The molecular role of fusion oncoproteins in myxoid liposarcoma

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Till min älskade son Noah

Abstract

Myxoid liposarcoma is a rare type of tumor characterized by fusion oncogenes, most commonly *FUS::DDIT3*. The FUS::DDIT3 oncoprotein retains functional properties of both parental proteins, enabling it to act as an aberrant transcription factor that disrupts epigenetic and transcriptional regulation, thereby promoting tumor development. However, the precise mechanism of the fusion oncoprotein remains to be defined. The aim of this thesis was to determine the molecular role of FUS::DDIT3 in myxoid liposarcoma by studying epigenetic and transcriptional effects caused by FUS::DDIT3 expression, primarily using multi-omics approaches. We found that FUS::DDIT3, like the normal DDIT3 protein, can bind promoter regions containing binding sites of transcription factors known to dimerize with DDIT3. We further discovered that FUS::DDIT3 interacts with some of these transcription factors, together affecting downstream gene expression. Furthermore, we found that FUS::DDIT3, in contrast to DDIT3, prefers binding at distal intergenic sites enriched in the repetitive GGAAT sequence. The binding of FUS::DDIT3 to these regions may play a role in the transcriptional reprogramming specific to myxoid liposarcoma. Moreover, we show that FUS::DDIT3 interacts with and affects the SWI/SNF complex, a chromatin remodeling complex controlling epigenetic regulation. The action of FUS::DDIT3 further results in extensive downstream transcriptional regulation, enabling the single oncoprotein to disrupt normal cellular functions, such as proliferation, migration and differentiation. Here, JAK-STAT signaling plays an important role in regulating cancer stem cell properties, linked to tumor heterogeneity and chemotherapy resistance. We showed that FUS::DDIT3 upregulates phosphorylated STAT3 and that both transcription factors co-localize on chromatin to regulate a shared set of genes. Another factor with great impact on tumor development, including tumor heterogeneity, is the

tumor microenvironment. Therefore, we developed an experimental model system that partly mimics *in vivo* conditions and showed that the myxoid liposarcoma-specific microenvironment contributes to the cellular properties. Still, data showed that FUS::DDIT3 expression, more than the microenvironment, determines the myxoid liposarcoma-specific cellular phenotype. In conclusion, the results in this thesis advance our understanding of FUS::DDIT3 and its molecular functions in myxoid liposarcoma and reveal mechanisms that may represent promising therapeutic targets for targeted therapies and improved treatment outcomes.

Keywords: Epigenetics, FET fusion oncogenes, FUS::DDIT3, JAK-STAT signaling, Myxoid liposarcoma, SWI/SNF, Tumor microenvironment

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Sammanfattning på svenska

Myxoid liposarkom är en ovanlig tumörsjukdom som drivs av en fusionsgen som uppstår då två olika gener, oftast *FUS* och *DDIT3*, felaktigt slås ihop. Det resulterande fusionsproteinet FUS::DDIT3 får nya unika egenskaper som påverkar tumörcellens funktioner och egenskaper vilket leder till okontrollerad celldelning och tumörtillväxt. De mekanismer FUS::DDIT3 utnyttjar för att orsaka och driva cancern är till stor del okända. Myxoid liposarkom-tumörer har utöver *FUS::DDIT3* vanligtvis få andra återkommande genetiska förändringar. Ändå är ett stort antal gener som styr ett flertal olika cellulära funktioner påverkade i denna cancertyp vilket pekar på att FUS::DDIT3 är en starkt drivande orsak till att myxoid liposarkom utvecklas. Delstudierna i denna avhandling syftade till att identifiera och förstå de molekylärbiologiska mekanismer som FUS::DDIT3 orsakar och som bidrar till att cancer utvecklas. Vi har visat att FUS::DDIT3 delvis binder andra delar av genomet än det normala DDIT3-proteinet. FUS::DDIT3 påverkar även vissa proteinkomplex som har till uppgift att packa arvsmassan och inverkar på vart de specifikt ska binda till på genomet. Vi tror att denna egenskap utgör delar av den molekylära mekanism som FUS::DDIT3 använder sig av för att påverka genuttryck kopplat till cancertypen. Vi har visat att gener vars uttryck påverkas av FUS::DDIT3 styr olika cellegenskaper såsom deras förmåga att dela sig, förflytta sig och specialisera sig till olika celltyper. Dessa egenskaper är även viktiga när en tumör utvecklas och blir aggressiv och svårbehandlad. Våra resultat tyder även på att FUS::DDIT3 agerar genom en signalväg som kallas JAK-STAT som påverkar dessa egenskaper och även är involverad i behandlingsresistens. Att påverka denna signalväg med utvecklade läkemedel skulle därför kunna utgöra en möjlig behandlingsmetod. Även mikromiljön i tumören påverkar cancercellerna och är viktig för tumörtillväxten. Genom att odla tumörceller i ett tredimensionellt

modellsystem som efterliknar tumörens naturliga mikromiljö har vi kunnat kartlägga hur cellernas egenskaper påverkas av sin omgivning. Våra forskningsfynd visar att FUS::DDIT3 har en starkare påverkan på tumörcellernas egenskaper än omgivningen, även om också mikromiljön bidrar med viktiga faktorer. Sammanfattningsvis har studierna i denna avhandling bidragit till ökad förståelse om FUS::DDIT3 och de molekylära funktioner som orsakar myxoid liposarkom. Denna kunskap är viktig och kan bidra till att nya och mer effektiva behandlingsmetoder kan utvecklas och att dessa terapier specifikt riktar sig mot cancercellerna.

List of Papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Ranji, P., Jonasson, E., **Andersson, L.**, Filges, S., Luna Santamaría, M., Vannas, C., Dolatabadi, S., Gustafsson, A., Myklebost, O., Håkansson, J., Fagman, H., Landberg, G., Åman, P., Ståhlberg, A. Deciphering the role of FUS::DDIT3 expression and tumor microenvironment in myxoid liposarcoma development. *Journal of translational medicine, 2024. 22(1), 38.*

II. Dolatabadi, S.*, Jonasson, E.*, **Andersson, L.**, Luna Santamaría, M., Lindén, M., Österlund, T., Åman, P., Ståhlberg, A. FUS-DDIT3 Fusion Oncoprotein Expression Affects JAK-STAT Signaling in Myxoid Liposarcoma. *Frontiers in oncology, 2022. 12, 816894.* *Shared first authorship

III. Osman, A., Lindén, M., Österlund, T., Vannas, C., **Andersson, L.**, Escobar, M., Ståhlberg, A., Åman, P. Identification of genomic binding sites and direct target genes for the transcription factor DDIT3/CHOP. *Experimental cell research, 2024. 422(1), 113418.*

IV. **Andersson, L.**, Leypold, N., Österlund, T., Jonasson, E., Grönqvist, K., Ranji, P., Fagman, H., Åman, P., Lindén, M., Ståhlberg, A. FUS::DDIT3 acts as an abnormal transcription factor causing epigenetic remodeling in myxoid liposarcoma. *Manuscript.*

V. Lindén, M., **Andersson, L.**, Albatrok, H., Canfjorden, V., Jonasson, E., Grönqvist, K., Sjövall, D., Jaako, P., Crescitelli, R., Fagman, H., Åman, P., Ståhlberg, A. FET fusion oncoproteins enrich SWI/SNF complex subtypes and interaction partners. *Cellular & Molecular Biology Letters, 2025. 30(1):107.*

Related but not included publications:

I. Jonasson, E., **Andersson, L.**, Dolatabadi, S., Ghannoum, S., Åman, P., Ståhlberg, A. Total mRNA Quantification in Single Cells: Sarcoma Cell Heterogeneity. *Cells*, 2020. *9*(3):759.

II. Lindén, M., Vannas, C., Österlund, T., **Andersson, L.**, Osman, A., Escobar, M., Fagman, H., Ståhlberg, A., Åman, P. FET fusion oncoproteins interact with BRD4 and SWI/SNF chromatin remodelling complex subtypes in sarcoma. *Molecular oncology*, 2022. *16*(13):2470.

III. Vannas, C., **Andersson, L.**, Dolatabadi, S., Ranji, P., Lindén, M., Jonasson, E., Ståhlberg, A., Fagman, H., Åman, P. Different HSP90 Inhibitors Exert Divergent Effect on Myxoid Liposarcoma In Vitro and In Vivo, 2022. *Biomedicines*, *10*(3):624.

IV. Filges, S., Jonasson, E., Leiva, MC., **Andersson, L.**, Gustafsson, A., Dhingra, D., Mendez, P., Ooi, A., Sciambi, A., Landberg, G., Ruff, D., Ståhlberg, A. Single-cell triomics analysis of tumor cells infiltrating patient-derived breast cancer scaffolds, 2026. *The American Journal of Pathology*.

Contents

Abbreviations

ATAC-Seq	Assay for Transposase-Accessible Chromatin using sequencing
CDX	Cell-derived xenograft
ChIP-Seq	Chromatin immunoprecipitation sequencing
ECM	Extracellular matrix
FET	FUS, EWSR1, TAF15
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
MLS	Myxoid liposarcoma
PDX	Patient-derived xenograft
PRC2	Polycomb Repressive Complex 2
RNA-Seq	RNA sequencing
SWI/SNF	SWItch/Sucrose Non-Fermentable

1

Introduction

Cancer is one of today's primary causes of death, with continuously growing numbers of incidents and deaths worldwide (1, 2). Cancer encompasses a broad spectrum of diseases commonly characterized by uncontrolled cell proliferation and an ability to invade surrounding tissues and metastasize to distant organs (3). A primary tumor is normally thought to be derived from a single cell with genetic changes, i.e., mutations, that provide selective advantages to survive and proliferate in the microenvironment (4). Cancer diseases are extraordinarily diverse at the genetic and molecular level. Different tumors of the same type, and even individual tumor cells within a single tumor, often display remarkable molecular variations that also may change over time (5). These inter- and intra-tumor heterogeneities make cancer a complex disease and introduce large challenges in discovering efficient treatments.

1.1 Cancer characteristics

Cancer cells need to overcome various regulatory mechanisms that multicellular organisms have developed to protect themselves from abnormal proliferating clones that invade normal healthy tissues. Hanahan and Weinberg described in 2000, six capabilities that tumor cells typically acquire during cancer development to defeat these mechanisms (Figure 1) (6). To keep proliferating, normal cells need to receive growth signals from growth factors secreted by other cells or via direct interactions with extracellular matrix (ECM) components or neighboring cells. Hence, proliferative cancer cells need to mimic this signaling, for example by inducing growth factor production, overexpressing transmembrane

receptors or by activating downstream growth signaling pathways. Tumor suppressor genes encode proteins that function to prevent cancer, for example by promoting DNA repair, inhibiting cell cycle progression, inducing programmed cell death (apoptosis) or triggering permanent growth arrest known as cellular senescence. Tumor suppressors acting by inhibiting proliferation are called growth suppressors, these allow a cell to divide only when it is ready for it, and not, for example, if the DNA is damaged. Proliferation can be blocked by letting the cells exit the cell cycle and enter the quiescent G_0 state, or by inducing a differentiated postmitotic state. Cancer cells force cell division even when severe abnormalities are present by inactivating growth suppressors and by inducing the expression of oncogenes promoting cell growth. Cancer cells also acquire resistance towards apoptosis, which is another protective function of normal cell populations, where potentially harmful damaged cells self-destruct. Cancer cells may avoid this by expressing anti-apoptotic proteins or by attenuating the expression of pro-apoptotic proteins (7). The fact that the tumor cells are not limited by the above-mentioned growth programs is not enough for them to become immortal. Normal cells are restricted to a limited number of doublings referred to as the Hayflick limit (8). When reaching this threshold, they stop growing and become senescent or die. This limitation is regulated by the DNA at the chromosome ends called telomeres, which are shortened with every cell division. Most cancer cells maintain the telomere length by upregulating the enzyme telomerase, extending the telomeres. Furthermore, to supply all cancer cells with oxygen and nutrients, growing tumors need to develop the ability to induce the formation of new blood vessels, a process called angiogenesis. Cancer cells achieve this by controlling the expression of angiogenic inducers and inhibitors (9). Tumors can be either malignant or benign, where the difference lies in the ability of malignant tumors to invade surrounding tissues and metastasize, i.e. spread through the vascular system to new distant locations. For the cancer cells to detach from the primary tumor and invade surrounding tissues, they need to go through a transformation

allowing for loss of cell-cell interactions and gain in mesenchymal characteristics, including increased migratory potential (10). Migration is also facilitated by degradation of ECM by matrix metalloproteinases secreted by the cancer cells or other normal cells. Additionally, the tumor microenvironment is an important player in creating favorable conditions to facilitate the migration of the cancer cells (11). In 2011, Hanahan and Weinberg presented two additional hallmarks (12). These involve the capability of cancer cells to shift their energy metabolism from the normal oxidative metabolism to a less efficient aerobic glycolysis, which allows rapid growth and survival in hypoxic conditions. Cancer cells also develop strategies to evade the immune system. Additional abilities, such as the capability of avoiding terminal differentiation and modulating gene expression through epigenetic regulation, have later been suggested as additional hallmarks of cancer (13).

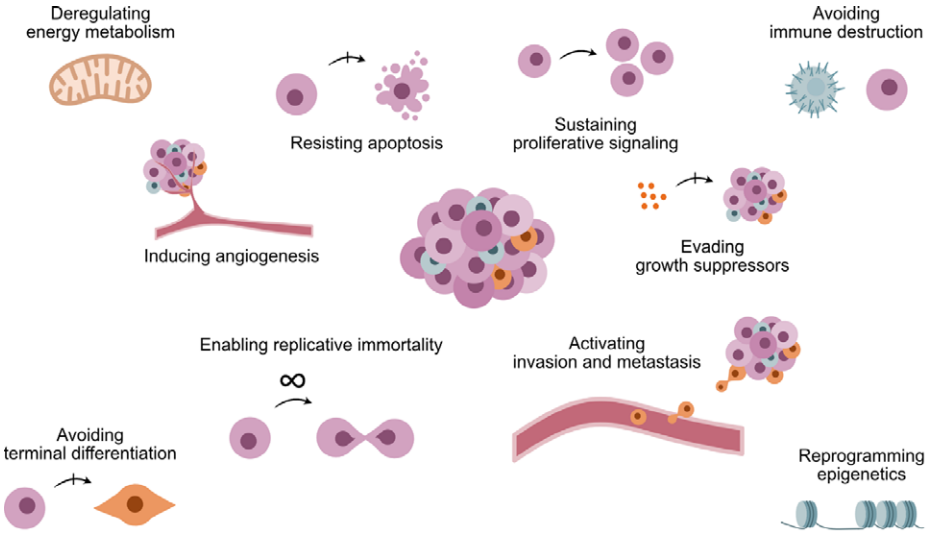

Figure 1. Hallmarks of cancer. Illustration depicting capabilities acquired by cancer cells during development that enable the transformation from normal to malignant cells, as described by Hanahan and Weinberg (6, 12, 13).

Cancers can arise in any tissue and are classified according to the tissue and/or cell of origin (14). Carcinomas originate from epithelial cells, sarcomas from connective tissues or muscles, and leukemias and lymphomas develop in hematopoietic cells. Sarcomas can roughly be subdivided into categories based on the specific tissue the tumor resembles or originates from, where liposarcoma for example recapitulates an adipocytic appearance. Further classification can be made based on morphology, where liposarcomas are grouped into well-differentiated liposarcoma, myxoid liposarcoma, de-differentiated liposarcoma, myxoid pleomorphic liposarcoma and pleomorphic liposarcoma (15).

1.1.1 Tumor heterogeneity

The abnormal genetic landscapes of cancer cells constitute the foundation for tumor growth and development. However, complex tumor biology can never be fully understood if studying cancer cells as a homogenous population. A tumor consists of a diverse mixture of tumor and stroma cells, and even individual tumor cells can show distinct molecular profiles that may change over time. Intra-tumor heterogeneity makes drug targeting challenging since subpopulations of tumor cells have different sensitivity and resistance to treatment (16). Variability among individual cells arises from multiple factors including genetic and epigenetic alterations, differences in cell-cycle state, transcriptional bursting, and heterogeneity in the tumor microenvironment (5). An increased mutation rate among cancer cells also contributes to genetic diversity through a gradual accumulation of mutations, which can result in subgroups of tumor cells with different characteristics (17). The presence of cancer stem cells is an additional source of tumor cell heterogeneity, as these cells, similarly to normal stem cells, can differentiate into progenitor cells and a range of more specialized cell types (18, 19). The interplay between the cancer cells and the microenvironment, consisting of stroma cells and ECM, is crucial for tumor growth and metastasis (20). The tumor microenvironment also contributes to tumor development through dysregulated immune

responses, by promoting angiogenesis and by functioning as a structural scaffold for the tumor cells to grow inside. Chemical and physical interactions between the tumor cells and their close surroundings affect gene expression and can even cause epigenetic changes resulting in altered cell plasticity and phenotype switch (21).

1.2 Myxoid liposarcoma

Myxoid liposarcoma (MLS) is a soft tissue sarcoma with slow-growing tumors typically appearing in the soft muscle tissues of the extremities, often in the thighs (22). The cell of origin has not been identified, but a mesenchymal stem or progenitor cell is most likely (23-25). Histologically, MLS tumors contain round or oval-shaped tumor cells mixed with lipoblasts at different stages of differentiation, surrounded by a myxoid stroma with a characteristic thin branching vasculature. High-grade MLS tumors contain an increased proportion of round cells (22). MLS accounts for about 5% of all adult soft tissue sarcomas and 20% of liposarcomas, making it the second most common liposarcoma in adults (15). The disease has a peak incidence of 40-50 years and is equally common in men and women. The general prognosis is good, with a five-year survival rate of about 80%, but some tumors show more aggressive behavior and about one-third of patients develop metastases, significantly lowering the five-year survival rate to approximately 40% (26, 27). Today's treatment approaches lack molecular targeted therapies and MLS is primarily treated by eliminating the tumors through surgery, often in combination with neoadjuvant radiotherapy (28). Chemotherapy, primarily with doxorubicin, is used for high-risk tumors and cases of metastatic or recurrent disease. Sometimes doxorubicin is used in combination with ifosfamide. In cases when initial treatment is no longer functional, Trabectedin and Eribulin can be used (29).

1.3 FET fusion oncogenes

MLS belongs to a subgroup of sarcomas characterized by FET fusion oncogenes which are formed through translocation events causing one of the FET genes (*FUS*, *EWSR1*, or *TAF15*) to fuse with a transcription factor-coding gene. Around 30 different types of FET fusion oncogenes have been identified, giving rise to around 20 different types of sarcomas and leukemia. The FET genes can replace each other in some cases, but the transcription factor partners are tumor-type specific. In more than 95% of cases, MLS is caused by the translocation t(12;16)(q13;p11) resulting in the fusion oncogene *FUS::DDIT3* (Figure 2), but it can also be caused by the less common translocation t(12;22)(q13;q12) that gives rise to the fusion oncogene *EWSR1::DDIT3* (30-33). FUS::DDIT3 also exists as different transcript variants and fusion proteins, depending on the breakpoint in the *FUS* gene (34). Unlike many other cancers, MLS and other sarcomas caused by FET fusion oncogenes are considered genetically stable, in some cases with no other mutations identified except for the fusion oncogene (35). Hence, *FUS::DDIT3* is considered a strong driver of tumor development, causing fundamental changes in the cell. Still, transforming non-malignant human cells into MLS with only *FUS::DDIT3* as driver mutation has been shown challenging. This suggests that a certain cell type with a certain level of differentiation is required for tumorigenesis to occur (36, 37).

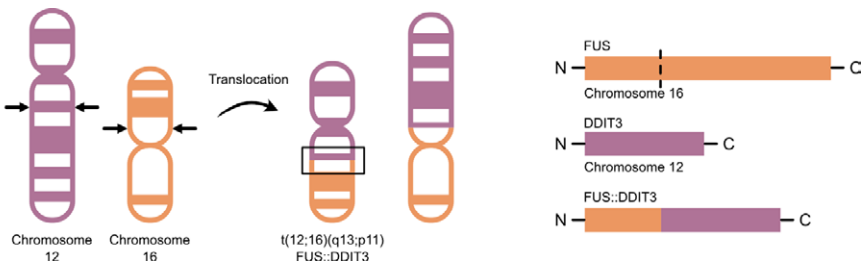

Figure 2. Illustration of the chromosomal translocation event t(12;22)(q13;q12) generating the FUS::DDIT3 fusion oncogene. Simultaneous double-stranded DNA breaks in distinct chromosomes can be incorrectly repaired through aberrant end-joining, leading to chromosomal rearrangements and exchange of genetic material. Such events may produce gene fusions that encode chimeric proteins with oncogenic potential.

1.3.1 FUS

The normal FUS (Fused in sarcoma), also called TLS (translocated in liposarcoma), is an RNA-binding protein involved in controlling processes related to the production and processing of mRNA, such as transcription, splicing and mRNA transport (38, 39). FUS and the other FET proteins, EWSR1 and TAF15, share a similar protein structure consisting of several domains with different properties that contribute to the multifunctional role of the proteins (39). The C-terminal contains several domains involved in nucleic acid binding such as an RNA recognition motif, several RGG (Arginine-Glycine-Glycine) repeat regions and a zinc finger domain. The N-terminal contains a structurally disordered prion-like domain rich in serine, tyrosine, glycine and glutamine (SYGQ). This composition of amino acids favors protein-protein interactions and the formation of many weak interactions makes the proteins prone to assemble and undergo so-called liquid-liquid phase separation (40). This process gives rise to membrane-less compartments called biomolecular condensates, which can hold high concentrations of proteins and nucleic acids. This way of compartmentalization is used by the cell to control spatial concentrations of e.g. reaction components involved in regulating cellular processes such as transcription, chromatin organization and intracellular signaling (41). The phase-separated state of FUS can for example recruit RNA polymerase II, contributing to the ability of FUS proteins to regulate transcription (42). The cellular processes regulated by liquid-liquid phase separation are also commonly deregulated in cancer cells, hence, aberrant types of phase separation may have important roles in tumorigenesis (41). When the fusion oncogene *FUS::DDIT3* is formed in MLS, most RNA-binding regions of FUS are usually lost in most FUS::DDIT3 types, while the N-terminal domain is conserved. Several recent studies have shown that FUS::DDIT3 inherits phase-separation properties from FUS, and is able to form biomolecular condensates with recruitment of transcriptional machinery

and epigenetic regulators including RNA polymerase II, BRD4 and SWI/SNF (43-45).

1.3.2 DDIT3

DDIT3 (DNA damage-inducible transcript 3), sometimes referred to as CHOP (CEBP homologous protein) or GADD153 (growth arrest and DNA damage-inducible gene 153), is a DNA-binding transcription factor protein. The original belief was that DDIT3 primarily acts as a negative inhibitor by preventing other members of the CEBP (CCATT/enhancer-binding protein) family from binding and promoting transcription of their targets through heterodimerization via their bZIP regions (46). Subsequent studies were however able to show that DDIT3 heterodimers are also capable of activating gene transcription (47). Expression of the *DDIT3* gene is normally suppressed, but when cells are exposed to endoplasmic reticulum stress, the expression is upregulated and the translated protein is involved in inducing pro-apoptotic signaling (48). Elevated DDIT3 expression is also observed during differentiation, where DDIT3 acts as a repressor of early adipogenesis by blocking CEBPB from binding its target genes, resulting in a loss of the adipocytic phenotype (49). The C-terminal of the FUS::DDIT3 fusion oncoprotein consists of the full-length DDIT3 protein, providing the fusion oncoprotein with DNA-binding properties and transcriptional targeting (50).

1.4 Mechanisms of tumor development in MLS

Unlike many other forms of cancer, tumors caused by FET fusion oncogenes are overall genetically stable and in addition to the fusion oncogene, they have few other recurrent genetic alterations (35). Tumor development is therefore not directly dependent on damaged tumor suppressor systems such as p53, which is often intact in MLS. To sustain a proliferative tumor cell population, these tumors instead rely on epigenetic and transcriptional reprogramming to acquire hallmarks of cancer,

including activation of growth signals (discussed below), dysregulation of the cell cycle (51, 52) and disrupted differentiation conferring a proliferative mesenchymal progenitor-like phenotype on the tumor cells (53).

1.4.1 Growth-promoting signaling in MLS

Several signaling pathways promoting survival, growth, proliferation and invasion are active in MLS. FUS::DDIT3 has been shown to induce expression of the IGF-I receptor (54) or the IGF-II ligand (55) in some MLS tumors, causing activation of PI3K/AKT signaling which regulates cellular processes, such as growth, survival and proliferation (56). Activation of this pathway can in some cases, especially in the more aggressive round cell variant, have a mutational cause. In approximately 20% of all MLS tumors an activating point mutation in *PIK3CA*, encoding the catalytic subunit of PI3K, has been found, and in some tumors loss of PTEN, a phosphatase counteracting PI3K, results in increased PI3K/AKT signaling (54, 57). Furthermore, through activation of IGF-IR/PI3K/AKT signaling, FUS::DDIT3 contributes to suppression of the Hippo pathway, allowing the transcriptional co-activator YAP1 to enter the nucleus and regulate genes involved in proliferation and survival by interacting with transcription factors, such as TEAD (58, 59). YAP1 and FUS::DDIT3 also seem to collaborate in the process of disrupting adipogenic differentiation (60). Terminal differentiation entails the cessation of cell proliferation and growth, so by disrupting this process, the tumor cell population maintains a proliferative progenitor phenotype. As a result of blocked adipogenesis, MLS tumors contain a mix of immature cells of the adipocyte lineage with different stages of differentiation (22). FUS::DDIT3 can prevent differentiation of preadipocytes by dimerizing with CEBPB, an early regulator of adipocyte differentiation, thereby preventing its binding and activation of target genes which results in repression of the late-stage regulators PPARG and CEBPA (53, 61). Furthermore, expression of FUS::DDIT3 has been linked to increased cell migration and invasion (62).

This enhanced metastatic potential has been connected to activation of SRC/FAK signaling (63) and upregulation of matrix metalloproteinases (62), which promote ECM degradation and thereby facilitate cancer cell migration through tissues.

We previously found that MLS tumors contain subpopulations of proliferating cancer stem cell-like cells, which are maintained by active JAK-STAT signaling (64). Cancer stem cells are cancer cells with the ability of self-renewal and the capacity of differentiating into progenitor cells and further differentiated cell types. They thereby contribute to tumor heterogeneity and growth and their slow proliferation rate is one reason for how they resist chemotherapy, which usually targets rapidly dividing cells (65). JAK-STAT constitutes a membrane-to-nucleus signaling cascade resulting in transcriptional activation of target genes, which stimulates cellular processes such as proliferation, differentiation, migration and apoptosis (66). The pathway is activated by binding of a ligand, usually a cytokine such as an interferon or interleukin, to a cell membrane receptor (Figure 3). This causes activation of Janus kinases (JAKs) followed by phosphorylation of Signal Transducer and Activator of Transcription proteins (STATs) which dimerize and translocate to the nucleus where they bind to enhancers in target genes and thereby regulate their transcription (67).

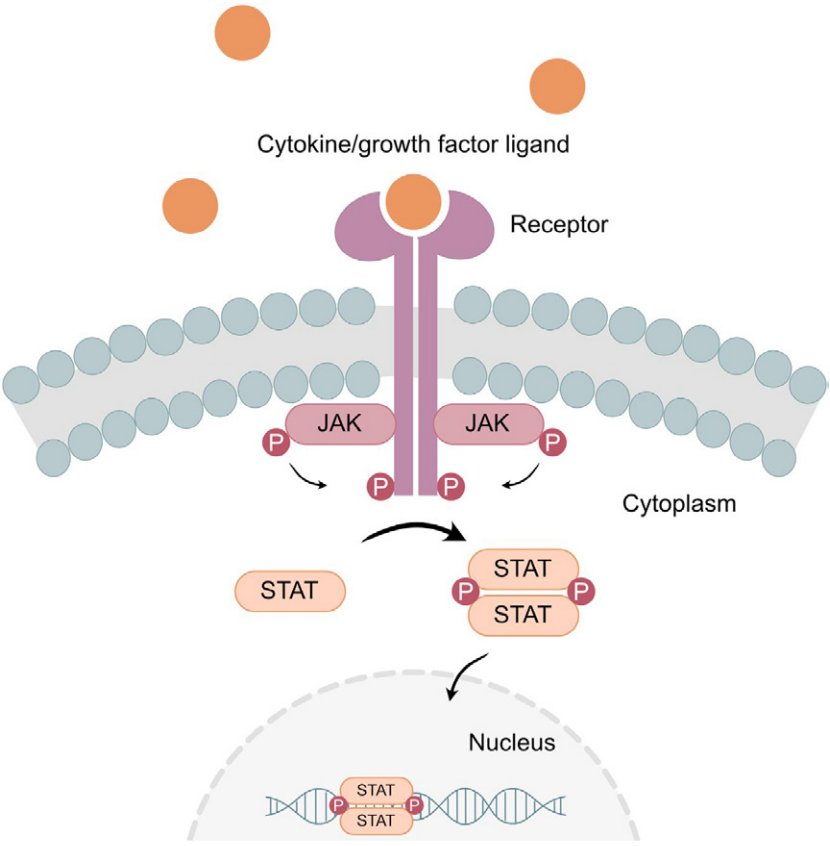

Figure 3. JAK-STAT signaling. Activation of the JAK-STAT signaling pathway is triggered by the binding of a ligand to a transmembrane receptor. This leads to activation of Janus kinases (JAKs) through phosphorylation, which in turn mediates activation of signal transducer and activator of transcription (STATs) through phosphorylation. Phosphorylated STATs dimerize and thereafter translocate into the nucleus where they act as transcription factors by binding and regulating the expression of target genes.

1.4.2 Epigenetic regulation in MLS

The DNA is organized in the nucleus through a systematized packing mechanism where the DNA is tightly wrapped around nucleosomes, consisting of eight histone proteins each (Figure 4). Compaction of DNA protects against genetic alterations and regulates the accessibility for different protein machineries involved in events, such as transcription,

recombination, replication and DNA repair. Genes can be activated or repressed through molecular mechanisms that temporarily alter the chromatin structure without changing the nucleotide sequence of DNA, referred to as epigenetic regulation. Epigenetics can be controlled through various mechanisms, including DNA methylation, histone modifications such as acetylation or methylation, ATP-dependent chromatin remodeling and RNA-associated silencing (68, 69).

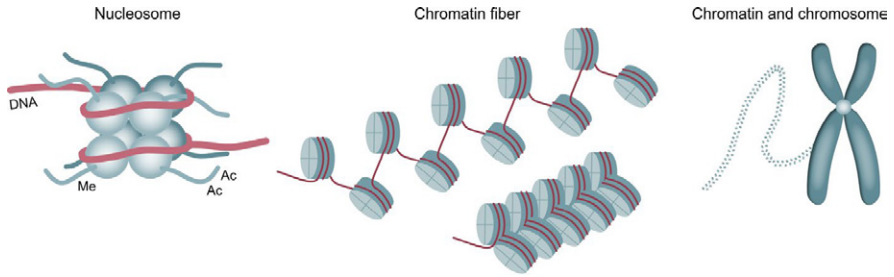

Figure 4. Organization of chromatin. The DNA double helix is wrapped around a nucleosome consisting of eight histones with post-translational-modified histone tails. A chain of nucleosomes makes up the chromatin fiber with a structure that can be loosely or tightly packed to regulate chromatin accessibility. The chromatin fiber is further organized into chromosomes.

The posttranslational addition and removal of acetyl-groups to lysine residues of the histone tails is carried out by two different families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). The addition of an acetyl group by HATs neutralizes the positive charge on lysine which results in a weaker interaction between the lysine and negatively charged DNA and less tightly packed DNA (70). HDACs counteract this by catalyzing the removal of acetyl groups which restores the DNA-lysine interaction, promoting a more compact DNA structure.

ATP-dependent chromatin remodeling is performed by large protein complexes that can eject nucleosomes or slide them along the DNA molecule by using the energy from ATP hydrolysis, to reduce or increase DNA accessibility. The mammalian switch/sucrose non-fermentable

(SWI/SNF) complexes, also called Brg-/Brama- associated factor (BAF) complexes, constitute a class of such protein complexes with the capability of exposing DNA for transcription (71). Three major subtypes of SWI/SNF complexes with varying subunit composition have so far been described: canonical BAF (cBAF), polybromo-BAF (PBAF) and GLTSCR/IL-BAF (GBAF) (72). Several of the genes encoding the SWI/SNF subunits are frequently mutated in almost 20% of all human cancers (73). In MLS, FUS::DDIT3 disrupts fundamental epigenetic regulatory mechanisms, leading to widespread dysregulation of genes involved in diverse biological processes (74). However, in these tumors, SWI/SNF complexes are usually not mutated. Instead, the FET fusion oncoproteins have been shown to interact with SWI/SNF complexes (including all three subtypes) through their N-terminal domain, to a much greater extent than normal FET proteins (75-78). In the EWSR1::FLI1-driven Ewing sarcoma, it has been shown that EWSR1::FLI1 recruits SWI/SNF complexes to GGAA repeats within enhancers. This mediates a transformation from closed to open chromatin with activation of target genes (76). A recent study showed that FUS::DDIT3 instead seems to partly inhibit the localization of the SWI/SNF complex at certain loci in MLS (50).

The functions of the SWI/SNF complex are balanced by the histone-modifying enzyme complex polycomb repressor complex 2 (PRC2)(71, 79). The catalytic domain of PRC2 called enhancer of zeste homolog 1/2 (EZH1/2) mainly catalyzes the methylation of lysine 27 on histone H3 (H3K27), resulting in more closed chromatin (71). Recently, our research group found that forced expression of FUS::DDIT3 and EWSR1::FLI1 results in higher H3K27me3 levels and that treatment with the EZH2 inhibitor tazemetostat reduces H3K27me3 levels (75). Increased expression of EZH2 in FET sarcomas and other fusion-oncogene-driven sarcomas has been repeatedly reported (80, 81), indicating an oncogenic function of EZH2 in FET sarcomas that potentially could be used as a therapeutic target.

2

Methodology and model systems

The following chapter will describe and discuss the selected methods and model systems used in the studies presented in this thesis. All methods used are described in detail in the attached papers and manuscripts.

2.1 Tumor model systems

There are many factors to take into consideration when selecting a suitable model system for studying tumor biology. In general, there is a trade-off between biological relevance and experimental simplicity, which influences the feasibility of studying specific cellular mechanisms. Simple model systems, such as *in vitro* 2D monocultures with cell lines, are well-suited for studying causal relationships since external factors are easily controlled. In addition, experiments are scalable, cost-efficient and provide reproducible results. Cancer cell lines are immortal cancer cells isolated from patient tumors, which are maintained in culture where they may proliferate indefinitely. Over time, these form homogenous populations of cells, often with new mutations and alterations in the transcriptome, which result in cell lines that deviate from the original tumor cells. Monolayer cultures further lack complexity in terms of the surrounding tumor microenvironment, which includes interactions with stromal and immune cells, mechanical forces, oxygen and nutrient gradients and other factors influencing tumor cell behavior. Hence, these simple model systems may be poor at predicting *in vivo* outcomes, for example in drug screening experiments, and results need to be further validated in more realistic contexts.

To better recapitulate the complex physiological conditions of the tumor, model systems such as *in vivo* mice models and *in vivo*-like 3D models are often applied. Patient-derived xenografts (PDX) and cell-derived xenografts (CDX) are *in vivo* models where tumor tissue or human cancer cell lines are implanted into mice that are typically immunodeficient to prevent rejection of the human cells. These model systems allow the tumor cells to grow in any direction, to interact with stromal cells and other extracellular components and allow for blood vessel development inside the tumor tissue. Additionally, the cell heterogeneity of the primary tumor is better preserved in PDX models. Xenograft models are, however, costly and time-consuming to establish and maintain. Furthermore, the infiltration of mice cells can influence tumor cell behavior and the immunodeficiency of the mice causes loss of interactions between cancer cells and immune cells. Ethical considerations are also essential in all types of animal-based studies. Research should always be conducted in line with the *Three Rs* principle, replacing animal use with alternative methods, reducing the number of animals used and refining procedures to minimize suffering. Hence, not all experiments can be ethically justified to be performed in animals. In recent years, various *in vitro* 3D models that recapitulate tumor conditions have been developed, enabling studies of tumor development and treatment response in systems that more accurately predict biological outcomes, compared to monolayer cultures, while reducing the need for animal experiments. The idea with these model systems is to provide tumor cells with a three-dimensional structure in which to grow, often referred to as a scaffold, together with ECM components that support cell–cell and cell–ECM interactions. The scaffold can be of synthetic origin, such as hydrogels of synthetic polymers, or of biological origin, being made from proteins, polysaccharides or decellularized tissue. Advantages with synthetic scaffolds include better control of their composition, stiffness and biodegradability which also increases reproducibility in contrast to scaffolds of natural origin which often show large variability (82). However, the inherited composition of decellularized tumor tissues is valuable since

it more closely mimics the *in vivo* tumor microenvironment, enabling studies of e.g. how tumor cells interact, move and respond to different stimuli in their natural environment. Growing cells in aggregates such as spheroids or organoids also allows for higher complexity than traditional monolayer cultures, enabling cells to grow in three dimensions with improved cellular interactions (83). Spheroids can be derived from a broad range of cells including cell lines, tumor cells or a sampling of cells from a tissue biopsy with multiple cell types and levels of maturation. Organoids are derived from tissue-specific stem cells or progenitor cells that proliferate, differentiate and self-organizes into structures that mimic the specific tissue when provided with an ECM and growth factors. However, these systems often lack important parts of the tumor microenvironment, such as immune cells, ECM components and functional vascularization. In summary, increased complexity of the model system replicates physiological conditions better and may hence predict in vivo outcomes in a more reliable manner. However, complex systems are more demanding in terms of time, cost and scalability. An increased number of parameters also introduces difficulties in identifying which factors are responsible for observed effects. For these reasons, the choice of model system should be based on the research question. Simple systems are for example suitable for initial high-throughput drug screenings, while more complex systems are useful for validation of promising candidates.

The studies included in this thesis apply basic research approaches to reveal the molecular role of the fusion oncoprotein FUS::DDIT3 in MLS. Cell lines are valuable for these types of studies since expression of the fusion oncoprotein can be expressed in the cells followed by evaluation of the effects on the cellular properties, here evaluated on DNA, RNA and protein level, compared to control cells with the same genetic background (Figure 5). The fibrosarcoma cell line HT1080 was used since these cells tolerate the expression of FUS::DDIT3 and FUS::DDIT3-expressing HT1080 cells have been shown to induce tumors with features characteristic of MLS when

injected into immunocompromised mice (84). The possibility of FUS::DDIT3 to carry out its function suggests that HT1080 cell line possesses an epigenetic environment similar to MLS. Still, some observed effects will be HT1080-specific as a consequence of epigenetic differences and the mutational background. HT1080 cells harbor mutations including a deletion in *CDKN2A*, which disrupts its role in cell-cycle regulation, an *IDH1* mutation affecting mitochondrial metabolism and an *NRAS* mutation that activates the N-Ras oncogene. To evaluate the relevance of FUS::DDIT3-related effects identified in the HT1080 system, the three MLS cell lines 2645-94, 1765-92 and 402-91 have been used in parallel (30, 85). In paper I, we developed a scaffold model system based on decellularized PDX tissue that we repopulated with different cell lines. This was used partly to study the impact of the tumor microenvironment, but also since these model systems will be highly valuable platforms for evaluating potential therapy targets in a more *in vivo*-like setting, when the supply of MLS tumor material is limited and the use of animal models should be minimized.

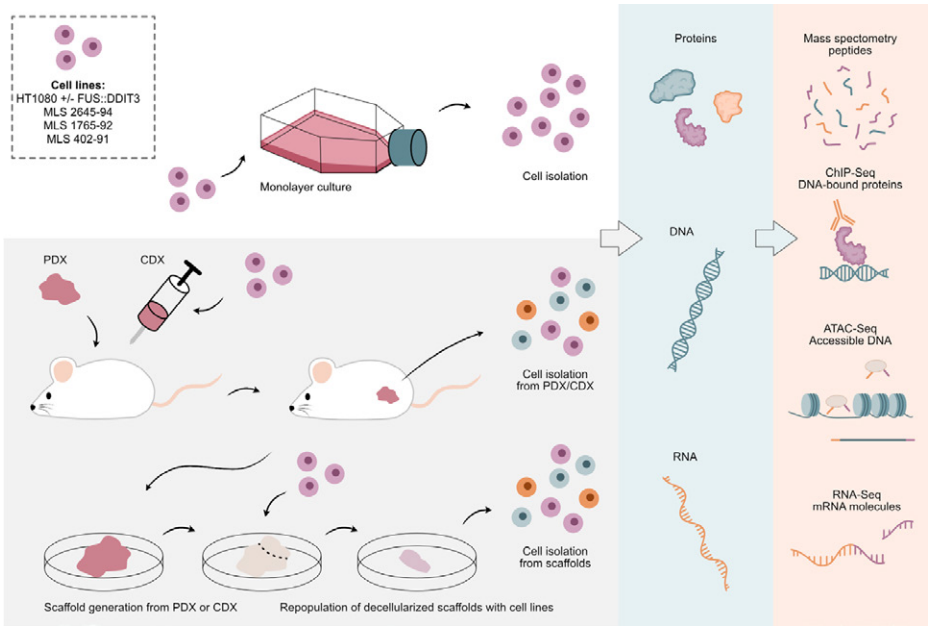

Figure 5. Schematic illustration of the methodology applied for studies of tumor development in this thesis. Cell lines (HT1080, MLS 2645-94, 1765-92 and 402-91) were either cultured in monolayers, as cell-derived xenografts (CDX) or in decellularized scaffolds generated from patient-derived xenografts (PDX) or CDX. Cells were thereafter isolated and analytes (proteins, DNA or RNA) extracted for analysis of peptide composition, protein binding to DNA, accessible DNA regions and gene expression (mRNA) using the molecular methods mass spectrometry, ChIP-Seq, ATAC-Seq and RNA-Seq.

2.2 Next-generation sequencing-based methods

2.2.1 Preparation of sequencing libraries

The methodology focus of the studies in this thesis includes techniques based on high-throughput next-generation sequencing (Figure 5). Chromatin immunoprecipitation sequencing (ChIP-Seq) relies on antibody detection of a protein of interest followed by sequencing of bound DNA fragments. Thereby, we could target FUS::DDIT3 and the normal DDIT3 protein to identify their DNA target sequences. Assay for

Transposase-Accessible Chromatin using sequencing (ATAC-Seq) captures euchromatin regions accessible for transcription factors and other proteins, such as the transcriptional machinery and RNA sequencing (RNA-Seq) identifies and quantifies the transcriptome. Here, we applied Smart-Seq2, which specifically detects polyadenylated full-length transcripts (86). Using these three methods, we could observe the effects of FUS::DDIT3 expression on chromatin architecture and gene expression in HT1080 cells. The workflows for preparing sequencing-ready libraries using these methods share some core concepts that are outlined in Figure 6.

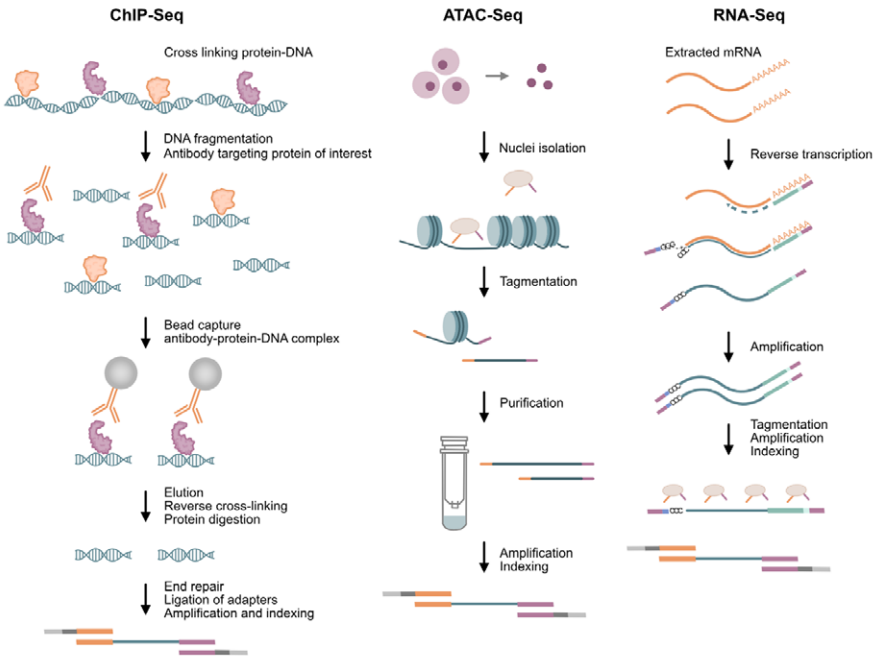

Figure 6. Illustration of the workflows for preparing sequencing-ready libraries using ChIP-Seq, ATAC-Seq and RNA-Seq.

Library preparation for ChIP-Seq starts with fixation of the protein-DNA interaction through cross-linking using formaldehyde, followed by lysis of the cells. The DNA with proteins attached is thereafter fragmented using sonication to a fragment size ranging between 100-600 base pairs. The

specific protein of interest is thereafter targeted using an antibody in a process called immunoprecipitation. Here, the quality of the antibody, in terms of sensitivity, affinity and specificity, is highly important to reliably detect the protein of interest and to minimize background noise (87). Next, magnetic beads that bind the antibody are used to pull out the whole protein-DNA complex, so non-bound proteins and other cellular components can be washed away. Purified antibody-protein-DNA complexes are then eluted from the beads, followed by reverse cross-linking to release the DNA from the attached protein. The irregular ends of the DNA molecules need to be repaired and thereafter sequencing adapters can be added through ligation. These adapters make the molecules compatible with the sequencing system, and to enable multiplexing, sample unique indexes are added. To distinguish real protein-DNA interactions from noise, some experimental controls are important to include. The first control is an input DNA control, consisting of cross-linked and fragmented but not immunoprecipitated DNA that is sequenced in parallel with the ChIP-Seq samples. This control is used as a baseline reference in the data analysis to distinguish true enrichment from background noise. Secondly, an Immunoglobulin G (lgG) control is a sample where a non-specific antibody is used to capture non-specific interactions. If a protein is truly bound to DNA at a certain site, the signal from the specific ChIP-Seq antibody should be larger than that from the non-specific lgG antibody. Thirdly, an empty-bead control, where no antibody is added, is used to detect DNA that non-specifically sticks to the magnetic beads.

The initial step for preparing ATAC-Seq sequencing libraries is to isolate nuclei containing intact chromatin in a mild lysis procedure. Thereafter, the chromatin is fragmented using tagmentation, a process where a Tn5 transposase enzyme cuts double-stranded DNA at accessible sites and simultaneously adds sequencing adapters to the fragment ends. Achieving an appropriate DNA-to-enzyme ratio is essential for optimal tagmentation efficiency and for generating the expected fragment size distribution

ranging between 200-1000 base pairs (88). The most abundant fragments are usually around 200 base pairs, representing nucleosome-free DNA. In high-quality libraries, larger fragments also appear with an approximate 160–200 base pairs periodicity, reflecting the number of nucleosomes present on each fragment. The fragment size also affects how well sequencing performs, which is generally true for different types of sequencing libraries. Overfragmentation, yielding too short fragments, can cause problems with PCR biases, poor mapping quality and reduced sequencing yield. Underfragmentation can also be problematic since large fragments do not cluster well on the flow cell during sequencing. Therefore, this experimental step might need optimization. DNA molecules with attached sequencing adapters are thereafter amplified and sample-unique indexes are added.

Different types of library preparation protocols for RNA-Seq exist, but for the studies in this thesis, a method capturing full-length mRNA by targeting the polyadenylated tail was applied (86). Hence, the workflow is described according to this method, even though many of the steps also apply for other protocols. RNA is first extracted from the cells, often using a column-based method. The use of high-quality RNA is important in transcriptomic analysis since degradation of RNA might cause skewed results with overrepresentation of genes that are less susceptible to degradation (89, 90). Therefore, proper quality control of RNA integrity, usually using electrophoresis-based methods, is required before further processing. In library preparation, polyadenylated RNA is first converted into complementary DNA using reverse transcription, followed by amplification. Thereafter, tagmentation is performed using the same enzymatic digestion as the ATAC-Seq protocol, but with random cutting across DNA, optimally generating fragments of approximately 300-800 base pairs. Fragmented complementary DNA is then further amplified in a PCR reaction where sample-unique indexes are added to the adapters already added by the Tn5 transposase enzyme.

2.2.2 Data analysis

The output of sequencing is a set of files containing raw reads, which correspond to the sequences of nucleotide bases that the sequencing machine identified for each DNA molecule. This data needs further processing before it can be used for biological interpretation. First, sequencing reads are matched to a reference genome to determine where in the genome they originate from, a process often referred to as alignment. In ChIP-Seq, reads map to genomic regions bound by the protein of interest, whereas in ATAC-Seq, reads cover regions of accessible chromatin. In RNA-Seq, the number of reads mapping to each gene instead reflects its expression level. The alignment for RNA-Seq data also needs to handle spliced reads, since mature messenger RNAs lack introns and sequencing reads can span multiple exons. Aligned ChIP-Seq and ATAC-Seq reads are forwarded for peak calling to identify regions of the genome where we have enrichment of aligned reads, compared to a background estimated from input control and/or background noise. If the ChIP or ATAC signal significantly exceeds the background, a peak will be called for that region and we can be relatively sure the enrichment of reads is of biological relevance and not just noise. For RNA-Seq data, the number of reads aligning to each gene, or other types of annotated genomic features, is counted and compiled into a count matrix. Thereafter, differential expression analysis can be performed to identify genes for which the expression level significantly differs between two biological conditions. The same method can be used for ChIP-Seq and ATAC-Seq to identify differential binding or differential accessible regions.

Many different downstream analyses exist to turn the above-described statistical results into biological insight, for example to understand which types of genomic regions the protein of interest binds and how this affects chromatin structure and gene regulation. Several of these analyses are based on overrepresentation tests using the hypergeometric distribution. Such analyses are, for example, used to determine whether genes in a list of

differentially expressed genes are functionally related, such as by participating in the same biological pathway. The test statistically compares the number of differentially expressed genes that belong to the pathway, to the number of genes that would belong to this pathway if this list was randomly selected from a background of genes. If the probability is low that the random gene list would have at least as many genes belonging to that pathway (we obtain a low and significant *p*-value), that means we have overrepresentation, or enrichment, of genes belonging to that pathway among the differentially expressed genes. Another analysis applying overrepresentation tests is HOMER motif analysis. It identifies enrichment of DNA sequences, also called motifs, among a list of genomic regions, such as binding sites of a certain protein of interest. Here, the background instead consists of randomly selected sequences from the reference genome.

3

Aims

The overall aim of this thesis was to investigate the molecular role of the abnormal transcription factor FUS::DDIT3 in MLS, with focus on transcriptional and epigenetic regulation.

The specific aims of the individual studies were the following:

Paper I: To develop an experimental model system that mimics *in vivo* conditions and define the role of FUS::DDIT3 expression in relation to the tumor microenvironment in MLS.

Paper II: To investigate the effects of FUS::DDIT3 expression on JAK-STAT signaling in MLS.

Paper III: To determine the DNA-binding properties of DDIT3 and its downstream transcriptional effects.

Paper VI: To determine the role of FUS::DDIT3 as an abnormal transcription factor in comparison with DDIT3 at epigenetic and transcriptional levels.

Paper V: To investigate how the fusion oncoproteins driving MLS and Ewing sarcoma influence SWI/SNF composition and interactome.

4

Results and discussion

4.1 The role of the tumor microenvironment in FUS::DDIT3-driven tumor development

The tumor microenvironment, including the ECM, has been shown to be an important factor for the regulation of many cellular functions involved in tumor development, but we currently lack understanding about the role of the tumor microenvironment in MLS. In paper I, we therefore aimed to determine the importance of the tumor microenvironment in relation to the contribution of FUS::DDIT3 expression in MLS.

To study how the tumor microenvironment affects MLS cells, we developed a three-dimensional culture system with a preserved ECM, which better mimics the environment in which the tumor cells naturally grow compared to traditional monolayer cultures. We used tissue from PDX in mice to generate the scaffold structure according to the protocol depicted in Figure 7. First, tumor tissue was cut into 6x6x6 mm pieces and passed through a decellularization procedure, including two rounds of washing with mild detergents. Cell-free tissue was verified histologically and by analyzing concentrations of remaining DNA. Pico-Serius red collagen staining was used to confirm an intact scaffold structure. The cell-free scaffolds were further cut into 2x2x2 mm pieces and thereafter repopulated with tumor cells. In this study, HT1080 cells with and without FUS::DDIT3 expression or MLS cell lines were used to enable studies of how fusion oncoprotein-driven processes are affected by the tumor microenvironment.

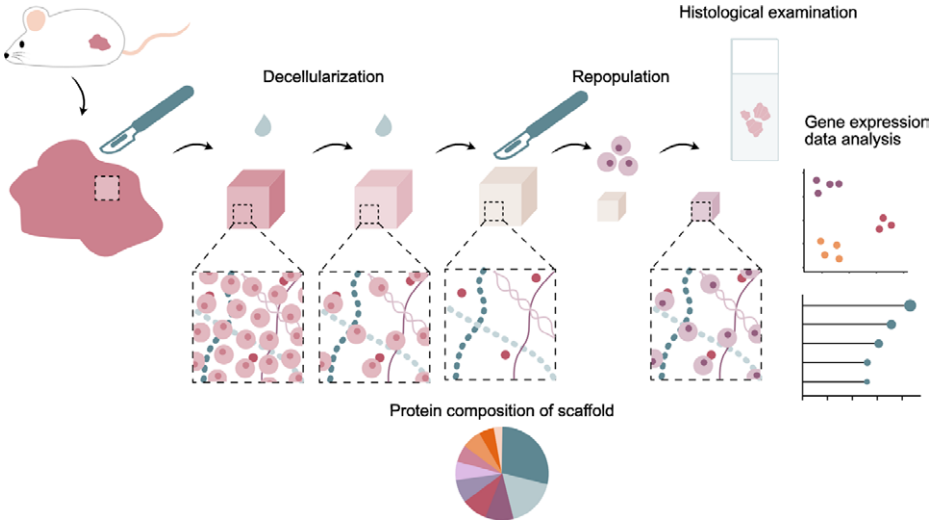

Figure 7. The procedure of generating scaffolds from PDX in mice. Decellularization is performed through two rounds of washing using mild detergents. The protein composition of the cell-free scaffolds is analyzed using liquid chromatography mass spectrometry. Cell-free tissue is cut into smaller pieces and repopulated with tumor cells/cell lines. After three weeks of culturing, tumor cell infiltration is examined histologically and cells are harvested for gene expression analysis.

We evaluated the infiltration of tumor cells in scaffolds by immunostaining after three weeks of culturing. HT1080 cells with FUS::DDIT3 expression, as well as MLS cells, showed poorer growth and capacity of infiltrating the scaffolds compared to HT1080 wild-type, which are considered highly invasive cells. These results agree with the fact that MLS tumors are considered slow-growing, and a previous single-cell study showed that increasing FUS::DDIT3 expression in MLS cells correlates with reduced cell proliferation (91). Furthermore, we characterized the protein composition of the cell-free MLS scaffolds with liquid chromatography mass spectrometry and identified about 3000 different scaffold proteins. Compared to a background of all protein-coding genes, these scaffold proteins were, as expected, overrepresented in protein classes related to ECM and membrane transport, but also classes of proteins involved in intracellular processes, such as translation, protein folding and

activation/deactivation of metabolites. Non-complete washing procedure, resulting in residual cell debris, could explain the presence of intracellular proteins in the scaffold structure. However, only very low levels of DNA were detected in the decellularized scaffolds, indicating that minimal cellular material remains. It is more likely that the intracellular proteins originate from dying cells that release proteins and other components into their surroundings, which in turn may influence the tumor microenvironment (92, 93). Some proteins annotated as intracellular might also have extracellular functions or be involved in interactions with the ECM.

Cells grown in MLS scaffolds showed a distinct gene expression profile compared to cells grown in monolayer cultures, where regulated genes could be connected to cellular processes such as hypoxia and glycolysis. A regulation of these processes is not surprising, since the oxygen and nutrient supply is much more limited when cells start infiltrating the scaffold structure compared to a monolayer culture, where cells are growing in nutrient-rich cell culturing medium. I*n vivo*, cancer cells will need to overcome these obstacles to survive, for example by promoting vascularization (9). The scaffold-regulated genes were related to both intracellular (e.g. cell-cycle, rRNA synthesis, nucleotide metabolism) and extracellular (e.g. ECM, cell adhesion, endocytosis) processes, indicating that cells cultured in MLS scaffolds are influenced by their surroundings and respond by adjusting internal processes, but also by regulating genes potentially impacting the tumor microenvironment. This interplay between the cells and the microenvironment further indicates that the cells grown in these PDXs acquire properties similar to *in vivo*-growing cells. We further identified a set of genes regulated by FUS::DDIT3 expression in scaffold-cultured HT1080 cells, which could be connected to several processes. First, genes related to proliferation were regulated by FUS::DDIT3 in agreement with a previous study reporting on decreased proliferation for FUS::DDIT3-expressing cells (91). Single-cell data also

confirmed reduced proliferation for FUS::DDIT3-expressing cells, with accumulation of cells in G1 phase based on the expression of known cell-cycle-associated genes. Furthermore, we observed regulation of genes related to epigenetics, which could be an effect of the known interactions between FUS::DDIT3 and the epigenetic regulators SWI/SNF and PRC2 (75, 94). Interestingly, FUS::DDIT3-regulated genes were also related to cellular interactions with ECM, as well as other processes involving ECM such as MMP14 targets and ECM organization, which could indicate that FUS::DDIT3-expressing cells interfere more or less with their surroundings compared to the HT1080 wild-type cells.

Single-cell analysis showed that, in contrast to HT1080 control cells, cells with FUS::DDIT3 expression had more similar gene expression signatures regardless of whether they were cultured in MLS scaffolds or as xenografts. For example, we observed high expression of genes related to adipogenesis in FUS::DDIT3-expressing cells, which is, as previously described, a process known to be disrupted by FUS::DDIT3 expression. Three-dimensional culturing systems contribute with an architecture and environmental composition similar to the ECM, affecting the cell behavior and thereby promoting cell heterogeneity. For example, the interaction with ECM is suggested to be essential for cells to acquire and preserve stem cell properties (95). Despite this, FUS::DDIT3 showed a unifying effect on gene expression, indicating that FUS::DDIT3, rather than the microenvironment, is the main driver of oncogenic processes such as differentiation, giving the cells the typical pre-adipocytic MLS phenotype.

The experimental approach developed in this study will represent an important asset for future studies of MLS aiming to evaluate biological effects in a more *in vivo*-like context where cell–environment interactions may influence cellular responses, such as for drug testing experiments. The large number of scaffolds that can be prepared from the same tumor also enables more large-scale drug screenings. The usability of tumor-derived

scaffold model systems within drug development has been evaluated by others. One group developed a scaffold model of pancreatic ductal adenocarcinoma and observed reduced treatment response for tumor cells grown in the scaffolds compared with monolayer cultures (96). Another group studied the effects of endocrine therapies on cells grown in scaffolds derived from breast cancer tumor tissue from patients (97). Similar to the other study, they found that cells grown in scaffolds showed a different treatment response with increased therapy resistance, compared to cells grown in monolayers. Through gene expression analysis and functional assays, they could further link the observed resistance to a gain in pluripotency and self-renewal, indicating that the scaffold model contributes with a tumor microenvironment that influences cell characteristics, hence supporting that these types of model systems add a dimension that can be valuable for therapy response studies. Our study used decellularized tumors from PDX in mice, but the experimental setup would work equally well on patient-unique tumor material, which would open up the field of personalized medicine. Additional layers of complexity could also be added to these scaffold model systems to more closely recapitulate the native tumor microenvironment. The tumor cells could for example be co-cultured with stromal cells, such as fibroblasts, which play an important role in tumor development, and/or with immune cells to study immune response (98, 99). Taken together, tumor-derived scaffolds offer great potential as *in vivo*-like model systems. Compared with traditional monolayer cell cultures, they enable biologically relevant interactions between tumor cells and their surrounding microenvironment, interactions that critically influence tumor development and treatment response and therefore represent essential features for models aiming to capture physiologically meaningful biological behavior.

4.2 FUS::DDIT3 activates JAK-STAT signaling, promoting chemotherapy resistance

As we observed in paper I, interactions between tumor cells and the microenvironment surrounding them can impact gene expression and epigenetics. The ECM is also believed to have a role in controlling the balance between stem cell renewal and differentiation by allowing stem cells to anchor to the local niche via cell surface receptors, both contributing to maintenance of stem cell properties but also allowing differentiation of daughter cells by introducing cell polarity, enabling asymmetric cell division (95, 100). This balance can be disturbed in tumors with abnormal ECM composition, which can result in overexpansion of stem cells (cancer stem cells) and/or disrupted differentiation.

In a previous study, we showed that MLS cell lines contain subpopulations of cells with cancer stem cell characteristics and that treatment with the chemotherapeutic agent doxorubicin enriches for these subpopulations, which can be linked to chemotherapy resistance (64). We showed that the size of the cancer stem cell population is controlled by JAK-STAT signaling, which is active in MLS, and can be attenuated by JAK1/2 inhibition using ruxolitinib. Hence, our previous data indicate that ruxolitinib could be useful for combinatorial treatment with doxorubicin to improve treatment outcome and reduce the risk of therapy resistance in MLS. However, it remains unclear how FUS::DDIT3 affects JAK-STAT signaling and how the chromatin remodeling complex SWI/SNF is involved in the downstream regulation of target genes, considering that the active phosphorylated STAT3 was shown to interact with the SWI/SNF subunit BRG1(64). The aim of paper II was therefore to determine how expression of FUS::DDIT3 affects JAK-STAT signaling to regulate drug resistance and subpopulations of cancer stem cell-like cells in MLS.

First, we showed with western blot analysis that when expressing FUS::DDIT3 in HT1080 cells, levels of STAT3 and pSTAT3 increased to

similar levels as in MLS cells, indicating that FUS::DDIT3 expression stimulates JAK-STAT signaling and confirms previous observations of an active JAK-STAT signaling in MLS.

Using RNA-Seq, we further evaluated the role of JAK-STAT in MLS and identified 126 genes that were regulated both by ruxolitinib treatment, causing JAK1/2 inhibition in HT1080 FUS::DDIT3 cells, and by FUS::DDIT3 expression in HT1080 cells. This suggests that FUS::DDIT3 and JAK-STAT signaling transcriptionally regulate partly the same cellular functions. Enrichment analysis confirmed the involvement of these genes in JAK-STAT signaling, but also revealed processes related to interactions with the ECM, adipocyte secretion of cytokines, stem cells and epigenetic regulation involving PRC2 and HDACs, processes that can be mechanistically connected. ChIP-Seq data showed that the majority of the regulated genes were bound by FUS::DDIT3 in MLS 402-91 cells, and thereby considered direct targets, while about one third were bound by STAT3, confirming that the regulatory effects observed for FUS::DDIT3 expression and JAK1/2 inhibition are mediated via the transcription factor STAT3. Furthermore, immunoprecipitation targeting FUS::DDIT3 identified a strong interaction between FUS::DDIT3 and pSTAT3 in nuclear extracts. In addition, FUS::DDIT3 interacted with the epigenetic complexes SWI/SNF and PRC2, as we and others have previously observed (75, 94). These findings together suggest a mechanism where FUS::DDIT3 and pSTAT3 co-localize on chromatin to regulate the expression of target genes, potentially with involvement from SWI/SNF and PRC2 (Figure 8).

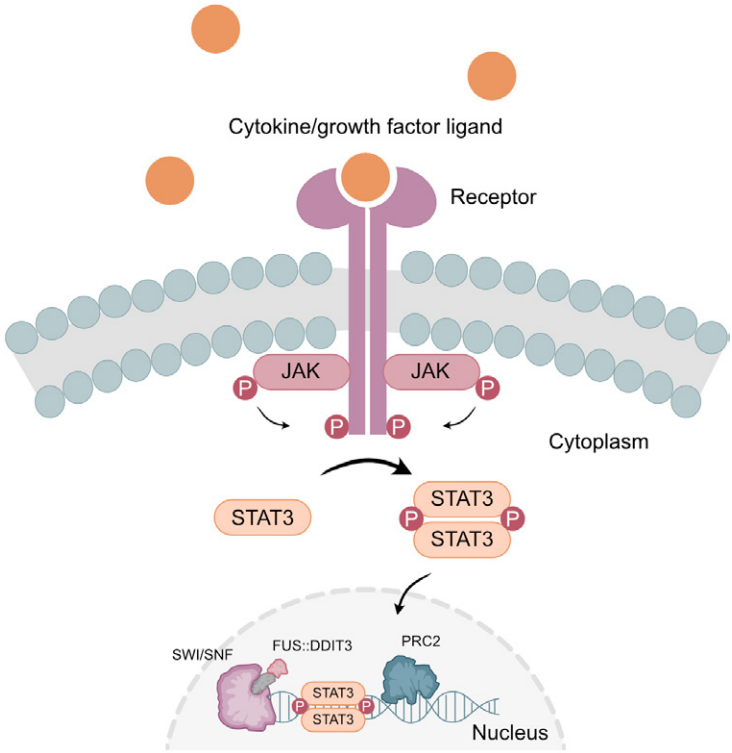

Figure 8. JAK-STAT signaling is active in MLS. FUS::DDIT3 and STAT3 co-localize on chromatin and regulate a shared set of target genes, potentially with involvement from the epigenetic regulators SWI/SNF and PRC2, which are both interacting with FUS::DDIT3 and phosphorylated STAT3.

Network analysis showed connections between about half of the proteins corresponding to the genes regulated by FUS::DDIT3 and JAK1/2 inhibition. Among the most central ones, we found the ECM-interacting cell surface receptor CD44, an established marker for cancer stem cells, which is involved in epithelial-mesenchymal transition and tumor development (101, 102). *CD44* gene expression was downregulated by JAK1/2 inhibition. Also on protein level, JAK1/2 inhibition resulted in decreased CD44 expression, both in HT1080 and MLS cells, even though FUS::DDIT3 expression showed no effect on CD44 protein levels in HT1080 cells. *CD44* was furthermore identified as a direct target of both FUS::DDIT3 and STAT3. We therefore decided to further examine if CD44

expression could be linked to cancer stem cell properties in MLS, which we assessed by evaluating anoikis resistance. Anoikis resistance is a feature that cancer stem cells develop to survive and proliferate anchorage-independently, and STAT3 has been reported to induce anoikis resistance (103). Indeed, anoikis-resistant MLS cells, enriched by growing in non-adherent cultures, showed up-regulation of CD44 protein levels, suggesting CD44 could potentially serve as a cancer stem cell marker in MLS. For future studies, enriching CD44-positive cells from an isolated population of MLS tumor cells would allow evaluation of their stem-cell potential in comparison with CD44-negative cells.

From these results, we can conclude that FUS::DDIT3 expression is directly connected to JAK-STAT signaling in MLS. Overactivation of JAK-STAT signaling has been reported to cause uncontrolled cell growth and survival, connected to therapy resistance, also in other cancer types. In many cases of the hematological malignancy Myelofibrosis, activation is caused by a mutation in the JAK2 gene (104). JAK inhibitors have shown effectiveness in reducing uncontrolled JAK2-mediated proliferation and disease-related symptoms and currently, Ruxolitinib and three other JAK inhibitors are approved for treatment of Myelofibrosis. Activation of JAK-STAT signaling has also been observed in multiple solid tumors such as triple-negative breast cancer, glioblastoma, colorectal cancer and lung adenocarcinoma (105-108). Preclinical studies have shown promising results from inhibition of several components of the pathway, including receptor tyrosine kinases, JAK2 and STAT3, but so far these inhibitors have shown very limited efficiency in clinical trials (107, 109, 110). Targeting JAK-STAT signaling still holds potential for future therapies. It is possible that targeting JAK-STAT signaling provides the greatest therapeutic benefit when used in combination with other therapies.

To conclude, we showed that FUS::DDIT3 activates JAK-STAT signaling. We further found that FUS::DDIT3 and STAT3 co-regulate target genes related to epigenetics and stemness and identified CD44 as a potential

cancer stem cell marker in MLS. These findings support a role of JAK-STAT signaling in controlling therapy resistance in MLS, further suggesting that inhibition of this pathway, in combination with current treatment strategies, may be an effective approach to reduce the risk of therapy resistance.

4.3 The involvement of FUS::DDIT3 and DDIT3 in epigenetic and transcriptional regulation

FUS::DDIT3 most likely uses DNA-binding properties of DDIT3 to function as an aberrant transcription factor, but how and to what extent remains to be elucidated. Differences in DNA binding between FUS::DDIT3 and wild-type DDIT3 may highlight the oncogenic mechanism of the fusion oncoprotein. In paper III, we aimed to explore the DNA-binding patterns of DDIT3, to identify DNA targets, and to evaluate the effect of DDIT3 expression on gene regulation. In paper IV we further mapped DNA-binding properties and downstream transcriptional effects of FUS::DDIT3 and compared these to DDIT3. We also analyzed the involvement of the two chromatin-modifying complexes SWI/SNF and PRC2 in relation to FUS::DDIT3 and DDIT3 regulation.

4.3.1 DDIT3 binds bZIP-specific motifs in promoter regions to regulate target genes

The bZIP DDIT3 from the CEBP protein family has been shown to not only inhibit the DNA binding of other bZIP transcription factors, as previously thought, but heterodimers containing DDIT3 can also function as classical transcription factors (111). CEBP homodimers are known to bind CAAT box sequences, but structural differences in the DNA-binding domain of DDIT3 suggest that its DNA affinity may differ from other CEBPs.

In paper III, we studied the DNA-binding profile of DDIT3 using ChIP-Seq analysis and identified 1200 DDIT3 binding sites in DDIT3-expressing HT1080 cells. The majority of these were located in promoter regions enriched in motifs usually bound by other bZIP transcription factors known to form heterodimers with DDIT3, such as ATF4, CEBP, FOS and JUN (46, 111, 112). This suggests that DDIT3 binds these DNA regions at least partly with these transcription factors as dimerization partners.

We further studied the effect of DDIT3 expression on transcriptional regulation by analyzing earlier published gene expression data (113). We found that most differentially expressed genes were downregulated by DDIT3 expression, indicating a more repressive function of DDIT3. To study this in more detail, we applied a Binding and Expression Target Analysis (BETA), which combines data on DNA-binding sites and transcriptional regulation to identify target genes and to predict a transcription factor's repressive or activating nature. DDIT3 binding showed a modest, though not statistically significant, association with downregulated genes. We then evaluated the distribution of histone marks across the genome, which provides insight into which regions are transcriptionally active and which are not. We found increased global levels of the histone mark H3K27me3 in DDIT3-expressing cells using western blot analysis, suggesting that DDIT3 in general promotes a more repressive chromatin state. However, DDIT3 binding sites rarely overlapped with H3K27me3 marks. Instead, DDIT3 binding overlapped with H3K27ac marks in promoter regions, suggesting that DDIT3 binds active promoter regions. About 100 genes were assigned as direct DDIT3 targets, i.e., regulated genes bound by DDIT3, using the BETA analysis. Compared to all DDIT3 binding sites, the direct targets, especially the upregulated ones, were more frequently located in promoter regions, suggesting a traditional role of DDIT3 as a transcription factor inducing transcription by binding regulatory regions in these promoters. Among direct DDIT3 targets, we found, as for all DDIT3 binding sites, enrichment of motif sequences known to be bound by bZIPs, including ATF4, CEBPB or DDIT3, suggesting that DDIT3 binding to these regions also induces transcriptional changes. These results suggest that DDIT3 can bind active promoter regions of target genes as a heterodimer with other bZIP transcription factors, resulting in downstream transcriptional regulation. However, this DNA-binding potential of DDIT3 does not rule out the possibility that a major part of DDIT3's function lies in blocking its dimerization partners from binding their target sequences.

4.3.2 The FUS N-terminal domain directs FUS::DDIT3 to bind preferentially at distal GGAAT-rich DNA regions

In paper IV, we studied the contribution of the N- and C-terminal domains, FUS and DDIT3, to FUS::DDIT3 genomic binding using ChIP-Seq in the HT1080 model cell line. FUS::DDIT3 predominantly bound DNA in intronic or intergenic regions located more than 10,000 base pairs upstream or downstream of transcription start sites, in contrast to the normal DDIT3 transcription factor, which we found in paper III binds mainly promoter regions. Still, almost one-third of the FUS::DDIT3 binding sites were also bound by DDIT3; these showed a more even genomic distribution across different types of genomic regions and were enriched in the known DDIT3 binding motif. Additionally, FUS::DDIT3 binding sites were enriched in sequences known to be bound by other bZIP transcription factors that DDIT3 usually dimerizes with, such as JUN, FOS, CEBP and ATF. This partly shared binding profile indicates that FUS::DDIT3 utilizes the DNA-binding capacity of DDIT3, but also that the FUS N-terminal domain impacts the DNA binding and redirects FUS::DDIT3 to new genomic targets. Interestingly, the FUS::DDIT3 binding sites not bound by DDIT3 were highly enriched in the repetitive sequence GGAAT, while DDIT3 binding sites were not in this analysis. Together with the results from paper III, where we identified an enrichment of GGAAT also among DDIT3 binding sites, this suggests that DDIT3 has the ability to bind the GGAAT repeat, but FUS::DDIT3 makes more frequent use of this ability. Furthermore, the repeats were located in distal regions and not promoters, which further strengthens that binding to these regions is more common for FUS::DDIT3, which primarily binds distally. The GGAAT repeat is characteristic of human class III satellite DNA, regions of condensed chromatin that can become transcriptionally active when cells are exposed to stress, primarily heat-shock (114). The resulting long non-coding poly-adenylated transcripts (HSATIII), transcribed by RNA polymerase II, accumulate close to the location of transcription and contribute as scaffolds

in the formation of membrane-less phase-separated structures called nuclear stress granules. This happens through the association with more than 100 different proteins, including RNA-binding proteins and splicing factors(115, 116). Nuclear stress granules have also been reported to incorporate transcription factors, such as CREB-binding protein and chromatin-remodeling factors such as BRG1, the enzymatic component of SWI/SNF, and BRD4 (117, 118). The binding of FUS::DDIT3 to these regions could possibly indicate a potential involvement of these long non-coding transcripts in the oncogenic program initiated by FUS::DDIT3. A speculative hypothesis could be that these transcripts contribute to the phase separation process that the N-terminal domains of FET proteins are known to induce via their prion-like domains, allowing for recruitment of epigenetic remodelers or different proteins involved in the transcriptional machinery. The repetitive GGAAT regions might have a similar role as the GGAA repeats in Ewing sarcoma, functioning as *de novo* enhancer regions (77, 119, 120), where phase separation appears to enable the long-range interactions required for the action of enhancers (121). Hence, further studies applying methods such as Hi-C (122) would be valuable to map the 3D chromatin structure and identify potential interactions between these GGAAT repeats and promoter regions. EWSR1::FLI1 binds microsatellite GGAA repetitive regions in usually condensed chromatin regions and recruits SWI/SNF chromatin remodeling complexes which open the DNA structure and enable aberrant transcription of adjacent genes (76). It is believed that this recruitment of SWI/SNF complexes and transcriptional machinery occurs through the phase separation process initiated by the accumulation of the aggregation-prone FET fusion oncoproteins (43, 44, 76). Similar characteristics have also been observed for other FET fusion oncoproteins. Desmoplastic small round cell tumors are characterized by the fusion oncoprotein EWSR1::WT1. This fusion oncoprotein was recently shown to bind primarily in distal intergenic regions at WT1 binding motifs, but repeats of TCC were also enriched among binding sites (123, 124). EWSR1::WT1 further affected the chromatin structure and 3D

chromatin looping was identified, especially at the repetitive sequences, suggesting these regions might participate in tumor-specific long-range gene regulation. The FET fusion oncoprotein EWSR1::ATF1 is expressed in the majority of clear cell sarcoma tumors (125). Recent studies found that the fused portion of EWSR1 confers the fusion with the capacity to bind more distal regions compared with the normal ATF1 protein, which mostly binds promoters (125, 126). Besides motifs known to be bound by ATF/CREB and AP1, repetitive TGA sequences were also enriched among EWSR1::ATF1 binding sites. Furthermore, EWSR1::ATF1 fusions were found to interact with the SWI/SNF chromatin remodeling complex and most distal EWSR1::ATF1 binding sites could be associated with active enhancer elements, marked by histone modifications characteristic of active enhancers.

In summary, FUS::DDIT3 can bind to promoter regions like DDIT3, at least partly via binding with bZIP dimerization partners. However, the FUS N-terminal domain greatly influences the fusion binding properties, and FUS::DDIT3 mostly binds to distal regions enriched in GGAAT repeats, similarly to what has been observed for other FET fusion oncoproteins. The reason for FUS::DDIT3 binding to these regions is still unknown, but they most likely play an important role in the oncogenic activity of FUS::DDIT3. Our results, in combination with the other studies described, suggest a generic mechanism common for the family of FET fusion oncogene-driven sarcomas, where FET proteins redirect the chromatin binding of their transcription factor partner to bind distal sites enriched in repetitive sequences, further altering epigenetics and gene expression.

4.3.3 FUS::DDIT3 and DDIT3 redistribute epigenetic complexes across DNA

Previous studies have identified interactions between FET fusion oncoproteins, including FUS::DDIT3, and the two epigenetic complexes SWI/SNF and PRC2 (75, 94). However, we still lack understanding about

how FUS::DDIT3 can affect their distribution across the genome to achieve chromatin remodeling at specific target sites. In paper IV, we therefore mapped the genomic binding pattern of SWI/SNF and PRC2 and their co-localization with FUS::DDIT3 and DDIT3. We first assessed how FUS::DDIT3 expression affects the global localization of the SWI/SNF complex by identifying the binding sites of BAF155 and BRG1, two SWI/SNF subunits of the core part of the complex. Compared to wild-type and DDIT3-expressing cells, where SWI/SNF complexes bound promoter regions primarily, they more often bound distal intergenic and intron regions in FUS::DDIT3-expressing cells, indicating that the fusion oncoprotein impacts the localization of SWI/SNF complexes in general to bind further away from the transcription start site. Secondly, we identified the sites bound by both FUS::DDIT3 and SWI/SNF and compared the SWI/SNF occupancy at the corresponding sites in wild-type cells. We also did the same comparison, but with EZH2, the enzymatic component of PRC2. A set of FUS::DDIT3 binding sites overlapped with both SWI/SNF and PRC2, and most of the corresponding sites in wild-type cells were also bound by both complexes. However, for some FUS::DDIT3 binding sites, we observed either more or less SWI/SNF binding compared to wild-type cells. Interestingly, FUS::DDIT3 binding sites, not overlapping with DDIT3 binding, were the ones overlapping more with SWI/SNF compared to the level of SWI/SNF occupancy in wild-type cells; about 40% of these sites also contained the GGAAT repetitive sequence. These were mostly present in distal intergenic regions. FUS::DDIT3 binding sites shared with DDIT3, instead overlapped less with SWI/SNF and we detected no enrichment of the repetitive sequence. These sites were more spread out across the genome. Hence, our data indicate that FUS::DDIT3 influences the distribution of SWI/SNF complexes across the genome and is capable of both recruiting SWI/SNF, often to GGAAT-rich regions, and displacing the complex at other sites. FUS::DDIT3 also occupied some non-accessible chromatin regions lacking both SWI/SNF and PRC2, indicating that FUS::DDIT3 might function as a pioneering transcription factor at these

sites. Pioneering factors are transcription factors with the capacity to bind silenced chromatin regions, often to initiate the process of increasing accessibility, but their binding can also lead to further compaction (127). The opening of DNA allows for the expression of genes, and these factors therefore have a key role in the activation of lineage-specific genes required for cell development and differentiation. Hence, aberrant pioneering factors can be very harmful and cause abnormal malignant cell phenotypes (128). As an example, the fusion oncoprotein PAX3::FOXO1, driving alveolar rhabdomyosarcoma, was recently reported to have pioneering activity (129).

DDIT3 expression did not affect the global localization of SWI/SNF as FUS::DDIT3 did. But instead, our data showed that DDIT3 expression affects the localization of PRC2 complexes, with enhanced binding in promoter regions compared to wild-type cells. Additionally, DDIT3 binding sites overlapped with EZH2 in general, while the same sites in wild-type cells, in principle, lacked EZH2 binding. PRC2 primarily functions to silence chromatin by adding methyl groups to histone H3 on lysine 27 (71). Surprisingly, these EZH2-bound regions lacked H3K27me3, but were acetylated and bound by SWI/SNF, indicating that DDIT3 recruits PRC2, but its enzymatic activity is blocked or remains inactive in these active chromatin regions. Another aspect that could affect these results is the difference in dynamics between the H3K27me3 and H3K27ac histone modifications. Histone acetylation is a rapid and dynamic process, regulated within minutes by histone acetyltransferases and deacetylases (130), allowing ChIP-Seq to capture transient changes associated with transcriptional activity. In contrast, H3K27me3 is a relatively stable histone mark that reflects a more established chromatin state that may change for example during cellular differentiation (131, 132). Additionally, RNA sequencing data supported a role of the PRC2 complex in DDIT3-mediated gene regulation, where genes differentially expressed by DDIT3 expression in HT1080 cells were enriched in known PRC2 target genes. For

FUS::DDIT3-regulated genes, only those also regulated by DDIT3 showed enrichment of PRC2 target genes. This further supports that FUS::DDIT3 preserves regulatory effects on a set of DDIT3-regulated genes, potentially involving PRC2-mediated mechanisms.

To summarize, FUS::DDIT3 can bind non-accessible chromatin, potentially acting as a pioneering factor, but it also binds GGAAT-rich sites occupied by SWI/SNF and PRC2. Furthermore, FUS::DDIT3 is capable of rearranging the binding of SWI/SNF complexes at various sites. In contrast, DDIT3 affects the general DNA-binding pattern of PRC2, directing it to promoter regions. This redistribution of epigenetic complexes most likely promotes epigenetic changes and further transcriptional effects.

4.3.4 FUS::DDIT3 regulates the expression of genes involved in cellular processes important in MLS tumorigenesis

To further study the downstream effects of FUS::DDIT3 and the redistribution of epigenetic complexes observed, we combined the ChIP-Seq data with ATAC-Seq data of accessible chromatin regions and RNA-Seq gene expression data (paper IV). By comparing HT1080 FUS::DDIT3 cells to wild-type, we identified differentially accessible DNA regions. These regions were mostly located in introns, distal intergenic or promoter regions and were enriched in motifs known to be bound by bZIP transcription factors, such as components of the API complex and CEPB proteins. Hence, our data show that FUS::DDIT3 not only binds these types of regions but also affects their DNA accessibility. We further identified 82 genes that were directly bound and regulated by FUS::DDIT3, both regarding chromatin accessibility and gene expression. To capture indirect effects on gene expression, we also analyzed the 377 genes that were regulated by FUS::DDIT3, both in terms of chromatin accessibility and gene expression, but not necessarily bound by FUS::DDIT3. For example, FUS::DDIT3 can act by blocking the binding of dimerization partners just

like DDIT3, resulting in loss of regulation by that transcription factor (61). The list of 82 FUS::DDIT3-regulated genes was enriched in genes containing binding motifs for a certain set of transcription factors. Fifteen of these were also identified as FUS::DDIT3 interaction partners when analyzing an immunoprecipitation and quantitative mass spectrometry data (paper V). As expected, the bZIP transcription factors JUN and CEBPB were among these, again suggesting that FUS::DDIT3 acts as a transcription factor at genes normally regulated by these transcription factors, potentially by forming heterodimers with them as does DDIT3 (46, 111). The interaction between FUS::DDIT3 and CEBPB is well known, where FUS::DDIT3 dimerizes with CEBPB and thereby prevents it from binding target genes involved in adipogenesis, resulting in blocked differentiation (51, 61, 94). A recent study suggested that the loss of CEBPB-mediated recruitment of SWI/SNF to the adipogenic target genes results in decreased expression of these genes, which is the reason for blocked adipogenic differentiation in MLS (50). This further suggests that the DDIT3 portion of FUS::DDIT3 is responsible for the poorly differentiated phenotype of MLS.

Through protein network analysis of FUS::DDIT3-regulated genes, we identified connections between 80% of the genes at protein level, where the majority were downregulated on gene level and connected to different cellular processes, such as cell proliferation and differentiation, tumor microenvironment and ECM, and immunological processes. These results are consistent with our observations in paper I, where data suggest that cells expressing FUS::DDIT3 interact more with the microenvironment surrounding them, possibly to create their own extracellular niche that favors the characteristic early adipocytic phenotype of MLS, induced by FUS::DDIT3 expression. That adipocyte differentiation is influenced by interactions with the microenvironment is previously known (133). Connected to adipogenesis, we identified HDAC targets among FUS::DDIT3-regulated genes and HDAC4 was also identified as a

FUS::DDIT3 interaction partner. HDACs induce transcriptional repression by enzymatic removal of acetyl groups from histones and some HDACs are involved in adipocyte differentiation and the process of white adipocyte browning (134, 135). Furthermore, others have shown (136), and we here confirmed, sensitivity to a pan-HDAC inhibitor, which increased when combined with an EZH2 inhibitor. Synergistic treatment effects from these two epigenetic regulators can be explained by their collaborative role in chromatin compaction, which could be relevant for FUS::DDIT3-driven gene regulation. The list of FUS::DDIT3-regulated genes was also enriched in genes related to cell migration, and the results from a transwell migration assay, evaluating the cells invasiveness and ability to metastasize, showed increased invasiveness for cells expressing FUS::DDIT3 or DDIT3 compared to wild-type. These results were in line with previous studies reporting on an increased migratory and invasive ability of cells with FUS::DDIT3 expression, which seems to involve activation of SRC-FAK signaling and elevated levels of matrix metalloproteinases, facilitating cell migration through tissue barriers by degradation of the ECM (62, 63). The decreased ability for FUS::DDIT3-expressing cells to invade scaffold tissue, which we observed in paper I, appears contradictory to these results. However, these model systems are imitating different biological settings with varying conditions, such as limited nutrient and oxygen supply in the more dense scaffold structure, while the transwell migration assay evaluates the cells ability to migrate through a thin basement membrane towards a higher serum concentration, which is supposed to mimic the process of initiated invasion when cancer cells adhere to and spread along the blood vessel wall. To conclude, FUS::DDIT3 expression caused regulation of a large set of genes involved in different processes related to MLS characteristics and tumor development, including epigenetics, differentiation, migration and tumor microenvironment. Dramatic epigenetic and transcriptional regulation affecting several of the hallmarks of cancer is expected in these tumors, with a generally low mutational burden, to induce oncogenesis.

4.4 The impact of FET fusion oncoproteins on the composition and interactome of SWI/SNF complexes

The chromatin remodeling complex SWI/SNF constitutes an important interaction partner of FET fusion oncoproteins due to its ability to remodel nucleosomes and expose chromatin. These complexes are therefore valuable tools for the oncoproteins to use to achieve a dramatic impact on gene regulation crucial for driving different oncogenic programs. The SWI/SNF complex has been reported to exist as three main subtypes (cBAF, PBAF, GBAF), and their subunit composition affects the DNA binding patterns and functions. In paper V, we aimed to determine if and how FUS::DDIT3-bound SWI/SNF complexes differ in their composition or interactome, compared to SWI/SNF complexes in general in MLS cells. We also compared SWI/SNF compositions and interaction partners in two different FET sarcoma subtypes, MLS and Ewing sarcoma, and investigated if the differences could be linked to FET sarcoma subtype-specific patterns in chromatin accessibility and gene expression.

The strategy applied to determine the protein composition of SWI/SNF complexes and their interaction partners was based on immunoprecipitation of nuclear extracts followed by quantitative mass spectrometry. The enzymatic component BRG1 was targeted to capture all SWI/SNF complexes in MLS and Ewing sarcoma cells, while a DDIT3 antibody was used to target FUS::DDIT3-bound SWI/SNF complexes in MLS cells (Figure 9). Results show that SWI/SNF complexes in MLS and Ewing sarcoma cells, in general, differ in their subunit compositions and in subtype proportions, where SWI/SNF complexes in MLS cells, for example, were enriched in components specific for the GBAF subtype. Also, the SWI/SNF interactomes differed between the two FET sarcoma subtypes. In MLS cells, we observed increased interaction between SWI/SNF complexes and several transcription factors known to form heterodimers with DDIT3,

such as JUN and FOSL2, as well as transcription factors involved in adipocyte differentiation and hence important for MLS tumorigenesis, such as RUNX1 and GATA2. SWI/SNF complexes in Ewing sarcoma cells instead showed enriched interactions with transcription factors reported as important in Ewing sarcoma tumor development, such as SOX6, MEIS1, HOXD11 and E2F6 (137-140). This suggests that SWI/SNF complexes interact with partly different sets of transcription factors in MLS and Ewing sarcoma cells, enabling divergent transcriptional regulation in the two cancer types. To further study downstream regulatory consequences, we applied ATAC-Seq to identify potential differences in DNA accessibility patterns and RNA-Seq to study gene expression in MLS and Ewing sarcoma cells. Results showed a divergent epigenetic landscape for MLS and Ewing sarcoma cells, primarily at introns and distal intergenic regions. DNA regions more accessible in MLS cells were rich in binding sites of known FUS::DDIT3 dimerization partners such as JUN and FOS, while in Ewing sarcoma cells, more accessible regions were instead enriched in the EWSR1::FLI1 binding motif, indicating that the FET fusions are driving the epigenetic regulation specific for each tumor type, perhaps by relocation of the SWI/SNF complex at different locations as shown in paper IV. Also, the transcriptional regulation differed between the two FET sarcoma subtypes, in correlation with chromatin accessibility, where genes with enhanced expression were connected to FET fusion-related targeting; in MLS with PRC2 targets and processes such as cell migration and biological adhesion and in Ewing sarcoma with EWSR1::FLI1 targets, PRC2 targets, H3K27me3 targets, neurogenesis and cell signaling. A set of genes up-regulated in each FET sarcoma subtype was also found to interact with SWI/SNF in their protein form, indicating that the FET sarcoma-specific transcriptome interacts with SWI/SNF and could thereby affect its function. By comparing transcription factor motifs among differentially accessible regions with transcription factors among regulated genes and SWI/SNF interactomes, we could identify a refined set of key transcription factors specific to MLS, such as JUN, RUNX1, FOSL2 and CREB5, and for

Ewing sarcoma, such as SOX6, MEIS1, E2F6 and L3MBTL3, as well as a set of shared ones, including e.g. CTCF. This suggests that FET sarcoma subtype-specific transcription factors can direct the SWI/SNF complex to their binding sites, thereby opening the DNA structure and enhancing transcription. It is important to remember that by comparing the two FET sarcoma subtypes, we capture the differences between them and may overlook shared fundamental mechanisms of tumor development involving interactions with SWI/SNF complexes.

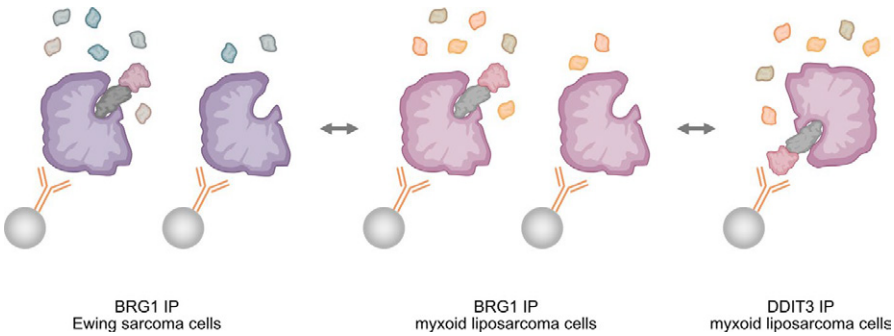

Figure 9. Visualization of the conditions captured using immunoprecipitation (IP) followed by quantitative mass spectrometry. IP using an antibody targeting BRG1 captures SWI/SNF complexes in general, including a subset bound to the fusion oncoprotein, either in Ewing sarcoma or MLS cells. IP using an antibody targeting DDIT3 captures FUS::DDIT3-bound SWI/SNF complexes in MLS cells. The comparisons made are indicated with arrows. Color differences reflect distinct SWI/SNF compositions and interaction partners in Ewing sarcoma and MLS.

The composition of SWI/SNF complexes affects their functions and genome binding preferences, and different subtypes localize to different genomic regions. Hence, we found it interesting to determine how FUS::DDIT3-bound SWI/SNF complexes differ in their composition and interactome compared to all SWI/SNF complexes in MLS cells. FUS::DDIT3 interaction caused no dramatic change in the composition of the core complex. However, FUS::DDIT3-bound SWI/SNF complexes were enriched in PBAF and GBAF-specific components, even though FUS::DDIT3 interacts with all SWI/SNF subtypes and we found cBAF to be

the most abundant subtype in MLS cells. Single-cell RNA-Seq data of MLS 402-91 cells generated no clusters based on the expression of SWI/SNF components, indicating MLS cells are relatively homogenous in their expression of SWI/SNF genes. We also observed an increase in SWI/SNF protein interactions when FUS::DDIT3 was bound. Increased protein interactions could be explained by FUS::DDIT3 proteins contributing with prion-like domain structures, prone to form protein aggregation through liquid-liquid phase separation processes.

To summarize, we showed that SWI/SNF complexes differ in their composition and interactome in MLS and Ewing sarcoma cells and could link these differences to a divergent epigenetic landscape and tumor type-specific regulation involving different sets of transcription factors. We further showed that FUS::DDIT3 bound all three main SWI/SNF subtypes; cBAF, PBAF and GBAF and FUS::DDIT3-bound SWIS/NF complexes had a larger interactome compared to SWI/SNF complexes in general in MLS cells.

5

Conclusions

The individual studies included in this thesis all contribute to increased insight into the molecular role of the fusion oncoprotein FUS::DDIT3 in MLS development, with focus on its role in disturbing epigenetic and transcriptional regulation.

Paper I: We developed an *in vivo*-like scaffold model system for studying the impact of the tumor microenvironment in MLS. We found that the microenvironment and FUS::DDIT3 expression both caused transcriptomic alterations linked to epigenetics, metabolism and cellular interactions with other cells or with the ECM. Our data suggest that the pre-adipocytic phenotype of MLS is more determined by FUS::DDIT3 expression than the microenvironment.

Paper II: We demonstrated that FUS::DDIT3 expression increases the activity of JAK-STAT signaling in MLS. We further identified an interaction between FUS::DDIT3 and active phosphorylated STAT3 and found that FUS::DDIT3 and STAT3 bind and regulate a shared set of target genes, potentially with involvement from SWI/SNF or PRC2. Lastly, we identified CD44 to be a potential cancer stem cell marker in MLS.

Paper III: We showed that DDIT3 acts as a normal transcription factor, which binds target genes in active promoter regions, at least partly via dimerization partners, to regulate the expression of target genes.

Paper IV: We showed that FUS::DDIT3 partly retains DNA-binding properties from DDIT3 and binds promoter regions at sites enriched in bZIP binding motifs. However, the FUS N-terminal domain redirects

FUS::DDIT3 binding to distal regions rich in the repetitive GGAAT sequence. FUS::DDIT3 is capable of both recruiting epigenetic complexes to GGAAT-rich regions and displacing them at other sites. We further showed that downstream effects include regulation of genes involved in different cellular processes relevant for MLS development.

Paper V: We demonstrated that the composition and interactome of SWI/SNF complexes differ between MLS and Ewing sarcoma cells and could link these variations to distinct epigenetic landscapes and tumor-specific gene regulation involving diverse sets of transcription factors. We further showed that FUS::DDIT3 engages all three major SWI/SNF subtypes, cBAF, PBAF, and GBAF and increases the SWI/SNF interactome.

6

Future perspectives and potential treatment strategies

The work in this thesis contributes with knowledge about the role of FUS::DDIT3 in MLS tumor development. We have observed that the combination of properties inherited from the parental proteins FUS and DDIT3 make FUS::DDIT3 a potent oncoprotein that induces widespread epigenetic and transcriptional alterations. These changes perturb essential cellular functions, thereby promoting tumorigenesis in accordance with the hallmarks of cancer. The ultimate goal within cancer research is to translate the newly acquired knowledge about tumor biology into more effective treatments. Current chemotherapy-based treatment of MLS does not specifically target cancer cells but rather rapidly dividing cells, other fast-dividing normal cells in the body are also affected, leading to numerous side effects. In addition, therapy resistance poses a threat to treatment failure. At present, no targeted therapies directed at the oncogenic mechanism of MLS exist, although such therapies are highly desired.

In theory, the fusion oncoprotein would be an excellent candidate to target due to the low mutation rate of these tumors and the fact that the fusion oncogene is the only recurrent mutation. A drug that targets the fusion oncoprotein exclusively would also be specific to the cancer cells and not harm healthy cells, which would minimize side effects. Degrading the fusion oncoprotein, rather than only inhibiting its activity, would be even more efficient, as it could in principle eradicate the fusion-addicted cancer cells and cure the disease. Examples of this strategy exist for other fusion oncoproteins such as PML-RARA, that cause acute promyelocyte

leukaemia, where a combination treatment with all-trans retinoic acid and arsenic trioxide causes degradation of the fusion oncoprotein (141). In many countries, this combination therapy is now the first-line treatment for acute promyelocytic leukaemia (142). Another well-known example is the BCR-ABL fusion oncoprotein driving chronic myelogenous leukemia, whose activity can be blocked by the binding of the drug Imatinib to the ATP-binding pocket of the fusion protein (143). However, targeting fusion oncoproteins with transcription factor characteristics and without enzymatic activity has been shown complicated since these lack natural binding pockets for a drug (144). Also, the intrinsically disordered N-terminal domain of FET oncoproteins makes the development of highly specific small-molecule inhibitors difficult (145). One promising strategy to degrade fusion oncoproteins is the use of the cell's normal protein degradation system, where proteins marked with a ubiquitin-tag are degraded by the proteosome. Molecules such as proteolysis-targeting chimeras (PROTACs) can be designed to target the protein of interest and induce the ubiquitination (146). However, this strategy still depends on the identification of a specific ligand that binds FUS::DDIT3, which requires further studies. Another future possibility would be to target the breakpoint of *FUS::DDIT3* itself or its target sequences at the DNA level using gene editing techniques such as CRISPR-CAS9. This was demonstrated in a study by Chen *et al*. where human prostate cancer cells were eliminated through apoptosis by inserting a suicide gene at the breakpoint of two fusion proteins using CRISPR-CAS9 (147). However, these approaches face significant challenges, including the development of effective delivery systems to ensure all tumor cells within the solid tumor are treated and minimizing off-target effects for therapeutic safety (148). Another aspect reducing the feasibility of this therapy strategy is that the exact breakpoint on the DNA level must be known and multiple *FUS::DDIT3* breakpoint variants exist in MLS tumors. Moreover, FUS::DDIT3 expression could be silenced by targeting the transcripts using techniques such as RNA interference or antisense oligonucleotides. Also for

these types of methods, the usability is currently limited by delivery challenges (149).

Indirect targeting of FUS::DDIT3 and its functions also constitutes a possible therapeutic option. As described in this thesis, the mechanism of FUS::DDIT3 epigenetically and transcriptionally affects many different cellular functions and signaling pathways central to tumor development. Hence, many potential therapeutic targets exist, such as various components of different signaling pathways. Several *in vitro* and *in vivo* studies have observed effects in terms of reduced cell viability and decreased tumor volume from the use of inhibitors targeting IGF-IR, PI3K and YAP1(55, 58, 150). Inhibition of SRC/FAK signaling further showed reduced invasiveness of MLS cells (151). At present, there are no MLS-specific clinical trials demonstrating clinical value for these treatment strategies (74). In paper II, we found that the transcription factor STAT3 and FUS::DDIT3 share a set of DNA binding sites and commonly regulate genes controlling stemness and thereby therapy resistance in MLS. Targeting JAK-STAT signaling in combination with chemotherapy could thereby be efficient in reducing the risk of therapy resistance, but further *in vivo* studies are needed to evaluate the clinical benefits (64). Additionally, further studies are required to determine how FUS::DDIT3 mechanistically activates JAK-STAT signaling, which could reveal additional targets. Targeting the core mechanism of FUS::DDIT3, as directly as possible, is obviously attractive compared to targeting the many branches of downstream transcriptional consequences resulting from FUS::DDIT3 activity. For example, if the fusion oncoprotein remains active, feedback mechanisms might restore transcriptional output via alternative routes of signaling, with the risk of developing therapy resistance. FET fusion oncoproteins seem to share a similar core mechanism for achieving dramatic transcriptional reprogramming in the tumor cells: the transcription factor partner leads the oncoproteins to different tumor type-specific chromatin sites, while the structurally disordered prion-like

domains of the FET partner contribute to protein interactions and phase separation with recruitment of chromatin regulatory complexes such as SWI/SNF and transcriptional machinery to promote abnormal gene regulation. By finding a way to target this mechanism, it is possible that we can find a universal treatment strategy, efficient for a whole family of tumor diseases.

FET fusion oncoproteins interfere with central epigenetic regulation, and several studies have confirmed an interaction between these oncoproteins and the epigenetic regulators SWI/SNF and PRC2, which hence constitute possible therapeutic targets (75). The work in paper IV brought further insight into how FUS::DDIT3 alters epigenetics by affecting the genomic localization of SWI/SNF complexes, affecting chromatin accessibility at different sites. When FUS::DDIT3 was present, SWI/SNF and PRC2 complexes were more frequently co-localized, probably disrupting the balance between their opposing functions. What consequences this entails need further investigation. So far, no FDA-approved drugs specifically targeting the SWI/SNF complex exist, but there are many ongoing studies evaluating different strategies, including small-molecule inhibitors and degraders targeting the catalytic subunit BRG1 or BRM (152). Tazemetostat is an FDA-approved inhibitor against EZH2, the enzymatic subunit of PRC2, that potentially could be used to replace the missing opposition from SWI/SNF remodeling at the sites where SWI/SNF complexes were displaced from chromatin by FUS::DDIT3 (153). In paper IV, we did not observe an effect on viability by treating the cells with this inhibitor. However, EZH2 inhibition in combination with inhibition of HDACs, enzymes catalyzing the removal of acetyl groups from histones, substantially reduced viability, especially for FUS::DDIT3-expressing cells. This observed effect might be related to the fact that HDACs and associated HDAC targets were repeatedly identified as being regulated by FUS::DDIT3 in our analyses. HDAC inhibitors have also shown effects on cell viability and tumor growth in MLS and Ewing sarcoma in preclinical

setups, but clinical trials have so far failed to show a significant response and clinical value (136, 154-156). Together, these data indicate that HDACs might have a role in FET fusion oncogene-induced epigenetic regulation and that we disturb this mechanism through HDAC inhibition. However, to reach therapeutic potential, combined treatment using for example other epigenetic drugs might be required. One such example could be the Bromodomain-containing protein 4 (BRD4), an epigenetic reader that accumulates at hyperacetylated sites where it acts as a scaffold to recruit protein complexes, RNA polymerase II and other factors involved in transcriptional regulation. Our research group and others have previously identified that FET fusion oncoproteins interact with BRD4 and we found that FET fusion oncoproteins co-localize on chromatin together with SWI/SNF, BRD4 and RNA polymerase II (78, 157, 158). We further found that both MLS and Ewing sarcoma cells are sensitive to BRD4 inhibition, demonstrating its therapeutic potential. Furthermore, preliminary data in the research group show an effect of BRD4 inhibition as a combination treatment with HDAC inhibition in MLS and Ewing sarcoma models. Although indirectly targeting the fusion oncoprotein via its interaction partners represents a promising therapeutic strategy, this approach also entails potential risks in terms of unintended side effects that may arise due to the involvement of these protein complexes in additional cellular processes (155). Therefore, the effects of such treatments must be carefully evaluated before they can be tested in clinical settings.

A low differentiation state of the cells appears to be important for FUS::DDIT3 to be able to induce tumorigenesis. Forcing maturation of pre-adipocytic MLS cells thereby represents one possible treatment strategy. One suggested approach for this is the use of ligands activating PPARG nuclear receptors, which is one of the late controllers in the adipocyte differentiation process (159). FUS::DDIT3 has been shown to reduce levels of PPARG to interrupt differentiation (53), hence, terminal differentiation could potentially be induced by activation of these receptors. A clinical

study including MLS patients failed to show clinical value from PPARG activation as monotherapy (160). However, more recent pre-clinical studies in mouse MLS PDX models demonstrated promising results, showing promoted terminal adipogenesis following combination treatment with PPARG and trabectedin, which is currently used as a second-line therapy in MLS (161). The exact mechanism of trabectedin in MLS is still not known, but it has been shown to block the fusion oncoprotein from binding target gene promoters, resulting in reactivation of adipocyte differentiation (162). The ongoing clinical study TRABEPIO is also evaluating the combinatorial effects of PPARG activation and trabectedin treatment in MLS patients, for which monotherapy with trabectedin was no longer functional, to see if PPARG activation can be valuable to overcome trabectedin resistance (163). It is possible that the action of the previously described epigenetic drugs inhibiting HDAC, EZH2 and BRD4, also results in differentiation of MLS cells, as they have all three have been shown to have roles in adipogenesis, where they regulate the expression of adipogenic factors such as PPARG and CEBPA (134, 164-166).

In paper IV, we observed that FUS::DDIT3 prefers binding at distal sites enriched in GGAAT repeats, in many cases accompanied by SWI/SNF and PRC2 binding. The functional implication of this needs further investigation, but possibly these regions could function as enhancer or repressor regions similar to the GGAA repeats in Ewing sarcoma (77, 119, 120), and/or the transcribed long non-coding RNAs could play a role in the transcriptional reprogramming initiated by FUS::DDIT3. In recent years, a growing interest in long non-coding RNAs has emerged, recognizing that these transcripts are not just non-coding noise but have important roles in cell and developmental biology (121). They for example interact with epigenetic regulators such as SWI/SNF and PRC2 to control chromatin architecture and contribute to the formation of biomolecular condensates (121, 167). As previously discussed, phase separation processes appear central to the oncogenic mechanism of FET fusion oncoproteins and the

involvement of long non-coding transcripts thereby constitutes a highly interesting future research topic. Potential interactions between FUS::DDIT3 and long non-coding RNAs could for example be identified using methods that isolate the fusion oncoprotein and associated RNAs with immunoprecipitation followed by sequencing-based or PCR-based detection of RNA transcripts. Insights could also be gained by disrupting these repetitive sequences at the DNA level using gene-editing approaches such as CRISPR–Cas9, or by targeting their transcripts for degradation and assessing the resulting consequences. If the oncogenic mechanism of FUS::DDIT3 relies on the binding to these repeats or the expression of their transcripts, they are potentially valuable therapeutic targets. Strategies for targeting the biomolecular condensates or preventing their formation also have therapeutic potential. Recent studies have challenged the existing idea that structurally disordered prion-like domains are impossible to target by identifying small molecules that bind these (41). Another possibility is to exploit the properties of biomolecular condensates to attract drug molecules, thereby increasing their local concentration, which could enhance their pharmacodynamic impact. This strategy of concentrating drugs was demonstrated for a set of small-molecule cancer therapeutics, including the BRD4 inhibitor JQ1 (168).

Taken together, significant progress over the past few years has been made in understanding how FET fusions initiate and drive tumor development, which has further revealed several potential targets for novel therapeutic strategies. Many of these hold great potential but are still at an early experimental stage and need further evaluation in more complex model systems and clinical trials. It is problematic that many potential therapies seem very promising in a preclinical setting, but when proceeding to clinical trials, they fail to show clinical relevance. This indicates that existing model systems routinely used in pre-clinical research are generally bad at predicting *in vivo* outcomes. Here, model systems such as the PDX-derived scaffolds we developed in paper I could be valuable, since these

more closely resemble the cellular context in which the drug will act *in vivo*. These model systems can also be further manipulated to make them even more physiologically relevant, for example by generating them from tumors of different patients to capture inter-patient variability, or by co-culturing different cell types, such as stromal or immune cells, to better predict how these interactions influence drug response. Nevertheless, this thesis has contributed to an increased understanding of the molecular role of FUS::DDIT3 in MLS, revealing several mechanisms that represent promising therapeutic targets for targeted therapies and improved treatment outcomes in MLS and other FET fusion oncogene-driven sarcomas.

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