Molecular analysis of the FET family fusion oncoprotein FUS-DDIT3

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ABSTRACT

Chromosomal translocations leading to formation of tumor type specific fusion oncogenes are frequently found in human cancers. The FET family genes (*FUS*, *EWSR1* and *TAF15*) occur in translocations with genes encoding various transcription factors in sarcomas and leukemias. The resultant fusion genes encode chimeric protein products containing the N-terminal domain (NTD) of a FET family protein juxtaposed to the DNA binding domain of a transcription factor. The constitutively expressed fusion proteins act as abnormal transcription factors and are considered to be the primary initiating and driving factors of tumorigenesis. Myxoid liposarcoma (MLS) is defined by the t(12;16)(q13;p11) that cause the fusion of the *FUS* and *DDIT3* genes and expression of a chimeric FUS-DDIT3 oncoprotein. The aim of this thesis was to functionally characterize the FUS-DDIT3 fusion oncoprotein and the normal FUS, EWS, TAF15 and DDIT3 counterparts. To understand the involvement of DDIT3 in cancer and other pathologies we defined the genes and cellular functions it regulates. We observed that DDIT3 may localize to both the cell cytoplasm and nucleus. Microarray analysis revealed that cytoplasmic or nuclear DDIT3 regulate distinct sets of target genes. However, cytoplasmic or nuclear DDIT3 control the same cellular functions of which cell migration, proliferation, apoptosis/survival and cell cycle were most clearly affected. The majority of target genes were repressed supporting a role for DDIT3 as a dominant negative factor that may sequester and block the action of cytoplasmic and nuclear transcription factors. Subcellular localization is likely to be an important regulatory mechanism for control of DDIT3 activity. We further studied the molecular function of the FUS, EWS and TAF15 N-terminal domains with focus on their involvement in protein-protein interactions. The NTDs of FET proteins were found to bind the cytolinker plectin and FUS was shown to form complexes with plectin in the cell cytoplasm. Plectin expression was required for normal FUS function as

plectin knockout cells revealed a shift in FUS nucleo-cytoplasmic distribution and a deregulation of FUS function in gene expression. Furthermore, we identified a conserved motif of the FUS, EWS and TAF15 NTDs that mediates interaction with full-length FET family proteins. The NTD of the FUS-DDIT3 fusion oncoprotein retained the capacity to bind wildtype FET proteins and disrupt their normal nuclear localization pattern. We also observed an involvement of wildtype FUS in the regulation of FUS-DDIT3 target genes. Finally, several components of the human SWI/SNF complex were isolated by pull-down with the FUS NTD. The SWI/SNF subunits Brg1 and ARID1A were further confirmed to form complexes with FUS-DDIT3 as well as wildtype FUS in the nuclei of MLS cells. SWI/SNF complexes control gene expression by modulating nucleosome positioning and are key players in cellular differentiation processes. Aberrant activity of SWI/SNF has further been linked with development of various cancers. Interaction with SWI/SNF is thus likely to be important for the impact of FUS-DDIT3 on gene regulation, differentiation processes and the transformed state of MLS.

Keywords: FUS-DDIT3, FUS, EWS, TAF15, DDIT3, myxoid liposarcoma, plectin, SWI/SNF.

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LIST OF PAPERS

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

I. Alexandra Jauhiainen, Christer Thomsen, Linda Strömbom, Pernilla Grundevik, Carola Andersson, Anna Danielsson, Mattias K. Andersson, Olle Nerman, Linda Rörkvist, Anders Ståhlberg, Pierre Åman. Distinct Cytoplasmic and Nuclear Functions of the Stress Induced Protein DDIT3/CHOP/GADD153. *PLoS One, 2012. 7(4): e33208.*

II. Christer Thomsen, Sameer Udhane, Rikard Runnberg, Gerhard Wiche, Anders Ståhlberg, Pierre Åman. Fused in sarcoma (FUS) interacts with the cytolinker protein plectin: implications for FUS subcellular localization and function. *Experimental Cell Research, 2012. 318(5):653-61.*

III. Christer Thomsen, Pernilla Grundevik, Per Elias, Anders Ståhlberg and Pierre Åman. A binding motif in the N-terminal domains of the FUS, EWSR1 and TAF15 fusion oncoproteins mediates interactions with full-length FET family proteins. *Manuscript.*

IV. Christer Thomsen, Anders Ståhlberg and Pierre Åman. Fused in sarcoma (FUS) and the FUS-DDIT3 fusion oncoprotein interacts with the human SWI/SNF complex. *Manuscript.*

CONTENT

ABBREVIATIONS

bZIP	Basic leucine zipper
CML	Chronic myeloid leukaemia
CTD	C-terminal domain
EB	Epidermolysis bullosa
EGFP	Enhanced green fluorescent protein
GST	Glutathione *S*-transferase
hnRNP	Heterogeneous nuclear ribonucleoprotein
IB	Immunoblot
Ig	Immunoglobulin
MLS	Myxoid liposarcoma
MPC	Mesenchymal progenitor cells
MS	Mass spectrometry
NTD	N-terminal domain
PLA	Proximity ligation assay
RFP	Red fluorescent protein
RGG	Arginine-glycine-glycine repeat/motif/box
RNP	Ribonucleoprotein domain
RRM	RNA recognition motif
SG	Stress granule
SG	Stress granule

SYGQ	Serine-tyrosine-glycine-glutamine rich
TAF	TBP associate factor
TBP	TATA binding protein
UTR	Untranslated region
Zf	Zinc finger domain

1 INTRODUCTION

1.1 Cancer

Cancer is one of our most common diseases and a major cause of death in the Western world. The disease is primarily associated with increased age, but is found in all age groups including children and young adults [1]. Cancer is a heterogeneous group of diseases in which abnormal cells accumulate without control and are able to spread to other sites of the body. The abnormal cells have lost their ability to assemble and create tissue of normal form and function and instead form tumors with less organized architecture [2]. Tumors can be either benign or malignant. Benign tumors constitute the majority of diagnosed cases and are not classified as cancer as they generally are slow growing, localized and non-invasive masses. Benign tumors are rarely harmful to the host unless they cause pressure on vital organs or secretion of excess hormones. In contrast, malignant tumors invade surrounding tissue and spread to distant sites by the seeding of metastases from the primary tumor. The vast majority of tumor-related deaths are associated with malignant tumors and more specifically metastasis.

Tumors can arise from tissues derived from any of the embryonic germ layers and there are examples of tumors originating from almost every cell type in the human body. The most common human malignant tumors originate from epithelia and are classified as carcinomas. These tumors are further separated in two main categories were squamous cell carcinomas are derived from the epithelia lining body surfaces and organs while adenocarcinomas originate from glandular epithelia. As the largest group of human malignancies carcinomas account for 80% of all cancer-related deaths in the Western world. In Sweden, the most common tumor types are carcinomas of the breast for women and carcinomas of the prostate for men representing almost one third of cancer cases. The remaining malignancies arise from non-epithelial tissue. Sarcomas originate in mesenchymal cell types and are derived from connective tissue e.g. bone, fat, connective tissue and muscle. Sarcomas make up less then 1% of all clinically diagnosed cases of human malignant tumors [3]. However, the group is highly heterogeneous and consists of numerous histological subtypes making diagnosis and treatment challenging. Cancer may also form from the blood forming hematopoietic tissues. Leukemias are the malignant growth of non-pigmented blood cells that move freely in the circulation. Lymphomas are tumors of the lymphoid lineages that aggregate and form solid tumor masses most commonly found

in lymph nodes. Finally, the cells from the central or peripheral nervous system form neuroectodermal tumors and astrocytomas.

Cancer treatment in its current form is heavily based on surgery, radiation and chemotherapy as well as hormone and immune therapy to a lesser extent. Advances in the design of targeted therapies have had great impact on the treatment of a few specific malignancies. Examples of targeted anti-tumor drugs used in the clinic are Iressa and Gleevec that target over expressed or constitutively activated tyrosine kinases [4].

1.1.1 Genetic aberrations in cancer

When Theodor Boveri published his original discovery of chromosome aberrations in tumor cells in 1914, it was the start of a long relay of research that have engaged a rapidly increasing number of scientists approaching the cancer problem from the genetic viewpoint [5]. With the development of molecular technology the consequences of the chromosome rearrangements discovered by Boveri and his followers has been revealed in detail for many tumor types. This research first led to the discovery and definition of oncogenes [6, 7]. Cellular oncogenes encode proteins that have important key functions in the regulation of many basic functions of cells. Mutations causing oncogenic activation change, or increase the biological activities of the resulting oncoproteins [8]. The oncogenes are thus considered as dominant acting genes since only one of the two gene copies in each cell has to be activated by mutation. The hundreds of oncogenes described today can be divided into groups based on their biological activities. Oncogenes are represented at all levels of cellular regulation for example growth factors, their receptors, cell-cell contact, signal transduction, transcription and RNA processing factors, chromatin modifiers and regulators of apoptosis. Continued research showed that in most tumor types, one activated oncogene was not enough for full malignant transformation but several activated oncogenes were required. Furthermore, experiments with fusion hybrid cells between normal and tumor cells of different types indicated the existence of genes that suppressed the tumor phenotype even when oncogenes were active in the cells [9, 10]. A number of tumor suppressor genes have subsequently been identified. In contrast to the dominantly acting oncogenes, the activity of tumor suppressor genes has to be eliminated and thus, in most cases both copies of a tumor suppressor gene have to be eliminated for a phenotypic change. A few dominant mutations have, however been reported where a mutated protein block the activity also of the remaining normal allele. Some tumor suppressor genes have been reported to be silenced in tumor cells by epigenetic mechanisms rather than regular DNA mutations. Tumor

suppressor genes are commonly classified into gatekeepers and caretakers, where gatekeepers, such as the RB gene, have functions that regulate cell proliferation and differentiation whereas caretakers such as TP53 maintain the integrity of the genome [8].

1.1.2 Cancer hallmarks

Carcinogenesis is a multistep process that initiates in a single normal cell and transforms its progeny into malignant counterparts by sequential genetic changes [2, 11, 12]. It has been proposed that 8 major alterations in cell physiology are required for development of a fully transformed malignant tumor [8, 13]. Thus, despite cancer being a group of genetically heterogeneous disorders there seems to be a common set of anti-cancer defense systems that need to be circumvented for a cell to obtain a fully malignant phenotype. The 8 characteristics suggested being essential for malignant human tumors, or the hallmarks of cancer, are as follows:

- Sustaining proliferative signaling
- Evading growth suppressors
- Activating invasion and metastasis
- Enabling replicative immortality
- Inducing angiogenesis
- Resisting cell death
- Avoiding immune destruction
- Deregulating cellular energetics

The mechanism, order and time scale of how these characteristics are acquired varies between different tumor types.

1.2 Fusion oncogenes

Recurrent balanced chromosomal rearrangements have been identified in almost every tumor type [14]. Chromosomal translocations leading to the formation of fusion genes are the most commonly encountered balanced rearrangements. Approximately 700 different fusion oncogenes are currently reported in human neoplasms [15]. Fusion genes are considered to be primary abnormalities and tumor initiating factors.

Balanced chromosome rearrangements may be formed by two alternative mechanisms and result in one of two types of cancer promoting gene fusions [16]. The first mechanism involves break points located upstream of coding regions and the fusion of an intact protein coding region with the regulatory

sequences of another gene. This process, sometimes referred to as promoter swapping, results in the constitutive overexpression of a normally strictly regulated tumor promoting 3' partner gene and is common in hematological malignancies. For example, in Burkitt lymphoma chromosomal translocations place the *MYC* gene on chromosome 8 under the control of a promoter region normally driving the expression of one of three immunoglobulin (Ig) chains [17, 18]. The regulatory elements of the Ig genes cause constitutive expression of the MYC protein promoting proliferation and development of lymphoma. The second mechanism involves fusion of break points located within the protein coding regions of two genes resulting in a hybrid gene and the production of a chimeric fusion protein. The classic example of a translocation producing a hybrid gene is the t(9;22)(q34;q11) that manifests as the Philadelphia chromosome, a cytogenetic hallmark of chronic myeloid leukemia (CML) and other leukemias [14]. The translocation causes the fusion of coding regions from the *BRC* and *ABL1* genes and the production of the BCR-ABL1 fusion protein [19, 20]. ABL1 is a tyrosine kinase that in the context of the fusion protein is hyper active and continuously triggers down stream signaling pathways and promote leukemia. The expression of both types of fusion genes is mainly regulated by the promoter region contributed by the 5' partner. In addition, the 5' and 3' untranslated regions (UTRs) that may regulate stability and translation of mRNA are combined in an abnormal way.

There are several observations suggesting that translocation derived fusion genes are important factors for initiation and maintenance of tumor development:

- Fusion genes generally show a high degree of tumor type specificity and are frequently associated with only on type of malignancy suggesting that their products interact with distinct differentiation controlling gene programs and cell types.
- In some tumor types fusion genes are frequently found to be the only cytogenetic change in tumors and most probably represent primary abnormalities and tumor initiating factors.
- Effective treatment of malignancies carrying balanced translocations are in some tumor types associated with a decrease in the fusion gene product.
- Many fusion genes have been shown to transform cells in culture and induce tumors of the same type that is observed in human cases in transgenic mice.

- Experimental depletion of fusion gene products *in vitro* cause tumor cell death, reduced tumorigenicity, decreased proliferation and/or differentiation.

The above findings have established fusion genes as key players in the development of human malignancies and enabled their use as important diagnostic and prognostic markers.

1.2.1 The FET group of fusion oncogenes

The FET group of fusion oncogenes is found primarily in sarcomas and leukemias were they are considered primary tumor initiating and driving factors. By definition, all fusion genes of this group contain 5' parts derived from one of the FET family genes *FUS*, *EWSR1* or *TAF15* (Section 1.2.3). The FET gene fragments are juxtaposed to one of several alternative transcription factor-encoding genes (Figure 1). The fusion genes show a high degree of tumor type specificity and in many cases they are the only genetic abnormality detectable by cytogenetic analysis [3]. The fusion gene products invariably consist of an N-terminal domain (NTD) derived from one of the FET proteins fused with the DNA binding domain from the transcription factor partner. The NTD of the FET proteins and the transcription factor domain are both required for full transforming activity of FET fusion proteins in cultured cells and mouse models [21, 22]. However, in some tumor types the 5' parts of the FET family genes are exchangeable and included in variant fusion genes indicating a similar function of the FET NTDs in tumorigenesis (Figure 1). The functional overlap of FET NTDs in cell transformation has been further demonstrated in experimental assays [21]. The FET NTDs share a high degree of sequence homology and are mainly composed of a degenerated repetitive sequence enriched for SYGQ. The domains probably have an intrinsically disordered structure and have been reported to form protein-protein interactions and mediate transactivation [21, 23-26]. In summary, the FET fusion proteins are considered to be abnormal transcription factors that cause aberrant regulation of downstream genes [27].

1.2.2 FUS-DDIT3

The t(12;16)(q13;p11) chromosome translocation is highly specific for myxoid liposarcoma [28-30] and involves an in-frame fusion of the 5' parts of *FUS/TLS* with the entire coding region of *DDIT3/CHOP* [31-33]. The resultant *FUS-DDIT3* (*TLS-CHOP*) fusion gene encodes a chimeric protein product were the central and C-terminal RRM and RGG domains of FUS are lost and replaced by the full-length DDIT3 protein (Figure 2). FUS-DDIT3 is a nuclear protein that is considered an abnormal transcription factor

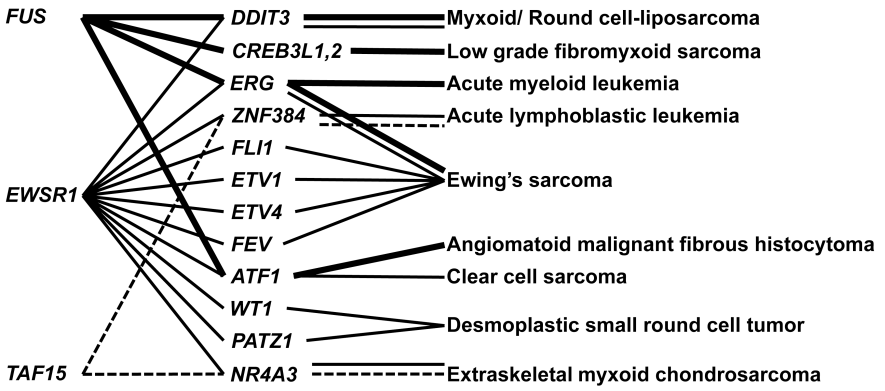

Figure 1. ***The FET group of fusion oncogenes.*** *The 5' parts of the FET family genes FUS, EWSR1 and TAF15 are fused to genes encoding various transcription factors (middle column). The respective fusion genes are found in the tumor types indicated in the right column. Observe the high degree of tumor type specificity for each FET fusion gene. Additional fusion genes involving the FET family genes exist.*

with the FUS NTD constituting a strong transcriptional activation domain fused to the dimerizing and DNA binding elements of DDIT3 [26]. The DDIT3 part retains its capacity to form dimers with other C/EBP family proteins but FUS-DDIT3 has distinct functions in comparison to wildtype DDIT3 and does not induce growth arrest [34]. Several potential target genes of FUS-DDIT3 have been identified by use of *in vitro* and *in vivo* systems [35-38]. In addition, FUS-DDIT3 interacts with splicing factors and inhibits alternative splicing [39].

Identifying the primary cell type(s) that support the expression of FUS-DDIT3 (and related FET fusion proteins) is important to understand their tumorigenic effects. Few permissive cell type(s) that support FUS-DDIT3 expression and transformation has been identified. FUS-DDIT3 has the capacity to transform murine fibroblasts and pre-adipocytes *in vitro* and these cells also establish tumors in nude mice when expressing the fusion protein [21, 40]. Transformation requires the dimerization and DNA binding domains of DDIT3 and the FUS NTD (or alternatively the EWS NTD). Transformation of primary cells by FUS-DDIT3 has been demonstrated in mouse mesenchymal progenitor cells (MPCs) that form MLS like tumors when introduced in SCID mice [38]. The causal role of FUS-DDIT3 in MLS development has been further demonstrated in transgenic mice [41]. Despite ubiquitous expression of FUS-DDIT3 in all tissues these mice only developed MLS like tumors confined to adipose tissue [41]. Transgenes with stable DDIT3 expression did not develop tumors, establishing that the NTD

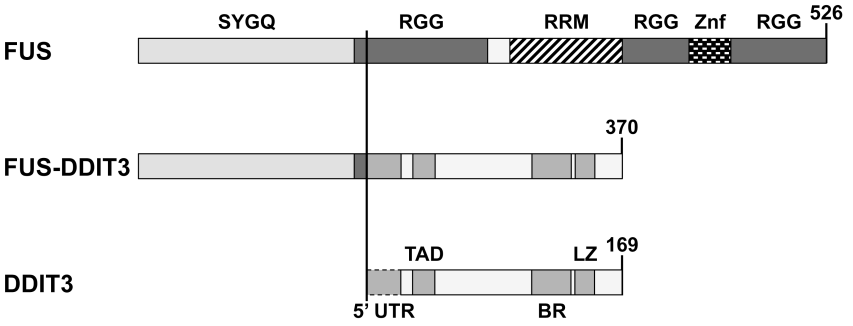

Figure 2. ***The FUS-DDIT3 fusion oncoprotein.*** *The MLS associated t(12;16)(q13;p11) cause the in frame fusion of FUS with DDIT3 and the expression of the FUS-DDIT3 fusion oncoprotein. Several FUS-DDIT3 (and EWSR1-DDIT3) transcripts have been identified due to different break points in the wildtype genes and alternative splicing [42-44]. However, no difference in transforming capabilities in vitro or in clinical outcome has been observed for variant FUS-DDIT3 fusion proteins [37, 42, 45]. The type II FUS-DDIT3 protein (shown above) is produced by the fusion of exons 1-5 of FUS with exons 2-4 of DDIT3 and found in two thirds of MLS cases [3, 43]. The fusion protein contains 175 residues of the intrinsically disordered FUS NTD fused with the entire DDIT3 protein. In addition, a varying number of amino acids encoded by the normally untranslated region (5' UTR) of DDIT3 exons 2 and 3 are included in the different types of FUS-DDIT3. Abbreviations for the regions of FUS are SYGQ: serine-tyrosine-glycine-glutamine rich; RGG: arginine-glycine-glycine repeat; RRM: RNA recognition motif; Znf: zinc finger. Abbreviations for DDIT3 are UTR: untranslated region; TAD: transcriptional activation domain; BR: basic region; LZ: leucine zipper. Protein regions were defined by UniProt-KB and drawn to scale.*

of FUS is required for the transformation both *in vitro* and *in vivo* [22]. A second transgenic model of MLS have been established were FUS-DDIT3 expression is driven by a mesodermally restricted promoter, however, this model require inactivation of p53 [46]. It should be further noted that no experimental setting has been reported that enable FUS-DDIT3 mediated transformation of human cells.

FUS-DDIT3 inhibit the development and terminal differentiation of murine adipocytes by dimerization with C/EBPβ and cancelling the induction of downstream transcription factors e.g. C/EBPα [40, 47]. This results in a cell population of partially committed pre-adipocytes that may progress to MLS. Transgenic mice have further demonstrated that FUS-DDIT3 blocks adipogenesis and that repression of PPARγ is a critical step for tumor formation in this setting [48, 49]. However, in addition to blocking adipogenesis FUS-DDIT3 also promote an adipocyte like phenotype when

expressed in unrelated primitive sarcoma cells [50]. MLS may thereby develop from cell types distinct from pre-adipocytes, which may explain the preference of human MLS for non-adipose tissue.

In summary, the ability of FUS-DDIT3 to promote liposarcoma development has so far been associated with deregulated transcriptional control of gene expression and interference with adipocytic differentiation.

1.2.3 FUS, EWS and TAF15

The FUS/TLS, EWS and TAF15/TAF_{II}68/TAF2N/RBP56 proteins are members of the FET protein family. The genes encoding FUS/TLS (fused/translocated in sarcoma) and EWS (*EWSR1;* Ewing sarcoma breakpoint region 1) were identified by their involvement in chromosomal translocations characteristic of myxoid liposarcoma and Ewing sarcoma respectively [31, 32, 51]. TAF15 (TBP associated factor 15) was later discovered as a subunit of transcription factor IID (TFIID) and by homology with *FUS* and *EWSR1* [52-54]. *TAF15* undergoes translocation in extra skeletal myxoid chondrosarcoma [55-57], establishing that all human FET family members are proto-oncogenes. FUS, EWS and TAF15 are abundantly expressed in nearly all human fetal and adult tissues examined [54, 58-61]. In agreement, the 5' flanking sequences of FET genes have features associated with housekeeping genes, e.g. the presence of CpG islands and the lack of TATA boxes [59-62].

The FET family proteins are grouped by extensive sequence homology and analogous domain architecture (Figure 3) and likely originate from the same ancestor gene [61, 63]. The proteins contain an N-terminal domain (NTD) with a degenerate repeat sequence rich in serine-tyrosine-glycine-glutamine (SYGQ), a central RNA recognition motif (RRM), a C-terminal RanBP2 type zinc finger and several regions with repeats of arginine-glycine-glycine (RGG) [63, 64]. The NTDs of FET proteins have been reported to function as strong transcriptional activation domains [23, 24, 26] in related oncogenic fusion proteins and experimental systems. However, the function of the NTD in the context of wildtype FET proteins is poorly defined. The RRM is the most conserved domain within the FET family [63] and a common feature of RNA binding proteins [65]. RNA recognition motifs are approximately 90 amino acids in size and form a four-stranded anti-parallel β-sheet with two closely associated α-helices [65]. The central strands of the β-sheet harbors two conserved motifs termed ribonucleoprotein domain 1 and 2 (RNP1 and RNP2) that in conjunction with other structural elements of the RRM defines the sequence specificity and affinity of RNA binding [65]. The RRM of FET

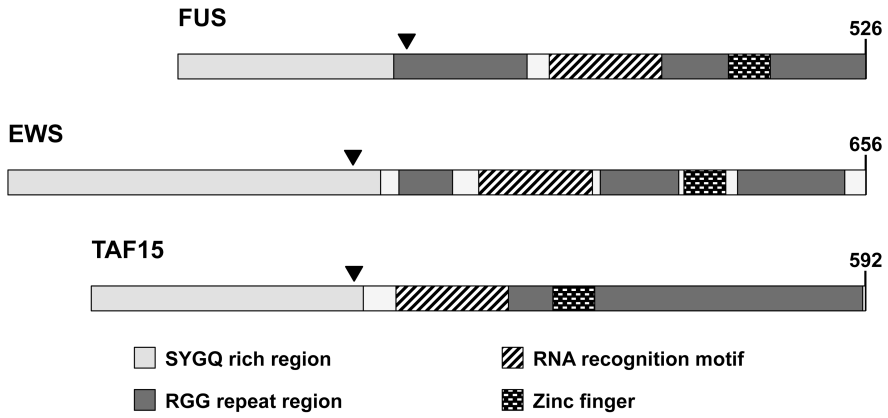

Figure 3. ***The FET family proteins.*** *Schematic illustration of FUS, EWS and TAF15 domain structure defined by UniProtKB/Swiss-Prot [64] (P35637, Q01844, Q92804) and Tan and Manley 2009 [66]. Numbers indicate amino acid residues. Arrowheads indicate frequent break points in fusion oncoproteins and truncation points of recombinant proteins used for protein-protein interaction studies in paper II-IV. Protein regions are drawn to scale.*

proteins have characteristic features with atypical amino acid substitutions at conserved residue positions in RNP1 and an extended loop structure between α-helix 1 and β-strand 2 [31, 52]. Following their identification, the FET proteins were shown to bind RNA *in vitro* [31, 52, 67, 68] and FUS was later confirmed to interact with RNA *in vivo* [69]. The mechanism of RNA recognition and binding is poorly defined but involve both the RRM and RGG domains [69, 70]. FET proteins preferentially bind RNA with homopolymeric regions of G or U and a GGUG binding motif has been reported for FUS [52, 67, 68, 70]. RNA recognition may also involve structural features as FET proteins bind G-quadruplex structures and AU rich stem loops [71-73].

FET proteins have been implicated in several aspects of gene regulation and may couple transcription with subsequent processing and transport of RNA products [63]. The FET proteins are implicated in transcriptional control by interaction with TFIID and RNAPII [52, 74]. TFIID consists of TATA binding protein (TBP) and various TBP associated factors (TAFs) that bind the TATA box in gene promoters followed by RNAPII recruitment. The FET proteins associate with different TFIID complexes and may promote specific functions. Furthermore, the FET proteins bind subunits of RNAPII [52, 74, 75] and TAF15 is incorporated in the RNAPII preinitiation complex *in vitro* [52]. FET proteins also interact with several specific activators and

repressors. FUS has been reported to interact with nuclear hormone receptors [76] and to act as a co-activator of the NF-κβ family member p65 [77]. FUS and EWS interact with the POU domain factors Brn-3a and Oct4 and modulate their capacity for transactivation [78-80]. Furthermore, all FET proteins interact with SF1 [81] and Runx transcription factors [82]. The FET proteins subsequently influence gene promoter activity by interacting with the general co-activators p300 and CBP. EWS mediated transcriptional activation is in some cases dependent on interaction with p300 or CBP [83, 84] and FUS down-regulates *CCND1* expression by inhibiting p300 and CBP histone acetyltransferase activity [85]. The recruitment of FET proteins to endogenous regulatory sequences may involve protein-protein interactions (as the above mentioned). However, FUS has also been reported to bind specific ssDNA response elements in promoter regions [86] and is recruited to the *CCND1* promoter by a novel mechanism involving ncRNAs [85]. In addition to the involvement in RNAPII mediated transcription FUS has been reported to repress transcription by RNA polymerase III (RNAPIII) that transcribes small structural and catalytic RNAs [87]. The FET proteins may thus control production of several functional classes of RNA.

The FET family proteins are further implicated in RNA processing and transport. FET proteins are found in complexes of RNA and heterogeneous nuclear ribonucleoproteins (hnRNPs) that assemble on pre-mRNAs during transcription [21, 88-90] and FUS and EWS are subunits of spliceosomes assembled on pre-mRNAs *in vitro* [89, 91]. The FET proteins have been reported to interact with various splicing factors and to modulate alternative splicing of pre-mRNAs. [92-95]. Furthermore, FUS and EWS bind SR splicing factors and YB-1 by their C-terminal domain (CTD) while their NTD interacts with RNAPII. By this mechanism FET proteins may function to connect splicing factors to the transcription machinery [75, 96-98]. Recent studies analyzing the interaction of FET proteins with endogenous RNAs have revealed a large repertoire of bound transcripts and preferential interaction with splice sites [73, 99, 100]. In addition to pre-mRNA splicing, FET proteins have been implicated in miRNA processing by their interaction with the Drosha complex [101, 102]. The FET proteins are involved in RNA transport over the nuclear barrier and delivery of mRNA to various cellular compartments supporting local translation [69, 103-105].

FET proteins have been implicated in maintenance of genomic integrity, DNA damage repair and recombination as they promote homologous DNA pairing and DNA D-loop formation *in vitro* [106-108]. In agreement, *FUS* and *EWSR1* knockout mice display genomic instability, decreased meiotic recombination, defective B-lymphocyte development and enhanced

sensitivity to ionizing radiation. Furthermore, FUS and EWS are both phosphorylated in response to DNA damage [109, 110] and EWS controls alternative splicing of genes involved in DNA repair and genotoxic stress signaling [111]. FUS activity in transcriptional regulation is controlled by ncRNAs induced by DNA damage [85].

In addition to their role as proto-oncogenes *FUS* and *TAF15* are mutated in amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases [112-115]. In the case of FUS, several mutations are clustered in the proximal CTD and interfere with transportin mediated nuclear import leading to increased levels and aggregation of FUS in the cytosol [116].

In summary, the FET proteins are ubiquitously expressed factors involved in several aspects of gene regulation and the maintenance of genomic integrity. The development of malignancies and neurodegenerative disorders underscore the importance of intact FET protein function.

1.2.4 DDIT3

DDIT3 (DNA-damage-inducible transcript 3) also known as *GADD153* (growth arrest and DNA damage-inducible protein GADD153) or *CHOP* (C/EBP-homologous protein) is a key factor in cellular stress response. *DDIT3* was identified by its transcriptional induction upon growth arrest and DNA damage and later shown to be regulated by oxidative stress, endoplasmatic reticulum (ER) stress, hypoxia, and nutrient deprivation [117-121]. In response to stress conditions *DDIT3* expression is up regulated at the level of transcription, mRNA stability and translation [121-124].

The DDIT3 protein (Figure 2) is a basic leucine zipper (bZIP) transcription factor belonging to the CCAAT-enhancer-binding protein (C/EBP) family. C/EBP proteins bind DNA as homo- or hetero-dimers formed with C/EBP family members or other bZIP factors and regulate target genes involved in several cellular functions e.g. proliferation, differentiation and metabolism [125]**.** DDIT3 deviates from other C/EBP proteins as it is unable to form homodimers and that two proline residues disrupt its DNA binding basic region. DDIT3 function as a dominant-negative factor by binding other C/EBP factors and forming heterodimers that are unable to bind consensus C/EBP sites in gene promoters [126]. In line with this, the majority of DDIT3 regulated target genes are repressed [127]. However, under stress conditions DDIT3-C/EBP heterodimers can bind a different DNA sequence in promoter regions and activate target genes [128]. The transactivation activity of DDIT3 is also enhanced in response to stress by serine phosphorylation [129, 130].

DDIT3 can also regulate transcription by forming dimers with other bZIP containing proteins or interact with other types of transcription factors by an intrinsically disordered N-terminal region [131-134].

Expression of DDIT3 promotes induction of cell-cycle arrest at the G1/S transition and ER stress associated apoptosis [34, 135]. DDIT3 has been implicated in the stress responses leading to apoptosis of insulin producing β-cells in mouse models of diabetes and in neurodegenerative disorders [121, 136, 137]. Furthermore, DDIT3 is involved in cell differentiation and inhibit adipogenesis and osteoblast differentiation [138, 139]. Finally, DDIT3 is also a proto-oncogene by its involvement in chromosomal translocations and gene amplifications in liposarcoma [31, 140, 141].

1.3 Myxoid liposarcoma

Myxoid liposarcoma (MLS) is the second most common subtype of liposarcoma and represent approximately 10% of all adult soft tissue sarcomas. The highest incidence of MLS is between 40-60 years of age with disease presentation approximately a decade earlier then other liposarcoma subtypes. Furthermore, MLS is the most common liposarcoma subtype in patients younger than 20 years. Primary MLS tumors occur predominantly in deep soft tissues of the extremities and the majority of cases arise in the musculature of the thigh. The tumor mass is composed of uniform round to oval shaped primitive non-lipogenic mesenchymal cells and small signet-ring lipoblasts. The tumor also contains a prominent myxoid stroma and a characteristic plexiform capillary network [3, 50]. In addition to the histological criteria, MLS is defined by presence of the t(12;16)(q13;p11) in 90% of cases or the rare t(12;22)(q13;q12) leading to gene fusion of *FUS* or *EWSR1* with *DDIT3*. Additional cytogenetic abnormalities are rarely observed in MLS.

MLS has a high tendency to recur locally and one third of patients develop distant metastases. Compared to sarcomas with similar histology MLS metastases presents mainly in unusual soft tissue locations (e.g. retroperitoneum) or bone (e.g. spine) and seem to settle in lung with low efficiency. A significant number of patients presents with multifocal disease, however, tumors at different sites are of monoclonal origin as demonstrated by analysis of *FUS-DDIT3* or *EWSR1-DDIT3* fusion genes [3]. A subset of MLS tumors has areas with primitive round cell morphology that lack intervening myxoid stroma and is associated with metastasis and significantly poorer prognosis. A gradual transition from myxoid to round cell morphology is commonly observed and the different compartments harbor

the same recurrent chromosomal translocations. High histological grade (generally defined as ≥5% round cell areas), multifocal disease, presence of necrosis, and TP53 overexpression are predictors of unfavorable outcome in MLS [3, 45, 142]. Recently, activation of the PI3K/Akt pathway in MLS by *PIK3CA* mutation or alternative mechanisms has been associated with an increase in round cell morphology and poor prognosis [143, 144].

Fine needle biopsies are generally used for histological diagnosis that may be further aided by detection of *FUS-DDIT3* or *EWSR1-DDIT3* fusion genes or transcripts with FISH or RT-PCR respectively [42, 44, 45, 145]. Treatment of MLS relies mainly on surgery sometimes combined with radiation while chemotherapy is used for advanced cases [146, 147]. The possible benefit of treating MLS with PPARγ agonists and the DNA binding alkaloid trabectedin have been investigated [46, 148-151].

2 AIMS

The overall objective of this thesis was to functionally characterize the MLS-associated FUS-DDIT3 fusion oncoprotein and the normal FUS, EWS, TAF15 and DDIT3 counterparts.

The specific aims included to (1) define the molecular function of the FUS, EWS and TAF15 N-terminal domains with focus on their involvement in protein-protein interactions; (2) characterize the target gene repertoire and cellular function of the FUS and EWS fusion partner DDIT3.

The main aims of paper I-IV were as follows:

Paper I – To identify target genes and cellular functions of the DDIT3 transcription factor and define the significance of DDIT3 subcellular localization.

Paper II – To study the functional role of an identified protein-protein interaction involving FUS and the cytolinker protein plectin.

Paper III – To characterize an interaction of the FUS N-terminal domain with the full-length FET family proteins and its significance for FUS-DDIT3 function.

Paper IV – To study the interaction of FUS-DDIT3 and FUS with the Brg1 and ARID1A subunits of the hSWI/SNF chromatin-remodeling complex.

3 MATERIAL AND METHODS

The myxoid liposarcoma derived cell lines 402-91, 1765-92 and 2645-94 [33, 35] as well as HT1080 fibrosarcoma cells and genetically modified subclones of this cell line [50, 127, 152, 153] were used for molecular analyses. In addition, human F470 fibroblasts [154], HEK293 and HeLa cells were utilized. The Raji lymphoma cell line was used for large-scale protein extractions utilized in GST pull-down experiments. The liposarcoma derived GOT3 cell line [140] was used for studies of DDIT3 (paper I). Primary fibroblasts from wildtype and plectin knockout mice [155] were used for protein and RNA analyses (paper II).

The results presented in this thesis are based on multiple molecular and cell biological methods including: cell culture and experimentation, cloning of EGFP/RFP/GST fusion constructs, recombinant protein expression and affinity chromatography, GST pull-down, cell fractionation, co-immunoprecipitation, RNA interference, microarray, real-time quantitative PCR, SDS-PAGE and immunoblot analysis, flow cytometry, liquid chromatography-mass spectrometry, cell transfection, immunofluorescence, co-localization, *in situ* proximity ligation assays, fluorescence microscopy, laser scanning confocal microscopy and protein sequence analysis.

The various methods used are described in detail in papers I-IV or in the included references and will not be further explained here.

4 RESULTS AND DISCUSSION

4.1 Paper I

Subcellular localization and gene regulatory function of DDIT3

DDIT3, also known as *GADD153* or *CHOP*, encodes a basic leucine zipper transcription factor of the dimer forming C/EBP family. DDIT3 is known as a key regulator of cellular stress response, but its target genes and functions are not well characterized. DDIT3 was initially described as a nuclear transcription factor, but later reports suggest that DDIT3 can be expressed in the cytoplasm [156, 157]. Here we show that GOT3 sarcoma cells and normal human fibroblasts accumulate cytoplasmic DDIT3 under tunicamycin and etoposide induced stress conditions. Many studies reporting nuclear localization of DDIT3 are made with cells transfected or transduced with DDIT3 expression plasmids or virus vectors. Transfection of fibroblasts and GOT3 cells with a DDIT3-EGFP expression vector also showed nuclear expression of the recombinant protein, indicating that these cells are capable of nuclear DDIT3 localization. With these results we hypothesized that transfection-induced stress could result in a nuclear DDIT3 expression. However, transient transfections of GOT3 cells with a GFP expression plasmid caused no change in the localization of endogenous DDIT3. We conclude that ER stress and genotoxic stress caused cytoplasmic accumulation of DDIT3 in the human cell types analyzed in this study.
The observation that DDIT3 can be expressed both in the cytoplasm and the nuclei has to be accounted for when biological functions of this protein is studied. Here, we applied a genome wide microarray based expression analysis to identify DDIT3 target genes and functions. By analyzing cells carrying tamoxifen inducible DDIT3 expression constructs (DDIT3morEGFP) we show distinct gene expression profiles for cells with cytoplasmic and nuclear localized DDIT3.

Analysis of microarray data from cells expressing cytoplasmic DDIT3morEGFP revealed 94 genes that were regulated at least 3-fold compared to their expression in morEGFP expressing cells. Genes regulated by cytoplasmic DDIT3 were obviously not direct targets. We speculate that cytoplasmic DDIT3 could bind and sequester other BZIP transcription factors and prevent their nuclear localization. Of the 94 genes, 33 genes were upregulated and 61 downregulated. 32 genes were selected and validated using reverse transcription quantitative real-time PCR analysis. Ontology

analysis of identified target genes showed that the functional categories *cellular movement, cell death, cellular development,* and *cellular growth and proliferation* were the most significantly affected. Twenty of 94 regulated genes for cytoplasmic DDIT3 were annotated to the functional category *cellular movement*. To experimentally address the hypothesis that DDIT3 negatively regulates migration, we used a modified scratch wound migration assay. Migration was inhibited with 48% ($P < 0.01$) for DDIT3morEGFP expressing HT1080 cells compared to morEGFP expressing cells. DDIT3 regulation of several migration/movement-associated genes may provide mechanistic explanations for the impaired migration. For example, *DSTN*, which encodes an actin depolymerizing protein, was downregulated in DDIT3 expressing cells.

After 2 hours, 45 genes were regulated. Eight hours after DDIT3 translocation, several initially regulated genes were back to their initial expression levels, but replaced by other response genes. In total 52 genes were regulated 8 hours after DDIT3 translocation. Functional analysis among the 2 hours response genes for nuclear DDIT3 showed the top categories *cell death*, *cellular development*, *cellular growth and proliferation* and *cell cycle*. *Cell death*, *gene expression*, *cellular development*, and *cell cycle* were the most significant categories enriched among the 8 hours response genes of nuclear DDIT3. Our results and ontogeny analysis suggest that nuclear DDIT3 controls cell growth. Forced expression of DDIT3 was reported to induce a G1 cell cycle arrest [158] and our experimental system recapitulated this effect when the protein was translocated to the nuclei. Several of the regulated genes reported here may execute the growth arrest but further investigations are needed to dissect the mechanism. In addition to the 94 genes regulated by cytoplasmic DDIT3, 84 more genes were regulated 2-fold or more by nuclear translocation of DDIT3. Moreover, these genes were in the same functional categories as the first 94 genes regulated by cytoplasmic DDIT3. This shows that nuclear translocation steps up and modulates functions already affected by cytoplasmic DDIT3.

In summary, we show that DDIT3 may be expressed both as a cytoplasmic and nuclear protein. Further, we show that cells expressing cytoplasmic and nuclear DDIT3 have distinct gene expression profiles, but that the target genes belong to the same functional categories.

4.2 Paper II

Plectin is required for FUS subcellular localization and function

In this paper we screened for protein-protein interactions involving the FUS NTD. The cytolinker plectin was isolated from a cell extract by its affinity for the FUS NTD in a GST pull-down assay and identified by mass spectrometry (MS) and immunoblot (IB). The N-terminal of EWS and TAF15 also bound plectin showing that the interaction is conserved for all human FET family proteins, at least under *in vitro* conditions.

Plectin is a high-molecular weight protein belonging to the Plakin family that control cytoskeleton organization and mechanical properties of cells [159-161]. Plectins involvement in cellular structure is reflected in interactions with actin, microtubule, intermediate filaments and cellular junctions [160-162]. Furthermore, Plectin mutations are associated with Epidermolysis bullosa (EB) were the structural integrity of the skin and the neuromuscular system is compromised [163]. Plectin also acts as a scaffold for proteins involved in signaling pathways suggesting that it also supports functions not directly related to cellular structure [164-166]. Furthermore, plectin is located at focal adhesion complexes and interacts with RACK1 [160, 167, 168], observations that are shared with the FET family proteins [58, 104].

In support of the results from pull-down we observed a partial co-localization of GFP tagged FUS and immunolabeled plectin in the cytoplasm of fixed HT1080 fibrosarcoma cells. The degree of co-localization was modest and we further analyzed fixed HT1080 cells by an *in situ* proximity ligation assay (PLA) with FUS and plectin specific antibodies. Quantification of PLA signals subsequently demonstrated complex formation of FUS and plectin in the cell cytoplasm.

With regard to plectin function we hypothesized that the FUS-plectin interaction was important for the stability, localization and/or function of FUS in the cytoplasm. To address the functional importance of the FUS-plectin interaction we compared FUS expression levels and subcellular localization in wildtype and plectin knockout mouse fibroblasts [155]. Analysis of FUS by immunofluorescence did not show any clear visual difference between wildtype and knockout cells. However, subcellular fractionation experiments revealed that the cytoplasmic to nuclear ratio of FUS was higher in cells lacking plectin. The total expression level of FUS protein was unaffected suggesting that the subcellular distribution FUS was

altered. FUS has been reported to shuttle between the nucleus and cytoplasm and participate in cellular transport of mRNA [69, 103-105]. The fact that FUS localization was altered in the absence of plectin prompted us to investigate FUS mediated gene regulation. We analyzed the expression levels of *Ash1l*, *Med12* and *Shock2* transcripts, which have been reported to interact with FUS [103], and found all to be up regulated in plectin knockout cells. The specific accumulation of FUS bound transcripts in plectin deficient cells may be caused by feedback regulation when transport of mRNA molecules is compromised. Alternatively, the change in FUS subcellular distribution may cause deregulation of FUS transcriptional activity in the nucleus. FUS and the other FET family proteins have been reported to associate with a substantial repertoire of transcripts [73], therefore, our observed deregulation of gene expression in plectin deficient cells probably extend beyond the transcripts evaluated in our study. Furthermore, several plectin isoforms exist that have different subcellular localization [169] and may recruit FET family proteins and associated mRNAs to various sites depending on the requirements of different cell types.

Mutations in *FUS* are causative of the neurodegenerative disorders ALS and FTLD [112-114] and linked to altered FUS nucleo-cytoplasmic transport, accumulation of FUS in the cytoplasm and subsequent formation of FUS immunoreactive inclusions that may constitute abnormal stress granules (SGs) [112, 113, 116, 170, 171]. Plectins involvement with the cytoskeleton and that stress granule assembly involves the microtubule network [172, 173] prompted us to investigate plectins role in the formation of SGs and the recruitment of FUS to SGs. However, SGs formed under oxidative stress conditions were not labeled by plectin specific antibodies in fixed cells. In addition, the number and morphology of SGs as well as the recruitment of FUS to SGs was unaffected in plectin knockout cells.

In summary, our results indicate that plectin functions as a scaffold that facilitate transport and targeting of FUS and associated mRNAs. The significance of the FUS-plectin interaction in relation to pathologies caused by plectin and FUS mutation warrant further investigation.

4.3 Paper III

The NTD of FUS, EWS and TAF15 bind full-length FET proteins

The FET group of fusion oncogenes found in human sarcomas and leukemias encode fusion oncoproteins that are considered as primary tumor initiating and driving factors. Their fusion protein products invariably consists of an N-terminal domain (NTD) from one of the FET family proteins FUS, EWS or TAF15, juxtaposed to the DNA binding domains from one of more than ten alternative transcription factor partners. The fusion oncoproteins act as abnormal transcription factors that cause aberrant regulation of target genes. The roles of the FET NTDs are not clear and structure modelling suggests that their structures are intrinsically disordered indicating possible functions as mediators of protein-protein interactions [174].

To search for proteins that bind FET NTDs, bacterially expressed GST tagged FUS NTD (residues 1-175) EWS NTD (residues 1-264) and TAF15 NTD (residues 1-201) were used as baits to immobilize proteins from Raji cell extracts. SDS-PAGE analysis revealed three prominent protein bands at 77, 68 and 61kDa specifically retained by all FET NTDs. Mass spectrometry analysis revealed That these band represented full length EWS (p77), TAF15 (p68) and FUS (p61). Immunoblot analysis confirmed the protein identities and their specific enrichment. Testing the binding quality with increasing salt concentrations showed a robust binding up to 1 molar NaCl. Co-immunoprecipitation studies confirm that the three FET NTDs are capable of binding all full length FET proteins both in homo or hetero complexes and that at least one of the FET fusion oncoproteins forms complexes with all three FET proteins.

Recruitment and hetero complex formation of normal FET proteins to promoters targeted by oncogenic FET proteins may thus be an important part in deregulation of target genes. This model was tested by knock down experiments of FUS and oncogenic FUS-DDIT3 in myxoid liposarcoma cells. The normal FET proteins are known to bind and regulate thousands of mRNAs as well as other RNA classes. They interact with also with DNA and act at many regulatory steps of gene expression. This makes any attempt to probe their functions and effects on specific genes and RNAs problematic. Our results, however, suggest that interaction with the normal FUS protein is involved in FUS-DDIT3 deregulation of target genes.

To map binding structures in the NTD parts, we studied the binding of FET-proteins to truncated recombinant FUS NTD baits. The results show that the FET NTDs contain a novel FET Binding Motif, FETBM1 that is necessary for binding of the full-length FET family proteins. The FETBM1 appears to be conserved among mammalians and is also present in Zebra fish FET proteins. FETBM1 contains specifically placed tyrosine residues that have been reported to facilitate homotypic complex formation of FUS [175]. A detailed study of the sequence requirement of the transactivating and transforming activity of EWS NTD further pointed out the importance of the tyrosine residues and underscored the potential effects of their phosphorylation [24] The identification of FETBMI is also important as it provides a sequence and structure that can be targeted in screens for future drugs. Modulation of the FETBM1 binding may be of importance, not only for the FET oncogene caused malignancies but also for neurodegenerative diseases caused by aggregation of FET proteins.

The FETBMI1 parts are not capable of self-binding since recombinant proteins containing only the NTDs fail to bind immobilized NTD fragments. This observation prompted a search for target sequences/ structures in the full-length FET proteins. A pull down assay with the FUS NTD as a bait against recombinant truncation mutants of FUS showed minor decreases in binding as increasing C-terminal parts were deleted. Deletion of amino acids 176-284 resulted in a major loss of binding indicating that this part contains a target structure for FETBM1. Alignment experiments with this part of FUS against TAF15 and EWS failed to highlight any specific region or sequence segment that was common for the three FET proteins. Instead we speculate that several parts of the FET proteins that contain stretches rich in G and RGG repeats may form structures that bind FETBM1. This also implies that many other proteins containing the common RGG repeats may be capable of binding the FET NTDs. Further studies are needed to test this hypothesis.

We conclude that the FET proteins forms homo and hetero complexes and that a novel motif FETBM1 is necessary for the binding. The binding function is retained in the FET fusion oncoproteins and at least one of them binds all the normal FET proteins. This binding is most probably also important for the transcription factor activities of the FET fusion oncoproteins. The FETBM1 motif and binding to full-length FET proteins has also implications for the pathogenesis of some neurodegenerative diseases.

4.4 Paper IV

FUS and FUS-DDIT3 interact with the human SWI/SNF complex

The FET family proteins FUS, EWS and TAF15 [63, 176] are ubiquitously expressed pre-dominantly nuclear proteins involved in transcription and RNA processing [63, 176]. Each of the FET proteins contain an enigmatic N-terminal domain (NTD) that invariably is expressed in the FET fusion oncoproteins and required for their oncogenic activity [21, 22]. The aim of this work was to identify proteins binding to the FET NTDs allowing a further insight into the contribution of these domains to the oncogenic activities of the FET fusion oncogenes.

A bacterially expressed GST tagged construct containing FUS residues 1-175 bound several high-molecular weight proteins in pull down assays. Mass spectrometry analysis of excised protein bands revealed peptides derived from ARID1A (BAF250a), Brg1 (SMARCA4), Brm (SMARCA2), BAF170 (SMARCC2) and BAF155 (SMARCC1). The identity of ARID1A and Brg1 was further confirmed by immunoblot. ARID1A, Brg1, Brm, BAF170 and BAF155 are all components of the large SWI/SNF multi subunit complex that control gene expression by ATP driven repositioning of nucleosomes [177, 178]. SWI/SNF thus control the activity of multiple genes by modulation of chromatin structure that determines the access of numerous DNA binding factors. SWI/SNF complexes also provide a link between chromatin remodeling and tumor suppression [179]. Interaction with SWI/SNF is thus likely to be an important aspect of wild type FUS and FUS-DDIT3 activities in gene regulation.

SWI/SNF complexes display variable subunit composition but always contain one of two mutually exclusive ATPases, designated Brg1 and Brm, responsible for SWI/SNF catalytic activity [177, 178]. The protein expression levels of ARID1A, Brg1 and Brm in the myxoid liposarcoma cell lines 402-91, 1765-92 and 2645-94 were examined by immunoblot. ARID1A and Brg1 were expressed in all MLS cell lines tested. Brm was not detected or of low abundance in 402-91 and 2645-94 cells. The preferential expression of BRG1 containing SWI/SNF complexes is associated with and necessary for maintenance of stemness in stem cells [180, 181] and this configuration of SWI/SNF in MLS cell is compatible with the low differentiated features of these cells. The interaction of FUS-DDIT3 with the SWI/SNF complex may thus affect differentiation of the muscle resident mesenchymal stem cells that most likely are the cell of origin for MLS cases. FUS-DDIT3 has been shown

to induce a pre-adipocytic differentiation but also block terminal adipocyte differentiation. This effect may depend on interactions with the SWI/SNF complex. Indeed, Brg1 and ARID1A containing complexes have been linked with adipogenesis and myogenesis [182-185], differentiation programs that are highly relevant to development of MLS. Further analysis of the SWI/SNF composition in MLS cells are planned and may reveal important information concerning the origin and nature of MLS tumors.

Although numerous mutations or deletion of SWI/SNF subunits have been reported in cancers, oncogenic transformation does not result from complete inactivation of SWI/SNF but rather is driven by aberrant activity [179, 186-188]. It is thus possible that aberrant protein-protein interactions, as those formed with FUS-DDIT3, affect SWI/SNF activity and/or targeting and cause the gene expression pattern behind the transformed state.

The 402-91 and 2645-94 cell lines were analyzed by *in situ* proximity ligation assay (PLA) with combinations of FUS or DDIT3 specific antibodies together with antibodies for Brg1 or ARID1A. The formation of PLA signals identified nuclear complexes containing FUS and Brg1 or FUS and ARID1A as well as complexes of the FUS-DDIT3 fusion oncoprotein with Brg1 or ARID1A. These results confirm that FUS-DDIT3 binds SWI/SNF complexes or parts thereof in MLS cells.

Pull-down experiments with serially truncated FUS NTD constructs showed that amino acids 31-66 of FUS are required for binding of the SWI/SNF components. This sequence contains the previously defined FETBM1 motif that mediates interaction with wildtype FET proteins (Paper III). It is thus possible that the FUS NTD binding of SWI/SNF components is indirect through the binding of the wild type FET proteins. FUS and its previously reported interaction partners as well as SWI/SNF subunits bind nucleic acids [63] making a DNA or RNA dependent binding plausible. However, DNase or RNase treatment did not cancel the interaction of ARID1A or Brg1 with FUS NTD thus ruling out this possibility.

In summary, the FUS NTD binds core components of the SWI/SNF complex *in vitro* and *in vivo* and this interaction provides new understanding of how FUS-DDIT3 may interact with core components of differentiation and gene regulatory systems. The identification FETBM1 as a required motif for binding of SWI/SNF components is an important and necessary step towards screens for novel drugs targeting MLS and other FET protein associated diseases.

5 CONCLUSIONS

The main findings of this thesis are concluded as follows:

Stress induced DDIT3 may localize to both the cell cytoplasm and nucleus and regulate gene expression. Cytoplasmic and nuclear DDIT3 control distinct gene repertoires, but the target genes are associated with the same cellular functions. The majority of target genes were repressed supporting a dominant negative function of DDIT3 in transcriptional regulation. Subcellular localization is an important regulatory mechanism for control of DDIT3 activity. In the context of tumor development it is interesting that only the nuclear DDIT3 block cell-cycle progression. However, the pathways that control DDIT3 localization remain to be elucidated (Paper I).

We show that the NTD of FUS interacts with the cytolinker protein plectin in the cell cytoplasm. Plectin is required for normal nucleo-cytoplasmic distribution of FUS and absence of plectin caused altered expression levels of FUS bound mRNAs. We propose that plectin serves as a scaffold for FUS in the cytoplasm and facilitate transport and targeting of FUS associated mRNAs. The precise mechanism by which the FUS-plectin interaction influence FUS function and its possible impact on FUS and plectin linked pathologies remains to be investigated (Paper II).

The NTD of FUS, EWS and TAF15 contain a conserved motif that mediates interaction with the full-length FET family proteins. The FUS-DDIT3 fusion oncoprotein retains the capacity to bind normal FET proteins and disrupt their nuclear localization pattern. Our results further suggest an involvement of FUS in the regulation of FUS-DDIT3 target genes. (Paper III).

The NTD of FUS interact with Brg1, ARID1A and several other subunits of the nucleosome remodeling SWI/SNF complex. Furthermore, Brg1 and ARID1A form complexes with both normal FUS and FUS-DDIT3 in the nuclear compartment of myxoid liposarcoma cells. Our results suggest that aberrant interaction of FUS-DDIT3 with SWI/SNF subunits cause deregulation of gene expression and contribute to tumorigenesis of MLS. However, the significance of the binding between FUS-DDIT3 and SWI/SNF subunits needs to be addressed by additional experiments (Paper IV).

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