0.25%) with phenol red (Gibco). After centrifugation, the cell pellet was obtained and either immediately processed or stored at -20°C . Total cellular extracts were prepared from these pellets using RIPA buffer (composed of 10 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 140 mM NaCl) supplemented with phosphatase inhibitors and the EDTA-free Protease Inhibitor Cocktail (Roche). To detect phosphorylated residues of some proteins, lysis was done with Lysis Buffer 6 (Bio-Techne). Cell lysates were incubated on ice for 30 minutes and then centrifuged at 14,000 rpm for 15 minutes at 4°C . Histone extracts from cultured cells were isolated using the EpiQuick Histone Extraction kit (Epigentek) according to the manufacturer's instructions.

To lysate proteins from mouse samples, small pieces of tumor samples were smashed in dry ice with a solution containing 0.5% Igepal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM TRIS-HCl (pH 7.5), and 150 mM NaCl. This homogenization process was carried out using stainless-steel beads (0.2 mm diameter) in a Bullet Blender (Nex Advance), at 12,000 rpm for 2-5 minutes until a uniform mixture was achieved. Supernatant was collected, and samples were lysed by three cycles of cooling in ice for 15 min and vortex. Samples were centrifuged at 14,000 rpm for 15 min at 4°C . All the protein supernatants, either from cell culture or mouse tissue, were collected and quantified using the Bradford assay (Sigma Aldrich) at 575 nm in the Infinite M Nano+ spectrophotometer (Tecan).

4.5. Western blotting

After Bradford quantification, 30–50 μg of protein extracts or 5 μg of histone extracts were combined with 5 $\times$ DTT-PLB sample buffer (containing 125 mM Tris-HCl pH 6.8, 0.2 M DTT, 30% glycerol, 5% SDS, and 0.1% Bromophenol blue) and heated to 95°C in a digital dry block heater to denature the protein structure. Samples were then loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels with varying concentrations of acrylamide (8–15%), depending on the protein samples weight to

detect. Electrophoresis was conducted at 35 mA/gel for approximately 1 hour in running buffer (containing 1% SDS, 25 mM Tris, and 192 mM glycine). The PageRuler Plus Prestained Protein Ladder (Bio-Rad) served as a molecular weight marker.

Subsequently, the gels were transferred onto 0.45 μ m pore nitrocellulose membranes (GE Healthcare Life Sciences) for 1.5-2 hours in transfer buffer (containing 20% methanol, 25 mM Tris, and 192 mM glycine) at 250-300 mA at 4°C. To prevent nonspecific binding, the membranes were blocked by incubating with 3% BSA or 5% skim milk powder (Sigma Aldrich) in PBS for at least 1 hour at room temperature under agitation. The membranes were then washed in PBS with 0.1% Tween 20 (PBST) (Sigma Aldrich) and incubated overnight at 4°C with the primary antibody (**Table III. 1**) under agitation. Following primary antibody hybridization, the membranes were washed in PBST three times for 10 minutes each and then incubated for 1 hour at room temperature with the corresponding secondary antibody, depending on the host species of the secondary antibody. After three additional washes in PBST, the membranes were visualized using the near infrared fluorescence-based imaging system Odyssey CLx (LI-COR Biosciences).

4.6. Protein immunoprecipitation

Protein immunoprecipitation (IP) is a technique utilized to identify physiologically relevant protein-protein interactions. This method involves the use of specific antibodies targeting proteins of interest within a whole cell lysate. By capturing the immunocomplex with high-affinity beads, such as agarose protein A or G beads, all unbound material is discarded. Subsequently, the immunocomplex is denatured, and the antigens are analyzed by SDS-PAGE and Western blotting.

To preserve protein-protein interactions, collected cell pellets are lysated using a soft buffer without SDS, such as IPH buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) supplemented with protease inhibitors (Roche). The cells are then lysated for 30 minutes on ice and centrifuged at 14,000 rpm for 15 minutes. A portion (10%) of the cell lysate is collected as the input fraction and stored at -20°C, while the remaining supernatant is incubated overnight with antibodies targeting the protein of interest. IgG is utilized as the negative control during the immunoprecipitation step. The

next day, samples are incubated with Dynabeads Protein A (Invitrogen) for 2 hours at 4°C in a rotating wheel. The supernatant is discarded, and the beads containing the immunocomplex are washed three times with IPH buffer containing protease inhibitors. Following washing, the beads are mixed with 5× DTT-PLB sample buffer and both input and immunoprecipitated samples are boiled at 95°C for 10 minutes to denature the protein structure and protein binding to the beads. In all immunoprecipitation experiments, protein-protein interactions are assessed using gradient SDS-PAGE 8–16% MINI Protean TGX Precast Gels (Biorad) to ensure detection of proteins with varying molecular weights.

4.7. Chromatin immunoprecipitation followed by qPCR

Chromatin Immunoprecipitation (ChIP) technique enables to identify interactions between DNA and associated proteins such as transcription factors (TFs), coactivators, and histones. This method involves fixation of DNA, then using specific antibodies, isolated DNA fragments are bound to the protein of interest and pulled down. These fragments can then be amplified via quantitative PCR (qPCR), providing a direct snapshot of protein-DNA interactions under specific experimental conditions.

Cultured cells were fixed using 1% methanol-free formaldehyde (Thermo Fisher Scientific) for 10 minutes at room temperature, and crosslinking was ended by adding glycine at 1.5 M. Depending on the cell type, different lysis buffers were employed for cell disruption. For EwS cell lines, lysis was performed using a mild lysis buffer (0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 1% Triton X-100, 1 mM Ethylenediamine tetraacetic acid (EDTA), and 20 mM Tris pH 8) supplemented with 1 mg/mL of protease inhibitors (Roche). For hpMSCs, a stronger lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris pH 8) with 1 mg/mL of protease inhibitors was used. Lysates were sonicated in a Bioruptor Pico (Diagenode) in four rounds of 10 min following cycles of 30s on/off to obtain an average fragment length of 200 bp. After centrifugation, a small portion of the eluted chromatin was quantified using QIAquick PCR Purification kit (Qiagen) and checked for fragment size. Immunoprecipitations were then carried out overnight using specific antibodies (**Table II. 1**), starting with a minimum of 30 µg of chromatin per

condition. Dynabeads Protein A (Invitrogen) were added the following day to capture the antibody-bound chromatin fragments after 2 hours of incubation. Samples were then washed consecutively to remove non-specifically bound material with buffers TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), TSE II (TSE I with 500 mM NaCl), and TSE III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8) and two times with Tris-EDTA buffer. All incubation and washing steps were performed in a rotating wheel at 4°C to avoid protein degradation. The DNA captured by the beads was released using a solution of 1% SDS, 0.1 M NaHCO₃ and Proteinase K (Sigma-Aldrich) in a decrosslinking step that heats the samples at 65°C during 3-5 hours at 1000 rpm of shaking. Genomic DNA fragments were purified using QIAquick PCR Purification kit (Qiagen) and quantified with Qubit dsDNA HS kit (ThermoFisher Scientific).

Quantitative PCR (qPCR) enables the determination of absolute or relative quantities of a known sequence amplified in a PCR reaction. By incorporating a fluorescent reporter, such as SYBR Green, which binds to DNA, the amplified DNA emits fluorescence proportional to its amount. This allows real-time monitoring of the reaction and measurement of DNA synthesis. Differences in DNA content from ChIP assays were assessed using qPCR with the 7500 Real-Time PCR System and SYBR Green master mix (both by Applied Biosystems). qPCR was conducted in 96- or 384-well plates (Thermo Fisher Scientific) using the QuantStudio 6 Flex (Applied Biosystems). Each reaction consisted of 2 µL of DNA from ChIP samples, 5 µL of SYBR Green PCR Master Mix and 0.2 µL each of forward and reverse primer pairs at 10 µM concentration (**Table II.3**), in a final volume of 10 µL per well. Each immunoprecipitation was performed in triplicate, and qPCRs were conducted using fixed amounts of input and immunoprecipitated DNA. The reported data represent qPCR values normalized to input DNAs using the bound/input signal percentage method. In experiments involving multiple conditions, data were calculated using the bound/input signal percentage method, with the corresponding IgG value subtracted from the immunoprecipitated samples.

4.8. Chromatin immunoprecipitation followed by sequencing

The DNA obtained from ChIP was utilized to study genome-wide DNA fragments bound to the protein of interest through sequencing techniques. Library preparation and quality control for the ChIP material were conducted by the Genomics Unit of the Centre for Genomic Regulation (CRG). Libraries were constructed using the NEBNext Ultra DNA Library Prep for Illumina (New England Biolabs) according to the manufacturer's protocol. Briefly, this protocol involved the use of 2–5 ng of input and ChIP DNA for end repair and addition of "A" bases to the 3' ends, followed by adapters ligation and excision. All purification steps were performed using Agen Court AMPure XP beads (Qiagen). Library amplification was carried out via PCR using NEBNext Multiplex oligonucleotides for Illumina (New England Biolabs). Final libraries underwent analysis using Agilent High Sensitivity Chips to estimate quality and assess size distribution. Quantification was performed by qPCR using the KAPA Library Quantification Kit (KapaBiosystems). Subsequently, libraries were amplified using Illumina's cBot and loaded onto the flowcell sequencer for a 1 × 50 run on Illumina's Hiseq 2500 platform.

ChIP-seq data analysis was conducted by Dr. Enrique Blanco from Dr. Luciano Di Croce's group (CRG, Barcelona). ChIP-seq samples were aligned to the hg19 human genome assembly using BowTie, with the option -m 1 to discard reads that could not be uniquely mapped to a single region (Langmead et al., 2009). MACS was then executed with default parameters, but with the shift-size adjusted to 100 bp, to perform peak calling against the corresponding control sample (Zhang et al., 2008). The genomic distribution of each set of peaks was determined by counting the number of peaks overlapping with different genomic regions based on RefSeq annotations. The promoter region was defined as the region within 2.5 Kb upstream and downstream of the transcription start site (TSS), while the remaining part of the gene, excluding the promoter region, was considered as a genic region. Peaks that did not belong to promoter or genic regions were classified as intergenic regions. Peaks overlapping with more than one genomic feature were proportionally counted. The set of target genes was identified by matching ChIP-seq peaks to regions 2.5 Kb upstream of the TSS and extending until the end of the transcripts

as annotated in RefSeq. Screenshots of each group of experiments throughout the thesis were generated using the UCSC genome browser (Kent et al., 2002).

4.9. RNA extraction followed by sequencing

RNA-seq libraries were prepared with 0,5-1 µg of total high quality RNA collected from samples and the Illumina Stranded Total RNA Prep kit (Illumina) according to manufacturer's instructions. Fastq files were analyzed using FastQC software to assess read quality. Adapters were removed and reads were trimmed using Cutadapt software according to per base Phred quality scores and minimum length. Reads were pseudoaligned to GRCh37 using Kallisto. Gene read counts from Kallisto were used to determine differential gene expression using R packages tximport and DESeq2. ERCC spike-ins were used for sample normalization and batch effect was removed using Limma package (Ritchie et al., 2015) . Heatmaps were performed with heatmap2 R package. Reports of functional enrichments of GO and other genomic libraries were generated using the EnrichR tool.

4.10. Proteomics

Three independent biological replicates were performed. For each replicate, SK-ES-1 EwS cell lines from 3 15-cm plates were collected by scraping in PBS plus protease inhibitors (Roche), pooled and centrifuged 5 min at 300 x g at 4°C. Cell pellets were resuspended in 6 ml hypotonic buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl, 15 mM MgCl₂ plus protease and phosphatase inhibitors), incubated 10 min on a rotating wheel at 4°C, and centrifuged 5 min at 700 x g at 4°C. Nuclear pellets were lysed in 2 ml nuclear lysis buffer (150 mM NaCl, 50 mM Hepes pH 7.5, 0.5% NP40, 2.5 mM MgCl₂, benzonase [Novagen, #71205] at a final concentration of 100 units/ml, and protease/phosphatase inhibitors) for 1 h on a rotation wheel at 4°C. Lysates were then clarified by centrifugation (16,000 3 g, 30 min, 4°C), and protein levels were quantified using BCA protein assay (Thermo Scientific). Cell lysates (3 mg per IP) were diluted 1/3 in dilution buffer (150 mM NaCl, 50 mM Hepes pH 7.5, 0.5% NP40, 5 mM EDTA plus protease/phosphatase inhibitors) and incubated with 5 µg RING1B antibody, or rabbit IgG as a negative control, together with 60 µl protein A dynabeads (Invitrogen #10002D), overnight at 4°C on a rotation wheel. After incubation,

beads were washed 3 x with dilution buffer. Beads used in immunoprecipitation were cleaned 33 with 500 ml of 200 mM ammonium bicarbonate, and 60 ml of 6M urea / 200 mM ammonium bicarbonate were added. Samples were then reduced with dithiothreitol (30 nmol, 37 C, 60 min), alkylated in the dark with iodoacetamide (60 nmol, 25 C, 30 min) and diluted to 1 M urea with 200 mM ammonium bicarbonate for trypsin digestion (1 mg, 37C, 8h) (Promega cat # V5113). After digestion, the peptide mixes were acidified with formic acid and desalted with a MicroSpin C18 column (The Nest Group, Inc) prior to LC-MS/MS analysis.

4.11. Immunohistochemistry

Mice tumor samples were characterized by immunohistological detection of anti-human nuclei antigen (1:200, MAB4383; Merck Millipore, Burlington, MA, USA), and counterstained with hematoxylin and eosin. Ki67 and phospho-Ser10 of histone 3 levels were also evaluated on mice samples. Tumor samples were fixed in 10% formalin and embedded in paraffin at Pathological Anatomy service of Hospital Sant Joan de Déu by Sandra Rincón Hoyos. Paraffin block samples were sliced into 2 µm sections using a microtome and heated at 56°C for 45 minutes. Standard protocols were followed for deparaffinization and rehydration using an alcohol series with xylene and ethanol baths. After rinsing with distilled water, antigen retrieval was conducted in a pressure cooker using Epitope Retrieval Solution pH 6 Novocastra (Leica Biosystems) at 100°C for 3 minutes. Following this step, slides were washed with distilled water and endogenous peroxidase activity was blocked with Peroxidase Block Novocastra (Leica Biosystems) for 5 minutes following manufacturer's instructions. Subsequently, slides were washed and incubated with Protein Block Novocastra (Leica Biosystems) for 5 minutes. Primary antibody was then applied at a 1:400 dilution with Bond Primary antibody diluent (Leica Biosystems) and left to incubate overnight at 4°C. The next day, slides were washed with Wash buffer and treated with the Post Primary component of the Polymer Kit (Post Primary Block + Polymer) Novocastra Novolink (Leica Biosystems) for 30 minutes. Following another wash, slides were incubated with the Novolink Polymer component of the kit for 30 minutes. The DAB (Polymer) Kit (Buffer + Chromogen) Novocastra (Leica Biosystems) was applied for 3–5 minutes until tissue staining was visually observed. Slides

were then washed, counterstained with hematoxylin for 20–40 seconds, washed again, and dehydrated using alcohol and xylene. Finally, slides were cover-slipped with dibutylphthalate polystyrene xylene (DPX) and air-dried at room temperature. Negative controls were processed without primary antibody, yielding no signal. IHC images were captured using a Leica DM5500B microscope and digital camera DFC000T (Leica Biosystems) at the Microscopy Unit of HSJD.

5. *In vivo* experiments

The study was conducted according to institutional and European guidelines (EU Directive 2010/63/EU) based on the principles of the 3Rs: replace, reduce, and refine the use of animals for scientific purposes. Animal procedures were approved by the Animal Experimental Ethics Committee (Comité de Ética de Experimentación Animal) at the University of Barcelona.

TC71 EwS cells collected from cell culture were resuspended in a mixture of 1:1 PBS and Matrigel (Corning® Basement Membrane Matrix, Cultek), and 200 μ L of the mixture containing 1×10^6 cells were inoculated into each flank of the mice. When tumor volumes reached 200 mm³, the mice were divided into four groups: a vehicle control group, an AZD1152-treated group (25 mg/kg), a BMS-986159-treated group (1 mg/kg), and an AZD1152 plus BMS-986158-treated group (25 mg/kg AZD1152 and 1 mg/kg BMS-986158). Both AZD1152 and BMS-986158 were diluted in DMSO 10% and vehicle (40% PEG-300, 5% Tween-80 and 45% Phosphate Buffer Saline) and freshly prepared daily before administration. Drug treatments and vehicle were administered to animals for five weeks with daily administration during 5 days and 2 days off each week.

AZD1152 was administered via intraperitoneal whereas an oral gavage was employed for BMS-986158 administration. Mice were weighed and tumors measured with a caliper three times per week during treatment and after treatment. The specific number of animals in each experimental group is indicated in the graphs.

Tumor volume was calculated as follows: (longer measurement x (shorter measurement)²) / 2. Animals were euthanized when the tumor volume exceeded 1000 mm³ or if body

weight loss was more than 20% of the starting weight. To analyze efficacy, percentage of tumor volume regression was calculated as: $((\text{Tumor volume at 10 doses}) - (\text{initial tumor volume})) / (\text{initial tumor volume}) * 100$. The study was concluded based on tumor volume criteria. Survival curves were calculated by Kaplan-Meier Method reporting survival event at 1000 mm³ tumor size. Log-rank statistic with Bonferroni correction test was used to compare statistical significances between treated groups, and performed with GraphPad Prism 10 software.

Defined parameters were Complete Response (CR): volume < 100 mm³ and > 50% reduction; Partial Response (PR): volume $\geq$ 100 mm³ and regression $\geq$ 50%; SD: volume $\leq$ 25% increase or < 50% regression; and PD: volume > 25% increase or < 50% regression.

IV. RESULTS

CHAPTER 1

1. EwS cell lines are sensitive to AURKB inhibition

1.1. AURKB is widely expressed in EwS

Due to the lack of drugs inhibiting the activating function of RING1B, our study aimed to elucidate the regulatory pathways modulating RING1B at active enhancers in Ewing Sarcoma (EwS) (Sánchez-Molina et al., 2020). Previous research has identified AURKB, MEK1, and CK2 kinases as modulators of RING1B's transcriptional activity (Gao et al., 2014; Rai et al., 2015). Consequently, we analyzed the expression levels of these kinases using data from a publicly available database comprising a cohort of 27 EwS tumor samples (Riggi et al., 2014) (accession number GSE7007).

Our analysis revealed that AURKB had the highest relative expression among the three kinases in EwS tumors (**Fig. IV. 1A**). To our knowledge, this is the first time that the expression levels of AURKB, MEK1 and CK2 kinases, which are described to perform important roles in cancer, are compared (Borgo et al., 2021; Caunt et al., 2015; Litchfield, 2003; Ohren et al., 2004). To further evaluate this evidence, we used the Gene Expression Workflow (GEW) platform, developed by Dr. Soledad Gómez, to analyze AURKB expression across multiple databases of pediatric tumors and mesenchymal stem cells. Results obtained showed that the relative expression of AURKB was high in EwS tumors and comparable to its level in the rest of pediatric tumors included in the analysis, those were medulloblastoma (MB), rhabdomyosarcoma (RMS), neuroblastoma (NB), osteosarcoma (OS) and synovial sarcoma (SS) (**Fig. IV. 1B**). Furthermore, we analyzed AURKB expression using the Cancer Dependency Map (DepMap) portal (Tsherniak et al., 2017), specifically the Expression Public 23Q4 database of cancer cell lines. In addition to EwS, we included in the analysis chondrosarcoma (CHS), RMS, NB, OS and SS cell lines. As it is shown in **Figure IV. 1C**, AURKB relative expression was considerably high in EwS cell lines, to a similar extent than the other pediatric cell lines evaluated. These findings align with previous reports of elevated AURKB expression in several cancer cell lines and tumors, including EwS (Riggi et al., 2014) (S. Wang et al., 2019).

Finally, we assessed AURKB protein levels in various pediatric tumor cell lines from our laboratory. Although most cell lines presented high AURKB protein levels, with slightly lower levels observed in osteosarcoma (OS) cell lines (Fig. IV. 1D), the EwS cell lines TC-71 and SK-ES-1 presented the highest levels of this kinase. Overall, our findings corroborate the high expression of AURKB in EwS cell lines and tumors and provide strong basis to study the modulation of AURKB in this disease.

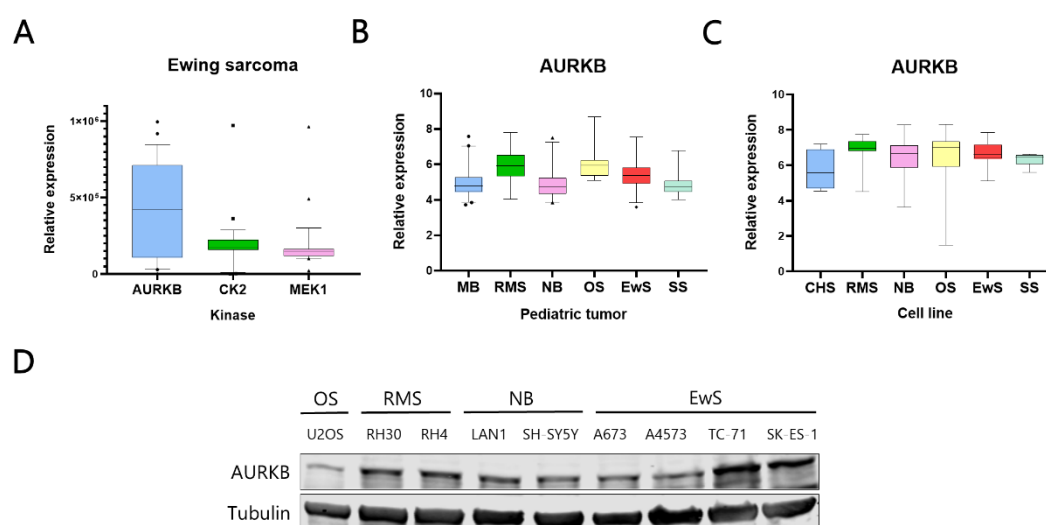


Figure IV. 1. AURKB is widely expressed in EwS. (A) Boxplot showing relative expression for AURKB (probe 209464_at), CK2 (probe 202670_at) and MEK1 (probe 212075_s_at) in a cohort of 27 primary tumors. TPM normalized expression values of the gene across samples is represented on Y axis. TPM counts represent Transcripts Per Kilobase Million. Values were obtained from GEO2R analysis of GSE7007. (B) Boxplot showing AURKB relative expression among several pediatric tumors based on GEW containing public databases. Resource available at https://pccb.shinyapps.io/GEW_V6/. Those tumors were medulloblastoma (MB), rhabdomyosarcoma (RMS), neuroblastoma (NB), osteosarcoma (OS), Ewing sarcoma (EwS) and synovial sarcoma (SS). (C) Boxplot showing AURKB relative expression among several pediatric cell lines based on Expression Public 23Q4 database from DepMap portal. Those cell lines were chondrosarcoma (CHS), RMS, NB, OS, EwS and SS. (D) Western blot showing AURKB levels of A673, TC-71, SK-ES-1 and A4573 EwS cell lines compared to OS, RMS and NB cell lines. Tubulin was used as loading control.

1.2. AURKB is a direct target of EWSR1-FLI1

EWSR1-FLI1 is described to directly regulate the transcription of AURKB promoter (Wakahara et al., 2008). Furthermore, knockdown of EWSR1-FLI1 confers resistance to AURKB inhibition (S. Wang et al., 2019). To study the effects of the oncogene in AURKB expression, we used two models of inducible knockdown and overexpression of EWSR1-

FLI1 in A673 cell line. Firstly, based on a public database of RNA-Seq data (accession number GSE27524) (Bilke et al., 2013), we analyzed AURKB expression levels upon EWSR1-FLI1 knockdown in A673 EwS cell lines using GEO2R. This database includes expression data at different times of doxycycline (dox) induction (18, 36, 53, 72 and 96 hours). As shown in **Figure IV. 2A**, AURKB relative expression decreased when the fusion oncogene is downregulated at every time of dox induction, except for 96 hours. We can observe that the sharpest decrease occurs after 72 hours, which is the standardized time point that we established in our lab. Following the expression data, AURKB protein levels decreased upon induction of EWSR1-FLI1 knockdown, analyzed at 72 hours after dox induction (**Fig. IV. 2B**). In order to confirm direct regulation of AURKB promoter by EWSR1-FLI1 (Wakahara et al., 2008), we performed ChIP-qPCR assay. Upon chromatin immunoprecipitation with two different oncogene antibodies, we observed that with antibody FLI1_66, we detected the EWSR1-FLI1 enrichment at *AURKB* promoter. We analyzed also *IGF1* as a positive control for being a direct target, and *DICER* as the negative control. (**Fig. IV. 2C**). We did not observe any enrichment when immunoprecipitating EWSR1-FLI1 with FLI1_67 antibody. These results demonstrated that AURKB expression is directly regulated by the EWSR1-FLI1.

The second model that we used to evaluate AURKB expression consisted on an ectopic EWSR1-FLI1 overexpression system under the control of a doxycycline-inducible promoter in the permissive HeLa cell line. In this system, the oncogene includes a C-terminal 3xFLAG tag that preserves the activity of the fusion protein (García-Domínguez et al., 2020). First, we analyzed AURKB expression in the HeLa cells before and after EWSR1-FLI1 induction with doxycycline by RT-qPCR. We observed an increase in AURKB relative expression when the oncogene is induced (**Fig. IV. 2D**). Nevertheless, when we evaluated the protein levels for AURKB upon oncogene induction the increase in expression was not translate in higher protein levels as we have expected. As a control, we detected EWSR1-FLI1 through FLAG antibody to confirm the induction after 48 hours of doxycycline incubation (**Fig. IV. 2E**). Last, we confirmed the ability of the ectopically expressed EWSR1-FLI1 to bind the promoter regions of AURKB. To that end, the 3xFLAG-tagged EWSR1-FLI1 in the HeLa system was immunoprecipitated as described in García-

Dominguez et al. After doxycycline induction, we observed that ectopically expressed EWSR1-FLI1 was enriched specifically at AURKB promoter at the same levels of other well described targets as *CAV1* and *EZH2*; with no binding to *DICER* promoter, confirming that the enrichment was specific (**Fig. IV. 2F**). Taken together, our results confirm that AURKB is directly regulated by the activity of EWSR1-FLI1.

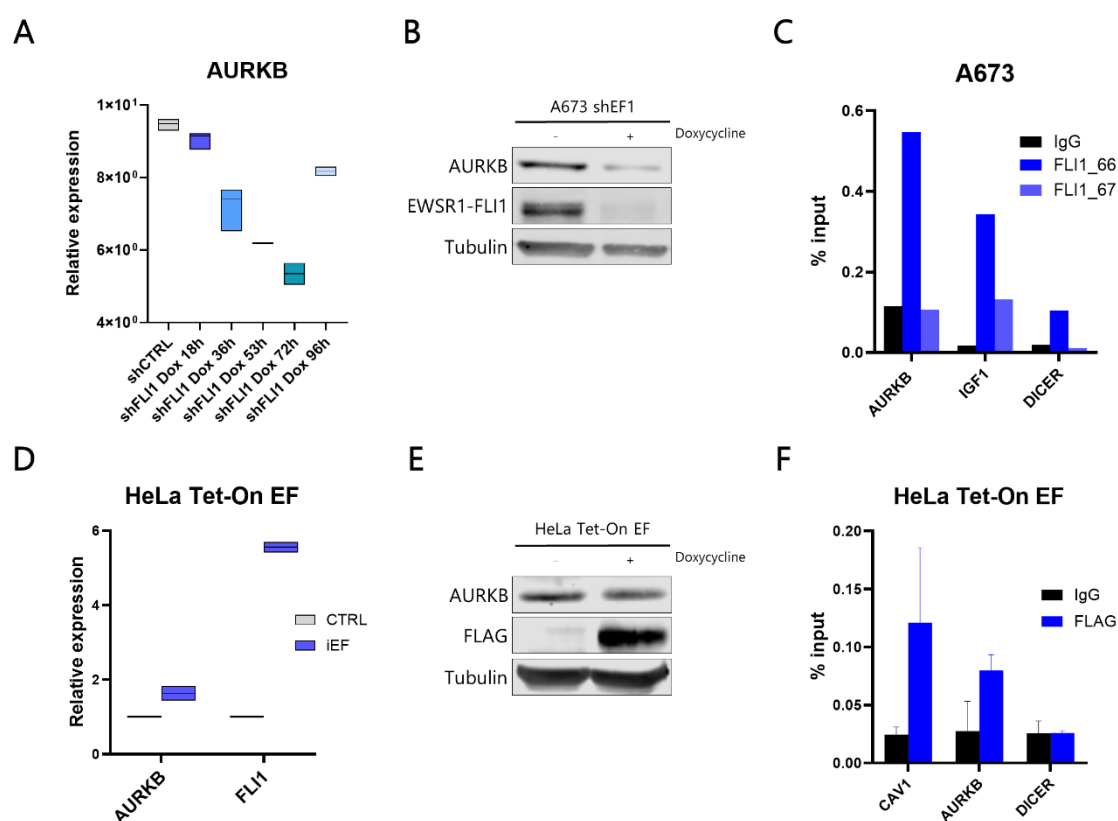


Figure IV. 2. AURKB is a direct target of EWSR1-FLI1. (A) Boxplot showing relative expression for AURKB in control and shFLI1 A673 EwS cell line after 18, 36, 53, 72 and 96 hours of doxycycline induction. Data obtained from GSE27524 (Bilke et al., 2013). (B) Western blot showing AURKB and EWSR1-FLI1 in A673 cells and A673 shFLI1. Doxycycline was added to induce the shFLI1 after 48 hours incubation. Tubulin was used as loading control. (C) FLI1 ChIP-qPCR with antibodies #66 and #67 at some EWSR1-FLI1 promoters in A673 cell line. The values of the Y-axis represent the enrichment ratio of immunoprecipitated samples relative to input. (D) Boxplot showing relative expression for AURKB and FLI1 HeLa Tet-On EF cells induced and non-induced with doxycycline for 48 hours. (E) Western blot showing AURKB and FLAG in HeLa Tet-On EF cells induced and non-induced with doxycycline for 48 hours. Tubulin was used as loading control. (F) 3xFLAG-tagged EWSR1-FLI1 ChIP-qPCR at EWSR1-FLI1 promoters and enhancers in HeLa Tet-On EF cell line after doxycycline induction. The values of the Y-axis represent the enrichment ratio of immunoprecipitated samples relative to input.

1.3. AZD-1152 is specifically effective on EwS cell lines causing cell death and apoptosis

Given the high expression of AURKB in several tumors and its role in tumorigenesis, an important development of targeted inhibitors has been addressed during the past 20 years. AZD-1152 is a specific AURKB inhibitor, with a median inhibitory concentration (IC_{50}) of ~20 nmol/L described in several EwS cell lines screenings (Teicher et al., 2015; S. Wang et al., 2019). To validate the effectiveness of this inhibitor in our EwS cells, we performed cell viability assays to calculate the IC_{50} after 72 hours of drug incubation. The optimal dose range for treating the cells, encompass the nanomolar concentration previously described in the literature. In agreement with the literature, we observed IC_{50} values of 29 nmol/L and 32 nmol/L in SK-ES-1 and TC-71 cells, respectively; in contrast, the IC_{50} for A673 was 1.34 μ mol/L. Importantly, AZD-1152 had no effect on the human embryonic kidney cells stably expressing Ag T from SV40 (HEK293T) that we used to assess EwS specificity against the drug (**Fig. IV. 3A**). Annexin V and Propidium iodide (PI) staining followed by flow cytometry were also performed to verify that AZD-1152 was causing apoptosis in EwS cell lines. We can observe that 20 nmol/L AZD-1152 treatment for 72 hours led EwS cell lines A673 and TC-71 to early and late apoptosis state. Around 13% of A673 cell lines became apoptotic and 21% of them went to necrosis as a consequence of the treatment. A sharper effect was observed in TC-71 cells, with more than 50% of apoptotic population and 11% of necrotic cells after 72 hours incubation with AZD-1152 (**Fig. IV. 3B**). The effect is higher in TC-71 cell line, which correlates with the lower IC_{50} previously shown. Finally, analysis of cleaved PARP levels further demonstrated that AZD-1152 stimulated apoptotic pathways in EwS cell lines at 10 and 20 nmol/L after 72 hours incubation, with TC-71 showing also higher protein levels for this apoptotic marker (**Fig. IV. 3C**).

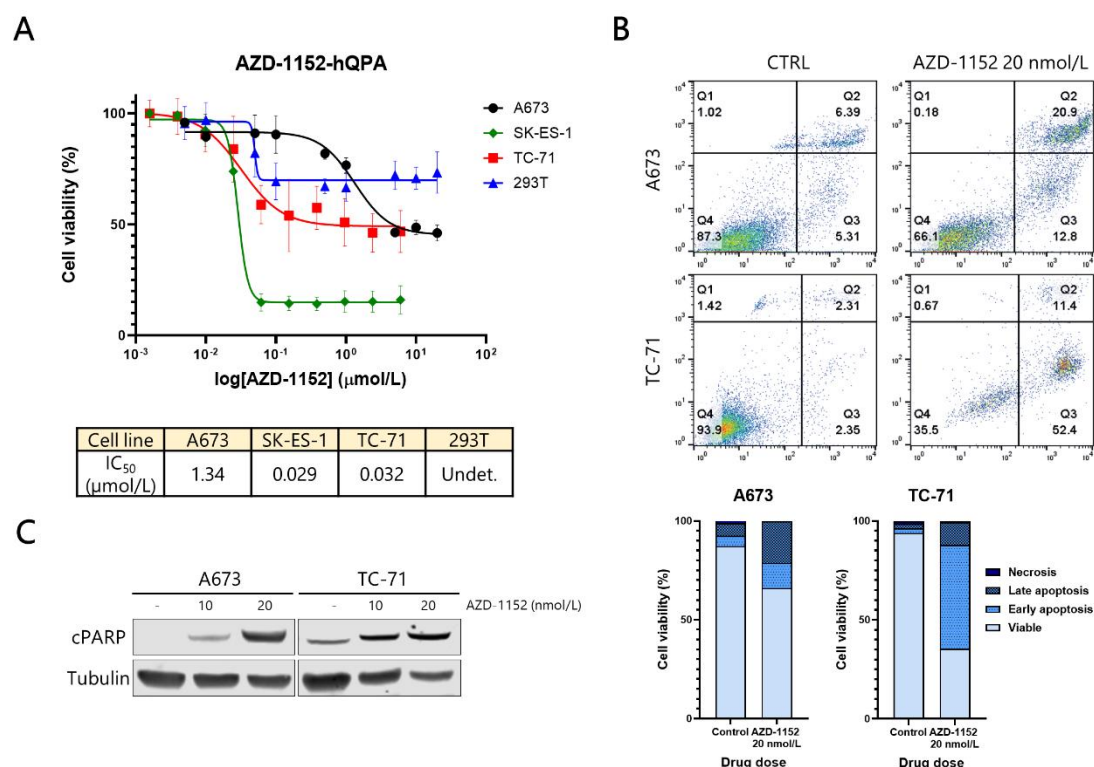


Figure IV. 3. AZD-1152 is specifically effective on EwS cell lines causing cell death and apoptosis. (A) Treatment dose-response curves of A673, SK-ES-1, TC-71 EwS cell lines and 293T, with different concentrations of the AURKB-specific inhibitor AZD-1152. % of cell viability normalized to the average viability of the vehicle (DMSO) treated cells is represented in the Y axis. Log₁₀ of AZD-1152 concentration expressed in μmol/L is shown in the X axis. Plotted is the mean normalized viability of three replicates ± the SD. (B) Dot-plots showing annexin V staining in A673 and TC-71 cell lines after 72 hours treatment with AZD-1152 (20 nmol/L). Bar graphs representing the different cell population percentages are shown below. (C) Western blot analysis of cleaved poly(ADP-ribose) polymerase (cPARP) after treatment with 10 or 20 nmol/L AZD-1152, in the A673 and TC-71 cell line. Tubulin was used as loading control.

2. Study of Aurora Kinase B distribution genome-wide

2.1. AURKB is enriched at active enhancers in A673 EwS cell line

AURKB and RING1B have been described to maintain the transcriptional activity at the TSSs of highly transcribed promoters in quiescent lymphocytes. AURKB phosphorylates histone H3S28 at active promoters and inhibits RING1B-mediated ubiquitination of histone H2A by enhancement of USP16 deubiquitinase activity, maintaining transcriptional activity (Frangini et al., 2013). Given the high levels of AURKB in comparison with other kinases that regulate RING1B activating role, we hypothesized

that AURKB is contributing to RING1B activating role at oncogenic enhancers, together with EWSR1-FLI1, a functionality that has never been proposed before. To evaluate this hypothesis, we analyzed genome-wide the distribution of AURKB, EWSR1-FLI1 and RING1B by chromatin immunoprecipitation followed by sequencing (ChIP-seq) in the A673 EwS cell line. Both EWSR1-FLI1 and RING1B ChIP-seq data were obtained from previous data from our lab, as described in Sánchez Molina et al. (Sánchez-Molina et al., 2020) Using differential binding analysis (DiffBind), which allows for the identification of statistically significant common peaks between replicates, we found 1653 AURKB peaks ($P < 0.05$, **Fig. IV. 4A**). Genomic distribution of peaks show that AURKB is more abundant in intergenic and intragenic regions than in promoters, supporting its enrichment at enhancers. (**Fig. IV. 4B**). Promoter is the region between 2.5 kb upstream and 2.5 kb downstream of the transcription start site (TSS). Genic regions correspond to the rest of the gene (the part that is not classified as promoter) and the rest of the genome is considered intergenic.

To further evaluate the possible contribution of AURKB at RING1B and EWSR1-FLI1 enhancers, we then categorized peaks for RING1B, AURKB and EWSR1-FLI1 in different regulatory elements following their histone marks. The classification of enhancers and promoters followed the criteria established by Blanco et al. (Blanco et al., 2020). We defined five classes of regulatory elements: active enhancers (H3K27ac), active promoters (H3K27ac + H3K4me3), poised enhancers (H3K27me3), primed enhancers (H3K4me1 without H3K27ac), and bivalent promoters (H3K27me3 + H3K4me3). To compile our collection of enhancers and promoters, we reanalyzed published ChIP-seq data for H3K4me1, H3K27ac, H3K27me3, and H3K4me3 in A673 cells (Riggi et al., 2014) (**Fig. IV. 4C**, left). We found that a significant fraction of AURKB peaks were located at transcriptionally active enhancers (60%) and primed enhancers (15%) in the A673 cell line (**Fig. IV. 4C**, right). Similarly, 40% of EWSR1-FLI1 peaks are located at these active enhancers. On the other hand, besides the enrichment at transcriptionally repressed regulatory regions, as expected, an important fraction of RING1B peaks are located at active promoters and enhancers, as previously describe by our group (Sánchez-Molina et al., 2020). (**Fig. IV. 4C**, right).

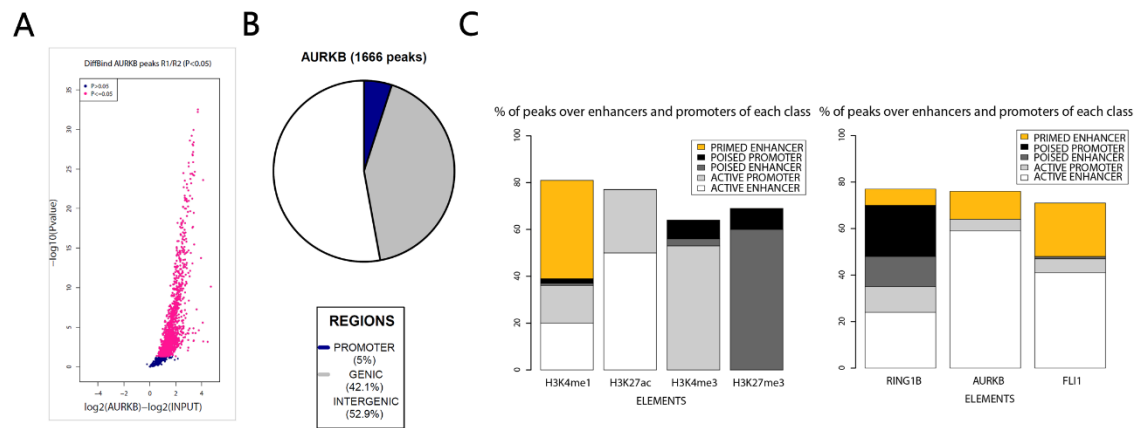


Figure IV. 4. AURKB is enriched at active enhancers in A673 EwS cell line. (A) Volcano plots of the 1,653 AURKB significant peaks identified by DiffBind (P value < 0.05) introducing two biological replicates. The X-axis represents the difference in the number of reads at each peak between ChIPs and the corresponding inputs. The Y-axis represents the significance of the peaks ($-\log P$ value). (B) Pie chart showing genomic distribution of AURKB peaks relative to functional categories including promoter (± 2.5 kb from TSS), gene body (intragenic region not overlapping with promoter), and intergenic (rest of the genome). (C) Boxplot depicting percentage of regulatory elements (active/poised enhancers and promoters and primed enhancers) in each group of peaks.

To confirm the association of AURKB peaks with active regions in the genome, we analyze the ChIP-seq signal of the epigenetic marks H3K27ac and H3K27me3 at AURKB, RING1B and EWSR1-FLI1 enriched regions (Fig. IV. 5A). H3K27ac exhibited the highest signal at AURKB peaks, reinforcing the notion that AURKB is positioned at active enhancers and supporting its role in the regulation of enhancers in EwS. Conversely, the repressive mark H3K27me3 was only enriched at RING1B peaks, consistent with our previous observations that RING1B retains its repressive function besides its activator role in EwS cell lines (Sánchez-Molina et al., 2020). This differential enrichment of histone marks highlights that besides both proteins have activating roles at promoters and enhancers within EwS, RING1B also maintains its transcriptional repressive role. Furthermore, AURKB, RING1B and EWSR1-FLI1 analysis of peak intensity confirms the enrichment of each protein in the other groups of peaks (Fig. IV. 5B), suggesting their colocalization genome-wide.

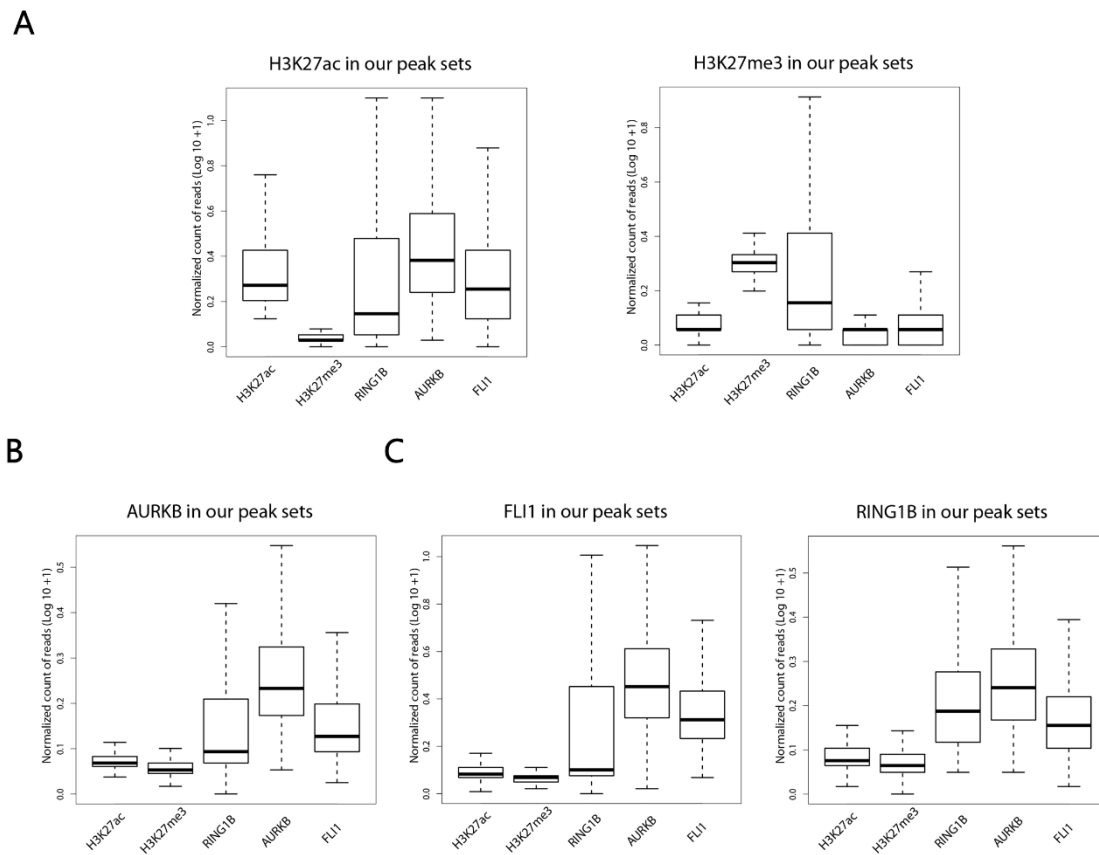


Figure IV. 5. AURKB is localized genome-wide at active enhancers. (A) Boxplot showing the average ChIPseq signal of H3K27ac and H3K27me3 at H3K27ac, H3K27me3, RING1B, FLI1 and AURKB peaks. (B) Boxplot showing the average ChIP-seq signal of AURKB at H3K27ac, H3K27me3, RING1B, AURKB and FLI1 peaks. (C) Boxplot showing the average ChIP-seq signal of FLI1 and RING1B at H3K27ac, H3K27me3, RING1B, AURKB and FLI1 peaks.

Due to the high percentage of AURKB at active enhancers associated with H3K27ac mark, we evaluated the correlation of these regions with associated gene expression levels. AURKB peaks were associated to 603 genes (2500 upstream of the TSS) and their expression using public databases was analyzed (Riggi et al., 2014). We compared these genes' expression levels with those associated with FLI1, RING1B, H3K27ac, and H3K27me3 peaks. The average expression of genes at AURKB-associated peaks in the A673 cell line was the highest compared to those at FLI1 or RING1B peaks and was similar to those associated with H3K27ac (Fig. IV. 6A). We further analyzed the biological functions and pathways linked to the AURKB-associated gene set using the EnrichR tool (E. Y. Chen et al., 2013; Kuleshov et al., 2016). The 'TF perturbations followed by expression' analysis revealed that the AURKB gene set was downregulated upon FLI1

knockdown, based on the array available with accession number GSE27524 (Bilke et al., 2013), indicating that these genes are activated by EWSR1-FLI1 (Fig. IV. 6B, left). Gene Ontology functional analysis, specifically 'GO Biological Process 2023', related the AURKB gene set with neural processes, suggesting a potential link to EwS neural activities (Fig. IV. 6B, right). Together with previous results, our data suggest that AURKB collaborates with RING1B at EWSR1-FLI1 target genes and contributes to enhancer regulation in EwS.

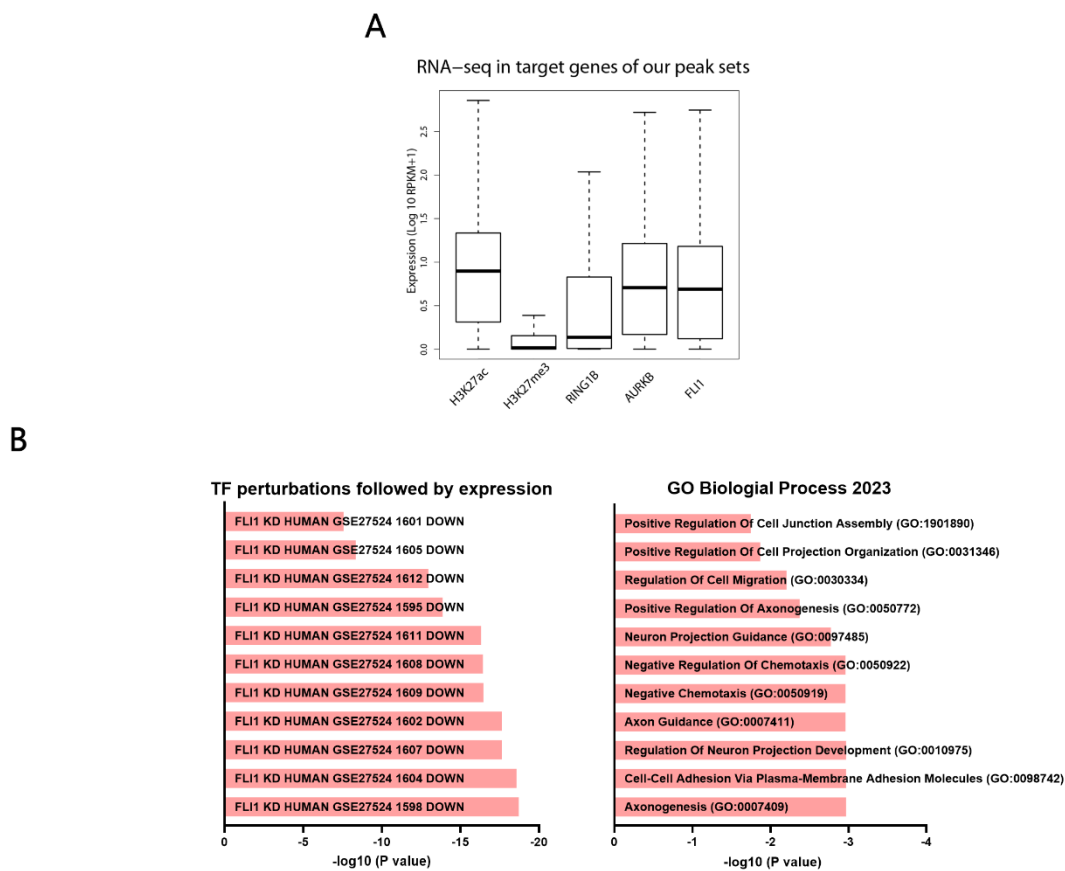


Figure IV. 6. Genes associated to AURKB peaks are highly expressed in EwS. (A) Boxplot showing the average expression (in RPKMs) of genes associated to H3K27ac, H3K27me3 RING1B, FLI1 and AURKB peaks in A673 cells. **(B)** Transcription factors (TF) perturbations followed by expression and Gene Ontology (GO) categories for 603 genes associated to AURKB peaks. The X-axis shows the statistical significance of the enrichment ($-\log_{10}(\text{P-value})$), and the Y-axis shows the top-10 enriched pathway categories provided by EnrichR tool (available at: <https://maayanlab.cloud/Enrichr/>).

2.2. AURKB co-localizes genome-wide with EWSR1-FLI1 and RING1B at active enhancers

As AURKB was mainly enriched at RING1B/EWSR1-FLI1 active enhancers, we further evaluated the rate of colocalization of the three proteins. To that end, we analyzed the triple intersection of AURKB, EWSR1-FLI1 and RING1B ChIP-seq signals in the A673 cell line and obtained 727 common peaks (sub-group 1) (**Fig. IV. 7A**). 779 common peaks were obtained from EWSR1-FLI1 and AURKB (sub-group 3) and only 18 common peaks between RING1B and AURKB (sub-group 4). We then performed the novo motif analysis on every sub-fraction obtained from the triple intersection. We observed that when AURKB is associated with EWSR1-FLI1 in the presence or absence of RING1B (sub-groups 1 and 3) there was a significantly enrichment for multimeric GGAA repeats DNA binding motif (**Fig. IV. 7B**). EWSR1-FLI1-specific sites contained predominantly one single occurrence of the canonical ETS motif GGAA alone or with RING1B (sub-groups 2 and 5) (**Fig. IV. 7B**). As we and others have previously described, we identified a group of RING1B peaks that were enriched for CG sequence (sub-group 6) besides the group that colocalizes with EWSR1-FLI1 at enhancers of actively transcribed genes (sub-group 2). Last, we observed a small fraction of AURKB-specific sites also enriched with the multimeric GGAA repeats (sub-group 7), strongly confirming AURKB enrichment at active enhancers. Known EWSR1-FLI1/RING1B target genes such *VRK1* or *CCND1* presented an intergenic peak of AURKB, which overlaps with defined super-enhancers (**Fig. IV. 7C**).

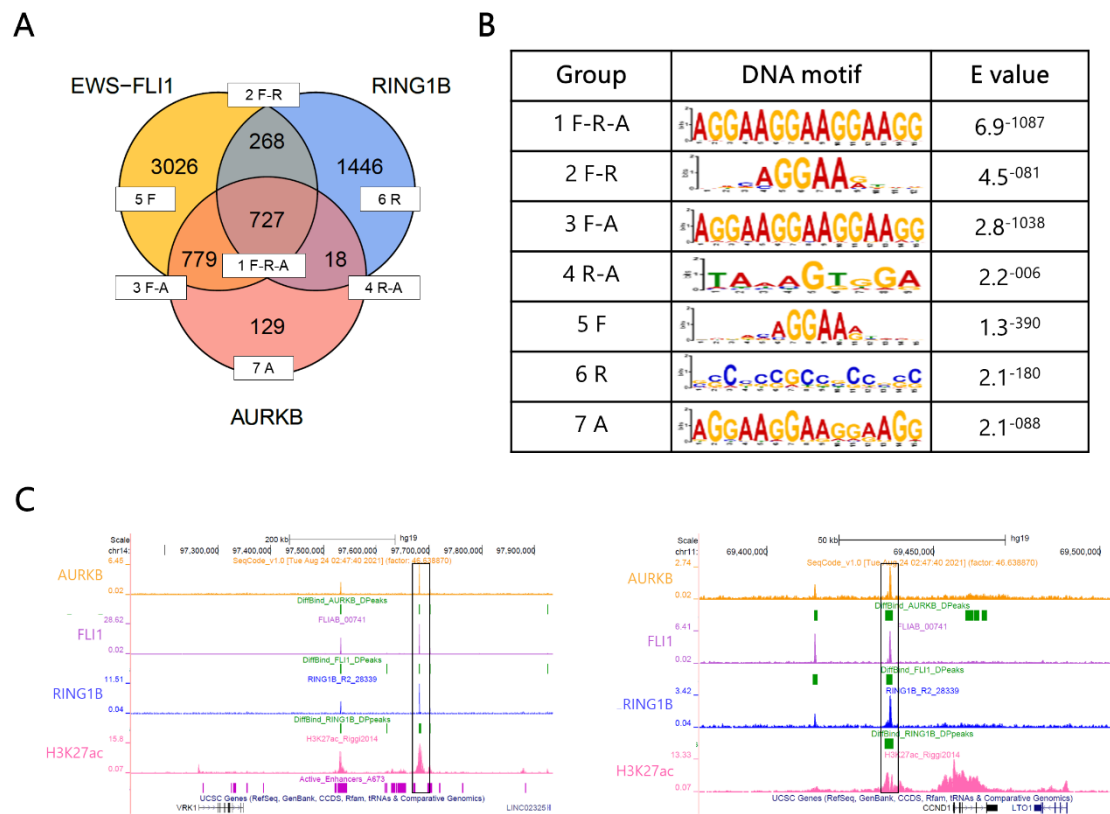


Figure IV. 7. AURKB co-localizes with EWSR1-FLI1 and RING1B at active enhancers. (A) Venn diagram depicting the overlap of genes containing AURKB, EWSR1-FLI1 and RING1B peaks. (B) Table showing top MEME DNA motifs and the corresponding E-value for every sub-group of peaks obtained from the triple intersection. (C) University of California Santa Cruz (UCSC) ChIP-seq signal tracks for RING1B, AURKB, EWSR1-FLI1 and H3K27ac in A673 cell line at VRK1 and CCND1 enhancer regions.

We then conducted a detailed analysis of the different sub-fractions obtained from the triple intersection of AURKB, EWSR1-FLI1, and RING1B peaks. The average levels of AURKB peaks were highest in sub-group 1, supporting the notion that AURKB cooperates with the oncogene EWSR1-FLI1 and RING1B in chromatin binding (Fig. IV. 8A). This was corroborated by the average ChIP-seq signals for both EWSR1-FLI1 and RING1B, which were highest at the intersections with AURKB (Fig. IV. 8B). These findings confirm that AURKB collaborates with RING1B at EWSR1-FLI1 target genes. We also assessed the levels of histone-associated epigenetic marks in each sub-group. The highest H3K27ac ChIP-seq signal, the mark defining active enhancer, along with the lowest H3K27me3 signal, the mark for repressive promoter, in intersections where AURKB co-localizes with EWSR1-FLI1 and/or RING1B (sub-groups 1 and 3), definitively confirms that AURKB is enriched at these EWSR1-FLI1/RING1B active enhancers (Fig. IV. 8C).

Lastly, we considered the possibility of the AURKA, the paralog member of AURKB that belongs to the same family of serine/threonine kinases, had also a contribution at EWSR1-FLI1/RING1B enhancers (Wakahara et al., 2008). AURKA transcription is also regulated in tumors such as astrocytoma and Rhabdoid tumor, where the member of SWI/SNF *INI1/hSNF5* associates with AURKA promoter (Lee et al., 2011; Yu et al., 2017). To that end, we analyzed AURKA levels across different peak groups and found no significant enrichment in any sub-group, confirming that the epigenetic role at EwS active enhancers is exclusive to AURKB (Fig. IV. 8D).

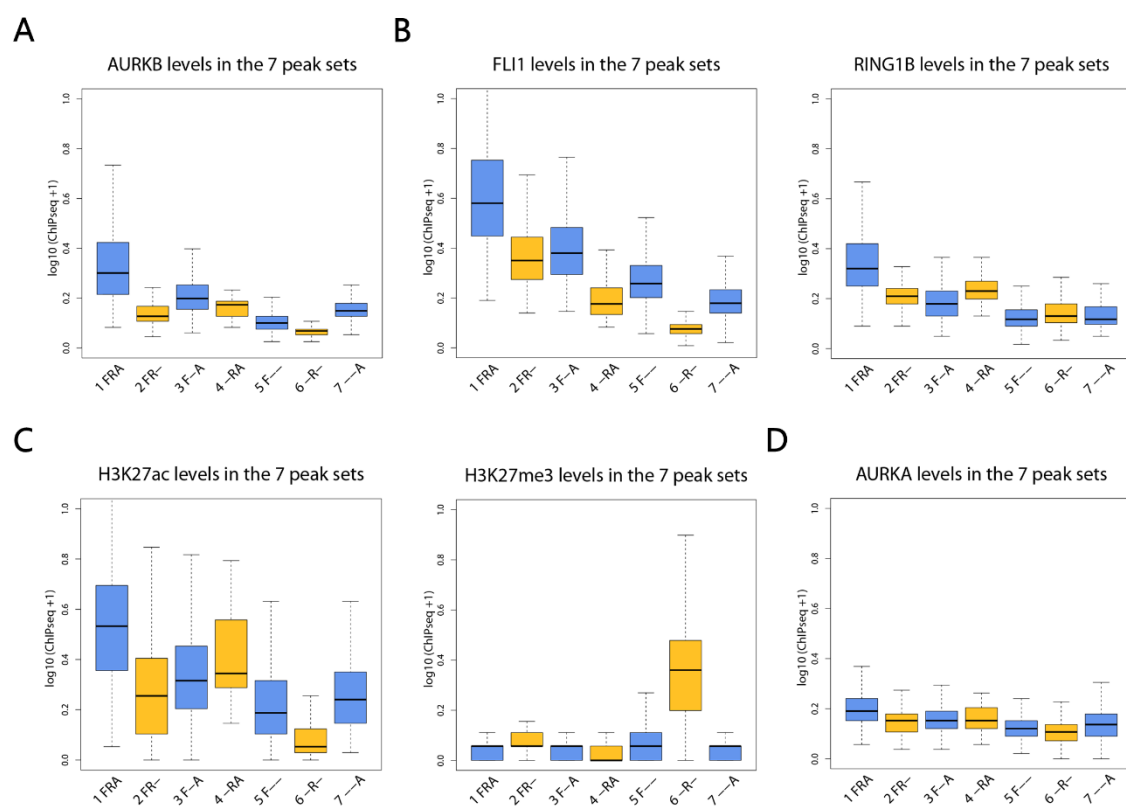


Figure IV. 8. AURKB co-localizes at EWSR1-FLI1/RING1B common active enhancers in a specific way. (A) Boxplot showing the average ChIP-seq signal of AURKB at each subgroup obtained upon intersection of AURKB, EWSR1-FLI1 and RING1B ChIP-seq signals. (B) Boxplot showing the average ChIP-seq signal of EWSR1-FLI1 and RING1B at each subgroup obtained upon intersection of AURKB, EWSR1-FLI1 and RING1B ChIP-seq signals. (C) Boxplot showing the average ChIP-seq signal of H3K27ac and H3K27me3 at each subgroup obtained upon intersection of AURKB, EWSR1-FLI1 and RING1B ChIP-seq signals. (D) Boxplot showing the average ChIP-seq signal of AURKA at each subgroup obtained upon intersection of AURKB, EWSR1-FLI1 and RING1B ChIP-seq signals.

As mentioned before, AURKB has been described to modulate the ubiquitin ligase activity of RING1B by enhancement of USP16 deubiquitinase activity, thus regulating transcription at active promoters in quiescent lymphocytes (Frangini et al., 2013). However, modulation of H2Aub and the mechanism behind the regulation of RING1B by AURKB was unresolved.

When we intersected AURKB and H2Aub peaks, we observed a very low number of common peaks (**Fig. IV. 9A**, top), while intersection between H2Aub and RING1B showed a few common peaks (**Fig. IV. 9A**, bottom), supporting our findings that RING1B retains part of its canonical repressive activity. We evaluated these findings at specific regions such as intronic *JARID2* enhancer and found an anti-correlation between AURKB peaks co-localizing with EWSR1-FLI1/RING1B and H2Aub enrichment at these positions (**Fig. IV. 9B**). Finally, we analyzed H2Aub levels in the different sub-fractions generated intersecting EWSR1-FLI1, RING1B and AURKB peaks, as previously shown. H2Aub levels were the lowest in the sub-fractions where AURKB peaks were intersected with EWSR1-FLI1 and RING1B peaks (sub-fractions 1 or 3) compared to sub-fraction 2, where AURKB is not present (**Fig. IV. 9C**). Sub-fraction 6, encompasses RING1B peaks when alone, presented the highest levels of H2Aub, supporting its repressive role in the absence of EWSR1-FLI1 (**Fig. IV. 9C**). These results support the contribution of AURKB to the activating role of RING1B together with EWSR1-FLI1 in the chromatin remodeling of EwS.

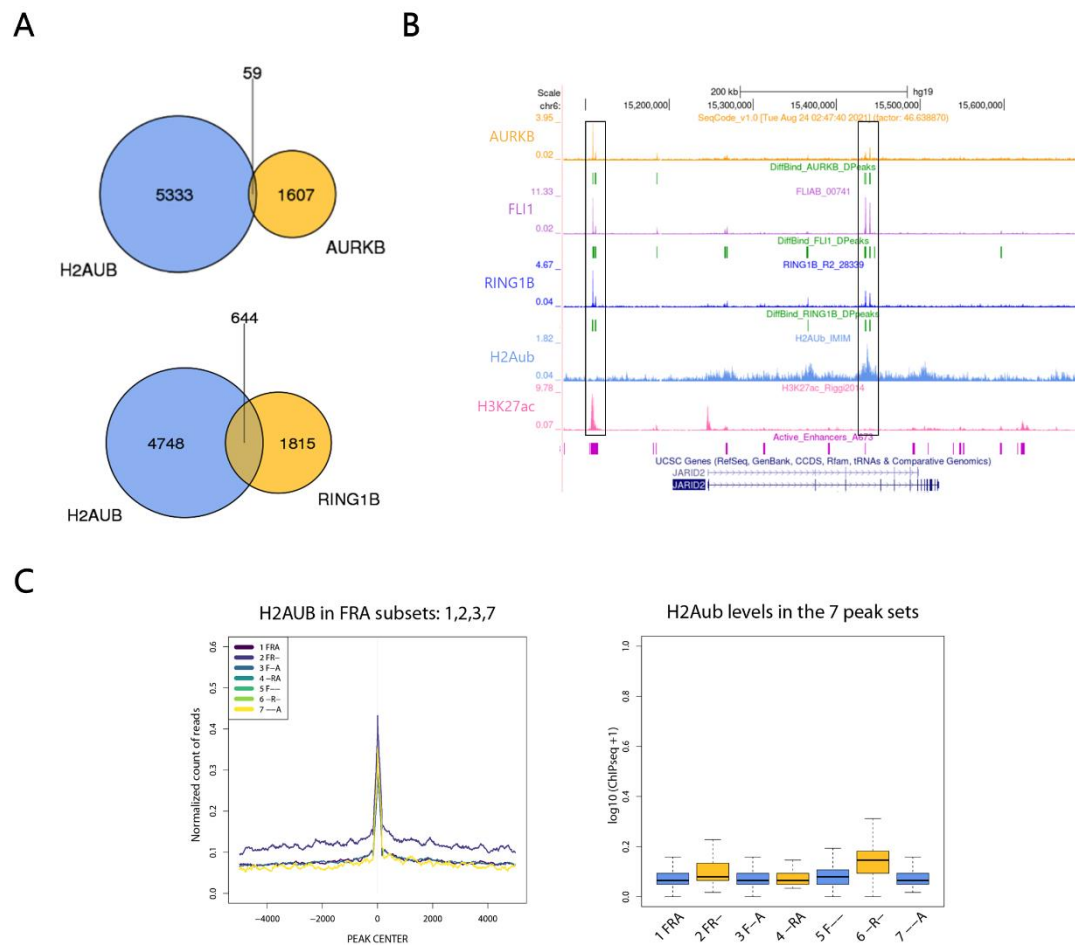
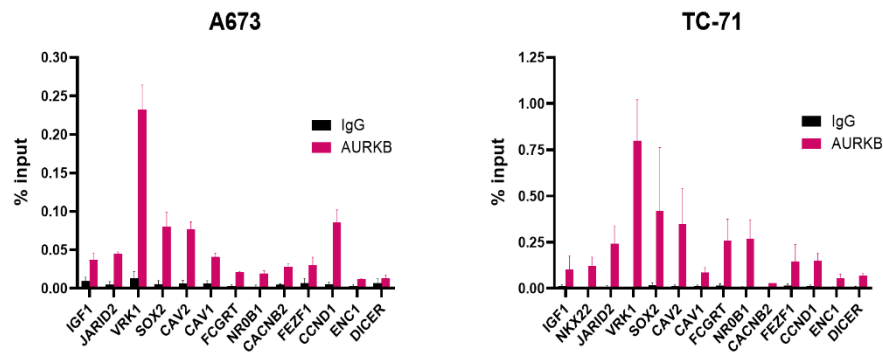


Figure IV. 9. H2Aub does not co-localize with AURKB genome wide. (A). Venn diagram depicting the overlap between AURKB and UbH2A and H2Aub and RING1B peaks in A673 cell line. (B) UCSC genome browser ChIP-seq signal tracks for RING1B, H2Aub, AURKB and EWSR1-FLI1 in A673 cell line at JARID2 enhancer position. (C). Aggregated plots showing the average H2Aub ChIP-seq signal segregating in the seven sets of RING1B, AURKB and EWSR1-FLI1 peaks in A673 cell line. In the left, boxplot showing the average ChIP-seq signal of H2Aub at each subgroup obtained upon intersection of AURKB, EWSR1-FLI1 and RING1B ChIP-seq signals in A673 cell line.

To validate AURKB enrichment at EWSR1-FLI1/RING1B binding regions, we analyzed several well described active promoters (*CAV1*, *FCGRT*, *NR0B1*, *CACNB2* and *FEZF1*) and enhancers (*CCND1*, *IGF1*, *JARID2*, *VRK1*, *SOX2*, *CAV2* and *NKX2-2*) by ChIP-qPCR (Fig. IV. 10A). We confirm that there was an enrichment of AURKB at these positions relative to input, which is higher at enhancers in comparison with promoters, both for TC-71 and A673 EwS cell lines. As we previously described genome wide, we performed the same confirmation upon AURKA chromatin immunoprecipitation and did not observed enrichment for this kinase at EWSR1-FLI1/RING1B/AURKB common target genes, compared to negative controls *ENC1* and *DICER* (Fig. IV. 10B). Taken together, our results

evidenced that role of AURKB at EWSR1-FLI1/RING1B enhancers was specific for this kinase. Nevertheless, further genome-wide analysis is being performed to confirm the specificity of AURKB at oncogenic enhancers in other pediatric tumors as NB.

A



B

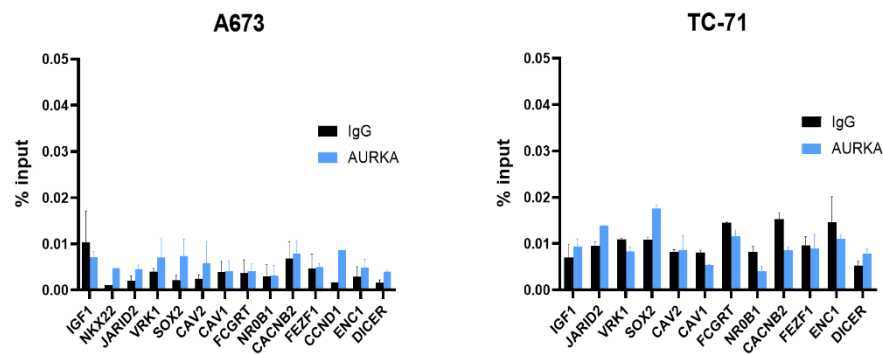


Figure IV. 10. AURKB is specifically enriched at EWSR1-FLI1/RING1B active promoters and enhancers. (A) AURKB ChIP-qPCR at EWSR1-FLI1/RING1B promoters and enhancers in the A673 and TC-71 cell lines. **(B)** AURKA ChIP-qPCR at EWSR1-FLI1/RING1B promoters and enhancers in A673 and TC-71 cell lines. The values of the Y-axis represent the enrichment ratio of immunoprecipitated samples relative to input. Error bars indicate SEM of at least two biological independent experiments.

2.3. AURKB distribution genome-wide in HeLa Tet-On cell line model

To understand whether EWSR1-FLI1 overexpression stimulates the distribution of AURKB to enhancer regions in the chromatin, we performed AURKB ChIP-seq in a HeLa cell model that express EWSR1-FLI1 under the control of a doxycycline-inducible promoter (García-Domínguez et al., 2020), as introduced in section 1.2. First, we analyzed by ChIP-seq using antibodies against EWSR1-FLI1 and FLAG the EWSR1-FLI1 peaks in induced HeLa cells (iHeLa), compared to the control HeLa cells. As shown in **Figure IV. 10A**, 8245 FLAG peaks and 21179 EWSR1-FLI1 peaks were significantly detected in the iHeLa cells.

Furthermore, the oncoprotein-specific sites were enriched with the multimeric GGAA repeats (**Fig. IV. 11A**, below). Finally, the higher intensity of FLAG and EWSR1-FLI1 peaks in iHeLa cells validated the oncogene overexpression and the finding of the GGAA repeat as DNA motif allowed to go further with AURKB analysis (**Fig. IV. 11B**).

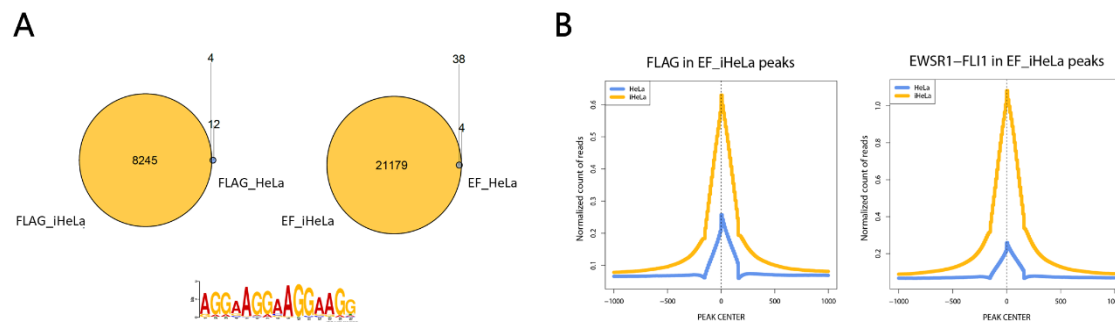


Figure IV. 11. HeLa cell line reproduce EWSR1-FLI1 chromatin binding upon oncogene induction. (A) Venn diagrams depicting the overlap between FLAG peaks (left) and EWSR1-FLI1 peaks (right) before (HeLa) and upon induction of the oncogene (iHeLa). Top MEME motif for EWSR1-FLI1 peaks obtained in iHeLa cells below. (B) Aggregated plots showing the average FLAG ChIP-seq signal (left) and the average EWSR1-FLI1 ChIP-seq signal (right) at iEWSR1-FLI1 binding sites HeLa and iHeLa cells.

As shown in **Figure IV. 12A**, AURKB peaks colocalize with oncogene induced peaks at positions like *CAV2* enhancer. However, we can appreciate that AURKB was already present in EWSR1-FLI1 bound positions before induction. It is remarkable that AURKB binding in iHeLa cell line showed a worse technical quality; consequently, lower number of these peaks were detected. Although EWSR1-FLI1 peak intensity is increased in both FLAG and EWSR1-FLI1 peaks once the oncogene has been overexpressed, AURKB ChIP-seq signal was maintained before and after EWSR1-FLI1 induction (**Fig. IV. 12B**). Nevertheless, when we plotted the triple intersection depicting AURKB peaks in HeLa and iHeLa with EF_iHeLa peaks (**Fig. IV. 12C**), the diagrams highlight an increased colocalization of AURKB peaks with EWSR1-FLI1 in iHeLa cells, emphasizing a potential interaction or co-regulation between AURKB and EWSR1-FLI1 following oncogene induction (**Fig. IV. 12C**). Similarly, AURKB_iHeLa peaks strong colocalized with AURKB peaks in A673, in a significant more extent than before oncogene induction (**Fig. IV. 12D**). This is particularly relevant considering the low number of AURKB peaks that were

obtained upon the oncogene induction. Taken together, these results suggest that, despite technical limitations in data quality of AURKB binding in iHeLa cells, oncogene induction may enhance the interaction between AURKB and EWSR1-FLI1 and cause changes in the genome wide occupancy for AURKB.

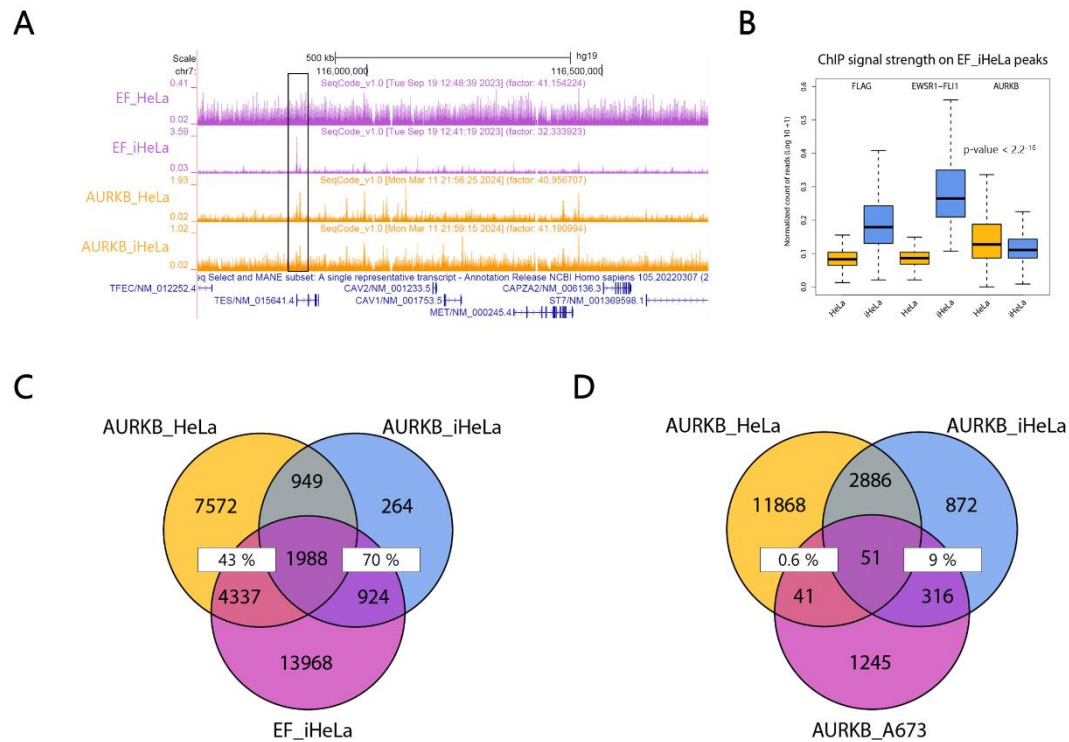


Figure IV. 12. AURKB chromatin distribution in HeLa cell line. (A) University of California Santa Cruz (UCSC) ChIP-seq signal tracks for EWSR1-FLI1 and AURKB before (HeLa) and upon induction of the oncogene (iHeLa) at a wide region including CAV2 enhancer. (B) Boxplot depicting the average ChIP-seq signal of 3xFLAG, EWSR1-FLI1 and AURKB at EWSR1-FLI1 induced peaks in control and induced HeLa cells. (C) Venn diagram depicting the overlap between AURKB peaks in control and induced HeLa cells and induced EWSR1-FLI1 peaks. (D) Venn diagram depicting the overlap between AURKB peaks in control and induced HeLa cells and AURKB peaks in A673 cell line.

3. EWSR1-FLI1/RING1B common target genes are regulated by AURKB modulation

3.1. AURKB is an essential gene in EwS

AURKB is crucial for the correct chromosome orientation and segregation during mitosis and cytokinesis. Since tumor cells are highly proliferative, they are very dependent on cell cycle and thus proteins such AURKB. In order to elucidate the function of AURKB in the

epigenetic program of EwS, we addressed different experiments to modulate AURKB level. Until now, only conditional AURKB knockout have been approached, predominantly in mice (Ah-Cann et al., 2021; Frangini et al., 2013; Hindriksen et al., 2017). Wang et al. (S. Wang et al., 2019) confirmed AURKB among the top expressed proteins in EwS and its specific inhibitor, AZD-1152, as a top drug for synergistic inhibition with FAK inhibitors in this disease. Consequently, they further validated AURKB as a target *in vitro* and *in vivo*, so they did conduct a knockout (KO) of AURKB, revealing the dependency of EwS cells on this protein. Their study demonstrated that the absence of AURKB significantly compromised cell viability, highlighting its critical role in the survival and proliferation of EwS cells. Analysis of AURKB dependency using the Cancer Dependency Map (DepMap) portal (Tsherniak et al., 2017), specifically the Public 24Q2+Score database, upon selection of pediatric cell lines revealed the lowest scores for EwS cell lines. (**Fig. IV. 13A**). Essential genes were previously determined in Cancer Cell Line Encyclopedia (CCLE) by calculating gene scores using the CHRONOS model, where gene score of 0 is equivalent to a gene which is not essential, whereas a score of ≤ -1 indicates this as an essential gene (Dempster et al., 2021).

Despite AURKB been considered a dependency for EwS, due to the feasibility of KO performed in Wang et al., we approached genome editing of this protein by transfection of A673 EwS cell line with ribonucleoprotein (RNP) complexes containing Cas-9/sgAURKB RNA. Limited dilution was performed and potential knockout (sgAURKB) clones were cultured, together with their respective control cells (sgCTRL), and the efficiency of the transfection was evaluated by Western Blot. AURKB protein levels in our candidates sgAURKB clones showed a decrease compared to the parental A673 cell line and the two sgCTRL cells, although a slight band was observed in most of them (**Fig. IV. 13B**). Consequently, we took these clones and performed DNA sequencing to validate the plausible AURKB KO. cDNA was extracted and AURKB target region was amplified with two suitable pair of primers by PCR, then revealed by agarose gel. Same size of sgAURKB #3D10 and #4B5 compared to A673 and sgCTRL cell lines revealed a non-successful KO generation (**Fig. IV. 13C**). We further proceed with the sequencing and subsequent analysis using ICE CRISPR Analysis Tool (<https://ice.synthego.com/#/>), which also

revealed that both sgAURKB #4B5 and #3D10 had a 99% of identity compared to the control sequence, which corresponds to AURKB wild type (Fig. IV. 13D). Altogether, these data suggest the impossibility to generate a viable KO of AURKB, corroborating that AURKB is an essential gene in EwS.

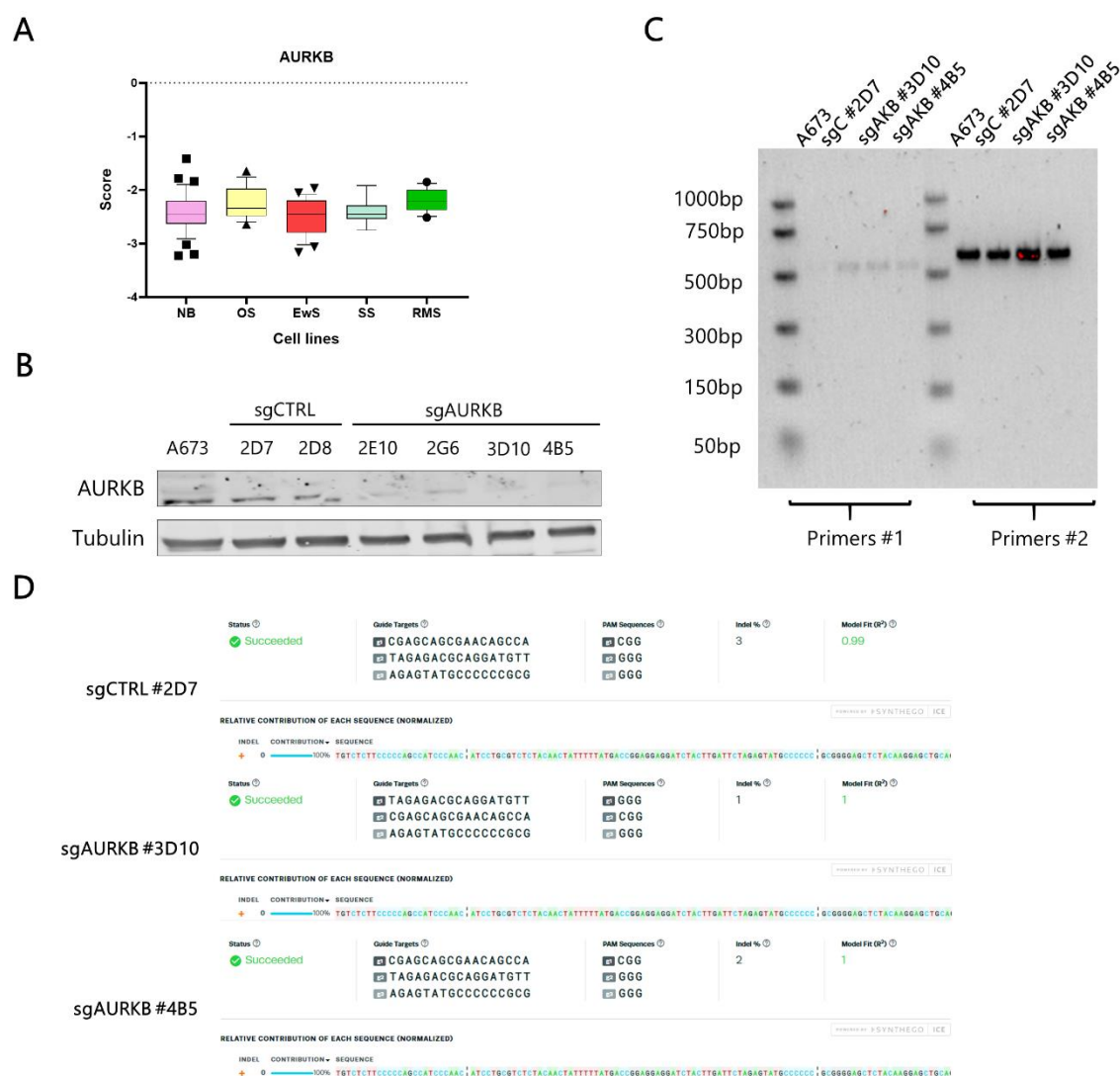


Figure IV. 13. AURKB is an essential gene in EwS. (A) Boxplot showing AURKB CRISPR dependency score among several pediatric cell lines based on Expression Public 24Q2+Score database from DepMap portal. (B) Western Blot analysis of AURKB protein levels in A673 parental cell line, sgCTRL and sgAURKB selected clones. Tubulin was used as loading control. (C) RT-PCR amplification resolved in agarose gel. (D) Screenshot of the ICE CRISPR analysis comparing sgCTRL sequence (AURKB) to sgAURKB #3D10 and #4B5 clones.

3.2. EWSR1-FLI1/RING1B targets are regulated by AURKB knockdown with small interference RNA and short hairpin RNA

In order to characterize the role of AURKB at EwS superenhancers, and due to the lack of a suitable KO model, we performed AURKB knockdown by two different strategies. First, transient small interfering RNA (siRNA) revealed a downregulation in the expression of most EWSR1-FLI1/RING1B common target genes in A673 and TC-71 EwS cell lines after 72 hours (**Fig. IV. 14A**). We observed a high decrease in the expression of several target genes, with a slight intensity at promoter level and a general stronger downregulation for A673 cell line. AURKB expression levels were evaluated as a control of the efficacy of the knockdown (**Fig. IV. 14A**, right).

We then proceeded with the expression analysis of the AURKB knockdown with two sequences (shAURKB-9 and shAURKB-10) in A673 cell line, provided by Crompton Lab and described in Wang et al. (S. Wang et al., 2019). Expression analysis showed a variable decrease at EWSR1-FLI1 enhancers that were analyzed, for both sequences shAURKB-9 and 10, after 48 hours. AURKB expression levels were evaluated as a control of the efficacy of the knockdown (**Fig. IV. 14B**, right). Finally, we performed Western Blot analysis to validate the loss of AURKB protein levels upon the knockdown, with no changes in the oncogene nor AURKA (**Fig. IV. 14C**). Together with our previous findings, these results confirm that AURKB has a modulator role in EWSR1-FLI1/RING1B activated promoters and enhancers. To further confirm the implication of AURKB EWSR1-FLI1 cell reprogramming RNA-seq in shAURKB-9 and 10 A673 cell line is being conducted.

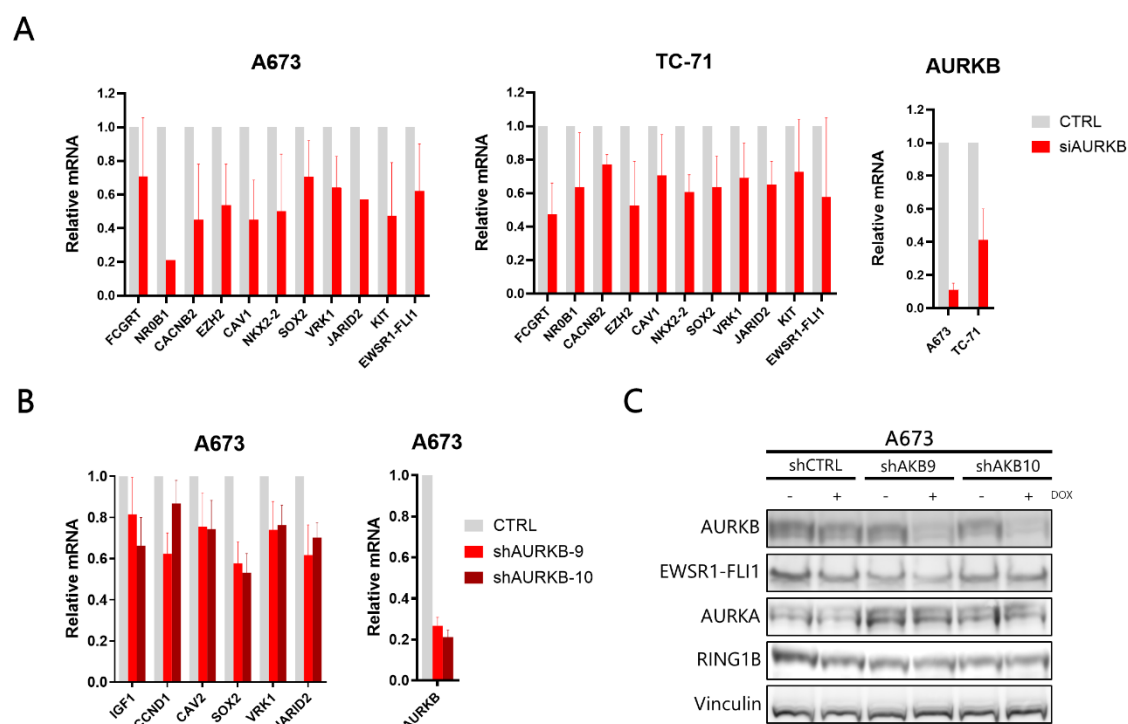


Figure IV. 14. AURKB silencing downregulates EWSR1-FLI1 and RING1B activated promoters and enhancers. (A) RT-qPCR of mRNA relative expression of common target genes in upon 72 hours AURKB knockdown by siRNA compared to control in A673 and TC-71 cell lines. AURKB expression showed on the right. TBP was used as housekeeping gene for normalization. (B) RT-qPCR of mRNA relative expression of common target genes in upon 48 hours AURKB knockdown by shRNA-9 and 10 compared to control in A673 cell line. AURKB expression showed on the right. (C) Western Blot analysis of AURKB, EWSR1-FLI1, AURKA and RING1B protein levels in shCTRL, shAURKB-9 and shAURKB-10 cells. Vinculin was used as loading control.

3.3. EWSR1-FLI1/RING1B targets are regulated by AURKB inhibition with AZD-1152

To go further insight into the effect of AURKB in EWSR1-FLI1 transcription regulation, we validated our genome-wide results. We examined by RT-qPCR the relative mRNA levels of EWSR1-FLI1/RING1B target genes regulated at promoter and enhancer level upon AZD-1152 treatment for 24 hours at 20 nM. A decrease in expression was observed in the majority of EWSR1-FLI1 targets in the A673 and TC-71 cell lines. In A673 cells, the treatment did not impact RING1B levels or the translocation levels (Fig. IV. 15A). However, in the TC-71 cell line, both RING1B and EWSR1-FLI1 levels were affected by the treatment, which may indicate the higher sensitivity this cell line to the drug (Fig. IV. 15B, right). For both cell lines, expression changes were less pronounced than those observed upon AURKB downregulation, suggesting that additional functions from the kinase activity

might be implicated in the EWSR1-FLI1 transcription regulation. Alternatively, using higher doses might better replicate the effects observed with AURKB knockdown. However, we aimed to avoid this approach to prevent the changes from being attributed to an increased apoptotic rate.

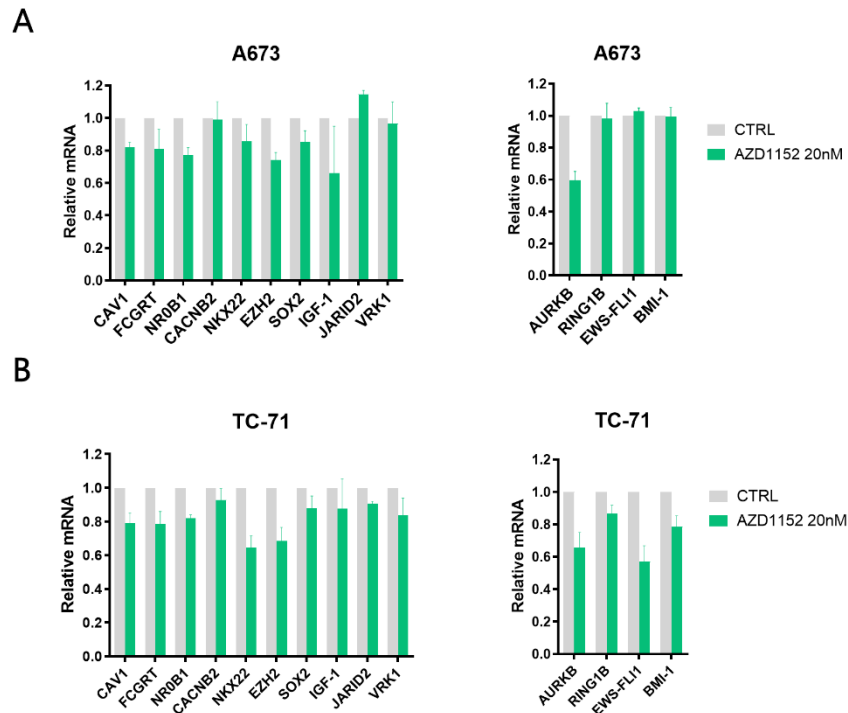


Figure IV. 15. EWSR1-FLI1/RING1B targets are regulated by AURKB inhibition. RT-qPCR of mRNA relative expression of EWSR1-FLI1/RING1B bound promoters and enhancers after 24 hours treatment with AZD1152 20 nM in (A) A673 and (B) TC71 cell lines. RPL27 was used as housekeeping gene for normalization. Dimethyl sulfoxide (DMSO) was used as the treatment control.

To analyze the implication of the enzymatic activity of AURKB in the oncogenic program, we targeted its kinase activity with the AURKB specific inhibitor AZD-1152 in two EwS cell lines. In order to study genome-wide expression changes, we carried out global transcriptomic analysis by RNA-seq upon AZD-1152 drug incubation. AURKB inhibition resulted predominantly in gene downregulation in both cell lines ($P_{adj} < 0.05$ and $|\log_2\text{Fold Change}| \geq 0.5$). We obtained 62 genes that were statistically significant differentially expressed in the A673 cell line (55% downregulated and 45% upregulated). In TC-71 cells, 144 genes were significantly regulated upon AZD-1152 inhibition (63% downregulated and 37% upregulated) (Fig. IV. 16A). The higher number of

downregulated genes in both cell lines support a role of AURKB in activation of transcription. Among the top downregulated genes in A673 cell line, we could find PRAME, BIRC5 and CDC20; for TC-71 cells we highlighted ROR1, SMAD5, WNT5A and E2F8 (**Fig. IV. 16B**), all of them being involved in EwS. GO analysis of downregulated genes reported cell cycle regulation processes in A673 cell line and, interestingly, neural processes and transcription regulation in TC-71 cells (**Fig. IV. 16C**). Finally, among the TFs perturbations we found as very relevant the downregulation in FLI1 KD genes in A673, what is, genes that are upregulated by the oncogene (**Fig. IV. 16D**).

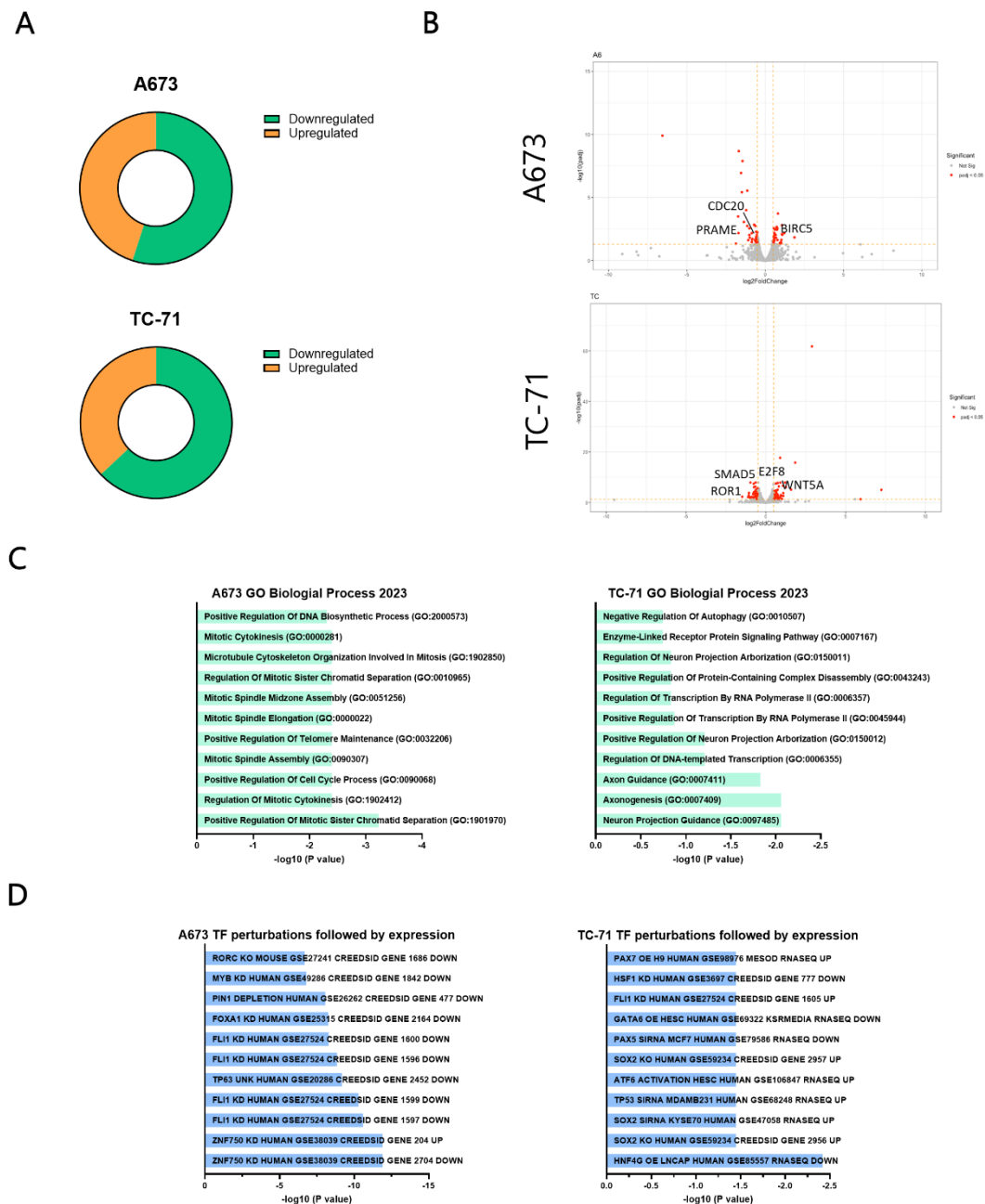


Figure IV. 16. Genome-wide expression analysis upon AURKB inhibition. (A) Donut charts representing the percentage of significantly upregulated or downregulated targets upon AURKB inhibition with AZD-1152 50 nM and 20nM in A673 and TC-71 cells, respectively, for 24 hours. (B) Volcano plots depicting log₂Fold change (x-axis) and the associated log P-adjusted value (y-axis) of deregulated targets AURKB inhibition in A673 and TC-71 cell lines. Statistically significant deregulated targets according to set cut-off are highlighted in red. (C) Bar chart representing the top eleven enriched gene ontology (GO) biological process and their associated Log₁₀ (P-value) of the 34 genes downregulated in A673 and the 91 downregulated genes in TC-71 cell line. (D) Bar chart representing the top eleven transcription factors (TFs) and their associated Log₁₀ (P-value) of the 34 genes downregulated in A673 and the 91 downregulated genes in TC-71 cell line.

Finally, to investigate potential alterations in the distribution and expression of the histone mark H3K27ac, which is associated with active enhancers and promoters, we

analyzed its genome-wide levels following AURKB inhibition. Given the observed decrease in gene expression with AURKB inhibition, we aimed to correlate these changes with H3K27ac levels. To that end, we evaluated by ChIP-seq the genome wide levels of H3K27ac upon AZD-1152 treatment. As shown in **Figure IV. 17A**, a modest reduction in H3K27ac signal was detected across the genome in the TC-71 cell line treated with 50 nM AZD-1152. However, in the more resistant A673 cell line, no significant change in global H3K27ac levels was observed after 24 hours of treatment with 200 nM AZD-1152. At the *SOX2* enhancer locus, no discernible change in peak intensity was observed between treated and control cells (**Fig. IV. 17B**). Additionally, ChIP-qPCR analysis of specific enhancers such as *CCDN1* and *CAV2* confirmed a reduction in H3K27ac enrichment following AZD-1152 treatment, consistent with our genome-wide findings (**Fig. IV. 17C**). These results, in conjunction with previous data, suggest that AURKB inhibition modulates EWSR1-FLI1 and RING1B-activated enhancers and promoters, further supporting the role of AURKB in regulating chromatin dynamics at key oncogenic loci.

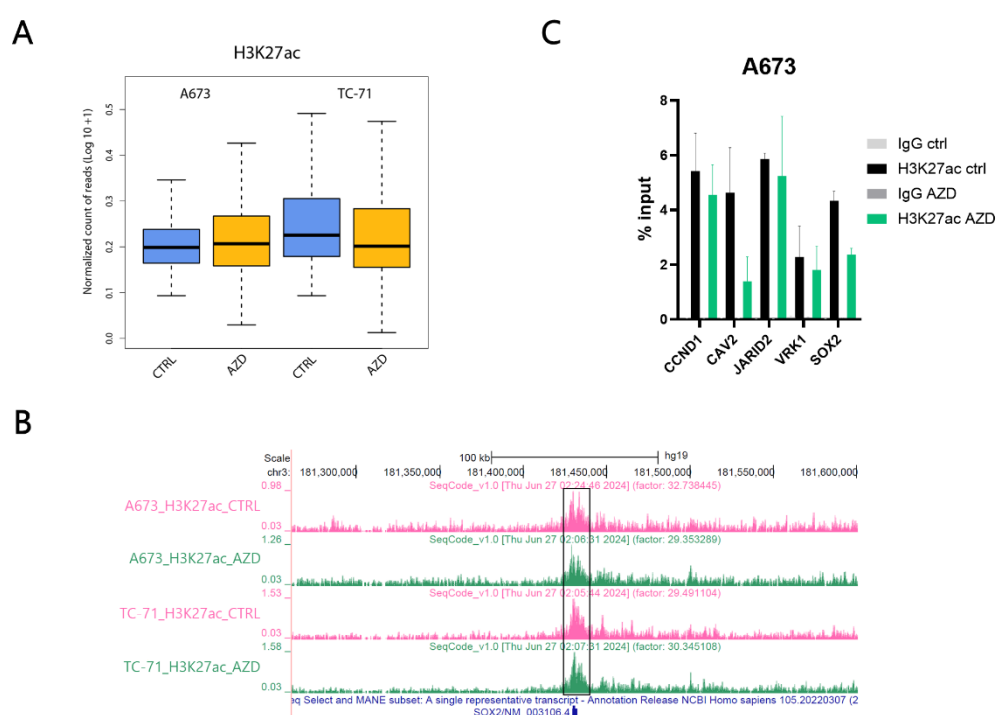


Figure IV. 17. AZD-1152 affects H3K27ac distribution genome-wide. (A) Boxplot depicting the average H3K27ac ChIP-seq signal at control and AZD-1152 treated A673 and TC-71 EwS cell lines. Treatment duration

was 24 hours. A673 were treated with 200 nM and TC-71 with 50 nM. **(B)** University of California Santa Cruz (UCSC) ChIP-seq signal tracks for H3K27ac in A673 and TC-71 control and AZD-1152 treated cells at SOX2 enhancer region. **(C)** H3K27ac ChIP-qPCR at EWSR1-FLI1/RING1B enhancers in control and AZD-1152 treated A673 cell line.

4. Underlying the mechanistic relation between AURKB, EWSR1-FLI1 and RING1B

4.1. AURKB modulation of RING1B E3 ubiquitin ligase activity

As mentioned before, AURKB has been described to modulate the ubiquitin ligase activity of RING1B by enhancement of USP16 deubiquitinase activity, thus regulating transcription at active promoters in quiescent lymphocytes (Frangini et al., 2013). However, modulation of H2Aub and the mechanism behind the regulation of RING1B by AURKB was unresolved.

Based on our previously genome-wide analysis where an anticorrelation of AURKB and H2Aub was observed and in order to delve deeper into the mechanism by which AURKB modulates EWSR1-FLI1/RING1B target genes, we evaluated whether AURKB modulates RING1B ubiquitin ligase activity upon AURKB inhibition with AZD-1152. To that end, we explore H2Aub levels upon AZD-1152 treatment, in a time course experiment by treating A673 and TC-71 cell lines with 200 nM and 50 nM of the drug, respectively, and collecting the cells after 24, 48, and 72 hours of treatment. Western Blot revealed that H2Aub protein levels slightly increased when inhibiting both A673 and TC-71 cell lines with AZD1152 for 72 hours (**Fig. IV. 18A**). The decrease in H3S10ph protein levels, a direct target of AURKB, confirmed the efficacy of the drug treatment. Furthermore, we conducted H2Aub ChIP-seq experiments to study possible changes in the distribution of this mark genome-wide upon AZD-1152 treatment. Looking at some regions such as *CAV1*, a gene activated by EWSR1-FLI1, we were able to detect very slight changes at peak level between H2Aub control and treated, in A673 and TC-71 cells (**Fig. IV. 18B**). Similarly, H2Aub enrichment was similar in control and AZD-1152 conditions in the two cell lines (**Fig. IV. 18C**). However, when analyzing by ChIP-qPCR specific enhancers such as *CCND1* or *CAV1*, we could appreciate an increase in H2Aub enrichment upon AZD-

1152 treatment in A673 cell line (**Fig. IV. 18D**). Several reasons might explain the discrepancies between H3K27ac supporting less transcription and H2Aub being maintained. Furthermore, the variability in our results could be explained considering the endpoint time of the experiments, which was 72 hours, to observe changes in protein levels (**Fig. IV. 18A**) compared to 24 hours for the genome-wide analysis. Is quite plausible that the deposition of repressive histone marks as H2Aub needs longer incubation times. Despite further studies are needed, we provide evidence that AURKB indeed inhibits at global level the ubiquitin ligase activity of RING1B in EwS.

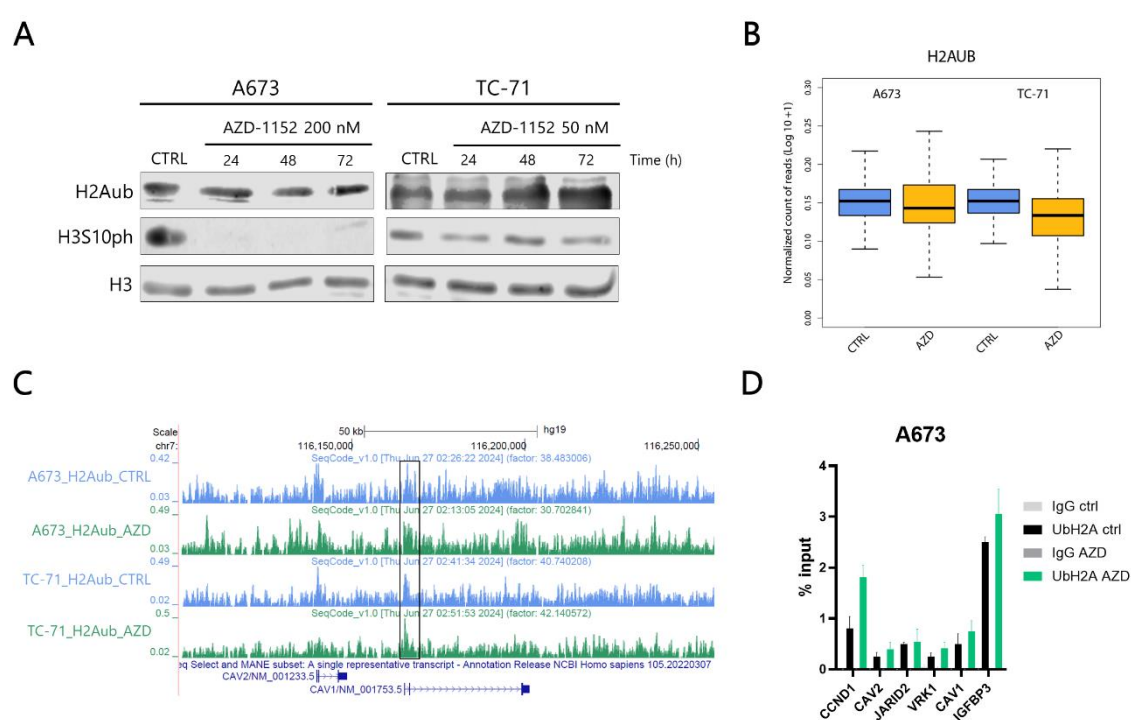


Figure IV. 18. AURKB inhibition by AZD1152 increases H2Aub levels in EwS cell lines. (A) Western blot analysis of H2Aub and H3S10ph in the A673 and TC-71 cell lines after 24, 48 or 72 hours treatment with AZD1152 200 nM or 50 nM. Histone H3 was used as loading control. (B) Boxplot depicting the average H2Aub ChIP-seq signal at control and AZD1152 treated A673 and TC-71 EwS cell lines. Treatment duration was 24 hours. A673 were treated with 200 nM and TC-71 with 50 nM. (C) University of California Santa Cruz (UCSC) ChIP-seq signal tracks for H2Aub in A673 and TC-71 control and AZD-1152 treated cells at CAV1 enhancer region (D) H2Aub ChIP-qPCR at EWSR1-FLI1/RING1B enhancers in control and AZD-1152 treated A673 cell line.

4.2. AURKB modulation of RING1B phosphorylation

To get further insight into the regulation of RING1B by AURKB, we also evaluated a potential direct mechanism. It is known that CK2 inhibits the monoubiquitination of

modification. Within the PRC1 complex, RING1B functions as an E3 ubiquitin ligase responsible for the ubiquitination of histone H2A at lysine 119 (H2AK119ub), a modification that is key to its repressive activity. RING1B typically interacts with core PRC1 components such as BMI1, which enhances its E3 ligase activity, and various Chromobox (CBX) proteins that recognize and bind to methylated histones. Additionally, Polyhomeotic homologs (PHC1, PHC2, or PHC3) and Polycomb group RING finger proteins (like PCGF2/MEL18 or PCGF4/BMI1) are also integral to PRC1 complexes (Aranda et al., 2015). Beyond the canonical PRC1, RING1B is involved in variant PRC1 complexes, such as PRC1.1, which includes KDM2B and BCOR, but lacks CBX proteins, and PRC1.2/PRC1.4, where RING1B associates with proteins like RYBP or YAF2 instead of CBX proteins. To further confirm the changes observed in RING1B phosphorylation and possible changes in its interactome we performed endogenous RING1B IP coupled with MS (IP-MS), following nuclear extraction, in TC-71 control (DMSO) and AZD-1152 50 nM cell lines. Firstly, we confirmed by RING1B immunoprecipitation (IP) followed by Western Blot that we were able to immunoprecipitate RING1B and detect phospho RING1B at S168 in whole and nuclear extract in TC-71 cell line (**Fig. IV. 20A**). Despite we confirmed the decrease in RING1B phospho in total extract, we could only observe a slight decrease in IP material upon 24 hours of AZD1152 50 nM treatment.

To identify possible changes in RING1B interactors we analyze three different conditions once the experiment is finished: AZD-1152 vs DMSO, AZD-1152 vs IgG and DMSO vs IgG and we compared the number of spectrums in every condition. However, it was extremely value for our group to characterize RING1B interactome. For this reason, we also compare TC-71 control cell line vs IgG. Using a probabilistic scoring by significance analysis of interactome (SAINT) we identified RING1B interactors setting a BFDR < 0.01 and then filtering a Fold Change > 120. When plotting them using STRING analysis, we applied the highest confidence in the minimum required score (0.9) and represented them as a physical network (**Fig. IV. 20B**). Although AURKB was not present in our list, the RING1B interactome include members of the PRC1.1 complex, such as BCOR and CBX4 and a large cluster of the Mediator complex, involved in the initiation of transcription by

recruiting RNA polymerase II to gene promoter. MLL complex and important interactors such as KDM6A were also identified.

In a sequential analysis of these data, we evaluated those changes produced in RING1B peptides and those produce from its interactors. Unfortunately, among the RING1B peptides obtained, we were not capable to detect a peptide containing RING1Bph S168 due to intrinsic characteristics of trypsin digestion. In **Figure IV. 20C**, we summarize the obtained peptides for RING1B (accession Q99496), which position in RING1B (the Master Protein) is shown in the first column and their annotated sequence in the second column. Amino acids between brackets (i.e. [S] in the first sequence) indicate the probability to be phosphorylated at those positions. The third column shows the phosphorylation positions that were indeed detected in our conditions. Since the detected peptides correspond only to amino acid number 149 (the highest position shown in the first column), S168 could have never been identified. Nevertheless, we could confirmed no alterations in the position S41. Although we observed changes in other peptides from interacting proteins, most of them corresponding to subunits of Mediator complex, they were not statistically significant. All these results confirm the activator role of RING1B and describe important interactions with complexes required for transcriptional activation.

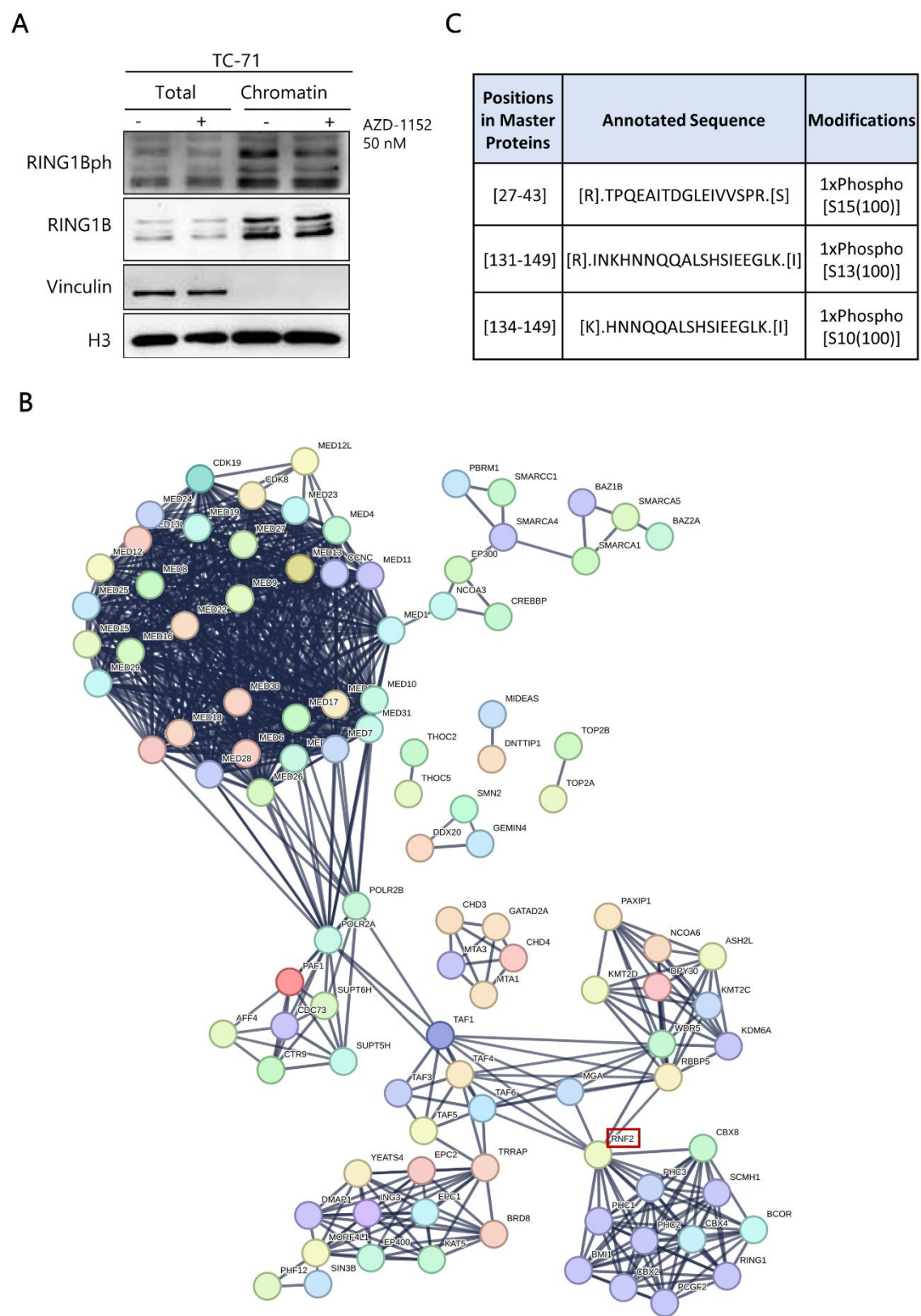


Figure IV. 20. Unraveling RING1B interactome in EwS. (A) Benzoylase-treated cell extracts immunoprecipitated with a specific anti-RING1B antibody in TC-71 control (DMSO) and AZD1152 50 nM for 24 hours. Vinculin was used as loading control for the whole fraction. H3 was used as loading control for

chromatin fraction. (B) The protein-protein interaction network of the RING1B interactome as defined by STRING analysis. (C) Table summarizing RING1B (Q99496) peptides obtained after trypsin digestion. Position, sequence and detected phosphorylations are indicated.

5. Evaluation of the synergistic combination of AZD-1152 and superenhancers disruptors *in vitro* and *in vivo*.

5.1. Combination of AURKB inhibition and BET inhibition is potentially synergistic in EwS cell lines

It has been widely described the difficulty to inhibit fusion oncoproteins like EWSR1-FLI1 is remarkably challenging. Besides, RING1B inhibitors only target its repressive role. We and others have demonstrated the antitumor effectiveness of AURKB inhibitor AZD-1152 in EwS (S. Wang et al., 2019) (Sánchez-Molina et al., 2020) (Teicher et al., 2015); however, its activity remains non-sufficient as single therapy, evidencing the need to find new combinatorial strategies. Superenhancers represent an Achilles heel in the chromatin remodelling necessary for EwS and enhancer disruption with BET inhibitors is efficient in EwS cell lines (Gollavilli et al., 2018). Furthermore, combinatorial of BRD4 and AURKA inhibition with JQ1 and alisertib respectively, is synergistic against preclinical models of EwS (Tomino et al., 2019). Given the unexpected role of AURKB at enhancers and considering BRD family of proteins as part of RING1B interactome, we aimed to combine AZD-1152 with the family of BET inhibitors. JQ1 is an early BET inhibitor with widespread use in research. The advanced version of JQ1, I-BET 151, has an improved bioavailability, a longer half-life, better tissue distribution, and a more favorable metabolic profile. The more recent inhibitor, BMS-986158, also considered more selective, presented already clinical applications which are being actively explored. Remarkably, it is currently on clinical trial in pediatric cancer (Stathis & Bertoni, 2018).

To combine AURKB inhibitor AZD-1152 with BET inhibitors, we started treating EwS cell lines with AZD-1152 and BMS-986158 across a wide range of concentrations that encompass 0.3 nmol/L to 30 μ mol/L for AZD-1152 and 0.08 μ mol/L to 0.4 μ mol/L for BMS-986158. Each combination of specific concentrations was tested in technical quadruplicates and, at least, biological duplicates. Cells were incubated with the two

drugs combination for 2 or 3 days and cell viability was measured by fluorescence, so we represented the Average ratios to untreated control (**Fig. IV. 21A**). We then evaluated whether each combination tested was synergistic. To that end, we first calculated the Excess over Bliss Independence, which formula is $C = X - [A + B - A \times B]$, which is a first approximation that indicates potential synergistic combinations. X is the fractional inhibition for combined compounds at given concentrations, A is the fractional inhibition for compound 1 (AZD-1152) at fixed concentrations of compound 2 (BMS-986158) and B is the fractional inhibition for compound 2 at fixed concentrations of compound 1. That fixed concentration is the DMSO value. A positive C value is considered potentially synergic, whereas a negative value did not. Finally, the most accurate parameter to evaluate is the combination index (CI), that was obtained based on the average cell viability inhibition of the four replicates through an R studio algorithm. A $CI < 0.7$ is indicative of synergy, a CI between 0.7 and 1.3 is considered as an additive effect and a $CI > 1.3$ indicates that the combination is antagonistic. We observed that EwS cell line A673 treated with AZD-1152 in combination with BMS-986158, showed a positive Excess over Bliss (**Fig. IV. 21A**) and a $CI < 0.7$ for a large range of drug concentrations (**Fig. IV. 21B**). Our findings conclude that the combination of AURKB inhibitor AZD-1152 with the BET inhibitor BMS-986158 is potentially synergistic in A673 EwS cell line at nanomolar concentrations.

A

		AZD1152													
		0,4	0,26	0,169	0,10985	0,071403	0,046412	0,030168	0,019609	0,012746	0,008285	DMSO			
BMS-986158	30	4,16%	5,00%	4,75%	5,01%	4,81%	4,93%	5,23%	4,43%	5,33%	6,81%	14,09%			
	12	4,28%	5,72%	4,99%	5,39%	5,90%	5,91%	5,56%	6,17%	7,45%	10,78%	29,47%			
	4,8	8,09%	7,05%	7,49%	8,54%	7,31%	7,95%	7,21%	9,17%	11,52%	19,87%	43,14%			
	1,92	8,43%	10,53%	8,69%	8,82%	9,52%	10,22%	9,07%	11,70%	18,91%	34,73%	55,17%			
	0,768	8,97%	10,84%	10,92%	10,89%	11,24%	10,44%	11,50%	14,13%	25,12%	49,70%	61,11%			
	0,3072	10,66%	10,99%	11,88%	12,70%	11,85%	11,93%	13,23%	15,86%	34,21%	56,16%	68,34%			
DMSO		35,84%	36,79%	37,90%	39,30%	38,52%	42,93%	38,11%	56,00%	97,98%	101,29%	100,00%			

		AZD1152													
		0,4	0,26	0,169	0,10985	0,071403	0,046412	0,030168	0,019609	0,012746	0,008285				
BMS-986158	30	0,008873	0,001816	0,005927	0,005231	0,006137	0,011233	0,001421	0,034628	0,084784	0,074653				
	12	0,062788	0,051192	0,061821	0,061926	0,054556	0,067432	0,056762	0,103375	0,214299	0,190733				
	4,8	0,07374	0,088199	0,08859	0,084144	0,093027	0,1057	0,092282	0,149837	0,30743	0,238197				
	1,92	0,113401	0,097617	0,122187	0,128597	0,117344	0,134639	0,119624	0,191999	0,351519	0,211556				
	0,768	0,129317	0,116381	0,122414	0,131232	0,123031	0,157956	0,117909	0,200875	0,347536	0,121903				
	0,3072	0,138363	0,141534	0,140153	0,141596	0,144737	0,174163	0,12819	0,224144	0,327501	0,130643				

B

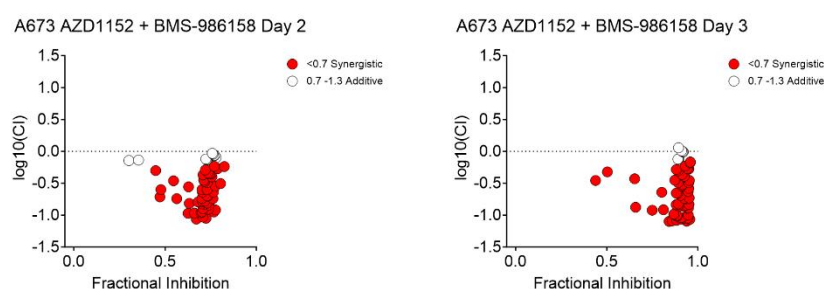
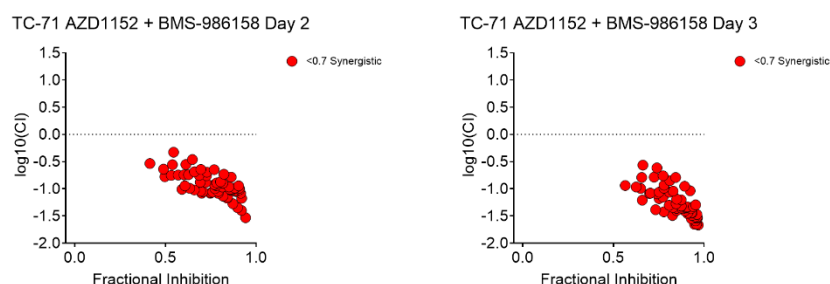


Figure IV. 21. Combination of AZD-1152 and BMS-986158 is potentially synergistic in A673 cell line. (A) Above, schematic representation of the average ratios of cell viability (expressed in percentage) compared to untreated control at day 2 after treatment. From left to right AZD-1152 effect is represented with a range of concentrations from 400 nmol/L to 8.3 mmol/L. From top to bottom BMS-986158 effect is represented with a range of concentrations from 30 μ mol/L to 0.31 μ mol/L. The obtained matrix contains every combination of each single drug in A673 cell line. Below, schematic representation of the Excess above Bliss based on the fractional inhibition of every compound. Disposition of the values follows same pattern than figure 16A. Cells in green show a positive result that correlates with synergism of the combination represented in A673 cell line. (B) Scatter plot of the $\log_{10}$ normalized combination index value versus fractional inhibition of viability for each treatment combinations of AZD-1152 and BMS-986158 at day 2 and day 3 post treatment in A673 cell line. Combinations with a CI < 0.7 (indicating synergy) were plotted in red, CI = 0.7–1.3 (indicating additivity) are plotted in white, and CI > 1.3 (indicating antagonism) are plotted in black.

We then moved to complete our findings and extended the study to test the combination of AZD-1152 and BMS-986158 in TC-71 and SK-ES-1 EwS cell lines. The experiments were conducted in the same way at the same range of concentrations, and CI was calculated following the same algorithm. Our results showed that CI was <0.7 for the vast majority of the doses tested in TC-71 cell line (Fig. IV. 22A) and for all of them in SK-ES-1 cell line (Fig. IV. 22B), at both day 2 and day 3 after treatment.

A



B

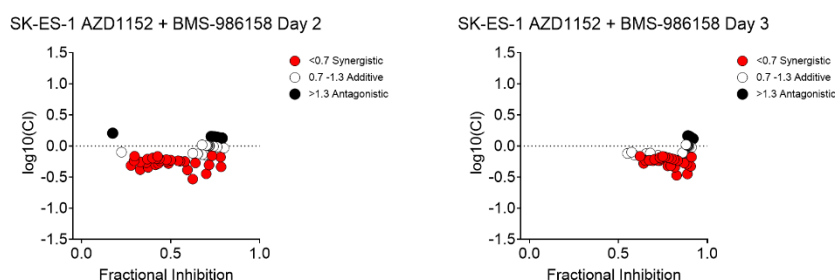


Figure IV. 22. Combination of AZD1152 and BMS-986158 is potentially synergistic in TC-71 and SK-ES-1 EwS cell lines. Scatter plot of the log₁₀ normalized combination index value versus fractional inhibition of viability for each treatment combinations of AZD-1152 and BMS-986158 at day 2 and day 3 post treatment in (A) TC-71 and (B) SK-ES-1 cell line. Combinations with a CI < 0.7 (indicating synergy) were plotted in red, CI = 0.7–1.3 (indicating additivity) are plotted in white, and CI > 1.3 (indicating antagonism) are plotted in black.

Finally, given the good results obtained for BMS-986158, we completed our screening with the BET inhibitors I-BET151 and JQ1 following the same workflow. We obtained a CI value for each combination of a wide range of drug doses. As previously observed, our results showed a potential synergistic effect on A673 and TC-71 EwS cell lines at day 2 and day 3, given by a CI < 0.7 (Fig. IV. 23A and IV. 23B). Results were less conclusive for SK-ES-1 cell line, with several CI between 0.7 and 1.3, showing an additive effect (Fig. IV. 23C). AZD-1152 combination with JQ1 resulted less promising, with combination indexes >0.7 or 1.3 (Fig. IV. 23D and IV. 23E), particularly in SK-ES-1 cell line (Fig. IV. 23F). Considering these screenings, we discarded JQ1 as a potential candidate to combine with AZD-1152, as well as SK-ES-1 cell line.

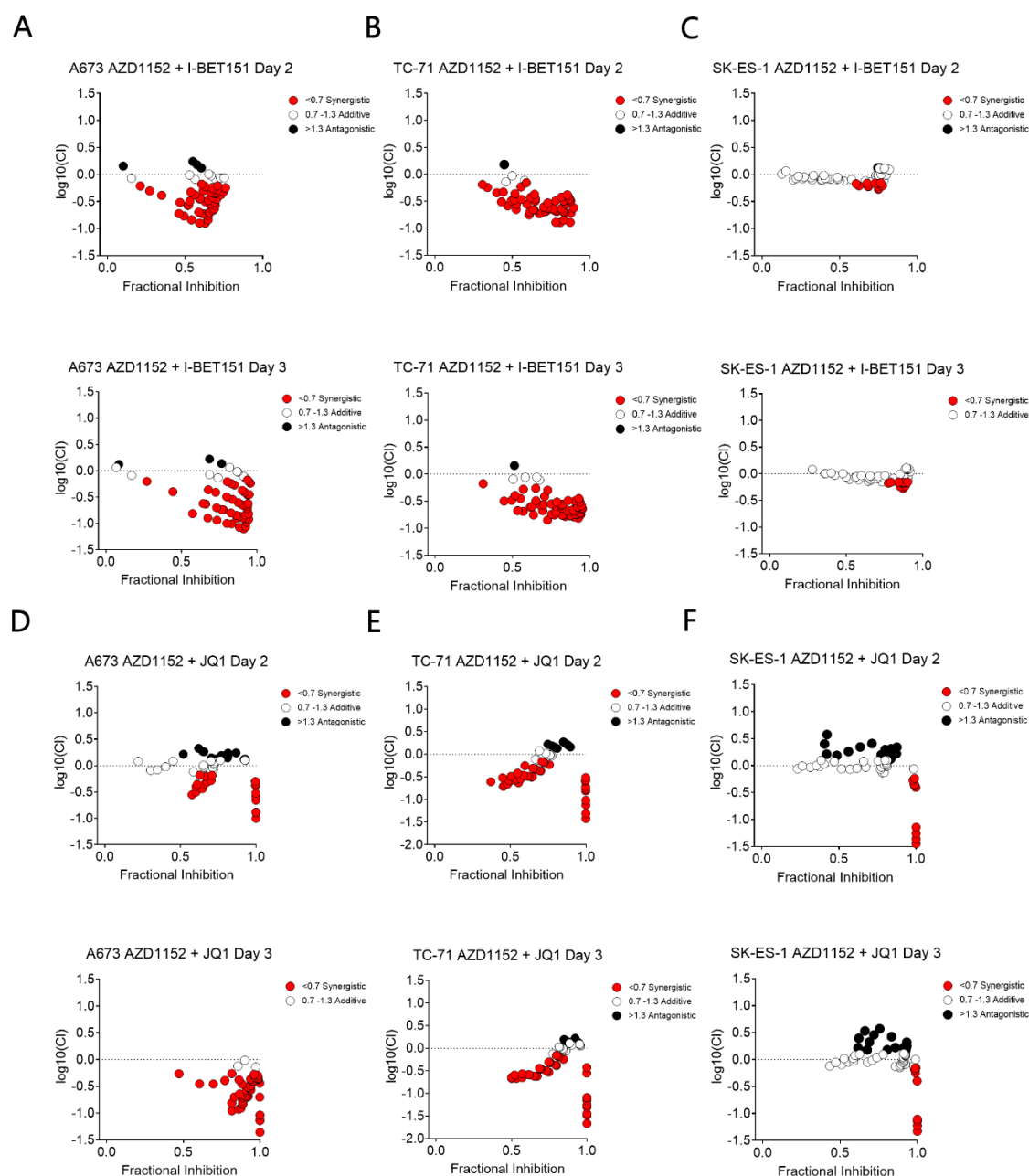


Figure IV. 23. Combination of AZD1152 and I-BET151 impairs viability in EwS cell lines. Scatter plot of the log₁₀ normalized combination index value versus fractional inhibition of viability for each treatment combinations of AZD-1152 and I-BET151 at day 2 and day 3 post treatment in (A) A673 (B) TC-71 and (C) SK-ES-1 and cell line. Scatter plot of the log₁₀ normalized combination index value versus fractional inhibition of viability for each treatment combinations of AZD-1152 and JQ1 at day 2 and day 3 post treatment in (D) A673 (E) TC-71 and (F) SK-ES-1 and cell line. Combinations with a CI < 0.7 (indicating synergy) were plotted in red, CI = 0.7–1.3 (indicating additivity) are plotted in white, and CI > 1.3 (indicating antagonism) are plotted in black.

To further elucidate the effect of the combination of AZD-1152 and BET inhibitors, cell death was analyzed by Annexin V staining. 48-hours post AZD-1152 and BMS-986158 treatment of A673 and TC-71 cell lines led to an increase in early apoptosis, compared to single drug (**Fig. IV. 24A** and **IV. 24B**). TC-71 cells were significantly more sensitive than A673 to both single drugs and the combination, in line with all our previous results. Very importantly, 293T cell viability was barely compromised with AZD-1152, BMS-986158 nor the combination, demonstrating the specificity of the treatment (**Fig. IV. 24C**). Viable, early and late apoptosis and necrosis cell percentages are plotted in bars in **Figure IV. 24D**. These findings confirm our previous results whereby the combination of AURKB inhibition with BET inhibition is synergistic in EwS cell lines. Analysis of cleaved PARP levels further demonstrated that the combination stimulated the apoptotic pathways at higher levels when EwS cell lines were treated with AURKB inhibitor and BMS-986158 (**Fig. IV. 24E**). 293T cells were also non-affected by the drugs.

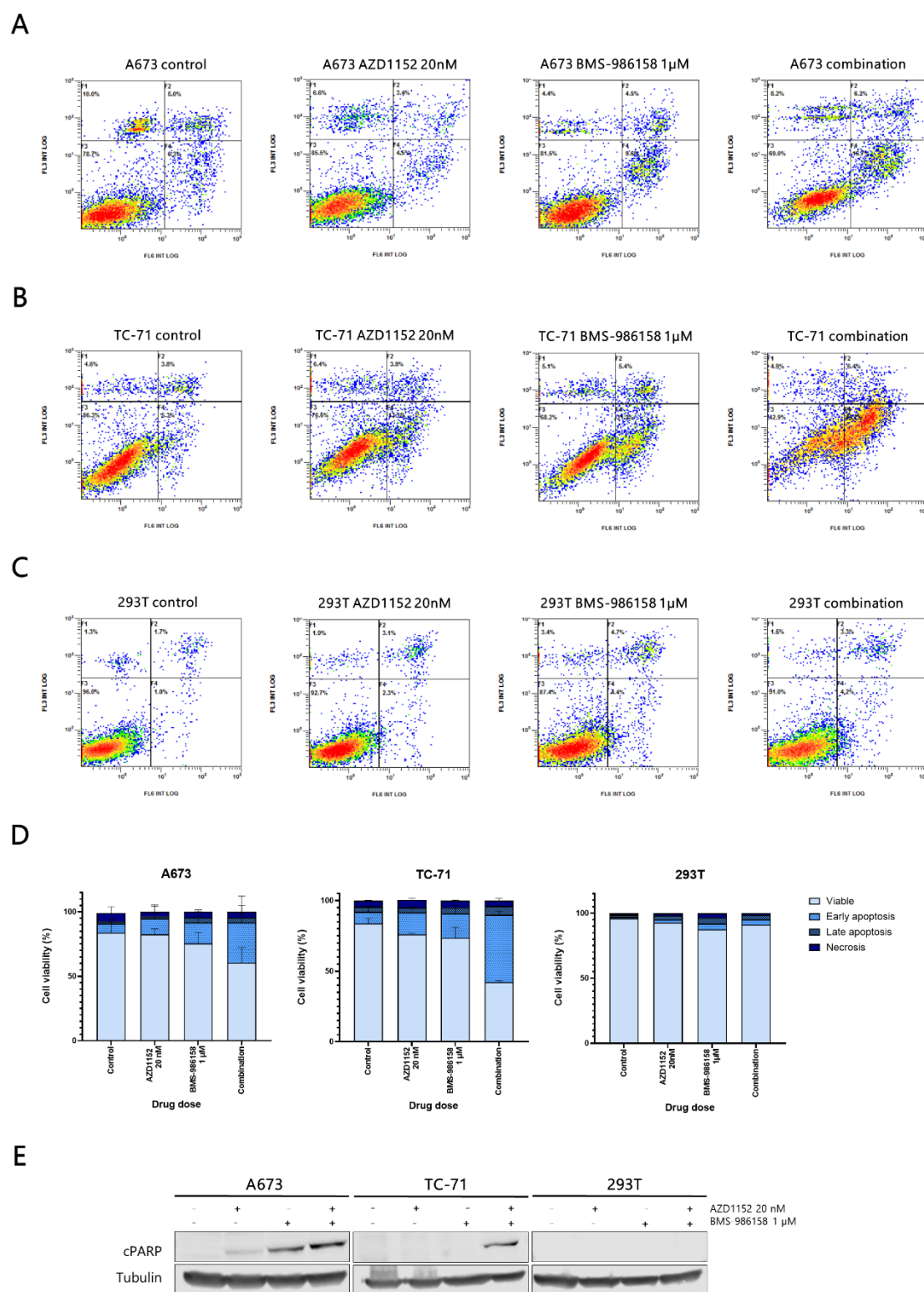


Figure IV. 24. Combination of AZD-1152 and BMS-986158 increase apoptosis in EwS cell lines. Dot-plots showing annexin V staining in (A) A673 (B) TC-71 and (C) 293T cell lines after 48 hours treatment with DMSO (control), AZD-1152 20 nM, BMS-986158 1 μ M and their combination. (D) Bar charts representing percentages of viable, early, and late apoptotic and necrotic cells analyzed by Annexin V staining by flow cytometry described in A-C. (E) Western Blot analysis of cPARP levels in A673, TC-71 and 293T cell lines

treated with AZD-1152 20 nM, BMS-986158 1 μ M and their combination for 48 hours. Tubulin was used as loading control.

We finally extended our *in vitro* results to the combination of AZD-1152 with the BET inhibitor I-BET151. We analyzed cell death using the same assay of Annexin V staining after 72 hours treatment with the two combination of the two drugs in A673, TC-71 and 293T cell lines (Fig. IV. 25A). We were not able to observe changes in the apoptotic cell populations when treating EwS cell lines with the drug combination versus each single agent (Fig. IV. 25A). 293T cells were not affected, demonstrating that the combination of AZD-1152 and I-BET151 was also selectively effective in EwS cell lines. Nevertheless, the analysis of cleaved PARP levels revealed that the combination stimulated the apoptotic pathways at higher levels when EwS cell lines were treated with AZD-1152 and I-BET151, compared to 293T control cells (Fig. IV. 25B). The discrepancies between synergy in IC₅₀ and Annexin V might be intrinsic to the different techniques.

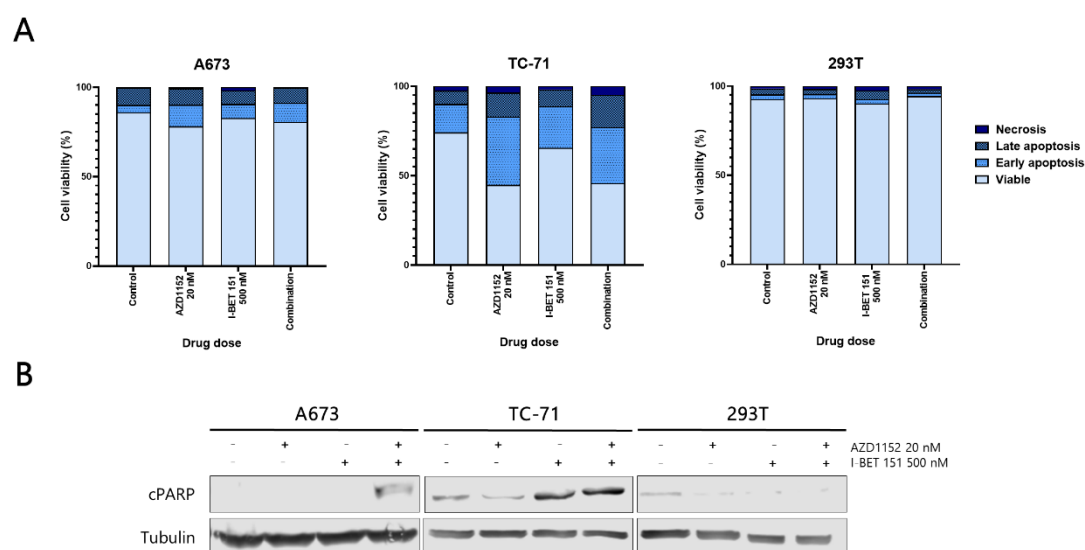


Figure IV. 25. Combination of AZD1152 and I-BET151 increase apoptosis in EwS cell lines. (A) Bar charts representing percentages of viable, early, and late apoptotic and necrotic cells analyzed by Annexin V staining by flow cytometry in TC-71, A4573 and 293T cell lines upon 72 hours treatment of 20 nM AZD1152, 500 nM I-BET151 and their combination. (B) Western Blot analysis of cPARP levels in TC-71, A4573 and 293T cell lines treated with AZD1152 20 nM, I-BET151 500 nM and their combination for 72 hours. Tubulin was used as loading control.

5.2. Combination of AZD-1152 and BMS-986158 is potentially synergistic in EwS *in vivo* models

Our results showing *in vitro* synergistic combination for AZD-1152 and BET inhibitors, prompted us to test this combinatorial treatment in EwS *in vivo* models. To this end, the most synergistic combination of AZD-1152 and BMS-986158 was translated to *in vivo* models. In brief, 1×10^6 TC-71 cells were injected per flank into athymic nude mice. When tumors reached a volume of 200 mm³, mice were randomly divided into 4 groups: vehicle, 25 mg/kg AZD-1152, 1 mg/kg BMS-986158 and the combination of both drugs. Mice were administered 5 days a week during a maximum of 5 weeks. At 35 days after injection, tumors treated with both the single agents and the combination were considerably smaller than those only treated with the vehicle (**Fig. IV. 26A**). Kaplan-Meier curves revealed that the combination treatment improved notably the survival of mice, highlighting the fact that all mice from this group were sacrificed while on treatment (**Fig. IV. 26B**). Despite further evidence needs to be provided, our results suggest the potential for the combination of AURKB inhibition and BET inhibition in EwS.

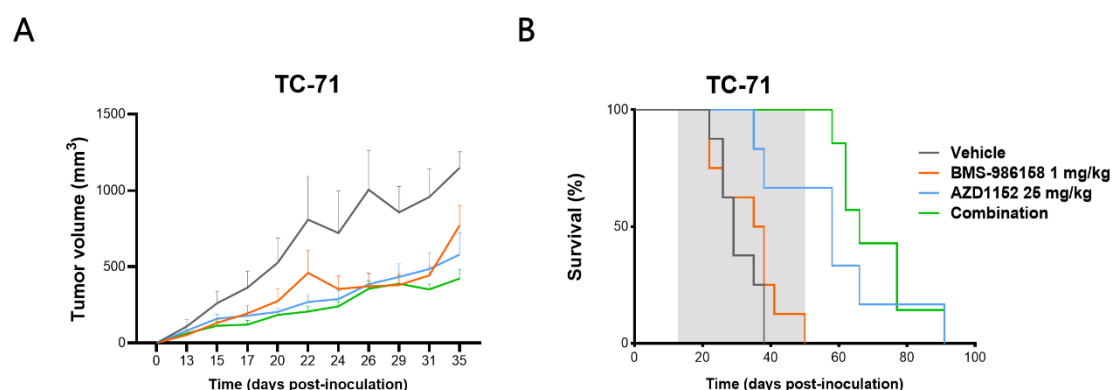


Figure IV. 26. Combination of AZD-1152 and BMS-986158 is potentially synergistic in EwS *in vivo* models. (A) Tumor volume curve in TC-71 subcutaneous xenografts in athymic nude mice treated with vehicle control (grey), 25 mg/kg per day of AZD1152 (blue), 1 mg/kg per day of BMS-986158 (orange) or the combination of the two drugs (green) at 35 days after injection. Mean $\pm$ SEM. (B) Kaplan-Meier xenograft survival curves for TC-71 tumors. Grey shadow depicts treatment time frame (37 days).

CHAPTER 2

6. Establishment and characterization of hpMSCs

EWSR1-FLI1 overexpression in adult hMSCs blocks their differentiation, and generates a transcriptome profile reminiscent of EwS (Riggi et al., 2008). Many of these EF-regulated genes are even more induced when the oncogene is expressed in pediatric hMSCs (hpMSCs), while numerous genes that are among the most prominent EwS markers are induced in hpMSCs but not in their adult counterparts (Riggi et al., 2010). Notably, although hpMSCs provide a far more permissive environment, hpMSCs that express EF (EF-hpMSCs) are unable to form tumors *in vivo*, which has led to the suggestion that a cooperating genetic event or a unique cellular phenotype is additionally required. Several clinical trials in EwS have consistently identified age as a prognostic feature, although the age cutoff has varied among studies. The GEIS-21 study was the first and only Spanish clinical trial in EwS including adults and children within the GEIS consortium. In this clinical trial it was confirmed in a multivariate analysis the importance of age (≥ 18 years cutoff) as an independent predictor of worse outcome, superior to the presence of metastasis (Mora et al., 2017).

Human MSCs (hMSCs) are multipotent precursors that can be differentiated *in vitro* into various cell types, typically osteoblasts, chondrocytes, and adipocytes, but also into endothelial lineage (Oswald et al., 2004). We have established 21 pediatric MSC from bone marrow of different age donors (Data summarized in **Fig. IV. 27A**), an extremely valuable resource for this and future studies. Models include lines derived from younger (pediatric) or older (adult) individuals including healthy donors (hpMSC-HD or hMSC-HD) and EwS patients (hpMSC-P or hMSC-P). Three of the 21 were from individuals 16-19 years of age and the rest were younger than 15 years. The lines from very young donors (1-5 years old) are extremely valuable since those models are not readily available in the scientific community. The older lines can be used to compare with EwS established lines since most of the commercial EwS lines come from old adolescents or young adults.

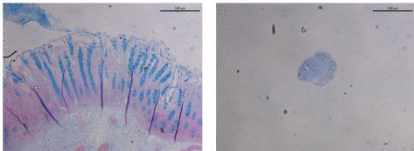
Once cells were isolated from bone marrow aspirates following established protocols, the MSC signature was evaluated considering their differentiation potential to chondrogenic, adipogenic and osteogenic lineages. Alcian Blue staining confirmed that isolated hpMSC have positively differentiated to chondrocytes, with positive control represented in **Figure IV. 27B**. hpMSC were also differentiated to adipocytes, as positive staining for Oil red was observed (**Fig. IV. 27C**). In the same way, with a buffered solution of BIP/NBT, we confirmed their osteogenic capacity (**Fig. IV. 27D**). To further validate our models, some candidates were also characterized using the Human Mesenchymal Stem Cell Verification Kit (n. FMC020 R&D Systems) by flow cytometry. hpMSC007 dot-plots showed that the hpMSCs are positive for their specific markers CD73, CD105 and CD90 as well as negative for their corresponding ones (CD45, CD34, CD11b, CD79A and HLA-DR) (**Fig. IV. 27E**). Positive isotypes are plotted below as a control of the experiment.

In order to further characterize our models, we performed RNA extraction followed by RT-qPCR to characterize the expression of some targets in a model of hpMSC derived from a patient <18Y, >18Y and a healthy donor (HD). mRNA analysis revealed that none of the hpMSCs expressed significant levels of the fusion gene, RING1B or EZH2, comparing with the SK-ES-1 EwS cell line as a reference (**Fig. IV. 27F**). We did not consider as significant the slight differences in the expression between the hpMSC models. Taken all together, the results obtained allowed us to verify the differentiation potential of isolated cells, confirming they are hpMSC.

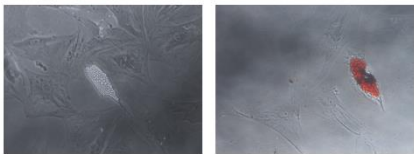
A

MODEL	AGE (Years)	GENDER	FUSION TYPE
hpMSC001	2Y	F	EWSR1-FLI1 type II
hpMSC002	7Y	M	FISH: EWSR1 rearrangement
hpMSC003	12Y	F	EWSR1-FLI1 type III
hpMSC004	12Y	M	(ex10-ex9) EwS-like
hpMSC005	13Y	M	Healthy
hpMSC006	5Y	F	EWSR1-ERG
hpMSC007	2Y	F	EWSR1-FLI1 type II
hpMSC008	-	-	Healthy
hpMSC009	10Y	F	EWSR1-FLI1 type III
hpMSC010	5Y	F	EWSR1-ERG
hpMSC011	17Y	F	EWSR1-ERG
hpMSC012	12Y	M	FISH: EWSR1 rearrangement
hpMSC013	-	M	EWSR1-FLI1 type III
hpMSC014	4Y	F	EWSR1-FLI1 type I
hpMSC015	19Y	M	EWSR1-FLI1 type II
hpMSC016	12Y	M	FISH: EWSR1 rearrangement
hpMSC017	10Y	M	EWSR1-FLI1 type I
hpMSC018	14Y	F	EWSR1-FLI1 type I
hpMSC019	2Y	F	
hpMSC020	16Y	F	FISH: EWSR1 rearrangement
hpMSC021	9Y	M	

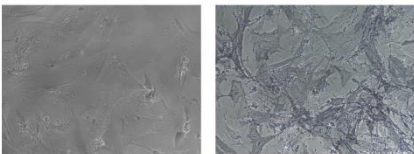
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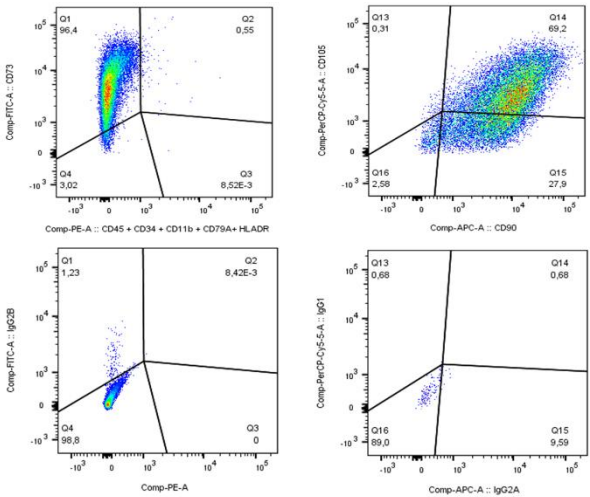
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D



E



F

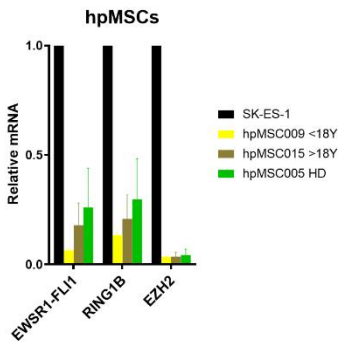


Figure IV. 27. Establishment and characterization of hpMSCs. (A) Table summarizing the 21 hpMSC models that were characterized. Age, gender and fusion type of the donor are shown. Highlighted in green the models that were used to perform the experiments contained in next sections. (B) In the right, microscope image of paraffin embedded tissue from one of our models of hpMSCs cultured with chondrogenic media and stained with Alcian blue, positive control image in the left. (C) In the right, phase-contrast images showing one of our models of hpMSCs after growing with their conditioned media and stained with Oil red revealing adipocytes, control image in the left. (D) Same as (C) showing alkaline phosphatase staining that evidence osteogenic lineage, control images in the left. (E) Dot-plot showing positive and negative markers staining in the model hpMSC007. (F) RT-qPCR of mRNA relative expression of EWSR1-FLI1, RING1B and EZH2 in hpMSC009, hpMSC015 and hpMSC005, compared to SK-ES-1 EwS cell line.

7. Genome-wide characterization of hpMSCs

Adult and pediatric human mesenchymal progenitor cells are permissive to EWSR1-FLI1 expression, although EF alone is insufficient to transform them (Riggi et al., 2008, 2010). Indeed, hpMSC expression patterns reproduce better those observed in EwS tumors than adults MSC upon EWSR1-FLI1 induction. However, there is no reported comparison between chromatin states generated by the oncogene in different types of mesenchymal cells. Furthermore, although 18 years cut off has been established as an important prognostic factor, age has not been correlated with the epigenetic background where the translocation occurs to explain its prognostic significance.

To evaluate the different chromatin state where the translocation might occur, we performed a genome wide of characteristic histone marks for active and repressed chromatin by analyzing the active enhancer mark H3K27ac and the poised promoter mark H3K27me3. First, ChIP-seq analysis was performed studying the distribution pattern of the H3K27me3 epigenetic mark in hpMSCs established from backgrounds below or above 18 years. Unfortunately, we were not able to obtain adequate profiles for all cases for this mark (models hpMSC005 and hpMSC015, HD and >18Y, respectively) and the protocol was optimized with a stronger lysis buffer containing higher percentages of SDS (1%). This technical problem might account for the condense state of the chromatin that this epigenetic mark recognizes. These data are now under analysis and should eventually complete Figure 28. Nevertheless, preliminary results revealed higher intensity levels of H3K27me3 peaks observed in hpMSC >18Y (model hpMSC020) in comparison with hpMSC019 (model for <18Y) (Fig. IV. 28A). This can also be

appreciated looking at HNF1B specific region in the genome (**Fig. IV. 28B**). Finally, the H3K27ac, a mark of active enhancers, was also evaluated by ChIP-seq. We could include here all the hpMSC models analyzed, included the HD. Interestingly, H3K27ac levels are lower in hpMSC >18Y (**Fig. IV. 28C**) in accordance with the increase of H3K27me3 observed. Looking at *CAV2* enhancer region, we can also observe a decrease in the H3K27ac peak in models hpMSC015 and hpMSC020, which come from to patients above 18 years (**Fig. IV. 28D**). However, the result in H3K27ac hpMSC005 (HD) suggests this could be not so general, making the ChIP-seq of H3K27me3 in this model necessary to be done. Although all the observations are currently further analyzed by ChIP-seq and ChIP-qPCR validation, our preliminary data suggest a more repressed chromatin state in those models that correspond to EwS patients above 18 years old.

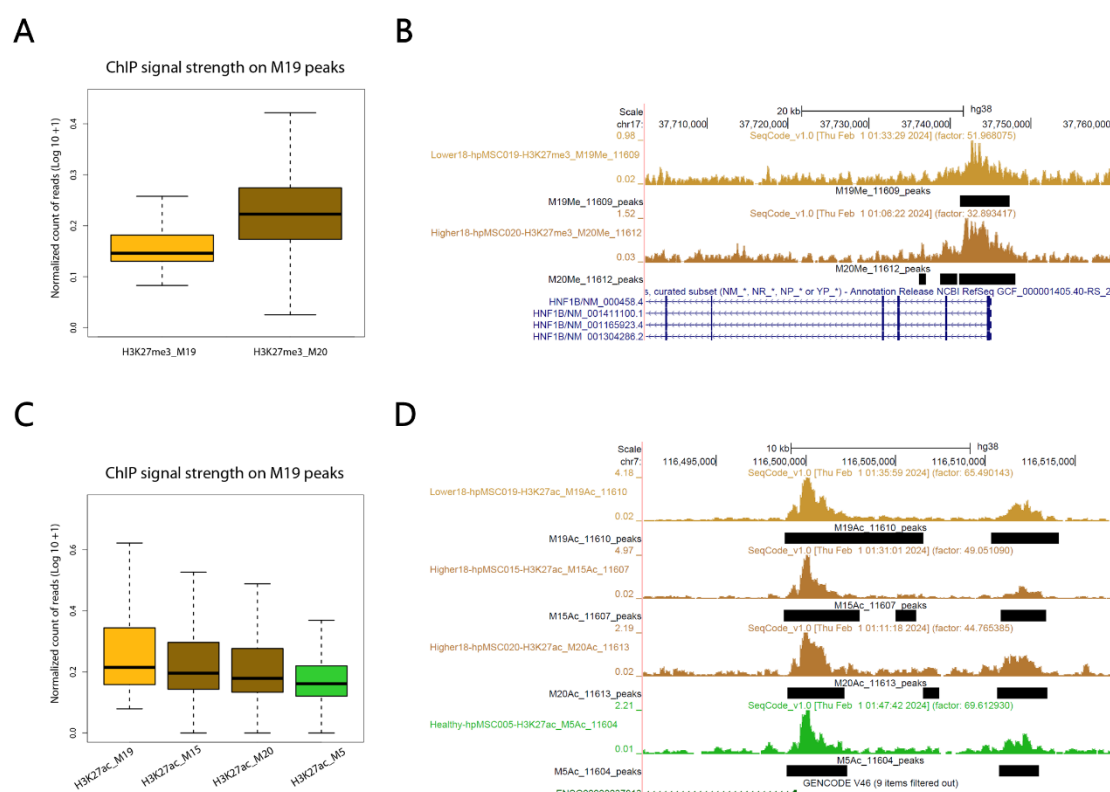


Figure IV. 28. Genome-wide characterization of hpMSCs. (A) Boxplot depicting the average H3K27me3 ChIP-seq signal in hpMSC0019 and hpMSC0020. (B) University of California Santa Cruz (UCSC) ChIP-seq signal tracks for H3K27me3 in hpMSC0019 and hpMSC0020 at HNF1B region. (C) Boxplot depicting the average H3K27ac ChIP-seq signal in hpMSC0019, hpMSC015, hpMSC0020 and hpMSC005. (D) University of California Santa Cruz (UCSC) ChIP-seq signal tracks for H3K27ac in hpMSC0019, hpMSC015, hpMSC0020 and hpMSC005 at CAV2 enhancer region.

To validate the epigenetic profile of our hpMSC models, we analyzed the enrichment of histone H3K27me3 and H3K27ac in an EwS defined set of promoters and enhancers. Different ChIP-qPCR experiments were performed in a model of hpMSC below 18 years old, an hpMSC-HD, and an hpMSC above 18 years. We evaluated the presence of H3K27me3 in **Figure IV. 29A** and H3K27ac in **Figure IV. 29B**. We observed minimal changes in both histone patterns when comparing each of the MSC models.

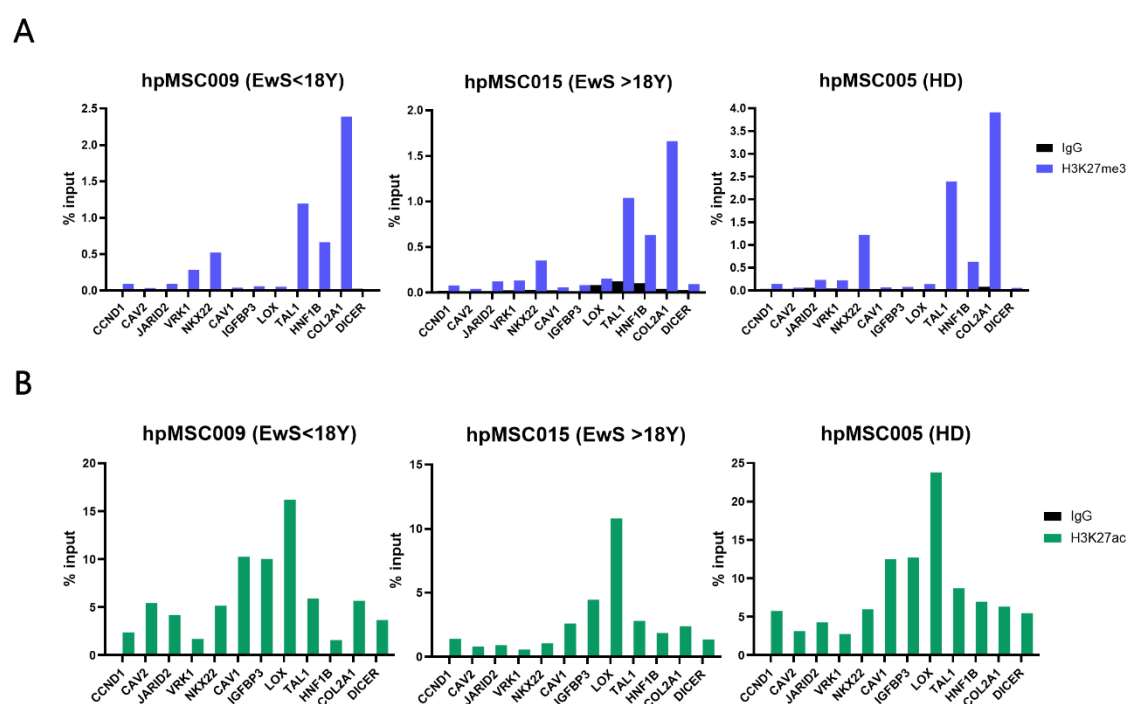


Figure IV. 29. Distribution of the H3K27me3 and H3K27ac histone marks at promoter and enhancer level in hpMSCs. (A) H3K27me3 and (B) H3K27ac ChIP-qPCR in three hpMSC cell lines corresponding to an <18Y EwS patient (left graph), a patient over 18Y (middle graph) and a healthy donor (right graph). The values of the Y-axis represent the enrichment ratio of immunoprecipitated samples relative to input. COL2A1 primers were used as a positive control region of an EwS target gene and DICER as the negative one.

V. DISCUSSION

Ewing sarcoma (EwS) is a disease that was firstly identified more than 100 years ago (Ewing, 1921). Since then, a lot of efforts have been done to approach in an integrative mode this disease.

Having a deep knowledge of EwS biology and tumorigenic mechanisms will be the key to find targeted therapies that overcome the relapses after current multi-drug systemic therapy. Due to the low mutational rate of EwS, epigenetic-based therapies offer a promising approach for treating this aggressive tumor by reversing the epigenomic changes caused by the fusion gene.

Regarding chromatin remodeling infringed by the oncogene, in which enhancers are an Achilles Heel for the tumor, our group added a crucial piece to the puzzle demonstrating that the Polycomb protein RING1B has an activator role in EwS recruiting EWSR1-FLI1 to enhancers containing GGAA repeats and activating key genes necessary for EwS tumorigenesis (Sánchez-Molina et al., 2020). However, since the classical inhibitors of RING1B, targeting E3 ubiquitin ligase activity, as PRT4165 (Ismail et al., 2013), are ineffective in EwS, we proposed the use of AZD-1152 (an AURKB inhibitor) as a plausible strategy to impair RING1B activity at superenhancers.

Although AURKB has been described an EWSR1-FLI1 target and a targetable dependency (Wakahara et al., 2008; S. Wang et al., 2019; Winter et al., 2011), the precise molecular function of this kinase remains uncovered. For these reasons, we aimed to decipher AURKB mechanisms involved in the epigenetics of EwS.

Consistently, our work reveals for the first time the role of AURKB at enhancers in EwS, establishes the basis for this protein as a modulator of RING1B activity and proposes a specific therapeutic strategy combining AZD-1152 with BET inhibitors, considered as superenhancers disruptors. The work presented in this thesis tried to encompass from fundamental aspects of the biology of this disease, including the study of the epigenetic background in the cell of origin, until a more translational approach that could be benefit to EwS patients.

1. Targeting AURKB in EwS cell lines

AURKB is overexpressed in multiple cancer types, including Ewing sarcoma (EwS) (Riggi et al., 2014) (S. Wang et al., 2019). This overexpression suggests that this may play a significant role in the progression of these malignancies. Previous data from our lab described RING1B colocalizing at active superenhancers with EWSR1-FLI1. As RING1B phosphorylation was previously described to inhibit RING1B E3 ubiquitin ligase activity, we performed a comparative expression analysis based on public databases between the three kinases previously described to modified RING1B phosphorylation status: AURKB, MEK1 and CK2. Although CK2 and MEK1 kinases have been described to develop important roles in EwS (Lagares-Tena et al., 2016; Nagulapally et al., 2024) their expression levels were not as high as AURKB (**Figure IV. 1** in results).

In concordance with the high expression of AURKB observed in our analysis, is previous data (Wakahara et al., 2008) and our own results show AURKB as a direct target of EWSR1-FLI1 in A673 and HeLa cells overexpressing the oncogene through direct interaction with their promoters (**Figure IV. 2** in results). In Burkitt's lymphoma cell lines, a positive regulatory region was identified between -74 and -104 upstream of the TSS in the AURKB promoter (Mori et al., 2011). In line with these findings, we demonstrated that inducible knockdown of EWSR1-FLI1 leads to a decrease in AURKB expression and protein levels in the A673 cell line (**Figure IV. 3A** and **IV. B** in results). Furthermore, the increased AURKB expression observed in the HeLa cell line upon oncogene induction (García-Domínguez et al., 2020) further supports the direct regulation of AURKB by EWSR1-FLI1 (**Figure IV. 3D** and **IV. E** in results).

Given that EwS mainly relies on the EWSR1-FLI1 translocation as the main genetic alteration (Riggi & Stamenkovic, 2007), an obvious therapeutic approach would be directly targeting this fusion protein. Indeed, strategies like antisense DNA, siRNA and dominant-negative approaches targeting EWSR1-FLI1 have shown significant impairment in EwS cell proliferation (Toub et al., 2006). However, the absence of specific enzymatic activity in transcription factors, along with the intrinsic disorder of certain domains that complicates structural determination, presents a significant challenge for

designing targeted pharmacological inhibitors (Darnell, 2002). As the indirect inhibition of oncogene activity by targeting downstream molecules has shown promising results in preclinical models, we hypothesized that inhibition of AURKB could be promising. Specific AURKB inhibitor AZD-1152 has been widely described as an anticancer drug across different tumors at nanomolar concentration (Löwenberg et al., 2011; Wilkinson et al., 2007). EwS cell lines and tumors are particularly and selectively sensitive to this inhibitor, that causes cell cycle impairment and apoptosis. Cell viability analysis in our cell lines confirmed the effectiveness of AURKB inhibitor across a range of nanomolar concentrations causing apoptosis (see **Figure IV. 2** in results). It is important to highlight the different sensitivity among EwS cell lines that were tested, with A673 cell lines being the most resistant. TP53 mutations are hypothesized to play a relevant role in treatment resistance (Crompton et al., 2014; Huang et al., 2005). TP53 status of each cell line must be then take into consideration as an explanation of the different AZD-1152 response observed. Aurora kinase inhibition primarily induces apoptosis in TP53-wildtype cancer cell lines while inducing cell-cycle arrest and a more delayed induction of apoptosis in TP53-mutated cancer cell lines (Ikezoe et al., 2010; Nair et al., 2012). In line with this, Wang et al. described 25% of apoptosis in p53-mutated cell line and 40% in case of wild type cells. Both A673 and TC-71 has mutant status for p53, whereas SK-ES-1 is p53 wild type. This is in concordance with our cell viability results, since we observed a higher apoptotic effect in SK-ES-1 cell lines compared to A673 and TC-71. It was also extremely valuable that control cells were unaffected, indicating the specificity of this drug for EwS cell lines. In agreement with our results, during the consecution of this thesis the synergy between AZD-1152 and FAK inhibitors was described in EwS. Besides the repurposing of our aims, the effectivity of AZD-1152 in EwS in vivo models and the dependency of AURKB were described. Very importantly, the downregulation of EWSR1-FLI1 expression decreases the dependency on AURKB, pointing to the oncogene for sensitization of cells to AURKB inhibition (S. Wang et al., 2019).

2. AURKB is enriched at active enhancers in A673 cell line

Many pediatric sarcomas are driven by transcription factor (TF) fusion proteins, resulting in oncogenic fusion genes. These cancers often feature a common dysregulated epigenetic axis that generates new super-enhancers, reshaping the transcriptional landscape of the affected cells. For example, in alveolar rhabdomyosarcoma (RMS), the PAX3-FOXO1 fusion protein drives chromatin remodeling by recruiting co-factors like p300 and BRD4 to super-enhancers (Gryder et al., 2017). These enhancers are marked by active histone modifications, such as H3K27ac, which coincide with the fusion protein binding sites. In synovial sarcoma (SS) the SS18-SSX fusion interacts with HDAC and components of the SWI/SNF complex (e.g., hBRM, hBRG1), leading to aberrant transcriptional activation (Kadoch & Crabtree, 2013; Nagai et al., 2001; Thaete et al., 1999). Similarly, in EwS, EWSR1-FLI1 recruits epigenetic regulators like p300, WDR5 from the MLL complex, and SWI/SNF's BAF subunit to GGAA repeats where the fusion oncoprotein binds (Boulay et al., 2017; Riggi et al., 2014). These regions also show strong enrichment of active histone marks, such as H3K27ac (Riggi et al., 2014; Tomazou et al., 2015). Furthermore, our group recently demonstrated that RING1B colocalizes with the oncogene and cooperates in gene activation at both active promoter and enhancer levels (Sánchez-Molina et al., 2020).

In our work for the first time AURKB is reported to be localized at active enhancers. As these regions are considered the Achilles Heel of EwS, this finding is key for our goal of rewiring the EwS epigenome. Notably, it was surprising to observe that the percentage of AURKB peaks at these enhancers was comparable to that of the oncogene, with an even stronger signal of the active enhancer histone mark H3K27ac (**Figures IV. 4C and IV. 5A** in results). The distribution of AURKB peaks within EWSR1-FLI1 and RING1B peaks suggests that enhancers enriched for AURKB are common to all three proteins, suggesting their colocalization (**Figure IV. 5B** in results). This evidence provides a strong rationale for exploring the mechanistic relationship between these proteins, which may help explain the potent effect of AZD-1152 at low nanomolar doses, beyond its role in cell cycle disruption. Furthermore, additional experiments might explore the possibility

of AURKB being in the superenhancers of other translocation as PAX-FOXO3 or SS18-SSX.

During the past 15 years, an important effort was made to identify a core set of EWSR1-FLI1 target genes, integrating ChIP-seq and RNA-seq data (Bilke et al., 2013; Riggi et al., 2014; Tomazou et al., 2015). Our results show that AURKB peaks were associated to a set of 603 genes, which expression was the highest among the A673 database defined by Riggi et al. This result supports AURKB colocalization with EWSR1-FLI1 peaks. Likewise, expression analysis of these genes confirmed that they are EWSR1-FLI1 up-regulated genes (**Figure IV. 6A** and **IV. 6B** in results). We then performed the triple intersection between EWSR1-FLI1, RING1B and AURKB peaks and confirmed their colocalization at around 86% of AURKB peaks (**Figure IV. 7A** and **Figure IV. 8A** and **IV. 8B** in results). Our results are in concordance with published genome wide AURKB ChIP-seq data in quiescent lymphocytes, showing enrichment in chromatin at active promoter level, in collaboration with RING1B. Nevertheless, in this context the presence at enhancer was not observed.

Notably, a higher proportion of EWSR1-FLI1 peaks overlap with AURKB compared to RING1B (737 in sub-fraction 3 vs. 288 in sub-fraction 2). This suggests that the oncogene may have a more prominent regulatory role on AURKB. Low intersection between AURKB and RING1B peaks also suggests that AURKB would be exclusively at active regions, while RING1B is also present at repressed regions, as previously described by our group. These results are supported by the AURKB containing fractions having the higher levels of H3K27ac and RING1B, with or without the oncogene, presenting H3K27me3. (**Figure IV. 8C** in results).

Finally, de novo motif analysis revealed that EWSR1-FLI1-specific sites contained predominantly ($P < 10^{-390}$) one single occurrence of the canonical ETS motif GGAA, while when associated with RING1B and AURKB, we observed a significant enrichment for multimeric GGAA repeats ($P < 10^{-1087}$) (**Figure IV. 8B** in results) (Gangwal et al., 2008, Riggi et al., 2014). These results corroborate the activator functions of RING1B and AURKB in the chromatin, as these repeats have been associated to very active superenhancers.

Single motif that we find associated to sub-fraction 2 and 5 are associated to EWSR1-FLI1 repressed binding sites (Riggi et al., 2014). The fact that AURKB is also recruited to GGAA repeats, even when it is not colocalizing with EWSR1-FLI1, could be explained due to technical issues, as sub-fraction 7 also contained EWSR1-FLI1 common peaks that were responsible to this motif enrichment. In the same sense, sub-fraction 2 might represent a different subset of RING1B and EWSR1-FLI1 common peaks than our group described in Sánchez-Molina et al., since this fraction would be in the triple intersection with AURKB peaks.

AURKB is a member of the serine/threonine family of Aurora kinases, together with Aurora kinase A and C (Carmena & Earnshaw, 2003). Our analysis of AURKA peaks showed no significant enrichment at any sub-fraction obtained from the triple intersection (**Figure IV. 8D** in results), while defined EWSR1-FLI1/RING1B target genes, are not enriched for this kinase (**Figure IV. 10B** in results) demonstrating that AURKB enrichment at enhancers is a specific feature not common for all the family. In this sense it has been widely described the non overlapping functions of these two proteins (Carmena & Earnshaw, 2003). Although further characterization of AURKA localization genome-wide and intersection with AURKB/EWSR1-FLI1/RING1B binding regions will confirm our data, the AURKB enrichment in chromatin mainly correlates with its enzymatic activity on H3S28 phosphorylation.

RING1B is an E3 ubiquitin ligase that monoubiquitinates histone H2A at lysine 119, which is ligated to its role in Polycomb silencing (H. Wang et al., 2004). AURKB has been described to indirectly inhibit PRC1-mediated ubiquitination of histone H2A while promoting deubiquitination by USP16, thereby maintaining transcription in resting lymphocytes (Frangini et al., 2013). Our results show AURKB and H2Aub peaks do not colocalize, whereas there is an overlap between H2Aub and RING1B (**Figure IV. 9A** in results). Due to RING1B retention of its repressive role

Although we observed elevated H2Aub protein levels following AZD-1152 treatment, along with greater enrichment at enhancers such as *CCND1* and *CAV1* (**Figure IV. 18A** and **C** in results) we did not detect significant genome-wide differences in H2Aub

enrichment (**Figure IV. 18B** in results). Of note, one of our most regulated genes is CCND1, being these data in concordance with the role of AURKB regulating this CCND1 in gastric tumors (Nie et al., 2020).

Although drastic changes were not expected given the indirect nature of the mechanism we are investigating, technical factors, such as the time point selection and the absence of a spike-in control for the ChIP experiment, may have contributed to these limitations. These results pushed us to investigate more direct mechanism of RING1B regulation as the direct phosphorylation by AURKB.

3. AURKB recruitment by EWSR1-FLI1

Identifying a permissive cell line that can tolerate the EWSR1-FLI1 translocation is essential for studying the oncogene molecular functions in Ewing Sarcoma. Upon induction of the oncogene in the permissive model generated by García-Domínguez in HeLa Cells, EWSR1-FLI1 is recruited to the chromatin, resulting in the detection of numerous EWSR1-FLI1 peaks enriched in GGAA repeat sequences. These data validates genome wide the model and allow us to confirm the presence of this kinase in the chromatin of other cell lines. Nevertheless, the presence of AURKB prior to EWSR1-FLI1 induction, does not reflect priority of AURKB in the recruitment as these positions are not the typical targets of the oncogene. Most importantly, although AURKB levels did not increase following EWSR1-FLI1 induction in the HeLa cell line (**Figure IV. 12B** in results), we observe relocalization with higher overlap of AURKB with EWSR1-FLI1 peaks in the induced condition. In this context, we first hypothesize that EWSR1-FLI1 might recruit AURKB to these sites, further contributing to the epigenetic changes driving tumor progression. Nevertheless, CoIP of EWSR1-FLI1 and AURKB did not work; for this reason, another plausible scenario might be the possibility of EWSR1 wild type recruiting AURKB to the repeats. It has been described that EWSR1 interacts with AURKB and collaborates in AURKB recruitment to the midzone in A673 and HeLa cell lines. Furthermore, EWSR1 colocalizes with EWSR1-FLI1 at GGAA repeats, supporting our hypothesis. (Park et al., 2014; Boulay et al., 2017) Indeed, EWSR1 has been describe to interact with AURKB and EWSR1-FLI1 has been reported to interfere with AURKB and EWSR1 functions during the

cell cycle, contributing to chromosomal instability (Park et al., 2014, 2021). Further genome-wide experiments downregulating EWSR1-FLI1 by knockdown and performing AURKB immunoprecipitation will be decisive to describe in what extent is the oncogene responsible for AURKB recruitment and binding to the chromatin.

4. AURKB downregulation and inhibition in Ewing sarcoma

The sequencing analysis of our CRISPR clones revealed 100% identical sequence than the control (**Figure IV. 13D** in results), while protein levels were decreased. Although a possible explanation could imply technical deficiencies during the sequencing protocol, with misinterpreted results when sequencing is not clean, being AURKB an essential gene, the impossibility to generate a KO is very feasible. In agreement, AURKB is considered an essential gene in several tumors, showing one of the highest dependency in EwS cell lines based on Public 24Q2+Score database (Tsherniak et al., 2017) (**Figure IV. 13A** in results). Our results showed a reduction in EWSR1-FLI1 expression levels when silencing AURKB. This unexpected downregulation could contribute to the switch off of EWSR1-FLI1 reprogramming. Nevertheless, evaluation of EWSR1 promoter did not retrieved any enrichment for AURKB suggesting indirect mechanism. As EwS cell lines are highly dependent on the oncogene, this downregulation could reinforce the high dependence of AURKB. Nevertheless, further analysis would be necessary to understand the mechanism behind.

We observed an important set of EWSR1-FLI1/RING1B target genes repressed by AURKB downregulation both by siRNA and shRNA (**Figure IV. 14A and IV. 14B** in results). NR0B1, *CAV1*, *NKX2-2* and *SOX2* are key players in EwS tumorigenesis (Kinsey et al., 2006; Riggi et al., 2010; R. Smith et al., 2006; Tirado et al., 2006). In the same sense, we observed that AZD-1152 treatment also downregulated the vast majority of EWSR1-FLI1/RING1B targets, reproducing the effect of AURKB knockdown. Genome-wide approach (RNA-seq undergoing) will help us to further elucidate the role of AURKB modulation in the chromatin reprogramming of EwS.

The higher percentage of downregulated genes for both A673 and TC-71 cells supported the activator role of AURKB that we hypothesized. Among the top downregulated genes,

we identified PRAME, BIRC5 (survivin) and CDC20 in A673 cell line, all of them described as important regulators of EwS program (Carmena, Wheelock, et al., 2012; De Hooge et al., 2005; R. Zhao et al., 2022). Indeed, CDC20 and survivin, this last being part of the chromosome passenger complex (CPC), directly interact with AURKB (Figure V. 1). In TC-71, Wnt/ β -catenin pathway members are downregulated such as ROR1, SMAD5, WNT5A, in agreement with their important role in EwS (Hawkins et al., 2020; Potratz et al., 2016).

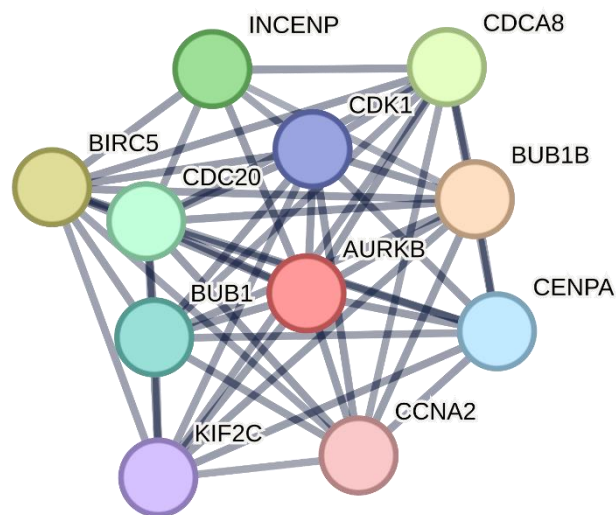


Figure V. 1. AURKB interactors.

5. Underlying AURKB's role in Ewing sarcoma

AURKB deregulation has been observed in cancer and is strongly associated with increased genetic instability. This suggests that misregulation of AURKB could lead to errors in the segregation of epigenetically marked chromatin, especially in multipotent tumor progenitors, thereby contributing to tumorigenesis (S. L. Smith et al., 2005). However, the extent to which epigenetic profiling can help predict and interpret chemical sensitivities, such as responses to AURKB inhibitors, remains unclear. Understanding these mechanisms could provide valuable insights into the role of AURKB in cancer progression and therapeutic resistance.

H3K27ac enrichment, associated to active enhancers and superenhancers, is decreased upon knockdown of the RMS fusion gene (Gryder et al., 2017). In neuroblastoma and

EwS, super enhancer-associated targets represent key vulnerabilities targetable with epigenetic disruptors such BET inhibitors (Gollavilli et al., 2018; Puissant et al., 2013). While a global decrease is observed in TC-71 cell line, for H3K27ac H2Aub is being maintained. (**Figure IV. 18** in results). While H3K27ac drop supports less transcription that correlates with genome wide expression changes, our data discard a recovery of H2Aub in positions where AURKB and the oncogene are bound. One possibility is the fact that histone marks associated with repressed chromatin might need more time to be deposited. Nevertheless, less defined profiles are obtained in the case of H2Aub, which are difficult to improve due to the lack of better antibodies. Besides, in order to quantify changes in intensity new experiments with addition of spike-in normalizations are being performed for both antibodies.

Our group has identified an activating role for RING1B in chromatin remodeling in EwS, where it aids in the recruitment of the fusion protein EWSR1-FLI1, thereby contributing to the development of the tumor (Sánchez-Molina et al., 2020). This shift from the traditional repressive function of the Polycomb Repressive Complex 1 (PRC1) to an activator role is thought to be linked either to changes in the composition of PRC1 subunits (Creppe et al., 2014) or to phosphorylation of its catalytic subunit, which diminishes its enzymatic activity (Gao et al., 2014; Rai et al., 2015; Frangini et al., 2013). Other kinases such as CK2 and MEK-1 phosphorylate RING1B and contribute to gene activation of transcription. The decrease in RING1Bph (at S168 position) levels upon AZD-1152 treatment supports, a direct mechanism of regulation that involved AURKB phosphorylating RING1B in the activating context of EwS chromatin remodeling (**Figure IV. 19A** in results).

Interestingly, AURKB is required for phosphorylation of H3S28 at promoters of active genes in resting B cells, being H3S28ph the predominant mark for active promoters in these cells. They described by ChIP analysis that there is a decrease in the enrichment of this mark at active genes upon AURKB KO (Frangini et al., 2013). In this context, H3S28ph has been associated with transcriptional activation and seems to occur independently of H3S10ph. H3S28ph has previously been shown to influence, and be influenced by, the dynamics of surrounding histone PTMs, especially the H3K27ac (J. Li et al., 2021). In

agreement, our results did reproduce these observations, since the enrichment of H3S28ph decreases at EWSR1-FLI1/RING1B activated targets upon AURKB inhibition with AZD-1152 (**Figure IV. 19 B** in results). A genome-wide analysis of the distribution of these chromatin marks following AZD-1152 treatment will provide valuable insights into the underlying mechanisms in the context of EwS.

Strategies that provides a better understanding of the underlying EwS biology allows for the discovery of novel agents with disease-relevant activity (Winter et al., 2011). Our proteomics analysis pursue two different aims: first, to characterize RING1B modifications upon AURKB inhibition with AZD-1152, such as phosphorylation changes and second, to evaluate possible changes in RING1B interactome upon drug incubation. Unfortunately, we were not able to detect changes between control and treated conditions at the level of phosphorylation due to technical issues like the peptides' size obtained after enzyme digestion.

However, we described RING1B protein-protein interactors in EwS. In that sense, our proteomics results provided valuable information characterizing RING1B interactome in EwS. Intriguingly, it has been described that phosphorylation of H3 S28 displaces binding of Polycomb group proteins and reduces the amount of H3K27me3 (Drobic et al., 2010; Lau & Cheung, 2011). As RING1B is present in active enhancers where S28 is phosphorylated, differential complex conformation for PRC1 should be necessary at these genomic positions. In this sense, proteomic data suggests the presence of the ncPRC1 complex containing RYBP. Besides PRC1.1 members, we identified MLL complex with important interactors such KDM6A (Figuerola-Bou et al., 2023) and several members of the Mediator complex as the most numerous cluster. We have described AURKB and RING1B colocalizing at the same positions genome-wide, together with EWSR1-FLI1 (**Figure IV. 7** in results). Proteomics data provides complementary information but does not exclude AURKB and RING1B being part of a big complex. As already suggested this complex might contain EWSR1, which has been described to interact with AURKB and to be localized at GGAA repeats. Indeed, although in a not significant way, we obtain peptides for this protein in our proteomic data. Plk1 is a critical mitotic kinase during the cell cycle is described to interact with AURKB, forming an activating phosphorylation loop

(Carmena, Pinson, et al., 2012). Interestingly, RING1B also colocalized with Plk1 at mitotic chromosomes in the prometaphase and metaphase (An et al., 2018). All this data might suggest common functions of AURKB and RING1B during mitosis.

Finally, among RING1B interactors we identified BRD4, a chromatin reader protein that recognizes and binds acetylated histones. Previous reports also observed a strong co-occupancy of BRD4 and cPRC1 at active genes and proposed that both epigenetic machineries are co-recruited to enhancer regions in breast cancer cells (Chan et al., 2018). This evidence supported our proposed synergy studies with BET inhibitors and AURKB inhibitor AZD-1152.

6. Synergistic combination of AZD-1152 and BET inhibitors

Although our understanding of the oncogenic mechanisms underlying Ewing sarcoma has advanced, there is still a notable lack of novel candidate drug targets for treating the disease. Therapeutic targeting of chromatin modifier proteins in cancer has been challenging due to the ubiquitous role these proteins play in normal healthy cells (Perry et al., 2019). To identify potential drug combinations for EwS we performed a screening of BET inhibitors combined with the AURKB inhibitor AZD-1152. We found that two of the three BET inhibitors tested demonstrated synergistic anti-Ewing activity when combined with AURKB inhibition. AURKB inhibitor AZD-1152 has demonstrated high efficacy alone in EwS cell lines (Winter et al., 2011; Teicher et al., 2015; Sánchez-Molina et al., 2020) and its combination with FAK inhibition resulted synergic against preclinical EwS models (S. Wang et al., 2019).

BET inhibitors, which act as mimetic compounds that selectively bind with high affinity to the acetyl-lysine binding pockets of the bromodomains of BET proteins, displace BRD4 from super-enhancer regions. Due to the vital role that BET proteins play in cancer development, pharmacological inhibition of bromodomains has demonstrated significant preclinical antitumor effects in various solid tumors and blood cancers (Z. Q. Wang et al., 2023). Indeed, the combination of BET inhibitors and AURKA inhibitors is highly synergistic in MYCN-amplified neuroblastoma cell lines (Yi et al., 2021).

Furthermore, combination of I-BET151 and the AURKA inhibitor Alisertib improved antitumor efficacy against EwS tumor xenografts (Tomino et al., 2019).

From the three BET inhibitors analyzed, obtained results with BMS-968158 were powerful enough in the three EwS cell lines tested, even at nanomolar concentrations, to proceed with an *in vivo* experiment. The initial *in vivo* assessment of the optimal combination we identified: AZD-1152 and BMS-986158, has not been previously proposed in the literature. Indeed, research on this BET inhibitor remains very limited in the existing literature. Notably, BMS-986158 is currently in the first dedicated pediatric trial of a BET inhibitor (NCT03936465) (Pearson et al., 2021). The combination treatment of AZD-1152 and BMS-986158 significantly inhibited tumor growth compared to each monotherapy, suggesting a potential synergistic interaction between the two drugs (**Figure IV. 26A** in results). They could be acting through complementary mechanisms, AZD-1152 may work by arresting cells in mitosis, while BMS-986158 could be modulating epigenetic pathways, enhancing the overall therapeutic effect. We hypothesize that these two epigenetic drugs could be inhibiting the same complex, thus is, same set of superenhancers, so the activation by these proteins would be completely affected. Another possibility is that AURKB and BRD4 regulate distinct superenhancer structures, that resulted compromised after the combination inhibition. This combination also provided a survival advantage, significantly outperforming monotherapy and control groups, a critical endpoint for clinical relevance (**Figure IV. 26B** in results).

Importantly, the combination therapy appeared to be well-tolerated, as suggested by the extended survival without signs of rapid toxicity. This points to the potential for using drug synergy to lower individual drug doses, thereby reducing side effects while maintaining efficacy. One of the possibilities that would improved the formulation of AZD1152, is the possibility to produce nanoparticles with this drug. Indeed, AZD1152 is a water soluble prodrug of AZD2811, which could be formulated into Accurins nanoparticles using ion-pairing agents, enhancing its efficacy and tolerability in preclinical models while allowing for less frequent dosing (Ashton et al., 2016). This approach mitigate the toxicity that has been a challenge in these treatments to date. Future work could focus on optimizing the dosing regimen and identifying biomarkers

predictive of response to this combination, which would be valuable for translating these findings into clinical settings.

7. Epigenetic background of hpMSC lines

Currently, it is still unclear which is the cell of origin of EwS, being neural crest stem cells (NCSC) and mesenchymal stem cells (MSCs) the putative candidates. Indeed, initial studies demonstrated that knockdown of EWSR1-FLI1 translocation in EwS cell lines made them cluster together with MSCs, according to their gene expression profiles (Tirode et al., 2007). In this sense, the translocation has been successfully introduced in bone marrow derived mouse MSCs, recapitulating initial tumorigenesis *in vivo* and reproducing a transcriptional program similar to EwS (Hancock & Lessnick, 2008; Riggi et al., 2008, 2010). However, EWSR1-FLI1 alone is insufficient to initiate EwS tumorigenesis, so hMSC-derived EwS animal models still lack in the field. For these reasons, human pediatric MSCs (hpMSCs) are a crucial model to demonstrate the importance of epigenetics and chromatin remodeling in EwS.

Age ≥ 18 years has been described as the most important predictor of outcome in EwS (Marina N., 2015; Mora J., 2017). Additionally, the fact that EWSR1-FLI1 alone is insufficient to transform MSC, highlights the epigenome as an important factor contributing to the activity of the oncogenic translocation. Bone marrow-derived MSCs are the most commonly isolated and characterized source of cells constituting the bone marrow microenvironment or niche. Our genome-wide analysis of the associated-repression mark H3K27me3 suggests a slight increase in the model corresponding to > 18 years old EwS patient (**Figure IV. 28A** and **IV. 28B** in results). In the same line, H3K27ac global enrichment is decreased in the models that belong to the patients above 18 years old (**Figure IV. 28C** and **IV. 28D** in results). All these data suggests a more closed chromatin in MSC from patients older than 18 years old, which implies for a less flexible chromatin structure. These high levels of compaction will abolished interaction of stem transcription factors as

The ability of MSCs to change is called plasticity. MSC plasticity refers to the cells' ability to differentiate into various cell types, secrete factors that influence tissue repair,

modulate immune responses, and adapt to different environmental signals. Growing bodies of evidences indicate that histone modifying enzymes significantly contribute to MSC lineage specification. Thus, open chromatin is essential for maintaining the undifferentiated state and the ability to activate lineage-specific genes during differentiation. Indeed, the "open chromatin hypothesis" was proposed where the ESC possess a more plastic and dynamic chromatin state in order to maintain an unlimited potential to differentiate. IN this context, we hypothesized that the appearance of the oncogene in a more plastic microenvironment (less than 18 years old) allows for an easier rewiring of the reprogramming infringed by the oncogene. The tumorigenic capacity of EWSR1-FLI1 would be limited and restricted to unique spatiotemporal conditions associated with pubertal growth. In these sense further studies of chromatin accessibility in MSc from patients below or above 18 years old will provide crucial information to complete our findings.

Recently, our group has reported how EF is extremely toxic in *Drosophila* and that toxicity can be avoided by a frameshift mutant (EWSR1-FLI1FS) that replaces the normal 69 amino acid C-terminal tail of EWSR1-FLI1 by a new 69 amino acid sequence (the FStail) (Mahnoor et al., 2024; Molnar et al., 2022). These experiments confirm decades long of failed experiments trying to model EwS in all sort of cells and animal models. Besides, we showed that EWSR1-FLI1FS reduced toxicity is owed to decreased protein concentration brought about by its frame-shifted C-terminal tail. Building on this observation, we tried to generate hpMSCs carrying the oncogene and the construct with the FStail tagging. Using these MSCs lines, we will aim to achieve a wide range of full-length, unmodified EWSR1-FLI1 protein levels, to confirm our observations about the different chromatin repressed state depending on 18 years old cut-off.

VI. CONCLUSIONS

The main conclusions of this work are summarized as follows:

Chapter 1

Aim 1:

- AURKB is highly expressed in pediatric cancer cell lines and tumors, particularly in EwS.
- EWSR1-FLI1 binds AURKB promoter and upregulates expression in A673 EwS cell line and HeLa Tet-On EF cell line.
- EwS cell lines are particularly and highly sensitive to AURKB inhibitor AZD-1152 at nanomolar concentrations, with an apoptotic effect.
- AURKB co-localizes genome-wide with EWSR1-FLI1/RING1B at active enhancers in A673 EwS cell line.

Aim 2:

- EWSR1-FLI1 induction in HeLa Tet-On EF cell line redistributes AURKB genome-wide occupancy.
- AURKB gene silencing and pharmacological inhibition with AZD-1152 downregulate the expression of EWSR1-FLI1/RING1B target genes.
- AURKB enrichment correlates with low levels of H2Aub histone mark.
- Downregulation of AURKB decreases RING1B phospho S168 protein levels in A673 EwS cell line, contributing to its role in gene activation.
- RING1B interactome in TC-71 EwS cell line revealed Mediator and MLL complexes as main interactors hubs.

Aim 3:

- Combination of AZD-1152 and I-BET151 *in vitro* is more effective than single therapy, suggesting a potential synergistic effect.
- Combination of AZD-1152 with BMS-986158 *in vitro* and *in vivo* is more effective than single therapy, suggesting a potential synergistic effect.

Chapter 2

- The hMSC models established from patients of our institution have the potential to differentiate multilineage, a specific property of these cells.
- Genome-wide analysis of H3K27me3 and H3K27ac pattern suggest a more repressed chromatin state in hMSCs models corresponding to EwS patients above 18 years old.
- There is no specific change in H3K27me3 and H3K27ac pattern at target level between our models of hMSC derived from below or above 18 years patients and healthy donors.

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