FET fusion oncoproteins in sarcoma tumorigenesis

Interactions with the SWI/SNF chromatin remodeling complex and epigenetic effects

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Cover illustration: FET-oncoprotein-bound SWI/SNF chromatin remodeling complex by Malin Lindén. Created with BioRender.com.

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To those who supported me along the way

ABSTRACT

The FET family of fusion oncoproteins containing the N-terminal of either FUS, EWSR1 or TAF15 together with a transcription factor partner is the characteristic oncogenic driver of more than 20 types of sarcoma and leukemia, including myxoid liposarcoma and Ewing sarcoma. The FET oncoproteins are thought to function as aberrant transcription factors, however, the exact oncogenic mechanism remains to be elucidated. The aim of this PhD project was to study the effects of the FET oncoproteins, especially on the SWI/SNF chromatin remodeling complex and downstream epigenetic effects. We therefore developed a comprehensive proteomic workflow, where subcellular fractionation by nuclear extraction and enrichment using immunoprecipitation was employed prior to western blot or mass spectrometry analysis to study nuclear interactions. First, we evaluated the contribution of each part of the fusion oncoprotein. The transcription factor partner DDIT3 bound specific genomic loci via its dimerization partners and repressed a set of target genes. The FET N-terminal domain in both normal and oncogenic FET proteins interacted with the SWI/SNF complex, however, in a dysregulated way for FET oncoproteins. Further analysis showed that FET oncoproteins interact with all three main subtypes of the SWI/SNF complex and that the core of the FET oncoprotein-bound SWI/SNF complexes remain intact. However, a minor effect on the complex composition cannot be excluded. We then showed that FET oncoproteins interact with the transcriptional coactivator BRD4, via the SWI/SNF complex. Importantly, further analysis revealed that FET oncoproteins, SWI/SNF components and BRD4 co-localize on chromatin and interact with Mediator and RNA polymerase II components thus providing a direct link to mechanisms in chromatin remodeling and transcriptional regulation. Downstream analyses indicated that FET oncoproteins affect the repressive histone mark H3K27me3 and disturb the important transcriptional balance between SWI/SNF and polycomb repressive complexes. Furthermore, the FET oncoprotein FUS-DDIT3 interacted with pSTAT3, a transcription factor activated by the JAK-STAT signaling pathway, indicating a role of this pathway in FET sarcoma. In conclusion, dysregulation of SWI/SNF and downstream epigenetic processes provide a unifying oncogenic mechanism for tumors caused by FET fusion oncoproteins thus providing an opportunity for development of new targeted therapies.

Keywords: Ewing sarcoma, Epigenetics, FET fusion oncoproteins, Myxoid liposarcoma, Proteomics, SWI/SNF (BAF) chromatin remodeling complex.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Sarkom är ett samlingsbegrepp för en sorts cancer som uppstår i kroppens skelett och stödjevävnader. Sarkom är relativt ovanliga med ungefär 400 nya patienter i Sverige varje år (lite mindre än en procent av antalet totala cancerfall). Vi studerar FET-familjen av sarkom som omfattar drygt 20 olika tumörtyper såsom myxoid liposarkom och Ewing sarkom. Till skillnad från de flesta andra cancerformer, orsakas inte dessa tumörer av en ackumulation av mutationer utan av specifika fusionsonkogener, som innehåller den främre delen av en av FET-generna FUS, EWSR1 eller TAF15 som fuseras med en gen för ett DNA-bindande protein. De senaste åren har allt större fokus inom cancerforskning lagts på betydelsen av epigenetiska förändringar, det vill säga styrning av hur DNA packas och uttrycks. Målet med detta doktorandprojekt var att studera effekten av FET-fusionsproteinerna och dess påverkan på olika epigenetiska processer. Därför odlade vi stora mängder tumörceller, extraherade proteiner från cellkärnan (där dessa processer äger rum) och använde bland annat FET fusionsproteinerna som lockbeten för att studera vilka proteiner de interagerade med. Vi upptäckte då att FET-fusionsproteiner interagerar med ett stort proteinkomplex som heter SWI/SNF. Komplexet omstrukturerar hur DNA packas i kromatinet, strukturen i kärnan som skyddar generna och reglerar deras aktivitet. Detta påverkar viktiga cancerprocesser såsom cellmognad och tillväxt. Vi gjorde sedan djupare analyser av de proteiner som interagerar med fusionsproteinerna och visade att FET-fusionsproteinerna binder till komponenter från alla tre huvudvarianterna av SWI/SNF-komplexet samt att kärnan av proteinkomplexet är intakt. Däremot är det möjligt att sammansättningen av komplexet påverkas av bindningen av FET-fusionsproteinerna. Detta är intressant eftersom varje liten förändring såsom byte av en av proteinkomponenterna i komplexet kan förändra funktionen på SWI/SNF. Våra försök tyder på att interaktionen med FET-onkoproteinerna leder till felreglering av SWI/SNF-komplexen och därmed störningar i cellernas genuttryck och funktioner. Därför studerade vi också betydelsen av ett protein som heter BRD4 som binder till specifika positioner i kromatinet och inducerar genuttryck. Vi kunde påvisa att FET-fusioner interagerar med BRD4, men de gör det inte direkt utan via SWI/SNF-komplexet. Detta indikerar att BRD4 och SWI/SNF är viktiga för FET-fusionernas roll i canceruppkomst och att både BRD4 och de felreglerade SWI/SNF-komplexen är möjliga läkemedelsmål. Tillsammans leder dessa resultat till ökad förståelse för hur FET sarkom uppstår. Förhoppningsvis kan det leda till utveckling av en gemensam molekylärt baserad behandling för patienter med dessa tumörsjukdomar som riktar sig specifikt mot cancercellerna.

LIST OF PAPERS

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

I. Osman, A., **Lindén, M.**, Österlund, T., Vannas, C., Andersson, L., Escobar, M., Ståhlberg, A. and Åman, P. *DDIT3 genomic binding sites in human sarcoma cells reveals dimerization partners and target genes*. (Manuscript).

II. **Lindén*, M.**, Thomsen*, C., Grundevik, P., Jonasson, E., Andersson, D., Runnberg, R., Dolatabadi, S., Vannas, C., Luna Santamaría, M., Fagman, H., Ståhlberg, A. and Åman, P. (2019) *FET family fusion oncoproteins target the SWI/SNF chromatin remodeling complex.* EMBO Reports, May; 20(5). DOI: 10.15252/embr.201845766. *Shared first authorship. These authors contributed equally.

III. **Lindén, M.**, Vannas, C., Österlund, T., Andersson, L., Osman, A., Escobar, M., Fagman, H., Ståhlberg, A. and Åman, P. *Interactions of FET fusion oncoproteins with BRD4 and SWI/SNF chromatin remodeling complex subtypes.* (Manuscript).

IV. Dolatabadi*, S., Jonasson*, E., Andersson, L., Luna Santamaría, M., **Lindén, M.**, Österlund, T., Åman, P. and Ståhlberg, A. *FUS-DDIT3 Fusion Oncoprotein Expression Affects JAK-STAT signaling in Myxoid Liposarcoma.* *Shared first authorship. These authors contributed equally. (Manuscript).

Additional publications, not part of this thesis:

i. Dolatabadi, S., Jonasson, E., **Lindén, M.**, Fereydouni, B., Bäcksten, K., Nilsson, M., Martner, A., Forootan, A., Fagman, H., Landberg, G., Åman, P. and Ståhlberg, A. (2019) *JAK-STAT signaling controls cancer stem cell properties including therapy resistance in myxoid liposarcoma.* International Journal of Cancer, July 145(2):435-449. DOI:10.1002/ijc.32123.

A Swedish popular scientific article based on paper II, originally published in “Onkologi i Sverige 2019:4”, is included as Appendix 1.

CONTENT

ABBREVIATIONS

BET	Bromodomain and Extra Terminal domain
ChIP-seq	Chromatin Immunoprecipitation sequencing
Co-IP	Co-Immunoprecipitation
EWS	Ewing sarcoma
FET	FUS, EWSR1 and TAF15
FET-FOP	FET fusion oncoprotein
IP	Immunoprecipitation
IDR	Intrinsically disordered region
MLS	Myxoid liposarcoma
MS	Mass spectrometry
NTD	N-terminal domain
PRC	Polycomb repressive complex
QWB	Quantitative western blot
RNA Pol II	RNA polymerase II
SSE	Sequential salt extraction
TSS	Transcription start site
WB	Western blot

SWI/SNF COMPONENTS – A GUIDE

SWI/SNF component	Alternative name	SWI/SNF subtype
ARID1A	BAF250A	cBAF-specific
ARID1B	BAF250B	cBAF-specific
ARID2	BAF200	PBAF-specific
BAF155	SMARCC1	All, core module
BAF170	SMARCC2	cBAF and PBAF, core module
BAF45A	PHF10	PBAF-specific
BAF45B	DPF1	cBAF-specific
BAF45C	DPF3	cBAF-specific
BAF45D	DPF2	cBAF-specific
BAF47	SMARCB1	cBAF and PBAF, core module
BAF53A	ACTL6A	All, ATPase module
BAF53B	ACTL6B	All, ATPase module
BAF57	SMARCE1	cBAF and PBAF, core module
BAF60A	SMARCD1	All, core module
BAF60B	SMARCD2	All, core module
BAF60C	SMARCD3	All, core module
BCL7A		All, ATPase module
BCL7B		All, ATPase module
BCL7C		All, ATPase module
BRD7		PBAF-specific
BRD9		GBAF-specific
BRG1	SMARCA4	All, ATPase
BRM	SMARCA2	All, ATPase
GLTSCR1	BICRA	GBAF-specific
GLTSCR1L	BICRAL	GBAF-specific
PBRM1	BAF180	PBAF-specific
SS18	SSXT	cBAF and GBAF, ATPase module
SS18L1	CREST	cBAF and GBAF, ATPase module
β-ACTIN	ACTB	All, ATPase module

1 INTRODUCTION

Sarcoma is a rare group of cancer arising from bone or connective soft tissue such as muscle or fat. Around 400 people in Sweden develop sarcoma every year, a little bit less than 1% of the yearly cancer incidence [1]. The classification of sarcomas is based on the specific tissue (or type of cell) the cancer originates from or recapitulates; tumors with osteogenic differentiation and malignant features are called osteosarcoma and tumors with adipocytic appearance are called liposarcoma. Further sub-classification is based on morphology and genetic alterations, for example liposarcoma can be divided into four subgroups: well-differentiated liposarcoma, myxoid liposarcoma, de-differentiated liposarcoma and pleomorphic liposarcoma. Ewing sarcoma, on the other hand, has an unknown origin and was therefore named after the pathologist James Ewing who discovered it around 100 years ago.

Most malignant tumors arise after accumulation of DNA damages, i.e. mutations, in a single cell and its resulting clone, a process that takes many years. In some tumor forms such as sarcoma, leukemia and lymphoma, special genetic aberrations called fusion oncogenes are overrepresented. They are formed during chromosomal rearrangements such as translocations, when two parts of chromosomes change place with each other after double-stranded breaks, resulting in a fusion gene and ultimately a fusion protein. Recently, more interest is placed on the importance of epigenetics, the regulation of how DNA is packed in chromatin and expressed, and how epigenetic changes might affect tumor development.

In cancer research, discovering the reasons for tumor formation and developing new therapies are the major aims. However, genomic instability and the big genetic variation between tumors of the same type and even within individual tumors are still major obstacles when trying to understand mechanisms behind tumorigenesis. Using a simple model system with maintained genomic stability therefore has considerable value when studying general tumor development. One such example is the group of tumors caused by FET fusion oncogenes (containing the N-terminal of either FUS, EWSR1 or TAF15; Figure 1). The most common of the around 20 tumor types carrying the FET fusion oncogenes are myxoid liposarcoma (MLS) and Ewing sarcoma (EWS). Modern cytogenetics and whole genome sequencing have shown that FET-oncogene-caused tumors contain few, or in some cases, no other mutations than the fusion oncogene, that these tumors are genetically stable (even at recurrences after many years or after many years of *in vitro* culture) and have, with few exceptions, an intact p53 system [2-5]. This indicates a “simple”

mechanism behind tumor development. Finding this mechanism has been a major goal of the research field for a long time. FET oncoproteins are thought to function as aberrant transcriptional regulators but the exact mechanism remains to be elucidated. We recently discovered that FET fusion oncoproteins bind to the SWI/SNF chromatin remodeling complex thus providing a direct link to mechanisms in chromatin remodeling and the global transcriptional effect [Paper II] [6]. Researching the effect of the FET fusion oncogenes and increasing our understanding of mechanisms driving oncogenesis is of upmost importance to be able to develop targeted therapies. This knowledge could also improve the general understanding of tumor development such as the importance of epigenetic changes and aberrant chromatin remodeling.

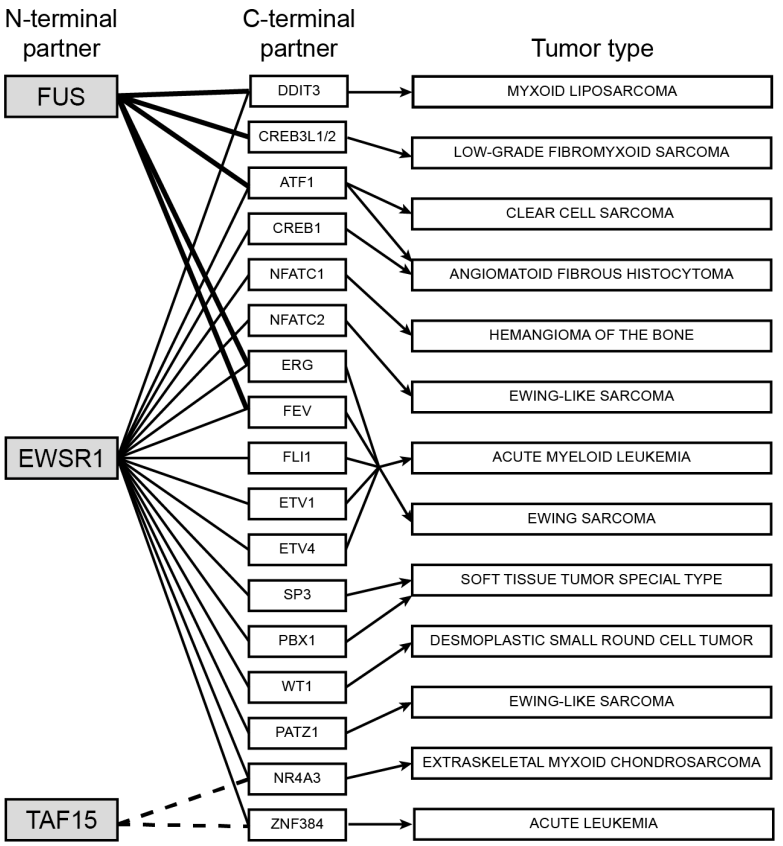

Figure 1. *FET fusion oncoproteins. Examples of FET N-terminal fusion partners, C-terminal transcription factor fusion partners and their associated tumor types caused by respective fusion oncogene. More FET oncoproteins are continuously discovered. Adapted from Paper II [6].*

1.1 FET family of fusion oncogenes

Fusion oncogenes are among the most common types of oncogenes in human cancers with over 30 000 distinct variants described [7]. The majority of these have only been detected once, but sarcomas are overrepresented in recurrent chromosomal translocations [8]. Here we focus on the FET family of fusion oncogenes that carries one of the genes *FUS, EWSR1* or *TAF15* as 5'-partners juxtaposed to one of various alternative DNA-binding transcription factor genes as 3'-partners (Figure 1). To date there are over 25 types of FET fusion oncoproteins described but with the improved diagnostic capabilities and use of sequencing in clinical practice, the list of FET oncoproteins is rapidly growing.

1.1.1 FET proteins

The normal FET proteins FUS (Fused in sarcoma, also called TLS), EWSR1 (Ewing sarcoma breakpoint 1) and TAF15 (TATA-binding protein-associated factor 15) are multifunctional RNA-binding proteins with high structural similarity (Figure 2). They are ubiquitously expressed in most cell types and tissues and can shuttle between the cytoplasm and nucleus, but are primarily localized in the nucleus [9, 10]. FET proteins are involved in many gene regulatory functions including transcription [11-14], splicing [15-17], DNA repair [18, 19] and possibly mRNA transport [9, 20]. FET proteins bind RNA with low sequence specificity, via two binding domains (RRM and RGG/ZF; Figure 2), thus binding thousands of mRNAs with extensive overlap between FUS, EWSR1 and TAF15 [20, 21].

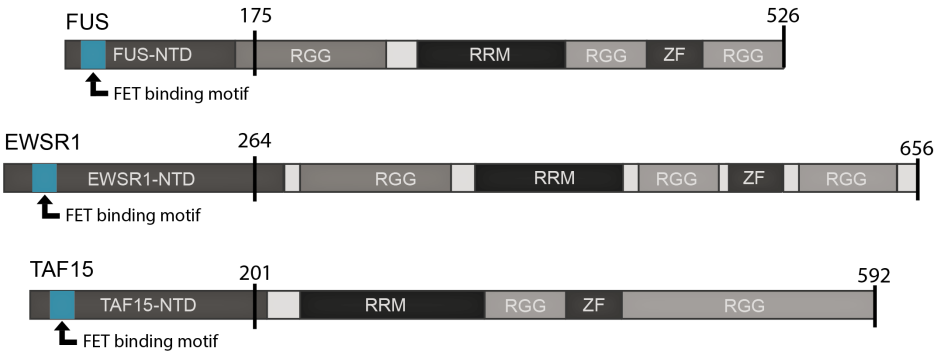

Figure 2. FET proteins contain the structurally disordered N-terminal domain (NTD), central RNA-binding domains (RRM: RNA recognition motif), zinc finger domains (ZF) and degenerated repeat regions (RGG: RGG repeat region). The FET binding motif is a conserved sequence required for binding between the normal FET proteins. Amino acid numbers are indicated. Adapted from Paper II [6].

1.1.2 FET N-terminal domain and phase separation properties

While the RNA-binding parts are lost when FET fusion oncoproteins are formed (in most cases), the conserved N-terminal domains (NTD) always remain. The NTDs consist of structurally disordered, prion-like domains with degenerated SYGQ-rich repeats (serine, tyrosine, glycine and glutamine), a composition that suggest a function in transactivation and protein-protein interactions. We previously showed that a conserved 26 amino acid sequence in the NTD called "FET binding motif" is required for binding between the normal FET proteins and interaction to FET oncoproteins, creating homo- and hetero-complexes [22].

FUS, EWSR1 and TAF15, together with around 240 human proteins, contain prion-like domains that make the proteins aggregation-prone and enable liquid-liquid phase separation through multivalent interactions (i.e. many "weak" interactions), creating so called biocondensates with a high local concentration of these proteins [23]. Liquid-liquid phase separation is a biophysical process utilized in cells, e.g. by formation of membraneless organelles such as stress granules, paraspeckles and nucleoli, which contribute to subcellular organization [23, 24]. The properties of the prion-like domain make these proteins, such as FUS, prone to pathological aggregation after additional mutations which can lead to fatal neurodegenerative diseases such as ALS (Amyotrophic Lateral Sclerosis) and FTD (FrontoTemporal Dementia) [25, 26]. In the literature, many terms are used for regions with increased phase-separation properties including prion-like domains (PLD), low-complexity domains (LCD) and intrinsically disordered region (IDR). From now on, I will use IDR since it best describes the biophysical properties contributing to self-interactions and phase separation. As mentioned above, the FET NTD contains an IDR enriched in uncharged polar and aromatic amino acids and especially Tyrosine (Y) have shown to be important for phase separation capacities [27, 28].

1.1.3 C-terminal transcription factor partners DDIT3 and FLI1

DDIT3 (DNA damage-inducible transcript 3, also known as CHOP; C/EBP homologous protein or GADD153; growth arrest and DNA damage-inducible gene 153) is a member of the C/EBP (CCATT/enhancer-binding protein) family of DNA-binding transcription factors. DDIT3 forms heterodimers with for example C/EBPα and C/EBPβ, through their basic leucine zipper region [29]. It is a tightly regulated protein, not expressed during normal growth conditions but induced by stress. DDIT3 is believed to be pro-apoptotic and forced expression of DDIT3 triggers cell cycle arrest and apoptosis [30, 31]. DDIT3 is thought to function as a dominant-negative inhibitor, formation of heterodimers with other

C/EBP members prevent their DNA-binding activity which leads to downregulation of C/EBP target genes [29]. Apart from its role as a C-terminal partner in FET oncoproteins, it is overexpressed in some tumor types as a result of amplification of the 12q13 chromosome region.

FLI1 (Friend leukemia integration 1) is a member of the ETS transcription factor family. FLI1 and its paralog ERG, together with other ETS transcription factors, contain the evolutionary conserved DNA-binding ETS domain, that binds DNA sites with a GGA(A/T) sequence. Although there are redundancy both in function and DNA binding between ETS factors, different binding partners contribute to their unique functions in differentiation, development, proliferation and apoptosis [32, 33]. Aberrant expression of ETS factors therefore impact crucial cell functions and can lead to tumorigenesis in both solid tumors and leukemia [33, 34].

1.1.4 FET fusion oncoproteins

FET fusion oncoproteins (FET-FOPs) are created after the in-frame fusion of one of the FET genes (*FUS, EWSR1* or *TAF15)* as 5'-partners juxtaposed to one of many alternative DNA-binding transcription factor genes as 3'-partners. The two most common FET oncoproteins, FUS-DDIT3 and EWSR1-FLI1, are shown in Figure 3. The many variants of FET-FOPs are, with few exceptions, specific for one tumor type each but the FET NTD can replace each other as the N-terminal partner in some fusion proteins showing their functional similarity (Figure 1). As mentioned above, the FET-FOPs are in numerous cases the only genetic alteration present in the tumor cells which indicate that they have the capacity to induce tumor development. Fusion proteins can retain properties from both parental proteins but can also possess neomorphic (novel) properties, potentially having an impact on both regulatory levels, stability and functions of their parental proteins. In FET oncogenes, the promoter and 5'-untranslated regions, and sometimes part of the N-terminal domain, of the 3' partner e.g. DDIT3 is replaced by the 5'-partner e.g. FUS [3, 35, 36]. Even though the abundantly expressed FET genes contribute with their promoters, FUS-DDIT3 was shown to have lower expression levels than FUS at both mRNA and protein levels in MLS cell lines, explained by lower stability of the fusion protein due to the contribution from DDIT3 [35]. FET oncoproteins are considered to act as aberrant transcription factors, inducing oncogenic gene expression [37, 38] but the exact mechanism(s) remain to be determined.

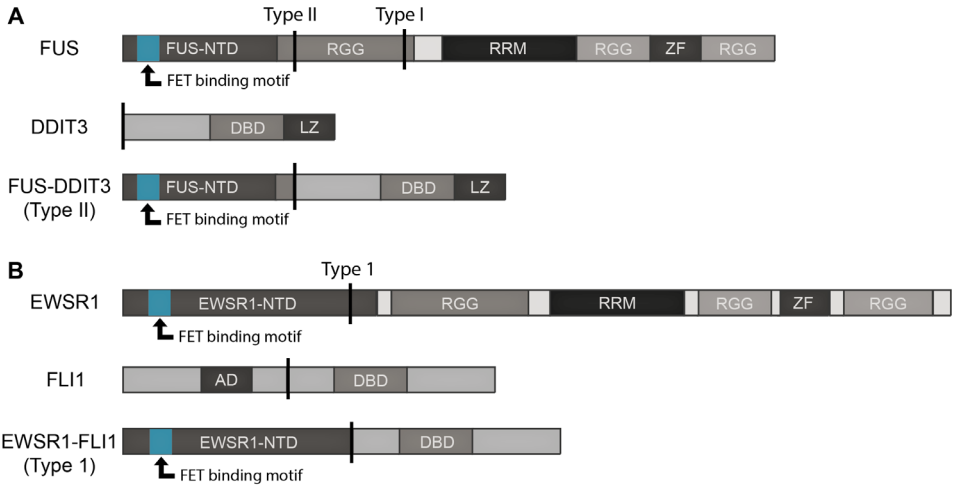

Figure 3. Schematic illustration of FET fusion oncoproteins FUS-DDIT3 type II (A) and EWR1-FLI1 type 1 (B) and their parental proteins. Type I and II show the location of the two most common MLS fusion breakpoints in FUS and Type 1 the most common breakpoint in EWSR1. NTD: N-terminal domain, RRM: RNA recognition motif, ZF: zinc finger domains, RGG: RGG repeat region, DBD: DNA-binding domain, LZ: leucine zipper domain. AD: activation domain. Part A adapted from Paper II [6].

1.2 FET-FOP-caused sarcoma

FET oncoproteins are the main cause of around 20 types of sarcoma and leukemia (Figure 1), with myxoid liposarcoma (MLS) and Ewing sarcoma (EWS) being the two most common FET-FOP-caused cancer types (hereby also referred to as FET sarcomas).

1.2.1 Myxoid Liposarcoma

Myxoid liposarcoma (MLS) accounts for about 20% of liposarcomas, constituting about 5% of adult soft tissue sarcomas [39]. MLS is equally common in males and females and has a peak incidence between 40 and 50 years. It usually occurs as a large, painless mass in deep soft tissues of the extremities, with more than two-thirds occurring in the musculature of the thigh. MLS has a tendency to recur locally but around one-third of patients develop distant metastases to other soft tissues such as retroperitoneum, bone or opposite extremity and axilla, before spreading to the lung. A subset of MLS with more round cell morphology (high-grade MLS, round-cell type) has a significantly poorer prognosis [39, 40]. MLS is mainly treated with surgery, but both neoadjuvant or adjuvant radiation treatment is also used. MLS tumors are

considered to be very radiosensitive. Palliative cytostatic treatment include Doxorubicin or, if the disease keeps progressing, Trabectedin and Eribulin [13, 41].

The karyotypic hallmark of myxoid liposarcoma is the t(12;16)(q13;p11) translocation, present in >95% of cases, leading to expression of the FUS-DDIT3 fusion protein (Figure 3) [42-44]. In some cases, DDIT3 is fused with EWSR1 in the t(12;22)(q13;q12) chromosomal translocation [45]. Presence of FUS-DDIT3 is highly specific for MLS and is absent in cancers with similar morphologies [46]. Several different FUS-DDIT3 transcripts exist with breakpoints in different parts of FUS introns, e.g. joining exon 7 to exon 2 of DDIT3 (7-2, type I, ~20%), exon 5-2 (type II, ~67%) and exon 8-2 (type III, ~10%) [47-49]. However, the diverse fusion transcripts do not seem to have a significant impact on grade or clinical outcome [47].

1.2.2 Ewing sarcoma

Ewing sarcoma (EWS) accounts for less than 10% of malignant bone tumors [39]. It is, however, the second most common bone and soft tissue sarcoma in children. EWS is more common in males and it has a peak incidence between 10 and 20 years. It occurs most prevalently in long bones, or in the pelvis and ribs. The most common clinical symptoms are pain and a mass in the affected area [39]. Ewing sarcoma is an aggressive disease with a tendency to recur after surgical removal, potentially forming early hematogenous metastasis in lung, bone and bone marrow [50]. EWS is normally treated with a comprehensive program of chemotherapy (including Doxorubicin, Ifosfamide and Actinomycin), surgery and often radiation. The 5-year survival is about 70-80% but significantly poorer with primary metastatic disease (<30%) [40]. The treatment for metastatic disease is focused on cytostatic drugs dependent on earlier therapies [41, 51].

Ewing sarcoma is characterized by a recurrent t(11;22)(q24;q12) chromosomal translocation present in 85% of cases, leading to the EWSR1-FLI1 fusion gene [52]. Around 10-15% of cases have the t(21;22)(q22,q12) translocation, producing the EWSR1-ERG fusion gene. FLI1 and ERG belong to the ETS family of transcriptions factors and in about 1-5% of cases, EWSR1 is fused to other ETS genes such as FEV, ETV1 and ETV4 [50].

1.3 Chromatin organization and remodeling

Chromatin is the 3D organization that package and protect DNA (Figure 4). First, DNA is wrapped around core particles called nucleosomes, an octamer that contains two copies of the four histone proteins H2A, H2B, H3 and H4. Nucleosomes are then compacted into a 30 nm fiber followed by higher order chromatin of various degree of compaction in the chromosome, with inactive heterochromatin as the most tightly packed form. Dynamic reorganization of chromatin, i.e. chromatin remodeling, is a vital process in the regulation of gene expression, DNA repair and DNA replication. Disruption of these important processes is involved in cancer development. In fact, almost all cellular processes require that proteins can access DNA in a dynamic, targeted and regulated way [53]. The dynamic regulation of chromatin organization includes addition and removal of histone marks by histone-modifying enzymes and reorganization of nucleosomes by chromatin remodeling complexes.

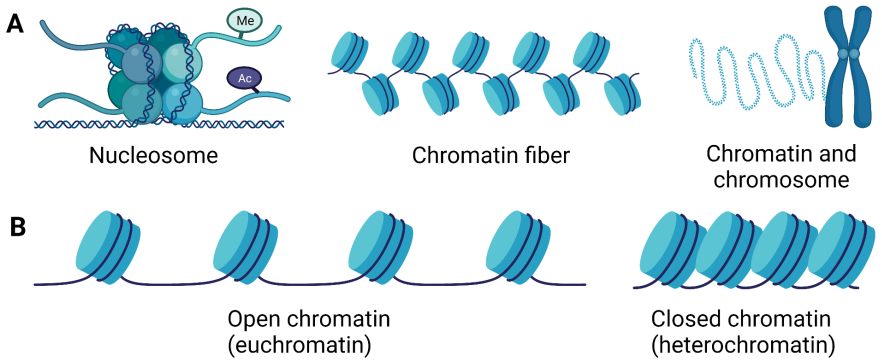

Figure 4. Chromatin. A. Organization of chromatin from the nucleosome octamer, with post-translational-modified histone tails, containing 147 base pairs of DNA to higher order chromatin fiber, condensed chromatin and chromosomes. B. Schematic visualization of open and closed chromatin (euchromatin and heterochromatin, respectively). Created with BioRender.com.

Histone-modifying enzymes alter the chromatin landscape by covalent addition or removal of post-translational modifications on the histone tails such as methylation, acetylation and phosphorylation (Figure 4A), which changes the packing of chromatin due to changes in electrostatic or steric interactions. Some of these histone marks, including trimethylation of H3K9 and H3K27, are associated with heterochromatin (closed chromatin, Figure 4B) and repression of gene expression. Other marks are enriched in euchromatin (open chromatin), such as methylations of H3K4 and H3K36 or acetylation of histone tails, and

associated with transcriptional activation [54]. The epigenetic landscape, the combined organization and expression of histone variants and modifications, are controlled by a large set of enzymes, some that add histone modifications (writers), some that remove the modification (erasers) and those that recognize and bind a specific histone mark (readers).

Chromatin is remodeled by ATP-dependent chromatin-remodeling enzymes which are highly conserved from yeast to human. They use the energy from ATP hydrolysis to disrupt histone-DNA interactions, move nucleosomes along DNA, evict histones or exchange histone variants which leads to changes in DNA accessibility. There are four groups of chromatin remodelers: the SWI/SNF family, the ISWI family, the NuRD family and the INO80 family. The activity of all groups of chromatin remodeling complexes can lead to both transcriptional repression and activation, and their mode of action is thus context-dependent [53, 55].

1.4 SWI/SNF chromatin remodeling complexes

The SWI/SNF (SWItch/Sucrose Non-Fermentable) chromatin remodeling complex was first discovered in yeast but exist in all eukaryotic organisms. The mammalian 1.5 MDa SWI/SNF complex consists of around 15 subunits encoded by 29 genes, including the mutually exclusive catalytic subunits BRG1 and BRM, and a number of BAFs (BRG1-Associated Factors). Some of the subunits exist in different paralogs and their combinatorial assembly leads to a large variety of complexes with diverse functions in different tissues and stages during development [56, 57]. The SWI/SNF complex utilizes the energy from ATP hydrolysis to remodel nucleosomes and expose DNA which often lead to active transcription [58]. However, due to the complexity of variant complexes and the contribution of each subunit to the function of the complex, the SWI/SNF complex can be expected to have diverse roles depending on many factors such as chromatin loci, cell type, or environmental cues.

1.4.1 General function of the SWI/SNF complex

SWI/SNF complexes remodel and control nucleosome landscapes, for example at transcription start sites, by sliding and evicting nucleosomes. They thereby have a role in different nuclear functions such as DNA transcription, replication and repair. SWI/SNF complexes also have essential roles in genome maintenance, and loss of for example BRG1 or PBRM1 leads to increased genomic instability [53]. The main function of SWI/SNF complexes is facilitating these molecular processes through chromatin remodeling. They also

have a role in other cellular processes such as cell differentiation, cell proliferation and cell adhesion, via interactions with different transcription factor partners [53]. It was estimated that between 5-20% of human genes are regulated directly by SWI/SNF complexes [53, 59]. They regulate gene expression by modifying chromatin accessibility at different chromatin loci including promoters, enhancers and transcription start sites [60]. However, SWI/SNF complexes do not function by itself but have an intricate balance with other chromatin remodeling complexes, histone readers and writers.

1.4.2 SWI/SNF complex subtypes

The combined assembly of SWI/SNF components leads to over 1000 theoretical combinations, however, with our current knowledge, these can be divided into three main SWI/SNF subtypes based on diverse size and biochemical composition [61]. Historically, two major types of SWI/SNF complexes were described: the more abundant canonical BAF complex (cBAF) and the larger (and less abundant) polybromo-BAF complex (PBAF). A third SWI/SNF subtype was then recently described, named GLTSCR1/1L-BAF (GBAF) [62] or non-canonical BAF (ncBAF) [61]. Determining the structure and composition of SWI/SNF complexes have been challenging but a lot of progress have occurred during the last five years. First, the modular assembly of SWI/SNF components was described based on cross-linking mass spectrometry and comprehensive biochemical analysis; from an initial dimer (BAF155/BAF170) to a larger core complex (with BAF60A/B/C, BAF57 and BAF47) followed by addition of subtype-specific components and lastly the ATPase module (catalytic subunit BRG1/BRM + accessory subunits) [61]. Many studies have then reported at least a partial structure of recombinant and endogenous cBAF complexes (the most common and studied subtype) with or without a nucleosome bound using Cryo-electron microscopy [63-66]. A schematic visualization of the SWI/SNF subtypes with comprehensive description of their different subunit composition is shown in Figure 5.

1.4.3 Antagonism between SWI/SNF and Polycomb repressive complexes

The remodeling activity of SWI/SNF complexes is generally believed to promote open euchromatin, with active transcription [58]. A large impact on the regulation of gene expression is achieved through antagonism of another epigenetic multi-subunit complex, the PRC2 polycomb repressor complex [58, 67, 68]. The PRC2 complex consists of the core components SUZ12, EED, RBBP4/7 and the enzymatic subunits EZH1 or EZH2, plus several accessory

chromatin-binding proteins [4]. It is a histone-modifying enzyme complex (writer) that post-translationally modifies the N-terminal tails of histone proteins which changes the structure of chromatin. The main catalytic subunit of PRC2, EZH2, catalyzes the methylation of lysine 27 on histone H3 (H3K27me3), a histone mark associated with repressive heterochromatin [57, 58]. Polycomb complexes have diverse repressive roles in tissue and embryonic development [69] and the balance between SWI/SNF and polycomb complexes enables expression of genes required for differentiation of progenitor cells into its committed lineage while repressing their alternate cell fates [4]. The SWI/SNF - Polycomb opposition occurs rapidly on chromatin, in an ATP-dependent way, and the opposition depend on BAF47; loss of this subunit (e.g. in malignant rhabdoid tumors) leads to reduced ability to oppose PRC complexes [70, 71]. Disturbing the delicate balance between SWI/SNF and PRC2 can thus play a role in oncogenesis.

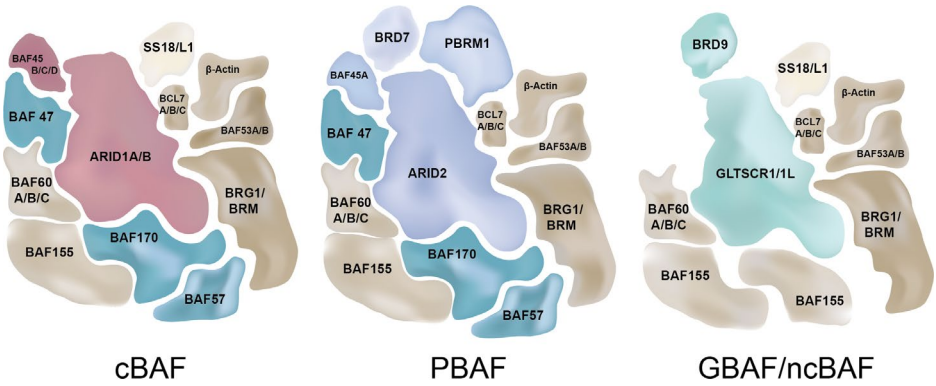

Figure 5. Schematic illustration of SWI/SNF subtypes. The SWI/SNF complex exists in three main subtypes; cBAF, PBAF and GBAF/ncBAF. All SWI/SNF complexes contain the structural core subunits BAF155 and BAF60A/B/C (light gold). Other core components are BAF170, BAF47 and BAF57 (cyan). The ATPase module containing one of the ATPase subunits BRG1 or BRM and BAF53A/B, BCL7A/B/C, β-actin (gold), and sometimes SS18/L1 (beige), is added to the complex last after addition of subtype-specific components; ARID1A/B and BAF45B/C/D for cBAF (red), and ARID2, PBRM1, BAF45A, and BRD7 for PBAF (blue). The two mutually exclusive paralogs GLTSCR1 and GLTSCR1L, and BRD9 are incorporated specifically in GBAF/ncBAF (green) that lacks both core components as well as ARID and BAF45 subunits. The reported SWI/SNF compositions differ slightly between studies, probably due to the analytical methods used or the cell types studied. Similarly depicted in Paper III.

1.4.4 SWI/SNF complexes and cancer

SWI/SNF components have recently been shown to be mutated in around 20% of human tumors, with different subunits protecting against cancer in specific tissues [72]. Genes encoding SWI/SNF subunits have been considered tumor suppressors, since loss of specific subunits on the protein level leads to oncogenesis in certain cellular contexts [4, 73]. In most cases, these mutations appear late in tumor progression, potentially permitting disease progression, metastasis and treatment resistance. Indeed, SWI/SNF mutations have been shown to be enriched in metastatic or relapsed disease [53]. However, in a few rare cancer types such as malignant rhabdoid tumors and synovial sarcoma, SWI/SNF mutations are driving mutations appearing early in tumor development. Mutations in SWI/SNF components can lead to either structural or functional alterations i.e. that loss of a subunit leads to altered composition and structural integrity, or to a change in activity and/or genomic targeting without affecting the structure [53, 74]. Although most mutations in SWI/SNF subunits lead to loss-of-function, it has been reported that the oncogenic activity arise from aberrant activity of residual SWI/SNF complexes rather than inactivation of the complex [75, 76]. In FET-fusion-oncogene-caused cancers, the SWI/SNF complex is not mutated. Instead, the SWI/SNF complex was shown to be aberrantly recruited to tumor-specific enhancers by the Ewing sarcoma EWSR1-FLI1 fusion protein leading to target gene activation [77]. The exact role of aberrant SWI/SNF complexes in FET sarcoma remains to be determined.

1.5 Transcriptional coactivator BRD4

BRD4 is a bromodomain protein of the BET (bromodomain and extra terminal domain) family together with BRD2 and BRD3. They contain two bromodomains that recognize acetylated histone tails such as the active histone mark H3K27ac. BRD4 exists in two main isoforms, the long (150 kDa) and the short (80 kDa). BRD4 is a reader protein that reads the histone code and accumulates at hyper-acetylated sites such as enhancers, double-stranded breaks and telomeres [78]. Acting as a scaffold, this leads to recruitment and stabilization of specialized multi-protein complexes and involvement in enhancer activity, transcriptional and splicing control as well as DNA damage repair [78-80]. The role in transcriptional activation is achieved through association with different transcription factors, the Mediator complex, and through interaction with the transcription elongation factor complex P-TEFb and RNA polymerase II (RNA pol II) [78, 81, 82]. The long isoform of BRD4 has histone acetyl transferase (HAT) activity, depositing acetyl groups at various histone tails, and is thus also a writer protein [83].

BRD4 is enriched at super-enhancers and therefore commonly used as a super-enhancer mark together with the Mediator complex or H3K27ac. In line with this, BRD4 has demonstrated different genome binding patterns depending on cell type and differentiation state [84]. Thus, BRD4 is important during development and for cell identity through regulation of lineage-specific genes [78]. In addition, BRD4 has been shown to occupy super-enhancers at key oncogenic drivers and BRD4 inhibition in cancer cells leads to preferential disruption of these cancer-dependent super-enhancers [85]. BRD4 has thus emerged as a potential target in several cancer types. Interestingly, BRD4 was reported to interact with FET oncoproteins and contribute to oncogenic gene expression in MLS [86] and EWS [79, 87-89]. BRD4 is not a common interaction partner of the SWI/SNF complex but the association between BRD4 and BRG1 has been reported in a few studies [90-92]. Rahman *et al.* identified GLTSCR1 as an interacting partner of BRD4 and Alpsoy *et al.* suggested that the interaction of BRD4 with BRG1 is specific to the SWI/SNF subunits GLTSCR1 and BRD9, indicating a role for the GBAF complex in BRD4 functions [62, 93].

1.6 Aberrant signaling and the JAK-STAT pathway

Various signaling pathways are dysregulated in FET-oncogene-caused cancers. FET oncoproteins were involved in aberrant IGF-IR (Type 1 insulin-like growth factor receptor) signaling through the PI3K/AKT pathway after induction of IGF2 in MLS [94-96] and through activation of IGF1 in EWS [97, 98]. In addition, Src signaling is activated in MLS [99] and EWS [100] potentially contributing to invasion and tumor progression. The Hippo pathway also plays a role in sarcoma oncogenesis: YAP1 signaling is overactive in MLS [101] and YAP/TAZ is activated in many other sarcomas including EWS [102].

Furthermore, a previous study in our group showed that JAK-STAT (janus kinase - signal transducer and activator of transcription) signaling (Figure 6) has an important role in MLS by controlling cancer stem cell properties including chemotherapy resistance [103]. We could see that a subset of MLS cells had active JAK-STAT signaling and by inducing the JAK-STAT pathway with the ligand LIF, the number of cells with cancer-stem-cell-like properties increased. Aberrant activation of the JAK-STAT pathway is associated with tumor development and progression of several solid tumors [104, 105].

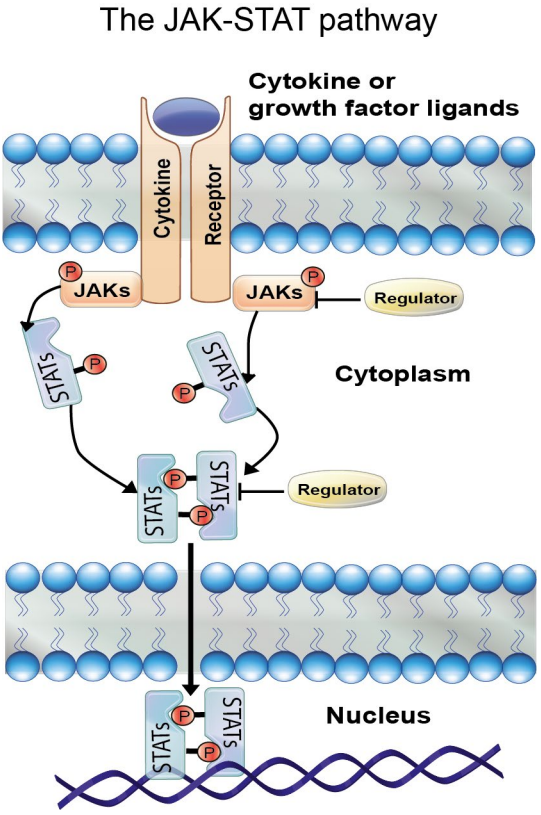

Figure 6. The JAK-STAT pathway. The JAK-STAT signaling pathway is activated after binding of ligands (various inflammatory cytokines such as interleukin IL-6, IL10-12 and IL22-23) to a transmembrane receptor. This leads to receptor multimerization and a closer interaction between receptor-bound JAKs (JAK1, JAK2, JAK3 and TYK2) that become activated by phosphorylation. The activated JAKs mediate intracellular signaling cascades by phosphorylating tyrosine residues of the receptor on the cytoplasmic side which allows binding of STAT proteins (STAT1-6). STAT proteins are then activated by phosphorylation (at highly conserved residues) which leads to dimerization and translocation to the nucleus. As a transcription factor, STAT3 (and other STATs) binds to target genes and regulate their expression. The JAK-STAT pathway is regulated at several levels, for example by different negative regulators such as tyrosine phosphatases and JAK inhibitors that limit the duration of JAK-STAT signaling [104, 105]. Adapted from [103].

2 SIGNIFICANCE

Although FET-oncogene-caused tumors are rare diseases, they mostly affect pediatric and young individuals. These patients are treated with more or less aggressive cytostatic treatment that in itself is DNA damaging and may lead to development of secondary cancers such as leukemia [106]. Today's treatment thus leads to severe side effects, often with lifelong problems for surviving patients. Since the FET-oncogene-caused tumors are genetically stable, it would be very attractive to develop new treatment strategies that are not dependent on DNA-damaging drugs or radiation. The epigenetic system involving the SWI/SNF complex and the transcriptional coactivator BRD4 may provide opportunities for such treatment strategies with many new drugs currently in development. The newly discovered connection to SWI/SNF provides a common explanation for tumorigenesis by all FET fusion oncogenes and might also shed light on other cancer types with perturbed SWI/SNF function such as when SWI/SNF subunits are mutated. The long-term goal is to develop a specific, effective treatment targeting the cancer cells with a perturbed SWI/SNF function, with less side effects as a result.

3 AIM

The major aim of this project is to study the effects of the FET fusion oncoproteins, especially on the SWI/SNF chromatin remodeling complex and downstream epigenetic effects, in FET sarcoma tumorigenesis.

Specific aims include:

Paper I: Determine the function of the transcription factor DDIT3; specifically its genomic binding sites, binding partners and target genes.

Paper II: Determine shared interaction partners of the FET NTDs, characterize the interaction of normal and oncogenic FET proteins to the SWI/SNF chromatin remodeling complex and evaluate downstream functional effects.

Paper III: Determine the interactions of FET fusion oncoproteins with SWI/SNF subtypes and the transcriptional coactivator BRD4, and further characterize their role in FET sarcoma oncogenesis.

Paper IV: Determine the effect of the FET fusion oncoprotein FUS-DDIT3 on aberrant JAK-STAT signaling in myxoid liposarcoma.

4 METHODS - ANALYZING NUCLEAR PROTEIN INTERACTIONS

This PhD project focuses on protein interactions of the FET fusion oncoproteins in the nuclei, especially with epigenetic modulators such as the SWI/SNF chromatin remodeling complex. We therefore developed a pipeline where subcellular fractionation by nuclear extraction and enrichment using immunoprecipitation (IP) is employed to study nuclear interactions and reduce sample complexity before Western blot (WB), quantitative Western blot (QWB) or mass spectrometry analysis (MS), see Figure 7. Important aspects of these methods and optimization attempts are discussed below. Other techniques have provided valuable insight for this PhD project such as drug testing/viability assays, RNA sequencing (RNA-seq) and Chromatin immunoprecipitation sequencing (ChIP-seq). The technical details and full method descriptions are provided in the attached published articles and manuscripts.

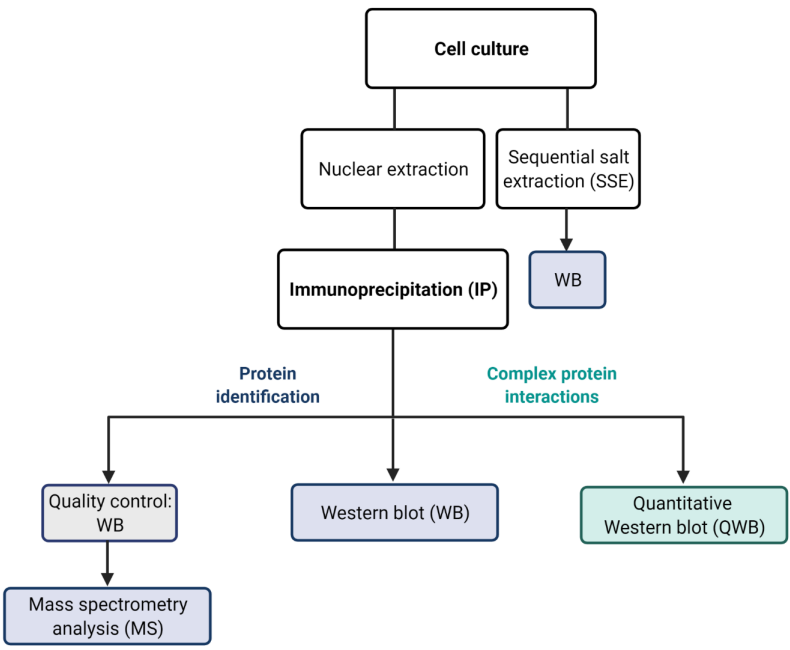

Figure 7. Method pipeline to analyze nuclear protein interactions. Western blot (WB) was used to detect proteins in nuclear extracts and sequential salt extracts and interaction partners were identified by WB or mass spectrometry after immunoprecipitation. For more complex analysis of proteins interactions, quantitative WB was employed.

4.1 Experimental systems

Here we studied the role of the FET fusion oncoproteins, especially FUS-DDIT3 in myxoid liposarcoma cell lines MLS 402, 1765 or 2645 (full name MLS 402-91, 1765-92 and 2645-94) and EWSR1-FLI1/ERG in Ewing sarcoma cell lines EWS TC-71 and IOR/CAR. The fibrosarcoma HT1080 cell line was used as a model system with ectopic expression (stable or transient) of EGFP-tagged FET fusion oncoproteins, their parental proteins or controls. Cancer cell lines are model systems for the specific cancer type, created from part of a tumor that have been cultured for a long time and adapted to a 2D laboratory setting. Other disadvantages include the lack of input from the cancer microenvironment and the tumor extracellular matrix. PDX mouse models (serial transplanted cancer tissue) and patient cancer tissues are available in the group but these were rarely used in the experiments presented in this thesis. This is partly because of the more limited supply of this material but also that it is more suited for verification of mechanisms and evaluation of drugs in the step after initial *in vitro* testing. This PhD project has focused on basic research, delineating molecular mechanisms induced by FET-FOPs for which cell lines are invaluable due to the large amount of cells that can be obtained in a short time span at different time points or cell passages, allowing for many different experiments at the RNA, DNA and protein level.

4.2 Nuclear protein extraction

Cultured cancer cell lines were expanded to large amounts and the cytoplasmic and nuclear fractions were extracted using a sophisticated nuclear extraction protocol. In short, the cells were harvested on ice by scraping in PBS and allowed to swell during incubation with a hypotonic lysis buffer (10 mM KCl). The outer cell membrane was disrupted with a syringe/needle and the cytoplasmic fraction was collected after incubation with Benzonase, which degrades accessible nucleic acids and potentially increases the release of nuclear proteins. The nuclear proteins were extracted through the nuclear membrane using a high-salt extraction buffer (420 mM KCl, final concentration ~250 mM). Nuclear extracts were diluted to physiological salt concentration (150 mM) before snap-freezing on dry ice and stored at -80°C to maintain protein quality and avoid degradation.

For more comprehensive studies of the binding properties of SWI/SNF components and FET proteins, a sequential salt extraction (SSE) procedure was developed based on our nuclear extraction protocol. After the first 250 mM KCl fraction was collected, the nuclear pellet was extracted twice more in the same high-salt extraction buffer but with 600 mM and 1200 mM KCl respectively.

Volumes corresponding to equal amount before dilution of the 250 mM, 500 mM and 1000 mM KCl fractions were then evaluated with WB. During these analyses, we noted that although the major parts of proteins (~65%) were extracted in the first fraction, increasing the salt concentration to 500 mM KCl would increase the recovery of nuclear proteins such as SWI/SNF components and FET proteins (~90%). Therefore, the most recent nuclear extractions were performed by extracting proteins directly in the high-salt extraction buffer but with 870 mM KCl (some of these experiments are included in Paper III). These 500 mM extracts were frozen without diluting to potentially minimize protein aggregation, and instead diluted prior to IP or WB experiments.

4.3 Western blot

Western blot was used frequently during this PhD project to detect proteins in nuclear extracts, sequential salt extracts and in IP samples. It is an invaluable method in proteomics and although simple, it is somewhat unpredictable. Problems can arise in each of the numerous steps; gel electrophoresis to size-separate proteins, transfer of proteins to membranes, primary and secondary antibody incubation, substrate chemi-luminescence incubation and detection, which can lead to both lack of signal at expected sizes or too many signals due to high background. Adequate protein detection relies on the quality of antibodies; therefore, new antibodies were optimized and evaluated thoroughly (Figure 8). More advanced considerations for detecting proteins in IP samples is covered in the section Co-immunoprecipitation.

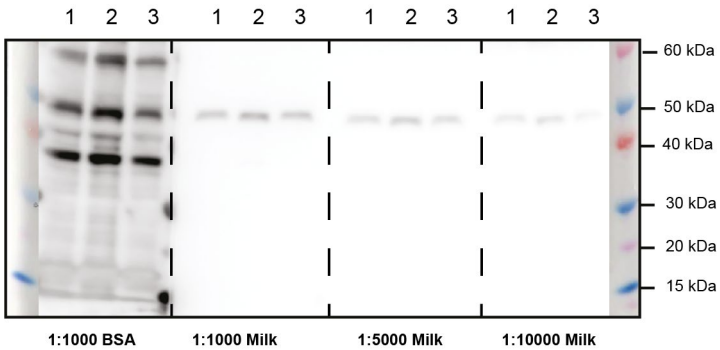

Figure 8. Example of antibody optimization. A BAF45D antibody (Abcam ab134942, 45 kDa) was evaluated by testing different antibody concentrations and blocking buffers (Milk or BSA), visualizing the importance of antibody optimization. These tests were combined with information from the manufacturer and the literature to determine if antibodies were suitable to use. In this case, either 1:1000 or 1:5000 dilution with milk as blocking buffer was suitable.

4.4 Co-immunoprecipitation

To study the nuclear protein interactions, FET oncoproteins or components of the SWI/SNF complex were pulled down using indirect immunoprecipitation (Figure 9A). The target protein and all interacting proteins end up in the bound fraction while remaining proteins, which do not bind to the target, end up in the nonbound fraction. It is important to include an IP with an IgG antibody matching the species of the target antibody as a negative control, to account for non-specific binding to the antibody and beads. To increase the yield of the immunoprecipitation procedure we used biotinylated antibodies and streptavidin beads (resulting in a very strong bond) and experimentally optimized the amount of beads, extract and antibody, all essential factors to consider for a successful IP. In addition, the buffer system used is important. It should be matched to the nuclear extracts and the beads need to be equalized in the same buffer so that protein binding is not due to changes in chemical or physical properties during the IP. The desired conditions, such as amount of detergent and salt, need to be determined before starting an experiment. The choice of elution buffer is also important and discussed in detail below (Co-IP: Considerations for MS detection).

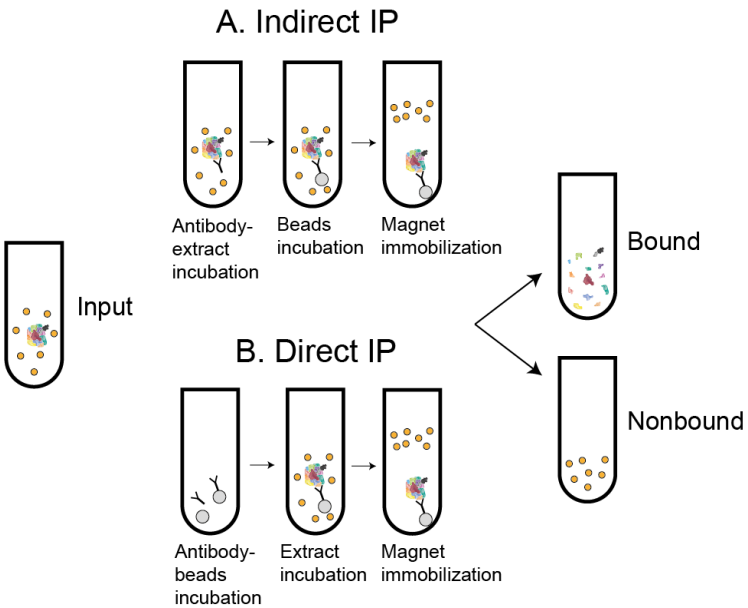

Figure 9. Imunoprecipitation (IP). A. Indirect immunoprecipitation: the input nuclear extract is incubated with antibody and then beads. The nonbound fraction is collected and the bound fraction is eluted from the beads. B. Direct immunoprecipitation: antibodies are first incubated with beads and then with input nuclear extract.

An indirect IP procedure is good to ensure optimal capture of interacting proteins, in particular those present at low amount. However, in the case of the indirect DDIT3 IP, the yield was quite low and only around 50% of the antibody was bound to the beads. In this case, antibody (with proteins of interest bound) remains in the nonbound thus making more advanced analysis difficult. In paper III and IV, we therefore utilized a direct IP method instead (Figure 9B) where the beads are incubated with antibody first, and then washed before incubation with extract to remove unbound antibody, potentially increasing the yield (Figure 10).

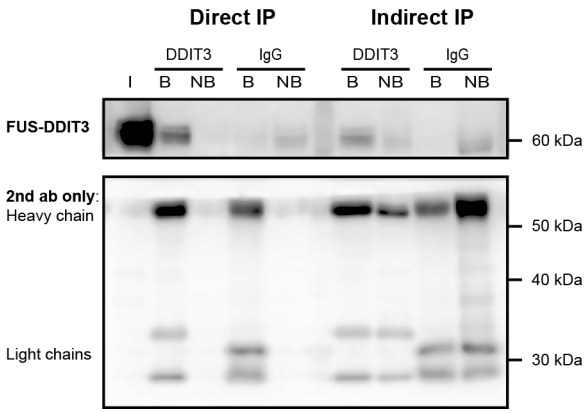

Figure 10. Optimization of DDIT3 IP: Direct vs Indirect IP. Quantitative WB of direct and indirect DDIT3 IP pulling down FUS-DDIT3 visualizes improved yield for the direct IP mainly due to improved capture of the antibody to the beads as visualized by the cross reaction of WB 2nd antibody with the denatured IP antibody heavy (55 kDa) and light (25 and 35 kDa) chains.

4.4.1 Co-IP: Considerations for MS detection

When performing immunoprecipitation intended for MS analysis, it is extra important to work as clean as possible to minimize background contamination e.g. from hair and skin. In addition, the buffers used need to be suitable for downstream mass spectrometry analysis; preferably no detergents should be used, especially not polyethylene glycol detergents such as NP40, Tween, and TritonX100, but small amounts of specific detergents such as CHAPS is possible if the last washes before elution is done in a pure triethylammonium bicarbonate buffer (TEAB). Remaining detergents will impact the chromatography step before MS-MS, as discussed below.

The choice of elution buffer is important to achieve optimal protein identification. We tested different elution buffers with the goal to elute proteins sufficiently from the beads while keeping it compatible with MS analysis. First, 0.2% Formic acid, a gentle elution buffer with low pH, was used but not all proteins of interest were eluted. When the proteomic analyses improved, allowing for MS-identification of proteins from LDS eluates (lithium dodecyl sulfate), the stronger 2x LDS sample buffer elution was the preferred elution buffer. LDS buffers elute more proteins, for example those that bind strongly to the beads, with the downside of increased background from the antibody (IgG heavy and light chains) as well as proteins binding the beads nonspecifically.

As a quality control, the yield of the target protein during immunoprecipitation and known interactions were verified with western blot before analyzing all co-immunoprecipitated proteins with mass spectrometry analysis at the proteomics core facility (University of Gothenburg). Both the LDS elution buffer, buffers used during IP and background proteins may provide problems during the LC (liquid chromatography)-separation before MS-MS, and therefore, all IP samples were treated with a "detergent-removal" step before analysis. The output from the MS analysis is a list of detected peptides and identified proteins (matched against the SwissProt database) which was used to analyze interaction partners of the target of interest in different samples. However, conventional MS analysis is not quantitative; the number of identified peptides depends on the size of the protein, tryptic cleavage sites/efficiency and the identification of ionized peptides. Therefore, we have also set up a comprehensive quantitative MS experiment, where immunoprecipitations are done in replicates and chemical labeling (TMT) of peptides are used to achieve relative global expression levels (see Future perspectives).

4.4.2 Co-IP: Considerations for WB detection

Western blot was used often in this PhD project to detect proteins in the bound fraction (eluate) after IP thereby confirming successful co-immunoprecipitation. When evaluating the IP with WB, an input control of nuclear extract is included as a positive control i.e. that the protein is present in the extract and is detected by the chosen antibody at the correct size. For regular WBs, the maximum amount of bound and nonbound samples for both the target antibody and the negative control IgG were loaded on the gel. This allows a strong detectable signal of interacting proteins. The input nuclear extract was diluted relative to nonbound (around 5% of total protein in IP), to achieve signals at the same level as the IP samples.

When analyzing proteins with WB after IP when a strong elution buffer is used, such as the LDS sample buffer, one have to be aware of the strong signals arising from the 2nd ab cross reactions that occur with the IP antibody if they are the same species. The IP antibody is denatured from the beads during elution and size-separates on the gel as the heavy chains (~55 kDa) and light chains (~25 and 35 kDa). It is therefore not possible to evaluate a protein with those protein sizes unless an antibody with a different species is used. However, you can use this to your advantage to analyze the yield of the IP, or more specifically, how much of the antibody binds to the beads. A good antibody with successful IP conditions should only display these bands in the bound sample and not in the nonbound (Figure 10).

4.4.3 Co-IP: Quantitative Western blot

Using western blot for protein identification after IP is very useful but it cannot quantitatively evaluate the amount of proteins bound to the target since much more of the bound fraction is loaded on the gel compared to the nonbound. We therefore developed a procedure to quantify the fraction of bound and nonbound protein directly by loading relative amounts of protein on the gel for each IP sample, taking the dilutions during the immunoprecipitation procedure into account (Figure 11, Appendix 2).

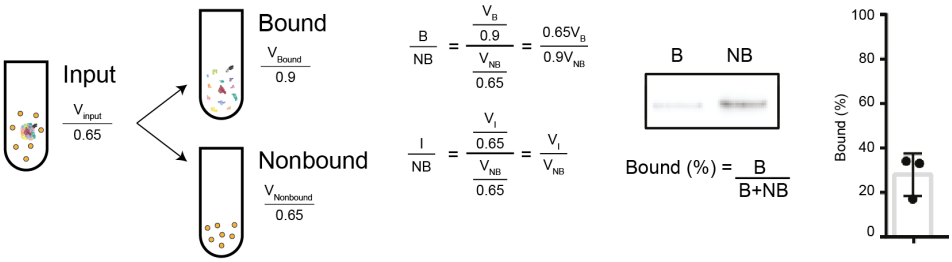

Figure 11. Quantitative western blot (QWB). During Immunoprecipitation (IP), nuclear proteins end up either in the bound (B) or nonbound (NB) fraction. Before QWB loading, input (I) samples and bound (B) samples are diluted relative NB, by taking into account dilutions during IP such as the different volumes (V) and WB-dilution factors (0.65 or 0.9: Input and nonbound are "diluted" in 25% LDS sample buffer and 10% sample reducing agent before WB while eluates are only diluted in 10% sample reducing agent). The detected WB signals can then be quantified and compared directly, because B+NB now equals 100%. Calculations are explained in Appendix 2. Similarly depicted in Paper III.

We call this quantitative western blot. The bound sample and input samples were diluted to comparable levels relative nonbound so that the signal strength of the bound plus nonbound bands equal the input (theoretically). Western blot bands were then quantified using image analysis and the fraction of bound proteins was calculated: B/(B+NB). Quantification of WB signals is semi-quantitative and relies on good laboratory practice with a standardized protocol where samples are treated the same, loaded on the same gel, detected and analyzed together. However, small differences, which very well may be biologically significant, are hard to detect using this method due to technical variation, and time-consuming biological replicates are then required.

5 RESULTS AND DISCUSSION

5.1 Oncogenic contribution of the transcription factor partner and FET NTD in FET-FOP-caused sarcoma

FET fusion oncogenes are the main driver of more than 20 different types of sarcoma and leukemia. All FET-FOPs contain the N-terminal domain (NTD) of the FET proteins and a DNA-binding transcription factor partner. FET oncoproteins are thought to function as aberrant transcription factors, thereby inducing oncogenic transcriptional profiles. Nevertheless, the exact mechanism behind transformation and the detailed contribution of each part of the oncoprotein is not known.

5.1.1 Role of transcription factor partner DDIT3

DDIT3 is a leucine zipper-containing transcription factor of the C/EBP family that interact with DNA as a heterodimer. DDIT3 is the C-terminal partner in FUS-DDIT3, the oncogenic driver in myxoid liposarcoma. It is likely that the oncogenic contribution of the DNA-binding transcription factor partner relies on its DNA-binding capacity but DDIT3 is considered to be a dominant negative inhibitor with few known binding sites [29]. We therefore evaluated the genomic binding of DDIT3 using chromatin immunoprecipitation sequencing (ChIP-seq) in HT1080 cells with stable ectopic expression of DDIT3-EGFP [Paper I]. Comprehensive data analysis identified 1200 specific binding peaks for DDIT3, after background subtraction using HT1080 wildtype ChIP signals. We used Homer analysis to identify target sequence motifs; the most common motif covered over 40% of all peaks and displayed substantial similarities with the C/EBP family core motif. In fact, when analyzing the DDIT3 binding sites against known sequence motifs, several leucine zipper transcription factors were among the top-ranked motifs including some known DDIT3 dimerization partners. The protein expression of known DDIT3 dimerization partners ATF4, C/EBPβ, JUN and FOS was verified by western blot (WB). These data suggest that DDIT3 binds to DNA, at least to some extent, via its dimerization partners which would explain the leucine zipper binding motifs identified.

We then utilized a previously published dataset from our group [30], analyzing gene expression after induction of DDIT3 expression in HT1080 cells, 2 and 8 hours after DDIT3 nuclear entry [Paper I]. Combined binding and expression analysis could therefore identify genes directly regulated by DDIT3 and avoid

secondary downstream-regulated targets; more than 100 genes were direct DDIT3 targets with 60 genes regulated at both time points. DDIT3 induction led to significant downregulation of target genes. To evaluate how this downregulation occurred, we evaluated the repressive histone mark H3K27me3 as well as the active mark H3K27ac. On global protein level, DDIT3 expression led to a significant increase in H3K27me3 but, interestingly, DDIT3 genomic binding rarely overlapped with H3K27me3 marks. This suggests another function besides PRC2-mediated repression i.e. methylation of lysine 27 at histone H3. Instead, DDIT3 genomic binding overlapped with H3K27ac at over 50% of the DDIT3 binding sites, for example at DDIT3 target genes BAX, ATF3 and RELB. This indicate a role of DDIT3 as a transcriptional suppressor at H3K27ac-marked genes in active chromatin regions [Paper I].

It seems that DDIT3 can bind DNA at specific genomic locations but at fewer sites than other transcription factors [Paper I]. The dimerization partners probably contribute to the DNA binding as suggested by the highly similar binding motifs. The question if DDIT3 functions as a dominant negative inhibitor hindering DNA binding of its dimerization partners at certain locations remains. The limited number of binding peaks could support this theory even though DNA binding and transcriptional repression was seen in DDIT3-expressing cells. DDIT3 hetero-dimers that bind DNA may have impaired transcriptional activation due to the lack of the transactivation domain in DDIT3 and may then outcompete other activating leucine zipper dimers. Exactly how DDIT3 leads to transcriptional repression remains to be elucidated. Importantly, which of these properties remain in the fusion protein and what effect the FET NTD contributes with determine the function of the FET oncoproteins. Expression of DDIT3 but not FUS-DDIT3 is known to cause growth arrest by blocking cell cycle progression from G1 to S phase [31]. This is likely not explained by disruption of protein interactions since FUS-DDIT3 can bind the normal DDIT3-dimerization partner C/EBPβ [31]. It is also known that transformation of cells by FUS-DDIT3 is dependent on both the leucine zipper dimerization domain of DDIT3 as well as the FET NTD [107]. Further studies are needed to elucidate the role of both the transcription factor partners and the FET N-terminal domain in FET sarcoma development.

5.1.2 Role of the FET N-terminal domain

One of the three FET NTDs is always present in FET oncoproteins. However, the role of the FET NTD was for long enigmatic. It is a prion-like domain, which contain unstructured low-complexity protein sequences sometimes referred to as IDR (intrinsically disordered region). These domains are known to promote

self-interactions and infer phase separation capacities, as extensively studied for FUS because of its role in pathologic aggregation in neurodevelopmental diseases. To assess the function of the FET NTD, we determined the interaction partners shared by the N-terminal domain of the three FET proteins using a GST pulldown assay [Paper II]. Bacterially expressed GST-tagged recombinant constructs of FUS-NTD-GST (amino acids 1-175), EWSR1-NTD-GST (1-264) and TAF15-NTD-GST (1-201) enriched several high-molecular-weight proteins from cell extracts. These were identified as core components of the SWI/SNF chromatin remodeling complex (ARID1A, BRG1, BAF170 and BAF155) by mass spectrometry analysis (MS). Due to the high similarity of the three FET NTDs, it was not surprising that they all share a major interaction partner, the SWI/SNF complex, that potentially have an important role in oncogenesis.

To pinpoint the interaction domain, deletion mutants of the FUS NTD (8-175, 31-175, 67-175 and 107-175) were used in the same GST pulldown assay. The interaction between FUS NTD and SWI/SNF was lost when amino acids 31-66 were removed [Paper II]. This part of the NTD contains the previously identified FET binding motif (FET BM1, Figure 2) required for binding between FET proteins [22], indicating that the FET BM1 was important also for the binding between FET proteins and SWI/SNF. Boulay *et al.* investigated which parts of the EWSR1 NTD was important for the EWSR1-FLI1 oncogenic function in Ewing sarcoma [77]. They identified two low-complexity regions; SYGQ1 (corresponding to FET BM1) and SYGQ2, that contained the characteristic [G/S]Y[G/S]Q motifs. Experiments using internal deletion mutants, where one or both domains were deleted, revealed similar properties as the full-length oncoprotein, with comparable binding to normal EWSR1 as well as the SWI/SNF component BRG1. Hence, their experiments did not identify any specific EWSR1 subdomain essential for EWSR1-FLI1 activity. Boulay *et al.* concluded that there is considerable functional redundancy between different EWSR1 NTD parts. Furthermore, Owen *et al.* evaluated when the phase separation capacity of FUS NTD diminished by evaluating when the subsequent formation of phase-separated nuclear puncta was lost by removing the first 25, 50, 75 and 125 amino acids of the FUS NTD [108]. Nuclear puncta structures were retained when removing up to 50 amino acids but then lost, suggesting that the region between amino acids 51 to 75 was important. However, in experiments with a 51-75 internal deletion mutant, nuclear puncta were formed. Owen *et al.* concluded that it is the total length of the NTD, not the specific location of the deletion that determines the self-interactions and phase separation capacity. These recent studies indicate that it is the total length of the low-complexity domain, not a specific sequence that is important for its functional properties [77, 108]. One could assume this also relates to the ability

of the FET NTD to form protein interactions with for example SWI/SNF. However, it remains to be elucidated if the FET BM1 has distinct properties that are important for these interactions.

5.1.3 Difference between normal and oncogenic FET proteins

The FET NTD is present in both normal and oncogenic FET proteins suggesting shared functions and interaction partners. The oncogenic role of FET-FOPs could be both a *de novo* oncogenic effect from the fusion protein (gain-of-function) but potentially also interfering with the function of the normal FET proteins (loss-of-function). We used sequential salt extraction (SSE) analysis to evaluate the properties of FET proteins and showed that normal FET proteins required higher salt concentration to be extracted from the chromatin environment than oncogenic FET proteins and thus bound stronger to chromatin [Paper III]. This suggests altered biochemical properties and functions of the FET oncoproteins. It is not surprising that FET proteins bind chromatin strongly due to its many roles in transcriptional regulation, splicing and DNA repair [20, 109]. In addition, FET oncoproteins reportedly bind to super-enhancers, i.e. more accessible regions of chromatin [77, 79, 86, 110] which may explain why they can be more easily extracted from chromatin compared to the normal FET proteins.

The normal and oncogenic FET proteins share some interaction partners e.g. the N-terminal domain of FET-FOPs can associate with the same RNA processing factors as normal EWSR1 [109]. However, the presence of the RNA-binding C-terminal domain impact on certain functions that make normal FET proteins behave differently compared to FET-FOPs. For example, the C-terminal domain of normal FET proteins can repress the FET NTD transcriptional activation by altering the structural conformation of the FET proteins so that the accessibility of the NTD for protein interactions is reduced [109, 111]. In addition, RNA binding has been shown to induce a conformational change in FUS that opens up the FUS NTD for protein interactions that repress transcription [112, 113]. Similarly, EWSR1 but not EWSR1-FLI1 could block activation of a transcription factor interaction partner [114].

Previously, our group showed that the FET NTD binds downstream C-terminal sequences in all FET proteins thereby mediating formation of homo- and hetero complexes involving FUS, EWSR1 and TAF15, as well as FUS-DDIT3 [22]. Another study showed that EWSR1-FLI1 can interact with normal EWSR1, and self-associate [115]. FUS-DDIT3 and EWSR1-FLI1 are mostly nuclear unlike the normal FET proteins that shuttle between the nuclei and cytoplasm [9, 116].

Importantly, in the presence of FET fusion oncoproteins, normal FET proteins were enriched in nuclear foci, while control experiments with only DDIT3 or normal FET proteins displayed smooth distribution without formation of nuclear foci [22]. This indicates that FET-FOPs can disturb the localization and potentially the function of FET proteins. The different functions and localization of FET proteins are usually tightly regulated to ensure appropriate activity [116]. In conclusion, normal FET proteins have both similar and divergent properties and functions compared to FET-FOPs and it is likely that part of the oncogenic effect of the fusion proteins is to sequester normal FET proteins from their normal localization leading to loss of some FET functions.

5.2 Interaction with the SWI/SNF chromatin remodeling complex

Since the evolutionary conserved FET NTD is present in all FET-FOPs, it was natural to speculate that a common oncogenic molecular mechanism, such as a common shared binding partner, could explain transformation. Earlier, we showed that the FET NTDs interact with the SWI/SNF chromatin remodeling complex. We then used proximity ligation assays and visualized nuclear interaction of FUS and oncogenic FUS-DDIT3 with components of SWI/SNF in myxoid liposarcoma cells [Paper II]. To confirm the interaction of both normal and oncogenic FET proteins to SWI/SNF, we performed BRG1 immunoprecipitation (IP), pulling down the catalytic subunit of the SWI/SNF complex, in MLS and EWS cells carrying four different types of FET-FOPs: FUS-DDIT3 type I in MLS 402, FUS-DDIT3 type II in MLS 2645, EWSR1-FLI1 in EWS TC-71 and EWSR1-ERG in EWS IOR/CAR. Western blot and mass spectrometry analysis of co-precipitated proteins verified the binding of normal FET proteins FUS and EWSR1 as well as all four FET oncoproteins with the SWI/SNF complex [Paper II].

5.2.1 FET oncoproteins have deregulated interaction with SWI/SNF

Since SWI/SNF complexes [117] and normal FET proteins [10] are abundant in most tissues and cell types, extensive interactions between them could be expected. On the other hand, FET-FOPs are more weakly expressed than normal FET proteins [35]. To further characterize the interactions between FET proteins and the SWI/SNF complex, we utilized our quantitative WB protocol and immunoprecipitated BRG1 from MLS and EWS nuclear extracts [Paper II]. Unexpectedly, only minor fractions of normal FET proteins bound to the

SWI/SNF complex, suggesting that this interaction is regulated and/or restricted to certain chromatin environments or SWI/SNF complex subtypes. In line with this, other studies have reported that post-translational modifications or mutations in the low complexity NTD can disrupt protein interactions and modulate function [24, 25, 77]. Quantification of the WB bands showed that <40% of normal FET proteins bound to SWI/SNF while about 80% of the FET-FOPs were bound, indicating a dysregulated interaction [Paper II]. In addition, a tougher elution buffer (2x LDS sample buffer instead of 0.2 % Formic acid) was required to elute the FET oncoproteins indicating that they bind stronger to SWI/SNF than normal FET proteins.

To evaluate this further, we performed BRG1 immunoprecipitation followed by quantitative WB of MLS and EWS sequential salt extracts (250, 500 and 1000 mM KCl) and showed that FET oncoproteins interact also with the fraction of SWI/SNF complexes extracted in high salt concentration (1000 mM KCl), indicating a robust interaction [Paper III]. In this fraction, the majority of FET-FOPs were bound to SWI/SNF compared to only a minor part of normal FET proteins. This highlights the different behavior and functions for normal and oncogenic FET proteins. Kadoch and colleagues reported a robust, yet transient interaction of fusion proteins EWSR1-FLI1 in Ewing sarcoma [77] and TMPRSS2-ERG in prostate cancer [118] with the SWI/SNF complex. These fusion proteins sediment mainly as monomers in glycerol density sedimentation assays and not together with the complex. In addition, the interaction of EWSR1 with EWSR1-FLI1 and SWI/SNF was lost above 0.5 M in urea denaturation assays. On the other hand, the fusion protein SS18-SSX in synovial sarcoma is incorporated into the SWI/SNF complex as a dedicated subunit as shown by co-sedimentation and a stable interaction above 2.5 M urea [119]. These results somewhat contradict our results since we always see a strong interaction of the FET oncoproteins with SWI/SNF. However, different extraction and analysis methods can contribute to the different conclusions as well as different views of what constitute a “strong” interaction.

Since both normal and oncogenic FET proteins bind SWI/SNF through their N-terminal domain, they could be expected to compete for the same binding site. If this is the case, more of normal FET proteins would bind SWI/SNF in the absence of FET oncoproteins. To test this hypothesis, we overexpressed FUS-DDIT3 and EWSR1-FLI1 in human HT1080 fibrosarcoma cells and quantified the normal and oncogenic FET proteins that co-precipitated with BRG1 [Paper II]. Surprisingly, no increase in BRG1-bound FUS or EWSR1 was seen without fusion oncoproteins compared to when the FET-FOPs were present. This lack of competition indicate that normal and oncogenic FET proteins bind different sites on SWI/SNF or to different variants of the SWI/SNF

complex. In Paper II, we introduced a set of binding models (Figure 12). We now know that the binding is not competitive and that a larger fraction of FET oncoproteins bind SWI/SNF in a robust way suggesting that they bind directly to SWI/SNF. Furthermore, all three normal FET proteins FUS, EWSR1 and TAF15 can bind each other and the FET oncoproteins through interaction of the FET NTD with a downstream C-terminal domain sequence [22]. Therefore, determining the direct interaction partner, that mediates the interaction with SWI/SNF, is problematic. Current data suggest that FET oncoproteins bind directly to SWI/SNF and either in a different position as normal FET proteins or to another subgroup of the SWI/SNF complex. It is likely that FET-FOPs oligomerize (with or without normal FET proteins) into clusters with high local concentration in certain contexts. In addition, normal FET proteins are most likely regulated by RNA interactions and possibly also post-translational modifications which may restrict binding to interaction partners such as SWI/SNF.

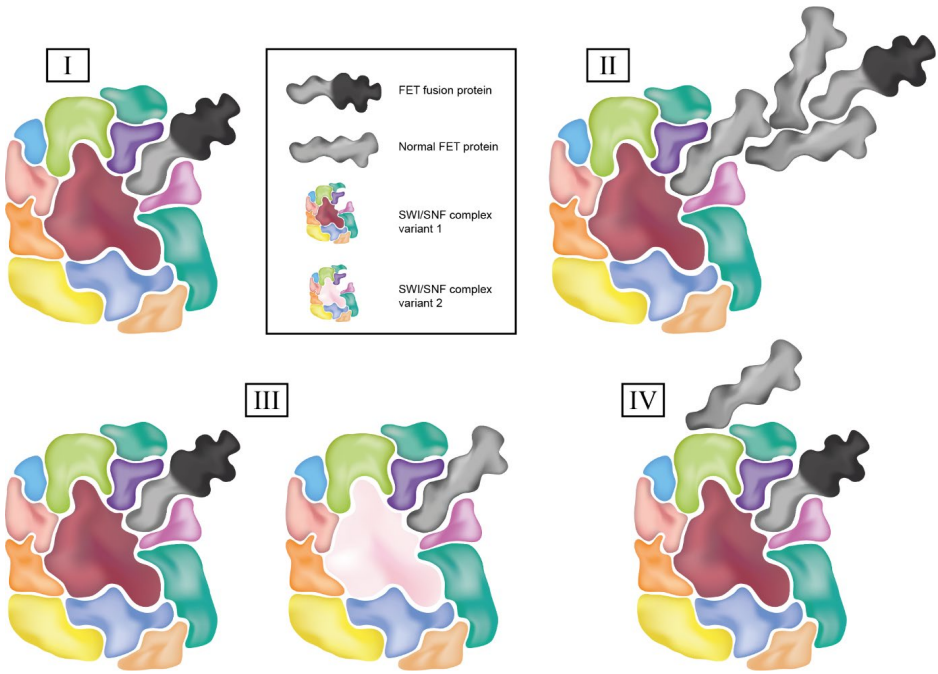

Figure 12. Four binding models. (I): FET oncoproteins bind directly to SWI/SNF. (II): normal FET proteins form homo- and hetero-complexes and mediate binding of FET oncoproteins. (III): Normal and oncogenic FET proteins bind to different SWI/SNF complex variants or subtypes. (IV): Normal and oncogenic FET proteins bind to different sites on the SWI/SNF complex. From Paper II [6].

5.2.2 Impact of FET oncoproteins on SWI/SNF complex stability and composition

The SWI/SNF complex is a robust protein complex that is intact at 2.5 M urea with interactions of similar strength to ribosomal subunits [119]. In our studies with recombinant FUS-NTD-GST assays, the strength of the FUS-NTD – SWI/SNF binding was evaluated by increasing the salt concentration during washes. Western blot analysis showed that all evaluated SWI/SNF proteins (ARID1A, BAF170, BAF155, BAF60A, SS18, BAF47 as well as normal FET proteins EWSR1 and FUS) remained in 1000 mM salt, except BRG1 that was lost above 250 mM salt [Paper II]. This suggests that the interaction of the FET NTD is through a subunit other than BRG1 and potentially that BRG1 is kicked out of the complex when FUS-NTD-GST is bound. This data indicates that binding of FUS NTD may affect the complex conformation and destabilize the complex. To evaluate the stability of the complex in FET sarcoma cells in high salt concentrations, we utilized the BRG1 IP of sequential salt extracts in both MLS 402 and EWS TC-71 nuclear extracts [Paper III]. SWI/SNF complexes with FET oncoproteins bound seemed to be intact at 1000 mM salt as indicated by co-IP of ARID1A, BAF155 and BAF57.

To analyze the composition of FET-FOP-bound SWI/SNF complexes, we performed reciprocal IP, targeting FUS-DDIT3 or EWSR1-FLI1, and analyzed co-precipitated proteins with MS analysis. In 2018, we concluded that a full set of core components (ATPase, BAF155, BAF170, BAF60, BAF47 and BAF57) precipitated with the fusion oncoproteins, ruling out the possibility that FET fusions cause a complete loss of a specific core protein resulting from a change in SWI/SNF conformation and stability [Paper II]. Since then, the experience from the scientific community has increased substantially and we now know that the SWI/SNF complex exist in three main subtypes and how these complexes are assembled [61, 62]. SWI/SNF assemble from an initial dimer (BAF155/BAF170) to a SWI/SNF core, with the subsequent addition of subtype-specific components and a final assembly of the ATPase module responsible for the catalytic activity [61]. Thus, re-inspection of our MS data in the context of the newly reported functional modules, showed that fusion-bound SWI/SNF complexes in myxoid liposarcoma and Ewing sarcoma cells may lack certain subunits of the ATPase module [Paper III]. In MLS, the alternative ATP-catalytic subunit BRM and the module components BCL7A-C were lacking while in EWS, SS18/SS18L1, an ATPase-module component present in cBAF and GBAF complexes was lacking. Co-immunoprecipitated SWI/SNF complexes in neither MLS nor EWS cell lines contained BAF53B while the isoform BAF53A was present. A small change in subunit composition can affect the function of the SWI/SNF complex; BAF53B was shown to be unique to

neuron-specific complexes (nBAF) in differentiated neurons while BAF53A was present in more neural progenitor cells [120]. In addition, incorporation of the fusion oncoprotein SS18-SSX into the SWI/SNF complex in synovial sarcoma leads to loss of BAF47 from the complexes [119]. Therefore, it is possible that the binding of FET-FOPs affects the SWI/SNF conformation so that the composition is changed. However, since these data are based on only two IP-MS analyses, more experiments are needed. Thus, a comprehensive experiment using IP and quantitative mass spectrometry has been performed and is currently being evaluated.

Interestingly, in a few experiments pulling down SWI/SNF complexes in MLS 2645 cells with the short version of FUS-DDIT3 (type II), no SWI/SNF proteins were seen neither in the bound nor nonbound fraction (unpublished observation). This might be due to destabilizing of the complex during immunoprecipitation leading to degradation of SWI/SNF components. A similar degradation was seen when BRG1 was pulled down together with truncated deletion versions of the FUS N-terminal domain transfected into HT1080 fibrosarcoma cells (unpublished observation). Even though our experiments show that the core of the SWI/SNF complex remain intact when FET-FOPs bind, even in high salt concentration, in a few instances, the binding of different versions of FET proteins or FET fusion oncoproteins seems to affect the conformation and stability of SWI/SNF. This observation needs to be evaluated further to elucidate the reason behind the complex destabilization and protein degradation.

5.2.3 SWI/SNF subtypes and interactions with FET oncoproteins

The SWI/SNF complex exists in three main subtypes called cBAF, PBAF and GBAF/ncBAF (Figure 5). We know that FET oncoproteins interact with the SWI/SNF chromatin remodeling complex, but it was not known if they interact with all SWI/SNF subtypes or just a subset. Therefore, we further analyzed the mass spectrometry-identified proteins after DDIT3 IP in MLS 402-91 or FLI1 IP in EWS TC-71 [Paper III]. Some subtype-specific SWI/SNF components were identified as interacting partners to FET oncoproteins; the cBAF-specific ARID1A/1B and BAF45D, the PBAF-specific subunits ARID2, PBRM1 and BAF45A, but not BRD7, and GBAF-specific components BRD9 and GLTSCR1L but not GLTSCR1. The fact that all core components are present and at least one of the subtype-specific components co-precipitated imply that all three main SWI/SNF subtypes are pulled down with FET fusion oncoproteins. To verify this, we immunoprecipitated FUS-DDIT3 in MLS 402

nuclear extracts using a direct IP followed by western blot analysis [Paper III]. Successful co-IP of the SWI/SNF complex was verified by detection of BRG1, BAF155, BAF57 and BAF47 in the DDIT3 bound fraction. Importantly, cBAF-specific ARID1A, PBAF-specific PRBM1 (and BRD7, weakly), and GBAF-specific GLTSCR1L and BRD9 co-IP with FUS-DDIT3 confirming that FET oncoproteins interact with all three main SWI/SNF subtypes. BRD7 was missing from the MS-identified proteins indicating a possibility that it is missing from FET-FOP-bound complexes. In the repeated experiment, BRD7 was detected in the DDIT3 bound fraction but a large signal was detected also in the negative control making it difficult to conclude whether BRD7 is part of FET-FOP-bound complexes or not. As with the potential loss of ATPase module components discussed above, our present analysis cannot completely rule out a loss of subtype-specific component. In summary, FET-FOPs bind to all three main SWI/SNF subtypes and may thus have an effect on the diverse functions of these complexes.

The different functions of SWI/SNF subtypes are not well known and several subcomplexes exist at the same time within the same cell, both with redundant and unique functions. The amount of available paralogs can affect the composition of the SWI/SNF complex and altered gene expression of SWI/SNF paralogs can result in subunit switching, leading to complexes with different functions. This may be important in determining cell identity and cell-type specific gene expression [120, 121]. Different subtypes may thus compete with each other for SWI/SNF subunits, and increased availability or mutations of subunits can shift the balance of subtypes as was shown in other cancer types. For example, in BAF47-mutant malignant rhabdoid tumors, the loss of BAF47 leads to increased incorporation of BRG1 into GBAF/ncBAF complexes [122] explaining the increased sensitivity of these cells to BRD9 inhibition [60, 123]. Other cancer types have shown increased dependency on GBAF such as the metastatic prostate cancer cell line PC3 [62], synovial sarcoma [60] and acute myeloid leukemia [92, 124]. Increased dependence of cancer cells on a specific SWI/SNF subtype provides interesting new therapeutic possibilities. Since each subunit contributes to the function of the complex, we evaluated the protein expression of SWI/SNF components using WB in nuclear extracts of FET sarcoma cell lines [Paper III]. All evaluated SWI/SNF core and subtype-specific components were present in the cell lines, and the expression levels of most SWI/SNF components were lower in MLS and EWS cell lines compared to HT1080. In addition, stable ectopic expression of FUS-DDIT3-EGFP displayed a more similar expression pattern to FET sarcoma cell lines compared to parental HT1080 but since this experiment was not repeated, no conclusive evidence was provided at this point. At mRNA level, expression of EGFP-tagged FET-FOPs did not significantly alter the expression levels of

SW/SNF components. We also used previously acquired, non-published microarray data from five MLS tumor tissues and evaluated SWI/SNF components using HT1080 cell line as a reference [Paper III]. These tumor tissues had expression levels of SWI/SNF components in the same range as HT1080, but there were slightly higher expression of cBAF-specific components and lower expression of GBAF-specific components compared to HT1080. Note that since the HT1080 fibrosarcoma cancer cell line was used as a reference in these experiments, differences might also be due to the oncogenic landscape in HT1080. In summary, the protein and gene expression analysis did not show loss of any of the evaluated SWI/SNF subunits and there was no clear shift in SWI/SNF subtypes. However, even for subunits that were identified and present in the complexes, FET fusions might still impact the functions or ratios of SWI/SNF variants. The overall function of collaborating SWI/SNF variants leads to a collective functional output and small changes to the SWI/SNF complex might still have a large impact in a cell. This change might be hard to notice using Co-IP studies or conventional MS-studies. We therefore setup a more comprehensive quantitative proteomic study to determine the detailed composition of FET-FOP-bound complexes and are waiting on the results.

To learn more about the properties of SWI/SNF subunits, including the subtype-specific ones, we used sequential salt extraction (SSE) to evaluate how strong they bind to chromatin [Paper III]. The stronger a protein binds, the higher salt concentration is required to extract the protein from the chromatin environment. Most SWI/SNF subunits, including subtype-specific components, had similar SSE binding profiles with most proteins extracted in the first two fractions (250 and 500 mM). Only small amounts of protein were extracted in the 1000 mM KCl fraction. Interestingly, the PBAF-specific components PBRM1 and BRD7 had differential binding profiles, with PBRM1 binding stronger to chromatin than BRD7. This indicate that PBAF complexes that bind strongly to chromatin do not include BRD7 or that PBRM1 also binds chromatin as a free subunit, as was reported by Mashtalir *et al.* [61]. This data can also imply that BRD7 do not bind as strong to PBAF complexes in FET sarcoma cells, perhaps due to the binding of FET oncoproteins. One could speculate if the properties of the SWI/SNF complex variants decide binding strength or if the chromatin environment where they bind is the main decider of how tightly SWI/SNF proteins bind to chromatin. Consistent with our data, previous studies have shown that PBAF binds more strongly to chromatin than the canonical BAF complex. In a study by Porter *et al.*, SSE binding profiles showed that the PBAF-component PBRM1 required stronger salt concentration to elute from chromatin than the cBAF-component ARID1A [125]. Using SSE, Alpsoy *et al.* showed that the GBAF-component GLTSCR1 had similar chromatin binding strength as ARID1A while PBRM1 bound stronger [62]. Consistent with the idea that

PBRM1 have additional functions, Porter *et al.* showed that PBRM1 bound tighter to chromatin after induction of DNA damage [125]. It is interesting to speculate whether FET fusions affect the SWI/SNF binding affinity to chromatin, similarly to what was seen upon rescue of BAF47 in malignant rhabdoid tumor cells [70]; BAF47-deficient SWI/SNF complexes in G401 cells was released from chromatin between 150 and 300 mM NaCl while after rescue, SWI/SNF subunits dissociated from chromatin at 500–1000 mM NaCl.

5.2.4 FET oncoproteins might bind to SWI/SNF variants with an accessible conformation

During the many immunoprecipitations performed using a BRG1 antibody, we repeatedly pulled down about 50% of BRG1 (and other SWI/SNF components) but around 80% of FET fusions [Paper II and III]. Our data thus suggest that the antibody preferably pulldown complexes bound to FET fusions proteins. This may be explained by a conformation change in the complex bound by FET-FOPs that makes the BRG1 bromodomain more accessible to the antibody, or that FET fusions bind to a certain type of SWI/SNF complex that have that accessible conformation. To evaluate this further, we performed immunoprecipitation with a BAF155 antibody, a SWI/SNF subunit that belongs to the inner core complex, in nuclear extracts from two MLS cell lines (402 and 1765). Unexpectedly, we saw similar trends as for the BRG1 IP; more FET fusions proteins were in the bound fraction than normal FET proteins and SWI/SNF components [Paper III]. This means it is not only an effect of the BRG1 antibody having more access to the bromodomain of BRG1 in complexes with FET-FOPs bound. Instead, binding of FET-FOPS to an undefined SWI/SNF subtype or an effect on SWI/SNF complex conformation that leads to a more accessible conformation may explain the preferential immunoprecipitation of FET-FOP bound SWI/SNF complexes. Indeed, over 1000 theoretical combinations of SWI/SNF complexes are possible based on the combination of mutually exclusive subunits in the three main SWI/SNF subtypes [61]. Based on the extensive research in the field of chromatin remodeling complexes, it would not be surprising if more functional subtypes are identified in the near future.

5.3 Role of the transcriptional coactivator BRD4

BRD4 is a known transcriptional coactivator that have a reported physical interaction with the FET oncoproteins FUS-DDIT3 [86] and EWSR1-FLI1/ERG [79] and could play a role in the oncogenic gene expression in FET sarcoma. BRD4 have also been reported to interact with components of

the SWI/SNF complex [62, 90-92], but only in certain conditions [93], which led us to speculate that this interaction is sensitive to buffer conditions such as strong detergents [Paper III].

Recent reports have shown the importance to distinguish between BRD4 isoforms; the short and the long isoform had opposite oncogenic effect in two recent studies [126, 127]. We first evaluated the mRNA expression levels of BRD4 in FET sarcoma cells [Paper III]. Both the long (150 kDa) and short (80 kDa) BRD4 isoforms were expressed in the same range in MLS tumor tissues as the HT1080 reference when evaluated by microarray analysis. At the protein level, the expression pattern of BRD4 isoforms were similar in FET sarcoma cell lines and HT1080. In addition to the short and long BRD4 isoform, two additional versions around 30 kDa larger than each isoform were detected. We suspect they are post-translationally modified versions e.g. SUMOylation [128]. The used BRD4 antibodies had different selectivity for the various BRD4 variants; ab128874 identified all BRD4 isoforms while the other two (Bethyl A301-985 and Cell signaling 13440) identified mainly the post-translationally modified long BRD4 isoform. Thus, choosing and evaluating antibodies are very important, as discussed in the methods section. In addition, treatment with a BRD4 degrader (ARV-825), specifically degraded the modified versions while the long isoform around 160 kDa proved harder to degrade. This verifies the detected post-translationally modified versions as being true BRD4 isoforms [Paper III].

5.3.1 FET oncoproteins interact with BRD4 via the SWI/SNF complex

To investigate the role of BRD4 in FET sarcoma oncogenesis, we first verified BRD4 as an interacting partner to the FET oncoproteins [Paper III]. We then confirmed that BRD4 (primarily the long isoform) was an interaction partner to the SWI/SNF complex in FET sarcoma cell lines and showed that the interaction was robust and remained in 1000 mM KCl [Paper III]. Since SWI/SNF interacts with FET oncoproteins, these experiments cannot determine which interactions are direct. Therefore, we evaluated the effect of FET-FOPs on the BRD4 - SWI/SNF interaction in HT1080 cells after transient transfection of FET oncoproteins or EGFP control followed by BRG1 immunoprecipitation. BRD4 interacted with the SWI/SNF complex in HT1080 cells without fusion protein and the amount of co-precipitated BRD4 was not visibly affected by presence of FET oncoproteins FUS-DDIT3-EGFP or EWSR1-FLI1-EGFP. This implies that FET oncoproteins are not involved in the BRD4 – SWI/SNF interaction and that the previously reported physical interaction of FET

oncoproteins with BRD4 is mediated by the SWI/SNF complex (or an unknown shared interaction partner).

5.3.2 FET sarcoma cells are sensitive to BRD4 inhibition

Due to its involvement in cancer development and potential as a therapeutic target in many cancer types [85, 129-131], we performed cell viability assays with the dual-bromodomain inhibitor AZD5153 from AstraZeneca using MLS, EWS, HT1080 cell lines and human fibroblasts F470 [Paper III]. FET sarcoma cells were more sensitive to BRD4 inhibition than fibrosarcoma cell line HT1080 and human fibroblasts, with IC-50 values in the lower nM range. This is in agreement with recent studies that reported a vulnerability of MLS and EWS cells to BRD4 inhibition [79, 86]. MLS cells were equally sensitive to BRD4 degradation by ARV-825 [Paper III]. To test if BRD4 inhibition affected the interaction between SWI/SNF and FET-FOPs, we immunoprecipitated nuclear extracts from MLS 402 control cells or cells treated with the BRD4 inhibitor [Paper III]. After pulldown of the SWI/SNF complex (via BRG1), no reduction in FUS-DDIT3 was seen in the bound fraction. However, the amount of BRD4 that bound to SWI/SNF was slightly reduced. Since the drug targets the bromodomain of BRD4 that binds chromatin and BRD4 do not interact with FET-FOPs directly, this result was not surprising. Instead, the effect of the drug is probably due to inhibition of chromatin binding, with reduced transcriptional activation as a result. Indeed, BRD4 inhibition was reported to reverse FET-FOP-induced transcriptional profiles [79, 87-89, 132]. We have initiated preliminary drug studies in MLS and EWS PDX tumors in mice using this BRD4 inhibitor which have provided promising initial results (unpublished observation).

5.4 Downstream epigenetic effects

We have thoroughly investigated the interactions between FET-FOPs and SWI/SNF, and showed that FET-FOPs have a dysregulated, robust interaction with the SWI/SNF complex that might affect the composition and function of the complex [Paper II and III]. However, to understand the functional consequences of this interaction, the impact on downstream epigenetic processes needs to be determined.

5.4.1 FET oncoproteins affect the chromatin landscape and the PRC2 complex

First, we aimed to investigate if the binding of FET oncoproteins to SWI/SNF leads to any functional effects. Global changes in histone-tail modifications such as acetylation and methylation, and especially aberrant expression levels of reader, writer and eraser enzymes have been reported in cancer [54, 67]. Therefore, we measured the global levels of the repressive histone mark H3K27me3 and the active marks H3K27ac and H3K4me3 as well as the amount of the PRC2 catalytic subunit EZH2 after ectopic expression of the FET oncoproteins FUS-DDIT3 or EWSR1-FLI1 in HT1080 cells [Paper II]. Recall that EZH2 methylates lysine 27 on histone H3 leading to addition of the repressive mark H3K27me3. The global amount of each histone mark relative loading control was quantified from western blots; the amount of H3K27me3 was significantly increased after forced expression of FUS-DDIT3 and EWSR1-FLI1. The active marks were largely unaffected or showed no consistent trend. In contrast, studies in Ewing sarcoma reported increased H3K27-acetylation levels at specific genomic loci using ChIP-seq but no effect on H3K27me3 levels [77, 133]. In addition, Tomazou *et al.* showed a global reduction of H3K4me3 and H3K27ac after EWSR1-FLI1 knockdown while no change was seen for H3K27me3 or other common histone marks [133]. The divergent results may be explained by different cell types and analysis methods. However, it is also possible that disturbed SWI/SNF function leads to increased active and repressive marks at different loci.

The increase in H3K27me3 in our study might be explained by a small but non-significant increase in EZH2 and/or that FET-FOPs disturbs the ability of SWI/SNF to oppose the PRC2 complex, leading to a shift in the SWI/SNF – PRC2 balance. In addition, analyzing RNA sequencing data of 2-fold regulated genes after ectopic expression of FET oncoproteins by enrichment analysis with the "chemical and perturbation" gene set collection, revealed that genes regulated by FUS-DDIT3 expression overlapped with PRC2-regulated genes [Paper II]. This supports the conclusion that binding of FET-FOPs disturb the SWI/SNF – PRC2 balance. Disruption of the SWI/SNF – Polycomb balance have been reported also in other cancer types. Oncogenic mutations of BRG1 lead to increased PRC2 activity and H3K27me3 levels [134]. In human malignant rhabdoid tumors, loss of BAF47 leads to elevated levels of EZH2 and H3K27me3, and gene repression at target genes [67]. Re-expression of BAF47 leads to removal of Polycomb from chromatin [70, 135]. In synovial sarcoma, the SS18-SSX fusion oncoprotein is incorporated into SWI/SNF instead of SS18 which lead to the loss of "core" subunit BAF47. The aberrant SWI/SNF complex is directed to silenced

Polycomb target genes, where they oppose the PRC2 complex to activate bivalent genes [119, 136]. Thus, part of the oncogenic function of mutated or aberrant SWI/SNF complexes might be due to disturbed repressive function of Polycomb complexes.

5.4.2 FET oncoproteins, SWI/SNF components and BRD4 co-localize on chromatin

We wanted to establish if the interactions between FET oncoproteins, the SWI/SNF complexes and BRD4 takes place at specific genomic loci, at the chromatin level, as suggested by the role of chromatin remodeling complexes in transcriptional regulation. We therefore performed chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) in MLS 402 cells to determine where SWI/SNF and FUS-DDIT3 localize in the genome, using antibodies against BRG1, BAF155 and DDIT3, respectively [Paper III]. Over 8000 binding sites of FUS-DDIT3 overlapped with binding of at least one SWI/SNF subunit. These genes were enriched in several biological processes including transcription and differentiation processes. We compared the genes with FUS-DDIT3 and SWI/SNF binding to genes regulated by FUS-DDIT3 (in HT1080 cells) and found a significant enrichment; 30% of regulated genes were bound by both FUS-DDIT3 and at least one SWI/SNF component, which led to slightly less upregulation than downregulated (44% vs 56%). This shows that FUS-DDIT3 and SWI/SNF co-localize in the genome and affect gene expression. In Ewing sarcoma, EWSR1-FLI1 genomic binding has been more extensively studied; it preferentially bind to chromatin regions enriched in microsatellite GGAA repeats and induce the formation of *de novo* enhancers and super-enhancers marked by H3K27ac to activate transcription of target genes [110, 133, 137]. In addition, the SWI/SNF complex was shown to be aberrantly recruited by the EWSR1-FLI1 fusion protein to these tumor-specific enhancers leading to activation of target genes [77]. In this study, Boulay *et al.* showed that EWSR1-FLI1 and BAF155 have a substantial binding overlap genome-wide at GGAA repeats; 72% of EWSR1-FLI1 sites overlapped with BAF155 while BAF155 had 87% of binding sites outside these genomic loci [77]. This is consistent with their Co-IP experiments which showed that only small amount of total SWI/SNF were bound to EWSR1-FLI1. The localization of BAF155 on GGAA repeats were dependent on EWSR1-FLI1 and lost after knockdown of the fusion protein. Recent studies showed that also FUS-DDIT3 can retarget chromatin remodeling complexes; FUS-DDIT3 recruited BRG1 to artificial DNA loci [138] and recruited the ISWI chromatin remodeling complex to enhancers [139]. Retargeting of SWI/SNF complexes thus emerge as a potential oncogenic mechanism in FET sarcoma.

In a study by Chen *et al.* in MLS 402 cells, FUS-DDIT3 was shown to co-localize with BRD4 at 40% of BRD4 binding sites [86]. Based on our data that FET oncoproteins interact with BRD4 via SWI/SNF, we speculated that SWI/SNF is also present at these locations. We therefore combined our dataset with the BRD4 dataset from Chen *et al.* and found that 45% of FUS-DDIT3 and BRD4 overlapping binding sites also overlapped with at least one SWI/SNF component [Paper III]. In summary, this supports the hypothesis that BRD4 and SWI/SNF is involved in the oncogenic function of the FET fusion oncoproteins, that they co-localize in the genome and potentially regulates gene expression. To our knowledge, no one have done BRD4 ChIP-seq in Ewing cells. In MLS, Chen *et al.* showed that many FUS-DDIT3 and BRD4 ChIP-seq peaks co-localize but also that the majority of super-enhancers had FUS-DDIT3 bound. They speculate that a potential function of FET fusion proteins is to establish an aberrant epigenetic landscape, regulating expression of super-enhancer-associated genes leading to malignant transformation. We wanted to evaluate how overlapping BRD4, FUS-DDIT3 (and SWI/SNF) binding sites matched with their super-enhancer genes in MLS 402 but unfortunately they had not disclosed lists of all of the super-enhancer-defined genes, nor did they evaluate how their BRD4 binding sites overlapped with the H3K27ac-defined super-enhancers. More studies are needed, especially in myxoid liposarcoma, to define super-enhancers and tumor-specific binding sites to see if FUS-DDIT3 share the function of EWSR1-FLI1 in creating *de novo* enhancers, and the roles of SWI/SNF complexes and BRD4 at these sites.

We were a bit surprised by the substantial amount of FUS-DDIT3 peaks in the study by Chen *et al.* as well as our study since DDIT3 is thought to have a dominant negative function with little DNA binding. We showed that many of the FUS-DDIT3 peaks were a contribution of the FET N-terminal domain and its interacting partners such as the SWI/SNF complex. However, a more comprehensive evaluation of FUS-DDIT3 chromatin binding together with SWI/SNF and chromatin marks is required comparing cells with and without the fusion protein. This is ongoing work in the lab.

5.4.3 Interactions with Mediator and RNA polymerase II provide a possible mechanism for transcriptional activation potentially via phase separation

We have shown that FET-FOPs, SWI/SNF and BRD4 interact with each other and co-localize on chromatin. In order to understand how the FET oncoprotein – SWI/SNF – BRD4 interaction contribute to oncogenesis, we further analyzed the MS-identified proteins that co-immunoprecipitate with

FET-FOPs and found several subunits of the Mediator complex and RNA polymerase II (RNA pol II) [Paper III]. Mediator is a large multi-subunit complex (~1.2 MDa) that is involved in transcription, working as a bridge between RNA pol II at transcription start sites and transcription factors or coactivators at enhancers. Our data suggest that FET oncoproteins and SWI/SNF complexes also interact with a complex containing Mediator/RNA pol II thus providing a link to transcriptional activation, see Figure 13. However, these data cannot determine if they all are located in one big protein complex or if FET-FOPs interact independently with different complexes. In Ewing sarcoma, Gollavilli *et al.* discovered EWSR1-FLI1 in a large transcriptional complex together with Mediator, BRD4 and RNA pol II [79]. They used gel filtration chromatography to size-separate nuclear proteins (and protein complexes) followed by western blot, and although the majority of EWSR1-FLI1 was found multimerized by themselves, a substantial fraction was found together with the transcriptional machinery. In addition, paradigm-shifting ideas that some of these interactions occur due to the biophysical process of phase separation have recently emerged. Both FUS-DDIT3 and EWSR1-FLI1 was shown to form biocondensates via their FET NTD, potentially at binding-specific loci in enhancer regions, which could recruit SWI/SNF and the transcriptional machinery (BRD4 and RNA pol II) and activate aberrant gene expression [28, 108, 138, 140]. The significance of these studies is discussed further in Future perspectives.

The FET oncoproteins as well as their parent proteins FUS, EWSR1 and TAF15 have been described to possess phase separation properties via their FET N-terminal domain. We used a phase separation predictor developed by Vernon *et al.* [141] to divide proteins into two groups; high or low phase separation propensity (PScore over or under 4, respectively) [Paper III]. Not surprisingly, all FET proteins and evaluated FET-FOPs had high phase separation propensity. In addition, including a larger part of the FUS NTD increased the PScore substantially from the short FUS-DDIT3 version (5-2, Type II) to the larger version (7-2, Type I) while addition of an even larger part (13-2), did not increase its predicted phase separation capacity. Even though this a just a theoretical prediction, these results imply different functions of the fusion types, or at least, a different tendency for phase separation, which might be influenced by the availability of proteins and their interactions with chromatin.

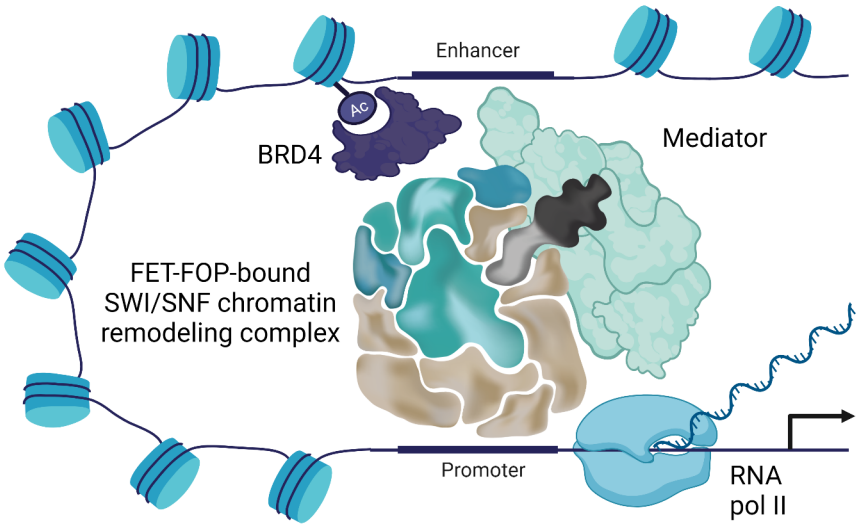

Figure 13. Schematic visualization of FET-FOP-bound SWI/SNF complexes, BRD4, Mediator and RNA polymerase II in oncogenic transcriptional regulation. Similarly depicted in Paper III. Created with BioRender.com.

Proteins with similar properties i.e. intrinsically disordered domains (IDR), were found to be more likely to interact with each other [141]. Indeed, the FUS-DDIT3 and EWSR1-FLI1 interactomes were enriched in proteins with phase separation capacity; over 10% of interacting proteins had PScore over 4 [Paper III]. This can be related to estimations that around 1.2% of the human proteins have a prion-like domain [23]. Also, in our evaluation, SWI/SNF components were enriched in phase separation properties with 34% above the PScore cutoff [Paper III]. A recent study by Davis *et al.* corroborates our finding; several SWI/SNF components were found to have IDR regions including BRG1, BAF155, ARID1A/B and SS18, as defined by the PLAAC algorithm (prion-like amino-acid composition) [138, 142]. Intriguingly, Davis *et al.* suggested that FUS-DDIT3 interacts with SWI/SNF through IDR-IDR interactions. Indeed, FUS-DDIT3 condensates could recruit the BRG1-IDR as well as the other tested IDR-containing SWI/SNF components, however, with different strength suggesting some specificity regarding the IDR sequence. This may very well answer the question of what subunits the FET-FOPs interact with. In addition, BRD4, Mediator components and RNA pol II share the phase separation capacity; the IDR of BRD4 or MED1 was enough to induce phase separation *in vitro* [143] and the C-terminal domain of RNA polymerase II contains a low complexity IDR domain sufficient for phase-separation [144].

The formation of transcriptional hubs at super-enhancers were recently attributed to formation of biocondensates through liquid-liquid phase separation, providing an explanation for the dynamic regulation of gene expression at super-enhancers, for example at oncogenes [143, 145].

5.5 Role of the JAK-STAT signaling pathway

Dysregulation of signaling pathways is one way for cancer cells to achieve and maintain expression patterns that favor survival. The JAK-STAT signaling pathway is aberrantly activated through JAK mutations in hematopoietic malignancies and through increased cytokine signaling or inhibition of negative regulators of the pathway in solid tumors [104]. The downstream transcription factor STAT3 is hard to target directly but small-molecule JAK inhibitors, such as the FDA-approved drug ruxolitinib, can be used to decrease STAT activation as was shown in preclinical studies where JAK-STAT inhibition led to decreased cell proliferation and survival of tumor cell lines [104]. We have previously shown that JAK-STAT signaling is active in MLS cells and controls cancer stem cell-like properties such as chemotherapy resistance (Additional publication: Dolatabadi, Jonasson, Lindén *et al.* 2019; [103]). The SWI/SNF complex is potentially involved in this mechanism as was shown by co-immunoprecipitation of active pSTAT3 with BRG1. Also, knocking down BRG1 decreased the levels of pSTAT3 and the number of cells with cancer-stem-cell-like properties.

5.5.1 FUS-DDIT3 interacts with phosphorylated STAT3 and affects its expression level

In order to evaluate if the fusion oncoprotein FUS-DDIT3 itself had any impact on the JAK-STAT signaling pathway, we first pulled down the fusion oncoprotein in nuclear extracts from three MLS cell lines (402, 1765 and 2645) and showed that FUS-DDIT3 interact with pSTAT3, the phosphorylated and active version of STAT3 [Paper IV]. As we have shown before [paper II and III], both SWI/SNF components (BAF57) and PRC2 components (EZH2) co-IP with FUS-DDIT3 [Paper IV], suggesting that these complexes can be involved in, or potentially mediate, the interaction between FUS-DDIT3 and pSTAT3. The interaction of pSTAT3 with FUS-DDIT3 was confirmed in HT1080 cells with stable ectopic expression of FUS-DDIT3. We then immunoprecipitated FUS-DDIT3 in MLS 1765 sequential salt extracts (250, 500 and 1000 mM KCl) and showed that the interaction with pSTAT3 remained even in the 1000 mM salt fraction, indicating a strong interaction [Paper IV].

To evaluate if the presence of FUS-DDIT3 had any effect on STAT3 protein levels, we ectopically expressed FUS-DDIT3 in HT1080 cells which led to a significant increase in STAT3 levels [Paper IV]. In addition, in nuclear extracts where the JAK-STAT pathway was induced by LIF-stimulation (leukemia inhibition factor), the pSTAT3 levels in HT1080 FUS-DDIT3 were increased to similar levels as in MLS cell lines, unlike HT1080 cells without FET oncoprotein [Paper IV]. Since pSTAT3 interacts with FUS-DDIT3 in the nuclei (or at least in nuclear extracts), one possible mechanism is that they co-localize on chromatin and regulate gene expression. Furthermore, STAT3 is a known transcriptional target of itself [146, 147]. Thus, the increased levels of STAT3 after FUS-DDIT3 expression points towards a possible positive feedback loop with STAT3 and FUS-DDIT3 (with or without SWI/SNF) binding at the STAT3 promotor/enhancer regions.

5.5.2 FUS-DDIT3 expression and JAK-STAT inhibition regulate overlapping genes

We were curious if FUS-DDIT3 and JAK-STAT signaling were connected in other ways such as having shared downstream targets. We therefore used RNA sequencing to analyze gene expression changes when the JAK-STAT signaling pathway was inhibited by ruxolitinib treatment (HT1080 FUS-DDIT3-EGFP with or without ruxolitinib treatment) and analyzed it together with the expression changes induced by ectopic FUS-DDIT3 expression (HT1080 with or without FUS-DDIT3-EGFP) [Paper IV]. In total, 126 genes were mutually regulated by JAK-STAT inhibition (JAK1/2) and FUS-DDIT3 expression, which was around 10% of the genes significantly regulated in each setting. The 126 genes were enriched with gene sets involved in cytokine signaling and chromatin remodeling. Evaluation of publicly available ChIP-seq data for STAT3 in three cell types revealed that the commonly regulated genes were enriched in STAT3 targets [Paper IV]. STAT3 binding was identified at 45 of the 126 genes in at least two of the cell types evaluated, indicating that STAT3 is a downstream mediator for FUS-DDIT3 and JAK-STAT signaling. Also, around 50% of the 126 genes were involved in a protein interaction network, including several proteins regulated by EZH2, suggesting overlapping functions of the shared target genes [Paper IV]. Most EZH2 target genes were downregulated by ruxolitinib treatment suggesting that the JAK-STAT pathway induces EZH2 target genes. On the other hand, expression of FUS-DDIT3 led to both up- and downregulation of EZH2 target genes. This may be because of increased antagonism from SWI/SNF at certain new loci (after possible relocalization by FUS-DDIT3) and loss at other locations.

Both EZH2 and SWI/SNF components have been reported to regulate STAT3 activity; phosphorylated EZH2 activated STAT3 via methylation (followed by phosphorylation) in glioblastoma cells [146]. However, downregulation of SWI/SNF components led to both increased [148-150] and decreased pSTAT3 [151] suggesting different mechanisms depending on tumor type. As the SWI/SNF complex seems to be the main binding partner for FUS-DDIT3, this interaction may impact not only PRC2 activity but also the activity of STAT3. In agreement with this, we previously showed that pSTAT3 was downregulated after knockdown of BRG1 in MLS cells [103]. In conclusion, FUS-DDIT3 expression is connected to JAK-STAT signaling at several levels opening up for a potential new targeted therapy in myxoid liposarcoma.

6 CONCLUSIONS

In this thesis, we delineated the function of the FET oncoproteins in FET sarcoma oncogenesis, focusing on their interactions with the SWI/SNF chromatin remodeling complexes and downstream epigenetic effects. We evaluated the contribution from both parts of the fusion oncoprotein, the low-complexity FET N-terminal domain and one of the C-terminal transcription factor partners DDIT3. Furthermore, the role of the transcriptional coactivator BRD4 and impact on aberrant JAK-STAT signaling were also evaluated.

Paper I: We identified DDIT3 genomic binding sites by ChIP-seq; the major binding motifs had homology with C/EBP transcription factor family and other leucine zipper transcription factor motifs, including both known and unknown dimerization partners of DDIT3. Binding and expression analysis after induction of nuclear DDIT3 entry identified direct DDIT3 targets genes and these were consistently downregulated. DDIT3 did not have extensive co-localization with the repressive histone mark H3K27me3 but instead co-localized substantially with the active mark H3K27Ac. In conclusion, DDIT3 binds DNA via its dimerization partners and may act as a transcriptional repressor at H3K27Ac-marked genes in active chromatin regions.

Paper II: We identified the SWI/SNF chromatin remodeling complex as a major interaction partner to FET oncoproteins. We showed that FET oncoproteins interact via the FET N-terminal domain, that a larger fraction of FET oncoproteins interact with SWI/SNF, and in a stronger way compared to normal FET proteins, indicating a dysregulated interaction. Furthermore, FET oncoproteins did not compete with normal FET proteins for the interaction with the SWI/SNF complex, suggesting that they bind to different parts of SWI/SNF or to different SWI/SNF variants. Downstream analyses revealed a changed chromatin landscape with a significant increase in global H3K27me3 repressive histone marks and overlap of genes regulated by EZH2 and FET oncoproteins, indicating a disruption of the SWI/SNF – PRC2 repressive complex balance. In conclusion, dysregulated SWI/SNF function may be a unifying oncogenic mechanism for tumors caused by FET oncoproteins.

Paper III: We showed that FET oncoproteins interact with the three main SWI/SNF subtypes cBAF, PBAF and GBAF and determined their composition with FET oncoproteins bound. We showed that FET proteins bind stronger to chromatin than FET oncoproteins and that the SWI/SNF subtypes have diverse binding patterns. FET oncoproteins interacted with the transcriptional coactivator BRD4 via the SWI/SNF complex. Furthermore, we showed that

FET oncoproteins, SWI/SNF components and BRD4 interact robustly, co-localize on chromatin and regulate a set of FUS-DDIT3 target genes. FET oncoproteins interacted with components of the Mediator complex and RNA polymerase II and enriched proteins with phase-separating capacity. In conclusion, these interactions provide a possible mechanism for the FET-oncoprotein-induced gene expression patterns. Both BRD4 and aberrant SWI/SNF complexes emerge as potential new targeted therapies.

Paper IV: We evaluated the role of the FET oncoprotein FUS-DDIT3 on aberrant JAK-STAT signaling and showed that expression of FUS-DDIT3 induces the active form of STAT3, pSTAT3. In addition, the oncoprotein bound to pSTAT3, potentially via the SWI/SNF complex or PRC2 repressive complex. We identified 126 commonly regulated genes after FUS-DDIT3 expression and inhibition of the JAK-STAT pathway in HT1080 fibrosarcoma cells, indicating overlapping functions of the target genes. These were related to cytokine signaling and chromatin remodeling processes and included a set of genes regulated by EZH2, the enzymatic component of PRC2. In conclusion, FUS-DDIT3 expression is connected to JAK-STAT signaling at several levels pointing to a new potential targeted therapy in myxoid liposarcoma.

7 CURRENT AND FUTURE PERSPECTIVES

In the first part of this chapter, current perspectives on the roles of FET fusion oncoproteins and SWI/SNF chromatin remodeling complexes in FET sarcoma tumorigenesis and the impact on epigenetic processes are summarized, leading to description of a model based on the current literature and results from this thesis. In the second part, potential future strategies to target the fusion proteins and epigenetic systems including SWI/SNF and BRD4 are described. Throughout this chapter, future perspectives are discussed, highlighting areas where more research is needed.

7.1 FET oncoproteins and SWI/SNF complexes infer aberrant epigenetic landscapes and regulate gene expression

FET fusion oncoproteins are tumor-type specific and often considered pathognomonic for the respective malignancy since they are present at large fractions in tumors that often lack secondary mutations. As such, they can be classified as driver mutations with a causative role. The specificity of FET-FOPs suggests that their oncogenic role is limited to certain cell types or developmental stages [152]. Indeed, FET oncoproteins, as well as other fusions, promote oncogenesis in certain context such as stem- and progenitor cells and expression of fusion proteins in other cell types leads to cell death [152]. The cells of origin are therefore likely tissue-specific progenitor or stem cells [3, 152-155]. FET oncoproteins are considered to act as aberrant transcriptional regulators, inducing oncogenic gene expression [3, 37, 38, 110, 154]. Although the FET N- terminal domain is described as a potent transactivation domain, FET-FOPs lead to both activation and repression of target genes [20, 133, 156]. The oncogenic gene expression guided by the DNA-binding partner can explain the large variety of phenotypes of FET sarcomas. Furthermore, many of the FET sarcomas are low-differentiated tumors and the FET-FOPs may block differentiation programs or activate/maintain stem cell programs [152]. Although FET oncoproteins have been studied for years, the exact oncogenic mechanism remains to be elucidated.

7.1.1 Aberrant chromatin remodeling and epigenetic landscapes

The fact that FET sarcomas in most cases contain few mutations beside the fusion oncoprotein, suggests that epigenetic mechanisms are involved in the deregulation of gene expression. We showed that FET oncoproteins interact with the SWI/SNF chromatin remodeling complex via their FET N-terminal domain and that the majority of FET-FOPs interact with SWI/SNF, unlike normal FET proteins. Our comprehensive proteomic analysis showed that the FET-FOP binding to SWI/SNF is robust but do not cause a massive change in the complex composition, such as in the case of synovial sarcoma where BAF47 is lost from the complex after incorporation of the SS18-SSX fusion protein [119]. The SWI/SNF core remain intact but our analysis cannot completely rule out a loss of a subtype-specific or ATPase-module component. A more detailed analysis is required. We therefore set up a comprehensive Quantitative mass spectrometry experiment, immunoprecipitating BRG1 and FET-FOPs FUS-DDIT3 and EWSR1-FLI1 in technical IP replicates followed by chemical labeling (TMT) to quantify relative protein amounts in the different samples. In this way, we can compare the composition of SWI/SNF complexes in MLS and EWS cells and compare complexes with FET-FOPs bound. This is ongoing work in the lab. Although the structural effect is unknown, most experiments point towards a functional effect of FET oncoproteins binding to SWI/SNF. For example, we showed that FET-FOPs and SWI/SNF co-localize on chromatin and regulate a set of FUS-DDIT3 target genes. We also showed that the chromatin landscape is altered by expression of FET oncoproteins, leading to a significant increase in H3K27me3 levels. However, studies in Ewing sarcoma have instead shown increased H3K27 acetylation levels at specific sites [77, 133].

FET oncoproteins can retarget SWI/SNF complexes to new, possibly oncogenic, loci leading to aberrant chromatin remodeling. Boulay *et al.* showed that SWI/SNF was aberrantly recruited by the EWSR1-FLI1 fusion protein to GGAA repeats, leading to target gene activation [77]. Other FET-FOPs may function in similar ways. Very recently, Davis *et al.* showed that FUS-DDIT3 could recruit BRG1 to artificial DNA loci [138] and Chen *et al.* showed that FUS-DDIT3 could recruit ISWI chromatin remodeling complexes (main subunit SNF2H) to target sites marked by active enhancers [139]. They also showed that phosphorylation of the FUS IDR by DNA-damage response kinases after radiation disrupted FUS-DDIT3 – SNF2H interactions that were necessary for MLS tumor maintenance [139]. One possible consequence of FET-FOP binding to SWI/SNF may be relocation to new genomic targets which cause changes in histone marks and gene expression both at the new location and old

location i.e. the “normal” binding spot where SWI/SNF would bind in cells without FET oncoproteins. This may be due to a direct effect on SWI/SNF function and/or altered ability to oppose the PRC2 polycomb repressive complex. Loss of SWI/SNF activity at “normal” sites leading to loss of PRC2 opposition i.e. increased PRC2 activity (H3K27me3) supports our finding of increased H3K27me3 levels in FET-FOP expressing cells (as also suggested by Davis *et al.* [138]). It remains to be determined exactly how this retargeting occur and if FET-FOPs have equal effect on the different SWI/SNF subtypes. It is probable that the C-terminal partner directs SWI/SNF complexes to new targets genes, which is consistent with the tumor-type specificity of FET-FOP-caused cancers.

7.1.2 Aberrant gene expression and role of phase separation

Increasing evidence suggests an altered epigenetic landscape in FET sarcomas with increased acetylation at specific loci and induction of super-enhancers [110, 133]. Super-enhancers induce gene expression, especially genes involved in cell identity and oncogenesis [157, 158]. We showed that the transcriptional coactivator BRD4 interacts with FET-FOPs via the SWI/SNF complex and co-localize at genomic loci. In addition, components of Mediator and RNA pol II associate with FET oncoproteins suggesting possible recruitment of the transcriptional machinery to FET-FOP-, SWI/SNF- and BRD4-bound genomic loci.

Gene expression is regulated by transcription factors that bind specific genomic loci through their DNA-binding domain and use their activation domain to activate transcription e.g. by recruiting regulatory coactivators and RNA Pol II [138]. Even though the mechanism of the transactivation was for long unknown, the biophysical process of phase separation is now suggested to play an important role [28]. So called transcriptional condensates (or transcriptional hubs) have been proposed to form by liquid-liquid phase separation at particular genomic loci and promote transcription locally [27]. In addition, BRD4, Mediator and RNA pol II can form stable clusters with phase-separation properties, located specifically at super-enhancers [143, 145]. Disruption of BRD4 chromatin binding with JQ1 treatment dissolved both Mediator and Pol II clusters [145] suggesting that BRD4 binding to chromatin can nucleate transcriptional condensates. In addition, disruption of phase separation by hexanediol treatment reduced BRD4, MED1 and RNA pol II presence at super-enhancers, and reduced RNA pol II binding at super-enhancer-associated genes, indicating adverse effects on transcription when transcriptional condensates were disrupted [143]. The composition of condensates can be regulated by

post-translational modification; RNA pol II was released from condensates after phosphorylation of its C-terminal domain, potentially to perform its role in transcriptional elongation [27].

Recent data suggest that part of the oncogenic function of FET-FOPs occur via liquid-liquid phase separation [28, 77, 108, 138, 140]. Early on, Boulay *et al*. suggested that phase separation was involved in SWI/SNF recruitment and transcriptional activation at GGAA microsatellites. A study by Chong *et al.* showed that EWSR1-FLI1 formed transactivation hubs at endogenous GGAA repeats, and that EWSR1-FLI1 were enriched at those sites (many more fusion proteins present per satellite than the two that would bind by direct DNA-protein interactions) [28]. This indicate that binding to genomic loci can nucleate EWSR1 IDR-IDR interactions forming transactivation hubs. Indeed, reducing phase-separating properties by replacing all tyrosines (Y) to serines (S) reduced hub formation and the interaction with endogenous EWSR1 was lost. In contrast, replacing tyrosines with phenylalanine (F) retained function suggesting that aromatic amino acids and hydrophobic contacts are important for EWSR1 IDR-IDR interactions [28]. Moreover, Zuo *et al.* showed that FET-FOPs can form biocondensates at specific loci where they recruit RNA polymerase II and enhance gene transcription [140]. Another study attributed the oncogenic role of FUS-DDIT3 to its phase-separating capacity; Owen *et al.* showed that FUS-DDIT3 have novel phase-separating properties compared to DDIT3, and that overexpressed FUS-DDIT3 in cells form nuclear puncta with liquid-like characteristics that co-localize with BRD4 [108]. Surprisingly, disruption of the DNA binding domain in ectopically expressed FUS-DDIT3 did not disrupt puncta formation suggesting that the DNA binding domain is not important for condensate formation when FUS-DDIT3 is overexpressed. Furthermore, endogenous FUS-DDIT3 in MLS cell lines formed smaller nuclear puncta distributed over the whole nucleus [108], potentially suggesting that nucleation at specific genomic loci is required for hub formation at endogenous oncoprotein concentrations [28]. One question that arise is whether phase separation is required for transcriptional activation, since the same properties that affect phase separation affects the important protein interactions. Zuo *et al.* stated that phase separation is required for recruitment of RNA pol II [140] but Chong *et al.* showed that transactivation hubs with high local concentration of transcription factor IDR at native genomic loci without apparent phase separation can recruit RNA pol II and activate transcription [28]. IDR domains in transcription factors or FET fusion oncoproteins can interact with each other in a dynamic and selective way which stabilize DNA binding at both synthetic DNA and endogenous genomic loci. When overexpressed they can liquid-liquid phase separate but at physiological levels they form transactivation hubs [28]. The concept of phase separation is a little bit controversial and perhaps it is a

matter of definition when the biophysical properties that lead to IDR-IDR interactions reach a substantial enrichment of proteins so phase separation occurs, and if it is the phase separation itself that is responsible for the transcriptional activation and recruitment of the transcriptional machinery.

In summary, proteins with low complexity prion-like domains such as the FET N-terminal domain are known to phase separate and these proteins are more inclined to interact with each other [141]. Indeed, the interactome of FUS-DDIT3 and EWSR1-FLI1 were enriched in proteins with phase-separating capacity. It is possible that the local concentration of FET-FOP and SWI/SNF at bound genomic loci is enough to induce phase separation which might lead to recruitment of other proteins such as the transcriptional machinery followed by oncogenic gene expression. The role of phase separation in these processes should be further assessed.

7.1.3 Role of FET NTD versus C-terminal partner

Both the FET NTD and the C-terminal transcription factor partner contribute to the oncogenic roles of FET-FOPs. FET oncoproteins bind chromatin via the DNA-binding domain in the C-terminal transcription factor partner. However, Boulay *et al.* showed that the EWSR1 IDR is necessary for stable binding at GGAA microsatellites, phase separation and de novo enhancer activation [77]. While FLI1 could bind GGAA repeats, EWSR1-FLI1 was substantially enriched at these loci and enhancer activation at these sites was a neomorphic property of EWSR1-FLI1. In addition, fusing FLI1 with either one of two EWSR1 SYGQ-rich domains (SYGQ1: 37 amino acids or SYGQ2: 64 amino acids) was sufficient to recapitulate EWSR1-FLI1 activity, indicating that at least parts of the FET NTD are required for oncogenic function [77]. Another study showed that FLI1 can bind GGAA repeats at shorter GGAA lengths but EWSR1 NTD was required for binding at so called “sweet-spots” lengths (18-22 GGAA repeats, with maximal EWSR1-FLI1 responsiveness to target genes) and part of the EWSR1 IDR (amino acids 1-82 and 246-262) was enough to recapitulate EWSR1-FLI1 function [159]. In addition, the FLI1 DNA-binding domain was required for EWSR1-FLI1 binding at GGAA repeats. These studies indicate that FLI1 can bind GGAA repeats by itself but the FET NTD (IDR region) is required for enrichment at these loci, as well as transactivation. Indeed, Chong *et al.* found that transcription factor IDRs stabilized transcription factor binding to its genomic loci, potentially via multivalent contacts such as DNA binding to chromatin and multiple IDR interactions with partner proteins [28].

The N-terminal prion-like domain of FUS (amino acids ~1–165) was shown to be required for FUS self-association, binding to chromatin and transcriptional activation [160]. However, the FUS NTD by itself require higher protein concentration to phase separate *in vitro*, compared to full-length FUS as well as FUS-DDIT3 [138, 161, 162]. *In vitro* experiments mixing FUS NTD with FUS RNA-binding domains facilitated phase separation at lower concentrations than for FUS NTD alone indicating that interactions between these domains cooperate to drive phase separation in normal FUS [161]. Indeed, protein interaction facilitates phase separation at lower IDR concentrations through increased valency via oligomerization [27]. The FET-FOP transcription factor partner may contribute to phase separation in other ways than providing DNA binding to chromatin; FUS-DDIT3 formed condensates at significantly lower protein concentration than FUS NTD *in vitro* suggesting that DDIT3 contributes to interactions between FUS-DDIT3 molecules [138]. Certainly, a previous report has shown that purified DDIT3 can homo-oligomerize [163]. Both parts of the fusion protein thus seem to be important for protein interactions as well as phase separation capacity. In support of this, FET-FOPs appear as nuclear puncta due to extensive self-interactions and possibly phase separation, while normal FET proteins and DDIT3 remain diffuse [10, 28, 77, 108, 164]. Normal FUS remain soluble in the nucleus under physiological concentrations due to interactions with RNA [138].

The DNA-binding domain of the C-terminal partner is necessary for FET-FOP binding to specific genomic sites [159], thereby nucleating i.e. enriching the oncoproteins to high local concentration forming transactivation hubs [28, 140]. FLI1 along with other ETS family transcription factors contain the conserved ETS DNA-binding domain, binding at conserved loci across the genome. We showed that DDIT3 binds genomic DNA through its leucine zipper dimerization partners at relatively few genomic loci, leading to repression of genes, potentially because of its lack of transactivation domain. We do not know exactly how the FET N-terminal domain affects the binding pattern and dimerization capacity of DDIT3. FUS-DDIT3 binds the genome at more sites than DDIT3 but no comprehensive ChIP-seq study analyzing both FUS-DDIT3, SWI/SNF and histone marks in cancer cells with and without fusion oncoprotein exist, as far as we know. This is ongoing work in the lab.

7.1.4 Aberrant function of normal FET proteins and interaction partners

One role of the FET-FOPs is possibly to interfere with the function of normal FET proteins or other proteins they normally collaborate with. A previous study

in our group showed that normal FET proteins were relocated to nuclear puncta in the presence of FET oncoproteins [22]. Changing the localization and interactions of FET proteins may have a large impact on their functions. Transformation of cells with FET-FOPs therefore leads to neomorphic gain of function from the fusion protein but potentially also loss of function for the normal FET proteins. FET proteins can normally interact with RNA polymerase II [11, 12]. Indeed, ChIP-seq analysis of FUS and EWSR1 showed co-localization with RNA polymerase II and that they bind a subset of genes with active transcription, mainly genes involved in RNA regulatory processes [165]. Furthermore, normal FET proteins can phase separate in living cells, via their FET NTD, recruit RNA pol II and drive transcription [27, 162]. Post-translational modification of the FUS IDR by phosphorylation was shown to disrupt phase separation capacity of normal FUS [25], suggesting that this is a way for the cell to regulate protein aggregation, transactivation and other functions of FET proteins. Interestingly, a recent report showed that FET proteins can have diverse function depending on biophysical state; Reber *et al.* developed a method to analyze the phase-separated FUS interactome by enriching FUS droplets from cell extracts and analyzing the protein and RNA content compared to whole cell extracts analyzed by immunoprecipitation [166]. They showed a substantial difference in interacting proteins depending on biophysical state; non-phase-separated FUS interacted mainly with pre-mRNA processing machinery while phase-separated FUS bound to proteins involved in chromatin remodeling and DNA damage response, including BRG1 and SNF2H (subunit of the ISWI chromatin remodeling complex). In addition, phase-separation-deficient FUS with all tyrosine mutated to serine had reduced interaction with chromatin. One can assume that the different functions depending on biophysical state also apply to FET oncoproteins. More studies are needed to determine the impact of FET oncoproteins on normal FET function and if FET-FOPs affect normal FET protein phase separation or genomic targeting. However, it is hard to separate the functions of normal and oncogenic FET proteins in cancer cells that contain both since they also interact with each other.

FET oncoproteins have numerous interaction partners besides normal FET proteins. They are known to impact on several signaling pathways such as PI3K/AKT, IGF-R, Hippo and JAK-STAT [94-102]. Here, we showed that FET-FOPs interfere with the JAK-STAT pathway and induce the active form of the pioneer transcription factor STAT3. The contribution of the JAK-STAT pathway to FET sarcoma oncogenesis needs to be further studied.

7.1.5 Concluding remarks

The interest in epigenetics and chromatin remodeling in cancer development has increased substantially the last decade and although research in this field is growing exponentially, these complex biological systems are far from understood even in healthy cells. Therefore, more research is needed to delineate these processes in healthy and malignant cells in order to develop targeted therapies in FET sarcomas. FET-FOPs bind to specific binding sites and possibly recruit both the SWI/SNF complex and the transcriptional machinery to these locations, possibly via liquid-liquid phase separation. Indeed, replacing tyrosines with serines in the EWSR1 IDR domain of the fusion protein disrupted phase separation, and failed to stabilize DNA binding so that targeting of SWI/SNF to tumor-specific DNA-binding sites repeats was lost [77]. The mechanisms involved are most clearly studied for EWSR1-FLI1 but other FET-FOPs may have similar modes of action. EWSR1-FLI1 bind GGAA microsatellites and, after recruitment of SWI/SNF, may act as a pioneer factor to convert closed chromatin to an open state, enabling transcriptional activation. EWSR1-FLI1 can bind to the same binding sites as FLI1 but the EWSR1 IDR domain induce oligomerization of fusion proteins and possibly normal FET proteins which leads to recruitment of SWI/SNF and the transcriptional machinery. The result is an aberrant epigenetic landscape with new super-enhancer regions marked by H3K27Ac and possibly places where loss of SWI/SNF leads to reduced polycomb opposition and increased H3K27me3 marks. EWSR1-FLI1 can activate *de novo* enhancers at GGAA repeats but also displace FLI1 at conserved ETS sites, leading to gene repression [110].

It is possible that SWI/SNF do not need to be retargeted to achieve aberrant gene expression. FET oncoproteins and normal FET proteins share many similar interaction partners but the majority of FET-FOPs are bound to the SWI/SNF chromatin remodeling complex compared to normal FET proteins. Since many SWI/SNF components (e.g. BRG1, BAF47, BAF57 and ARIDs) can bind DNA or chromatin domains [53, 117], one possible scenario is that FET-FOPs bind SWI/SNF at their “normal” binding sites but induce abnormal IDR interactions (and possibly phase separation), contributing to aberrant gene expression. Furthermore, BRD4 and Mediator at super-enhancers might also contribute to phase-separation and transactivation; Cho *et al.* suggested that large Mediator clusters at enhancers associate with the transcription machinery at promotors and that the distance between enhancer and promoter can be large (>300 nm), and potentially reach several promoters simultaneously [145]. A similar mechanism as proposed above for EWSR1-FLI1 was described for regulation of lineage-specific genes in B cells; the pioneer transcription factor EBF1 bound to inaccessible chromatin and via its IDR domain recruited FUS and the

SWI/SNF complex, and formed phase-separated condensates that led to chromatin opening at target sites [167]. The order of these events was not delineated fully, however, BRG1 could partition into phase-separated FUS condensates suggesting that FUS contributed to phase separation at target loci and recruited BRG1 leading to chromatin remodeling activity. Recently, Davis *et al.* reported a possible mechanism for recruitment of the SWI/SNF complex to FET-FOP condensates by interactions of the IDR of FET proteins to IDRs in several SWI/SNF components such as BRG1, BAF155 and ARID1A [138]. Mislocalization of biocondensates through loci-specific liquid-liquid phase separation, or at least through IDR protein interactions, emerge as a possible explanation for the aberrant transcription induced by FET oncoproteins.

7.2 Targeting FET fusion oncoproteins and epigenetic dependencies

Fusion oncoproteins are a common cause of cancer that often establish a state of oncogene addiction, meaning that cancer cells are dependent on the function of the oncoprotein. Direct pharmacological inhibition of the fusion oncoprotein has been effective for some kinase fusion proteins but have proven difficult for transcription factor fusions [133]. Many fusion-driven malignancies have poor prognosis, potentially because they are treated with non-targeted therapies that do not inhibit the oncogenic mechanism [152]. Since FET-FOPs are potent oncogenes capable of transformation without further mutations, they are suitable for targeted therapies where the goal is to target mechanisms specific to cancer cells. In other words, the neomorphic properties of the fusion proteins should therefore be exploited. The data presented in this thesis along with other studies suggest a common oncogenic mechanism for FET-FOPs involving the SWI/SNF chromatin remodeling complex. Finding a way to directly target this mechanism and developing targeted therapies in FET sarcoma remains an urgent goal.

7.2.1 Direct and indirect targeting of fusion oncoproteins

Pediatric bone- and soft-tissue sarcoma including FET sarcoma where fusion oncoproteins is the main oncogenic driver exhibit strong oncogene-dependence and direct targeting of fusion oncogenes have therefore been the focus of many studies but with limited success [168]. The fusion proteins can be targeted directly, or indirectly via targeting of protein or DNA interactions [152, 168]. Another possible strategy could be to target the fusion oncogene breakpoint DNA or regulatory elements, such as microsatellites in Ewing sarcoma, through CRISPR-CAS9-strategies or targeting its RNA transcript via RNA interference

[168]. One promising future strategy is the development of small molecule protein degraders that can induce degradation of the target protein by coupling it to molecules that recruit the proteasome [152, 168]. Protein degraders have great potential for "undruggable" targets since it completely removes the target protein.

The fusion oncoproteins can be targeted indirectly by disrupting their protein-DNA interactions for example using various epigenetic drugs, such as histone deacetylases (HDACs) [168]. Targeting the epigenetic regulatory system that controls expression of the fusions might be another strategy. We believe that interfering with the epigenetic machinery involving the SWI/SNF complex has potential as a targetable system (see next section). Epigenetic changes are reversible and can thus be a good drug target. A problem with indirect targeting is the lower specificity and potential side effects [168]. Another challenge for designing targeted therapies in FET sarcoma is that the chromatin regulators still have many diverse functions that are not understood completely, neither in healthy cells or cancer cells, and controlling the effect of small molecules targeting them is therefore complicated. Yet another challenge is reversing the specific aberrant chromatin changes. Perhaps combinations of epigenetic and chromatin-targeting drugs are needed to reverse the aberrant epigenetic landscape in these sarcomas [169]. FET oncoproteins could also be targeted by targeting the signaling pathways that are aberrantly regulated in cancer, for example the JAK-STAT pathway in MLS. However, elucidating the exact mechanism in pre-clinical studies is needed before progressing to clinical trials and hopefully the clinic.

7.2.2 Targeting aberrant SWI/SNF complexes

We believe that aberrant SWI/SNF complexes are potential therapeutic targets in FET sarcoma since the SWI/SNF complex is bound and affected by FET oncoproteins. The SWI/SNF complex has, during the last decade, emerged as one of the most commonly mutated gene families in cancer, with 20% of SWI/SNF components mutated in human tumors [72]. The most commonly mutated components are BRG1, PBRM1, ARID1A and BAF47 [53]. However, due to high variety of alterations, there is no unique mechanism that explains tumorigenesis for all aberrant SWI/SNF complexes. Research about targeting aberrant SWI/SNF complexes is in its infancy, and the majority of research is pre-clinical, delineating the mechanism behind tumor formation, but a few clinical trials using targeted therapies are ongoing [53, 170]. Current therapeutic strategies against aberrant SWI/SNF complexes target epigenetic antagonism, synthetic lethality or oncogene addiction [53]. One such approach is to target

the epigenetic antagonism between SWI/SNF and PRC2 complexes. Loss of certain subunits, such as BAF47 in malignant rhabdoid tumors, leads to disturbed ability to oppose PRC2 and thereby increased dependency on the PRC2 complex in cancer cells. In these cases, targeting the enzymatic component EZH2 with the drug tazemetostat has provided initial promising results *in vitro* [53]. In fact, tazemetostat was recently approved for treatment of Epithelioid sarcoma, an aggressive cancer characterized by BAF47 loss [171, 172].

Synthetic lethalities can be identified by RNAi or CRISPR-based screenings of cancer models where cancer cells and not normal cells are dependent on a specific gene. Interestingly, several synthetic lethal relationships between SWI/SNF paralog subunits have been reported. Cancer cells with mutated ARID1A specifically depend on its paralog ARID1B [173] and cancer cells with mutated BRG1 specifically depends on its paralog BRM [174, 175]. In these cases, absence of one of the paralogs leads to incorporation of the other SWI/SNF paralog into all remaining SWI/SNF complexes. The essential function of residual aberrant SWI/SNF complexes in SWI/SNF-mutant cancers have opened up for potential new therapies by targeting the remaining paralog, although the exact oncogenic mechanisms of these aberrant complexes remain poorly understood. Recent studies have described a dependency of BAF47-deficient tumors (malignant rhabdoid tumor, synovial sarcoma) on GBAF function. Without BAF47, the function or stability of cBAF and PBAF complexes is disrupted and cancer cells then rely on the function of GBAF, the only SWI/SNF subtype without BAF47. The GBAF-specific component BRD9 is therefore a potential target in these tumor types, and several selective small-molecule inhibitors or chemical degraders of BRD9 have been developed [53, 176]. Other potential dependencies for SWI/SNF-deficient cancers include the DNA damage response system or downstream pathways [53]. In addition, some SWI/SNF-mutations infer sensitivity to immune-checkpoint inhibition [53, 176]. Although targeting synthetic lethalities in aberrant SWI/SNF complexes hold great potential, SWI/SNF components are considered tumor suppressors and knocking out the remaining paralog may even promote cancer in certain contexts [176]. Targeting synthetic lethalities of aberrant SWI/SNF complexes such as the PCR2-component EZH2, paralog lethalities and dependency on a specific SWI/SNF subtype emerge as interesting future therapeutic options in many cancer types. The future will tell if this is a viable option also in tumors caused by FET fusion oncoproteins.

7.2.3 Targeting the transcriptional coactivator BRD4

BRD4 has been shown to occupy super-enhancers at key cell identity genes and oncogenic drivers [78, 85]. BRD4 has therefore gained interest as a therapeutic target in several tumor types since it is a novel way to target aberrantly activated oncogenic transcription factors. Young and colleagues showed that BET bromodomain inhibition with JQ1 disrupted super-enhancers in multiple myeloma cells by preferential reduction of BRD4, Mediator and P-TEFb (transcription elongation factor complex) followed by a reduction in transcription at super-enhancers connected to oncogenes such as MYC [85]. They speculated that super-enhancers regulate certain oncogenic drivers thus making some tumor cells more sensitive to BRD4 inhibition. Recently, it was reported that BRD4 is a target in cancers with aberrant SWI/SNF function such as when the SWI/SNF subunits BRG1 or BRM are mutated [131]. The authors hypothesize that BRD4 is the sole driver of an oncogenic network otherwise controlled by BRG1 and BRD4. The connection between BRD4 and GBAF raises the question whether GBAF-dependent cancers are susceptible to BRD4 inhibition. JQ1 bromodomain inhibition of embryonic stem cells showed that the bromodomain of BRD4 was important for BRD9 (GBAF) localization on chromatin [93] indicating that BRD4 might be a suitable target in cancer forms dependent on GBAF function such as synovial sarcoma and malignant rhabdoid tumors [60].

We showed that the transcriptional coactivator BRD4 interacts with SWI/SNF, FET oncoproteins, and possibly Mediator in FET sarcoma cells, and that they co-localize in the genome, potentially leading to oncogenic gene expression. FET sarcoma cells were sensitive to BRD4 inhibition by dual-bromodomain inhibition with AZD5153 or BRD4 degradation with ARV-825. In addition, MLS cells with acquired resistance to chemotherapy were shown to be sensitive to BRD4 inhibition/depletion [86]. Inhibition of the BRD4 bromodomain disrupts its interaction with chromatin and has been reported to reverse the FET-FOP-induced transcriptional profiles [79, 87-89, 132]. Thus, BRD4 emerges as a potential therapeutic target also in FET sarcoma.

As mentioned above, BRD4 has increased occupancy at super-enhancers which induces gene expression. Paradigm-shifting reports studying the biophysical properties of super-enhancers and transcriptional hubs suggest that BRD4 and Mediator are present in phase-separated biocondensates at super-enhancers [143]. The enrichment of biomolecules into these condensates may explain why BRD4 inhibition selectively disturb BRD4 at super-enhancers. Indeed, a recent study by Young and colleagues showed that small molecule inhibitors such as JQ1 can specifically partition into biocondensates and elicit a drug response

[177]. In conclusion, BRD4 is a possible therapeutic target in FET sarcoma and other cancer types. Several BET inhibitors have been developed and some of these are currently in clinical trials. Targeting BRD4 in combination with chemotherapy, DNA damage repair or epigenetic therapies have been proposed as a way to improve efficacy and avoid drug resistance [178].

7.2.4 Concluding remarks

Trabectedin is a second line chemotherapy drug in myxoid liposarcoma. It is an alkaloid drug that binds the minor groove of DNA and changes its structure. Although DNA-damaging, trabectedin is an interesting drug target in myxoid liposarcoma since soft-tissue sarcomas and MLS in particular is especially sensitive to this drug. It has a complicated mode of action, affecting many biological processes and its exact functions remains unknown [13, 179]. Interestingly, several studies suggest a more direct effect of trabectedin by targeting the FET oncoproteins. In MLS, trabectedin treatment was reported to disrupt FUS-DDIT3 binding from target promoters, reverse oncogenic expression and lead to adipogenic differentiation [180, 181]. In EWS, trabectedin reversed the EWSR1-FLI1 gene signature [182]. In addition, treatment of Ewing sarcoma cells with a relatively high dose of trabectedin lead to redistribution of EWSR1-FLI1 and loss of the SWI/SNF complex from chromatin, especially at target genes, leading to a global increase in repressive histone marks H3K27me3 and H3K9me3 [183]. Harlow *et al.* suggested that the eviction of SWI/SNF after trabectedin treatment was explained by both trabectedin and SWI/SNF binding to the minor groove of DNA [183]. These studies are surprising, that a DNA-damaging cytotoxic drug can directly target the function and chromatin binding of the FET-FOPs as well as the SWI/SNF complex. In addition, there seem to be some specificity of eviction at FET-FOP target genes which may be due to open chromatin regions at those locations or perhaps enrichment of the drug in potential phase-separated biocondensates at binding-specific loci. Furthermore, both trabectedin and BRD4 have been reported to reverse oncogenic transcription and BRD4 inhibition displayed a similar transcriptional response as trabectedin [86]. We envision the development of a non-DNA-damaging drug with less side effects and reduced risk of secondary cancers, based on some of the mechanisms described in this thesis, involving BRD4, SWI/SNF or other epigenetic processes. Elucidating the complicated mode of action of trabectedin and how it interferes with SWI/SNF and FET oncoprotein binding at chromatin could aid the development of drugs targeting FET-FOP – SWI/SNF interactions at chromatin without the DNA-damaging properties.

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APPENDIX

Appendix 1 68-72

Reprint: Lindén, M. and Vannas, C. *Okänd interaktion bakom genombrott i förståelsen av hur vissa sarkom uppstår*. Onkologi i Sverige, 2019: 4.

Available in the printed thesis.

Appendix 2 73-74

Calculations for IP-QWB dilution.

Appendix 2. Calculations for IP-QWB dilution

Dilutions factor from IP sample to WB:

	+25% 4x LDS Sample buffer	+10% Sample reducing agent	WB Dilution factor
Input (I)	+	+	0.65
Bound (B, Eluate)		+	0.9
Nonbound (NB)	+	+	0.65

There are two ways to calculate how to dilute the input and eluate (bound) sample before QWB to be able to directly compare with the nonbound sample, taking into account the different volumes during IP and the WB dilution factors.

1: Dilution ratio

"Amount" in WB samples/µl:

$$Input = \frac{V_{Input,WB}}{V_{tot}} * \frac{1}{V_{Input,WB}} * 0.65 = \frac{0.65}{V_{tot}}$$

$$Bound = \frac{V_I}{V_{tot}} * \frac{1}{V_B} * 0.9$$

$$Nonbound = \frac{V_I}{V_{tot}} * \frac{1}{V_{NB}} * 0.65$$

Amount relative NB = Dilution ratio:

$$\frac{Bound}{Nonbound} = \frac{\frac{0.9V_I}{V_{tot} * V_B}}{\frac{0.65V_I}{V_{tot} * V_{NB}}} = \boldsymbol{\frac{0.9V_{NB}}{0.65V_B}}$$

$$\frac{lnput}{Nonbound} = \frac{\frac{0.65}{V_{tot}}}{\frac{0.65V_{IP}}{V_{tot} * V_{NB}}} = \boldsymbol{\frac{V_{NB}}{V_I}}$$

Example IP-WB 15-well gel:

$$V_I = 20\ \mu l\ nuclear\ extract, V_{NB} = 250\ \mu l, V_{Bound} = 50\ \mu l$$

$$\frac{Bound}{Nonbound} = \frac{0.9V_{NB}}{0.65V_B} = 6.9$$

$$\frac{lnput}{Nonbound} = \frac{V_{NB}}{V_I} = 12.5$$

Maximize load of Nonbound: 14 µl (+10% sra)

=> (14/12.5) = 1.1 µl input (dilute to 14 µl+ sra)
=> (14/6.923) = 2.0 µl eluate (dilute to 14 µl+ sra)

2: Per µl Nonbound

Dilution of WB samples:

$$Input = \frac{V_I}{0.65}$$

$$Bound = \frac{V_B}{0.9}$$

$$Nonbound = \frac{V_{NB}}{0.65}$$

Volume per µl Nonbound:

$$\frac{Bound}{Nonbound} = \frac{\frac{V_B}{0.9}}{\frac{V_{NB}}{0.65}} = \frac{\mathbf{0.65}\boldsymbol{V_B}}{\mathbf{0.9}\boldsymbol{V_{NB}}}$$

$$\frac{lnput}{Nonbound} = \frac{\frac{V_I}{0.65}}{\frac{V_{NB}}{0.65}} = \frac{\boldsymbol{V_I}}{\boldsymbol{V_{NB}}}$$

Example IP-WB 15-well gel:

$V_I = 20\ \mu l\ nuclear\ extract,\ V_{NB} = 250\ \mu l,\ V_{Bound} = 50\ \mu l$

$$\frac{Bound}{Nonbound} = \frac{0.65V_B}{0.9V_{NB}} = 0.14\ \mu l\ per\ \mu l\ NB$$

$$\frac{lnput}{Nonbound} = \frac{V_I}{V_{NB}} = 0.08\ \mu l\ per\ \mu l\ NB$$

Maximize load of Nonbound: 14 µl (+10% sra)

=> (14*0.08) = 1.1 µl input (dilute to 14 µl+ sra)
=> (14*0.14) = 2.0 µl eluate (dilute to 14 µl+ sra

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