FET proteins in cancer and development

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Front cover
The FUS-DDIT3 protein (in this case tagged with DsRed1) displays a characteristic pattern of nuclear speckles when ectopically expressed in human HT1080 fibrosarcoma cells. The further co-expression of NFKBIZ tagged with green fluorescent protein results in a significant overlap between FUS-DDIT3 and NFKBIZ in such foci. Adapted from Göransson *et al*. Oncogene 2009 Jan 15.

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Till mor och far

Dans les champs de l'observation, le hasard ne favorise que les esprits préparés.

Louis Pasteur, French chemist and microbiologist, 1854

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Abstract

Chromosomal translocations leading to rearrangements of FET family genes (*FUS*, *EWSR1* and *TAF15*) are found in numerous human cancers. These genetic alterations result in the formation of fusion oncogenes that express potent chimeric oncoproteins able to promote tumor development. In order to further understand the function of the FET genes, we have characterized the expression of their encoded proteins products in human tissues and cells. By immunohistochemical analyses, we here demonstrate that the FUS, EWS and TAF15 proteins are expressed in a cell type-specific manner in human tissues. In experiments using cultured cells, we show that their expression is altered upon differentiation and that they localize to stress granules in response to cellular stress. Furthermore, FUS and TAF15 localize to spreading initiation centers upon early cell spreading. These results point to multiple cell type-specific functions for the FET proteins during both normal and stress conditions. We further attempted to elucidate the molecular mechanisms by which the aberrant FUS-DDIT3 protein gives rise to myxoid/round-cell liposarcoma (MLS/RCLS), a malignant soft-tissue tumor. FUS-DDIT3 expression results from a t(12;16)(q13;p11) translocation that is highly specific for MLS/RCLS and several studies have shown a causative role for FUS-DDIT3 in the development of these tumors. In FUS-DDIT3, the N-terminal parts of the RNA-binding FUS protein is fused to the entire DDIT3 protein, a transcription factor involved in endoplasmatic reticulum stress and programmed cell death. In the context of MLS/RCLS, the chimeric FUS-DDIT3 protein acts as an aberrant transcription factor able to deregulate multiple target genes. We have previously shown that FUS-DDIT3 and DDIT3 have opposing effects on *IL8* transcription in cells stably expressing these proteins. Here we demonstrate, by using multiple molecular methods, that FUS-DDIT3 interacts with the NF-κB system, specifically the NFKBIZ protein, and thereby activates *IL8* expression. These findings propose a role for inflammation-related processes in MLS/RCLS development. We further show that the growth factor receptor FLT1 and its ligand PGF are expressed in MLS/RCLS cells, which suggests the existence of an intracrine signaling loop in these cells. Moreover, through co-immunoprecipitation studies, we show that DDIT3 binds cyclin-dependent kinase 2 (CDK2), a protein involved in cell cycle regulation. This binding apparently alters the protein affinity of CDK2 and enhances its association with components of the cytoskeleton. The involvement of normal FET proteins in multiple regulatory pathways within a cell may explain why FET fusion genes are often the sole detectable abnormalities in their associated tumors. Specifically, the *FUS-DDIT3* gene studied in this thesis can promote several of the physical characteristics associated with cancer and thereby drive malignancy. In summary, the work included in this thesis suggests that agents which induce cellular differentiation, inhibit inflammatory processes (in particular the NF-κB system) or block FLT1 signaling may aid current treatments and thereby improve survival of patients afflicted with myxoid liposarcoma.

Keywords: FUS, EWS, TAF15, FUS-DDIT3, myxoid liposarcoma, NF-κB, NFKBIZ, FLT1, CDK2

List of papers

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

I. Mattias K. Andersson, Anders Ståhlberg, Yvonne Arvidsson, Anita Olofsson, Henrik Semb, Göran Stenman, Ola Nilsson and Pierre Åman. The multifunctional FUS, EWS and TAF15 proto-oncoproteins show cell type-specific expression patterns and involvement in cell spreading and stress response. *BMC Cell Biology. 2008 Jul 11;9:37*.

II. Melker Göransson, Mattias K. Andersson, Claudia Forni, Anders Ståhlberg, Carola Andersson, Anita Olofsson, Roberto Mantovani and Pierre Åman. The myxoid liposarcoma FUS-DDIT3 fusion oncoprotein deregulates NF-κB target genes by interaction with NFKBIZ. *Oncogene. 2009 Jan 15;28(2):270-8. Epub 2008 Oct 13*.

III. Mattias K. Andersson, Melker Göransson, Anita Olofsson, Carola Andersson and Pierre Åman. Expression of FLT1 and its ligand PGF in *FUS-DDIT3* carrying myxoid liposarcomas suggests the existence of an intracrine signaling loop. *Manuscript*.

IV. Christoffer Bento*, Mattias K. Andersson*, Carola Andersson, Anita Olofsson och Pierre Åman. DDIT3 and the sarcoma fusion oncoprotein FUS-DDIT3 bind cyclin-dependent kinase 2. *Manuscript*.

* These authors contributed equally.

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Introduction

1. Cancer

Cancer is a heterogeneous group of diseases characterized by uncontrolled growth and spread of abnormal cells. It is the second most common cause of death in the Western world and its incidence increases with age. Cancer is currently treated with combinations of surgery, radiation, chemotherapy, hormone therapy, biological therapy and targeted therapy[1].

The majority of cancer forms are associated with the emergence of malignant tumors*. These tumors are composed of a mass of abnormal cells that can spread out and invade nearby tissue or spawn metastases at distant sites in the body. Most human cancers arise from epithelial tissues, which consist of sheets of cells that line the walls of cavities and channels, or in the case of skin, serve as outside covering of the body. The malignant tumors originating from epithelial tissues are classified as carcinomas and these tumors are responsible for more than 80% of the cancer-related deaths in the West. The remaining malignant tumors arise from nonepithelial tissues throughout the body. Sarcomas, which constitute about 1% of the malignant tumors encountered in the oncology clinic, derive from tissues consisting of mesenchymal cell types. These include muscle, fat, bone, fibrous tissue, blood vessels and other tissues supporting the body. Even though rarely occuring, sarcomas are life-threatening and often pose a significant diagnostic and therapeutic challenge due to their histological heterogeneity. A second group of nonepithelial cancers arise in the various cell types that make up the blood-forming tissues and in cells of the immune system. These are called leukemias and lymphomas respectively. The final group of nonepithelial tumors develops from cells that form components of the central and peripheral nervous system. These are termed neuroectodermal tumors[2].

Carcinogenesis, the development of cancer, is a multistep process that initiates in a single normal cell and gradually transforms its progeny into highly malignant counterparts[3,4]. In an exceedingly cited review article from 2000[5], Hanahan and Weinberg propose that six essential alterations in cell physiology are required for cancer development:

* Malignant tumors constitute a minority of all human tumors and most tumors are classified as benign (localized, noninvasive). Benign tumors are only rarely dangerous to their hosts but may in some cases progress to malignant forms if left untreated [2].

Self-sufficiency in growth signals

Normal cells require growth-promoting signals from the extracellular environment before they can begin to actively proliferate. No known normal cell type can proliferate in the absence of such signals. Tumor cells on the other hand generate many of their own signals, thereby reducing their dependence on the normal tissue microenvironment. This ability disrupts one critical homeostatic control that preserves a proper behavior of the various cells constituting a tissue in the multicellular organism.

Insensitivity to anti-growth signals

Normal tissue homeostasis is also maintained in large part by antiproliferative signals that induce cellular quiescence or differentiation. Tumor cells acquire traits that make them unresponsive to such signals, which results in unconstrained proliferation.

Evasion of apoptosis

The expansion of cells in a tumor depends not only on the rate of proliferation but also on the rate of attrition. Programmed cell death, apoptosis, represents a major source of this attrition. A normal cell responds to signals of normality or abnormality originating from the extracellular and intracellular environment that influence whether it should live or die. Tumor cells gain resistance toward such signals and thereby avoid programmed cell death.

Limitless replicative potential

Most mammalian cells carry an intrinsic program that limits their multiplication and they can not divide indefinitely. This program must be disrupted if a cell population is to expand to form a macroscopic, life-threatening tumor.

Sustained angiogenesis

Oxygen and nutrient supply, as well as waste disposal, are crucial for proper cell function and survival in tissues. These processes are supported by the vasculature and cells are required to reside within 100 μm of a capillary blood vessel to utilize it. The growth of new blood vessels, angiogenesis, is carefully regulated in developed tissues and premalignant cells lack angiogenic ability, which halt their expansion. Thus, in order to progress to a macroscopically detectable size, tumors must obtain the ability to recruit new blood vessels.

Tissue invasion and metastasis

Eventually, during the development of human cancer, primary tumors spawn pioneer cells that gain the ability to move out and invade adjacent tissues or travel to distant sites in the body where they may found new colonies. These settlements of tumor cells, metastases, are the cause of 90% of human cancer deaths.

Each of the above characteristics, termed the hallmarks of cancer, represent a breach of the built-in anticancer defense mechanisms present in normal cells and tissues, and are shared by most, if not all, malignant human tumors. The order and timescale over which these features are obtained varies between individual tumors. So, how do normal cells acquire these capabilities?

2. The genetic basis of cancer

In 1890, David von Hansemann concluded, through detailed analysis of carcinoma samples, that cancer cells show abnormal mitotic figures*. He proposed that these result in an asymmetric distribution of chromosomes to daughter cells following cell division[6]. These findings were further pursued by Theodor Boveri who showed that experimentally induced multipolar mitoses in sea-urchin eggs lead to an unequal distribution of chromosomes in post-mitotic cells[7]. Some of the cells die from this imbalance while others survive but develop abnormally. From these results, Boveri suggested that tumors arise as a consequence of abnormal chromosome segregation to daughter cells.

The normal configuration of chromosomes is termed euploid karyotypic state. In humans, autosomes are present in 22 normally structured pairs and the X and Y chromosomes are present in numbers according to the sex of the individual. Cancer cells often differ from this normal configuration, showing presence of extra chromosomes or loss of others, in a state termed aneuploidy[8]. In addition, cancer cells generally contain abnormally structured chromosomes with rearrangements such as translocations and inversions. Furthermore, segments of a given chromosome may be copied many times over and present in multiple copies on a given chromosome through a process called amplification. Such segments can also exist as separate extrachromosomal entities in the nucleus. On occasion, parts of chromosomes are deleted and lost in subsequent cell divisions[2]. These structural rearrangements and changes in chromosome number affect the genes residing in the altered chromosomal regions[9].

2.1 Oncogenes

The relevance of genetic alterations for cancer development became clear with the discovery of the first proto-oncogene *src*[10]. This remarkable finding demonstrated that genes carried by normal cells have the potential, under certain circumstances, to turn into potent oncogenes with the capacity to induce cell transformation and thus cancer. Oncogenes invoke one or several of the previously mentioned hallmarks of cancer in a dominant manner. Their ability to do so stems from the fact that their normal proto-oncogenic counterparts have a range of growth-promoting functions at the cellular level. These genes encode proteins that act as growth factors (e.g. FGF and PDGF), growth factor receptors (e.g. RET and the ERBB family), inhibitors of apoptosis (e.g. MDM2 and the BCL2 family), signal transducers (e.g. SRC and the RAS family), transcription factors (e.g. MYC and the ETS family) and chromatin remodellers (e.g. ALL1). Hence, structural alteration or

* The microscopic appearance of cells undergoing mitosis (somatic cell division).

deregulation of expression of such genes can augment normal processes involved in cell proliferation and hereby promote malignancy[11].

Proto-oncogenes can be converted into oncogenes in several different ways. The amplification of chromosomal segments found in some cancer cells often result in multiple copies of certain genes being present in the cellular DNA, commonly followed by an increase in expression of their encoded proteins[12]. Translocations, i.e. fusions between nonhomologous chromosomes, can also cause oncogene activation[13] (see section 3). In other cases, a single point mutation* (e.g. affecting a critical residue responsible for regulating the activity of the protein product) is all that is needed to turn a proto-oncogene into a fully fledged oncogene[14].

2.2 Tumor suppressor genes

The discovery of proto-oncogenes provided a simple yet powerful explanation of how genetic alterations can fuel the uncontrolled cell proliferation seen in cancer cells. However, the underlying logic of any well-functioning control system dictates that components that promote a given process must be counterbalanced by other components opposing the same process. In the 1970s and early 1980s, evidence for the existence of a fundamentally different set of growth-controlling genes surfaced[15]. These tumor suppressor genes (TSGs) operate to constrain or suppress cell proliferation. Quite opposite to the case of proto-oncogenes, the inactivation or loss of TSGs is associated with tumor development.

The cancer phenotype is recessive at the cellular level[16] and in most cases the loss of both allelic copies of a tumor suppressor gene is required for a complete inactivation of its growth-repressing functions[17]. The inactivation of a TSG allele can occur either through genetic mutations or by epigenetic† silencing[18]. The remaining gene copy can subsequently be lost through different mechanisms collectively termed loss of heterozygosity‡ (LOH)[19]. Repeated LOH events in a given chromosomal region in independently arising tumors can indicate the presence of a TSG in that region. When a defective copy of a tumor suppressor gene is inherited through the germ line, the result is often a greatly increased susceptibility to one or another type of cancer and mutant alleles of specific TSGs have been found in families with hereditary cancer[20]. Since mutations in TSG alleles are commonly recessive, the loss of a TSG may occur far more frequently

* A nucleobase replacement in DNA (or RNA).

† Chemical modifications of chromatin proteins or DNA bases that do not alter the underlying DNA sequence but can result in changes of gene expression.

‡ Loss of heterozygosity can result from gene conversion, deletion, mitotic recombination or mitotic nondisjunction.

than the activation of a dominant-acting oncogene during both normal and neoplastic development.

Tumor suppressor genes regulate cell proliferation through many different biochemical mechanisms and they are unified only by the fact that their loss increases the likelihood of cancer development. The *TP53* gene is lost or mutated in approximately half of all human cancers[21]. Germ line mutations in *TP53* predispose affected individuals to a wide spectrum of cancers[20] and *TP53* knockout mice show susceptibility to spontaneous tumors[22]. In the normal case, the p53 protein[23,24] is transiently stabilized and activated in response to stress, DNA damage and chronic mitogenic stimulation. This activation leads to an inhibition of cell cycle progression, induction of senescence, differentiation or apoptosis[25]. Hence, p53 responds to signals of cellular imbalance and further orchestrates measures to repair or eliminate cells that could potentially pose a threat to the organism as a whole. Consequently, the loss of normal p53 function puts a cell at risk of accumulating cellular damage that could promote cancer development. The first TSG that was characterized was the *RB1* gene[26]. Children bearing germ line mutations in this gene are predisposed to retinal tumors at a young age and osteosarcomas as adolescents. The protein encoded by *RB1* governs the progress of a wide variety of cells through their growth and division cycles, and the growth control imposed by the *RB1* circuit appears to be disrupted in most human tumors. A large number of additional TSGs have subsequently been identified, often associated with specific cancer forms[2].

Acquired mutations in proto-oncogenes and tumor suppressor genes provide a straightforward concept of how normal cells can obtain the physical characteristics associated with cancer cells. Clones of such cells are progressively capable of competing for space and nutrients within a species in a process highly analogous to Darwinian evolution. However, this model has turned out to be too simplistic, in part by the findings of epigenetically inactivated tumor suppressor genes but also by the high frequency of randomly distributed mutations seen in most malignant tumors[27].

2.3 Genomic maintenance genes

Our genome is under constant attack by a variety of agents and processes that damage DNA. These mutagenic processes can be divided in three categories. First, the replication of DNA sequences prior to cell division is subject to a low, but significant level of intrinsic error. Second, the nucleotides within DNA undergo spontaneous chemical changes that often alter the base sequence and thus the information content of the DNA. Finally, DNA molecules are attacked by various mutagenic agents, including molecules generated by endogenous cellular

metabolism as well as those of exogenous origin, both chemical (e.g. tobacco tar and alkylating agents) and physical (e.g. X-rays and UV-light) mutagens. To counter these detrimental processes, an elaborate DNA repair system exists that constantly monitors the integrity of the genome. This system, associated with more than hundred different genes, is able to remove and replace inappropriate bases as well as repair strand breaks in DNA. In cases where the damage is too severe, the result is an induction of apoptosis in the damaged cell[28].

Defects in DNA repair genes lead to an increase in the overall mutation rate of the genome and inherited deficiencies in such genes predispose individuals to certain types of cancer[29]. Mice carrying mutated *BRCA2*[*] alleles show a high frequency of malignant thymic lymphomas and defects in DNA repair[31]. Moreover, a "mutator phenotype" acquired by cancer cells has been proposed to contribute to the morphologic and functional heterogeneity of human cancers and to be the reason why subpopulations of cells in a tumor can confer resistance to therapy[27]. Also, aberrant repair of DNA double-strand breaks is believed to be a major source of chromosomal translocations, attributing functions for DNA repair in keeping chromosomal structures intact[32]. These findings suggest that defects in DNA repair systems are causally linked to the development of many human cancers.

Aneuploidy, abnormal chromosome numbers, is proposed to result from missegregation of chromosomes during mitosis. Mutations in a set of genes with functions in mitosis have been associated with cancer formation and such defects are believed to be responsible for the aneuploidy seen in many human cancer cells[33].

However, not all genetic changes contribute to cancer development and a distinction between primary causal aberrations and those accumulated as a consequence of tumor progression is in most cases difficult to reconcile. Fortunately, recent efforts have shown that such issues can be resolved by the use of high-throughput methods[34].

* Women carrying mutated *BRCA1* or *BRCA2* genes have a high risk of developing breast and ovarian cancer [30].

3. Fusion oncogenes

Recurrent balanced rearrangements* have been found in almost every tumor type[36] and many of these changes are explicitly associated with distinct tumor phenotypes, clinical features and gene expression profiles. Recurrent balanced rearrangements are considered important early events during tumorigenesis and successful treatment of the associated disease is often paralleled by a decrease or loss of rearrangement-specific gene products. Furthermore, artificially constructed rearrangements in animal models give rise to tumors of the same kind as those sporadic human neoplasms that carry the corresponding rearrangements. In addition, experimental silencing of transcripts originating from recurrent rearrangements leads to a reversal of tumorigenicity, decreased proliferation and/or differentiation[35]. Recently, recurrent rearrangements were shown to occur in high frequencies in human prostate cancer[37]. These findings highlight the importance of such rearrangements for cancer progression and clinical outcome of many human cancers.

Chromosomal translocations are the most commonly encountered balanced rearrangements and they exert their action through two alternative mechanisms. The first, deregulation of a gene through exchange of regulatory elements is well-documented in hematological malignancies. In this type of translocation the promoter region of one gene is fused to the intact coding parts another gene by a process called promoter swapping (see Figure 1a). For example, in Burkitt lymphoma, which harbors one of three translocations, the *MYC* proto-oncogene is placed under the control of regulatory elements of an immunoglobulin gene and hereby becomes constitutively activated[13,38]. The second mechanism of fusion gene formation results in the creation of a chimeric fusion gene that comprises the coding regions of two different genes (see Figure 1b). The most famous example of a translocation creating a chimeric fusion gene came with the cloning of the Philadelphia chromosome† breakpoint. The Philadelphia chromosome was found to consist of material from both chromosomes 9 and 22[41], and the later cloning of the breakpoint revealed a fusion between the 5' part of *BCR* and the 3' part of the *ABL1* tyrosine kinase gene[42,43]. The rearrangement leads to the expression of a

* Chromosome abnormalities that result in structurally altered chromosomes without the gain or loss of genetic material. Reciprocal translocations, inversions and insertions comprise such changes [35].

† In 1960, Peter Nowell and David Hungerford reported that cells from patients diagnosed with chronic myeologenous leukemia (CML) had a normal number of chromosomes but that one chromosome was too small [39]. The findings were quickly confirmed by other cancer researchers and this marker became known as the Philadelphia chromosome, Ph. This was the first chromosome abnormality that was consistently associated with human malignancy [40].

hybrid BCR-ABL1 protein with increased tyrosine kinase activity[44]. Treatment of CML patients with the ABL1-targeted receptor tyrosine kinase inhibitor imatinib induces complete remission in most cases[45], demonstrating the central role of the *BCR-ABL1* fusion in this disease.

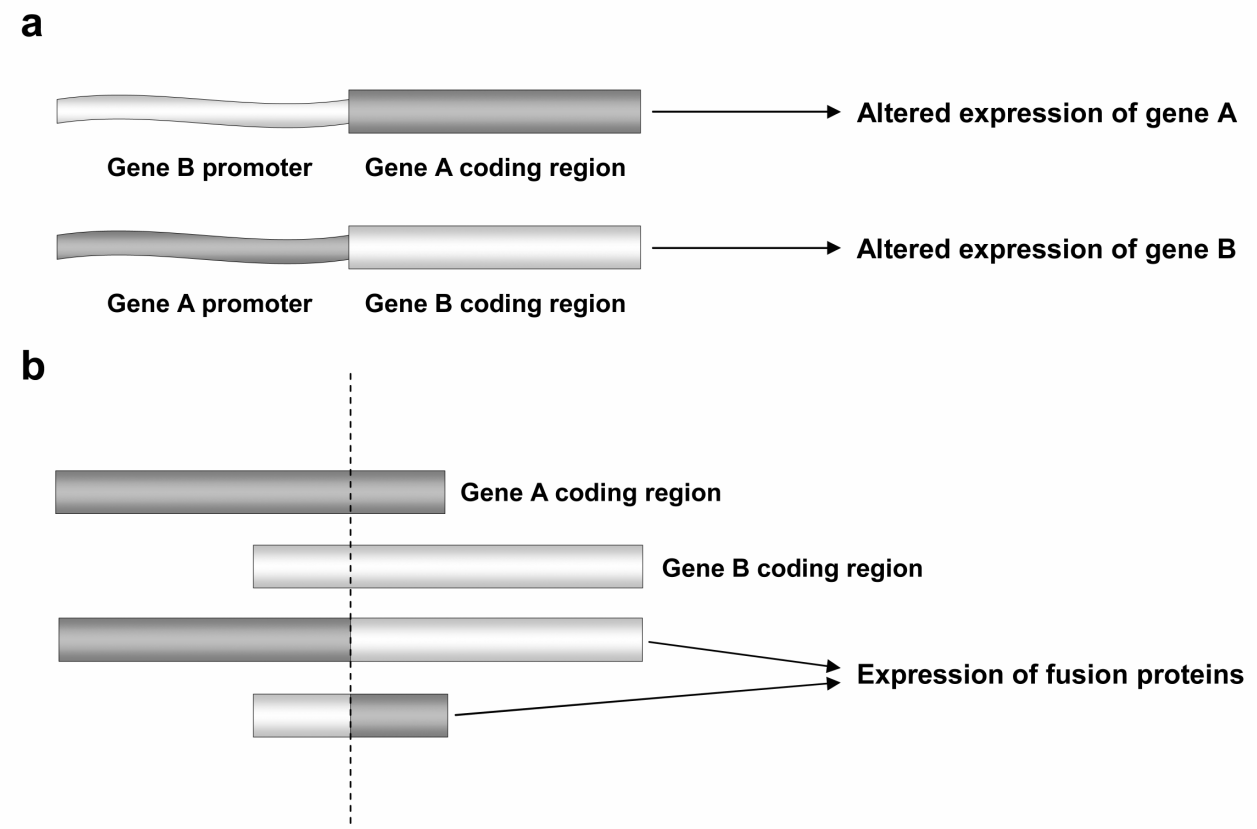

Figure 1. Mechanisms of fusion gene formation. a) Promoter swapping. b) Creation of chimeric genes. The figure illustrates reciprocal rearrangements between different chromosomes. Often only one functional gene product is expressed as a result of such rearrangements. Reciprocal translocations have to date not been linked to deficiencies in DNA repair systems. Adapted from Rowley 2001[40].

3.1 FUS-DDIT3

The *FUS-DDIT3* fusion oncogene is formed by a t(12;16)(q13;p11) chromosome rearrangement[46,47] that is highly specific for myxoid/round-cell liposarcomas (MLS/RCLS, see section 4) and detected in more than 90% of all cases[*,53]. The rearrangement results in the expression of an abnormal chimeric transcription factor[54] (see Figure 2).

* Rare cases present clinically with the *EWSR1-DDIT3* fusion oncogene [48]. To date there are nine *FUS-DDIT3* and four *EWS-DDIT3* variant fusion transcripts identified [49, 50]. However, the different transcript variants have not shown divergence in the ability to promote tumor development or affect the clinical outcome of MLS/RCLS [49, 51, 52].

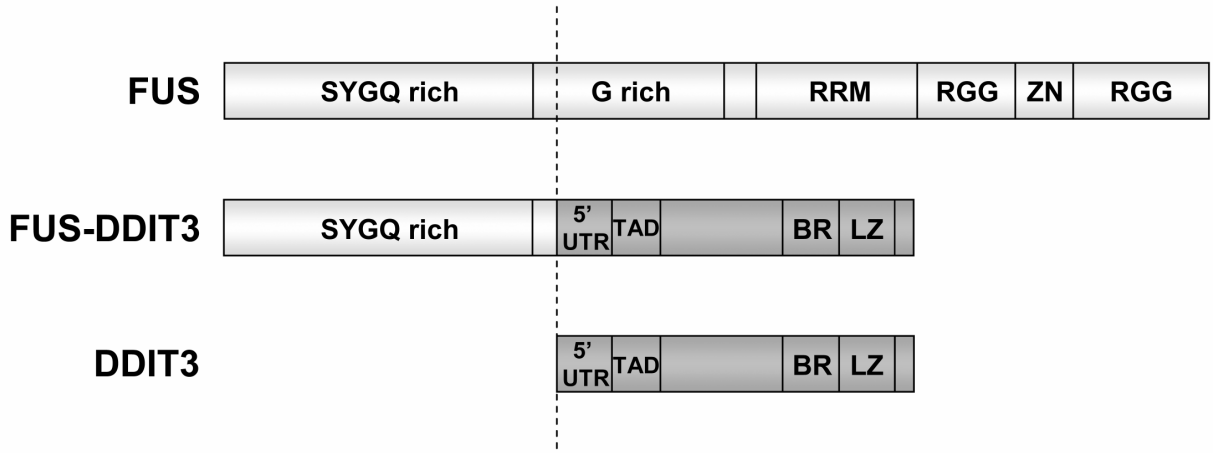

Figure 2. The t(12;16)(q13;p11) translocation, which results from a fusion of chromosomes 12 and 16, leads to the expression of a chimeric fusion protein having the N-terminal FUS domain fused to the entire DDIT3 protein. The figure illustrates the most common fusion variant (type II) found in two thirds of all cases[53]. Characteristic sequence motifs such as Alu repetitive sequences, Translin[55] binding sites and topoisomerase II cleavage sites have been found near the genomic breakpoints, which suggest that these regions are prone to somatic recombination and implicate Translin and topoisomerase II in the translocation process[56-59]. BR – basic region; G – glycine; LZ – leucine zipper domain; RGG – arginine, glycine, glycine repeats; RRM – RNA recognition motif; SYGQ – serine, tyrosine, glycine, glutamine; TAD – transactivation domain; UTR – untranslated region; ZN – zinc finger motif. Regions were defined using UniProtKB/Swiss-Prot (http://www.uniprot.org/)[60] and Ohaka 2007[61]. Regions in DDIT3 are not drawn to scale.

An aberrant transcription factor activity of FUS-DDIT3 has been demonstrated *in vivo* by studies showing distinct transcription profiles of tumor cells expressing the chimeric oncoprotein[52,62,63]. The FUS-DDIT3 protein is also implied in aberrant splicing and shown to inhibit YB-1-induced splicing in a dominant-negative manner[64]. We have previously identified a region in the N-terminal of FUS-DDIT3 that is responsible for its temperature-dependent localization to splicing compartments defined by the SC-35 protein[65], further implicating FUS-DDIT3 in abnormal RNA processing.

Soon after its discovery, the *FUS-DDIT3* gene was found to have transforming properties when expressed in cultured mouse fibroblasts[66]. It failed however to transform other cell types and was therefore suggested to elicit its oncogenic effects only in specific target cells. Further studies indicated that the N-terminal part of FUS was needed for realizing the full oncogenic potential of the chimera. Interestingly, when the FUS part was replaced with the N-terminal region of the highly homologous EWS protein this resulted in similar transforming properties. Conversely, the C-terminal was discovered to influence the tumor phenotype.

Tumors that emerged in nude mice upon injection with cells expressing fusion oncoproteins having different C-terminals showed distinct morphologies depending on the C-terminal transcription factor partner. From these experiments, it was concluded that the N-terminal is necessary for the transformation event while the C-terminal determines the specific tumor phenotype. These findings were later confirmed with FUS-DDIT3 transgenic mice[67]. In these mice, myxoid liposarcomas specifically developed in adipose tissues even though a ubiquitously activated promoter was used to drive FUS-DDIT3 expression in all tissues. Tumor formation required the co-expression of both FUS and DDIT3 domains but not necessarily in the form of a fusion protein[68,69]. A target cell population where FUS-DDIT3 could exert its transforming properties was subsequently identified using isolated mouse mesenchymal progenitor cells transduced with retroviral vectors carrying *FUS-DDIT3*[62]. These cells gave rise to myxoid liposarcomas resembling human counterparts when transplanted into SCID mice. Hence, FUS-DDIT3 was proposed to be the single causative factor of MLS/RCLS tumor formation in these mice. Concurrently, our group obtained evidence for an instructive role of the FUS-DDIT3 protein, showing that FUS-DDIT3 expression induces a liposarcoma-like phenotype in tumors arising from primitive human fibrosarcoma cells implanted in SCID mice[70]. We and others have also demonstrated that FUS-DDIT3 can block adipogenesis[70-73], the process whereby a mesenchymal stem cell matures into a post-mitotic, fat-laden adipocyte[74]. Thus, the ability of FUS-DDIT3 to transform pluripotent mesenchymal progenitor cells, FUS-DDIT3 transgenic mice showing adipose tumors, and the induced liposarcoma-like phenotype seen in xenografts, support a model wherein FUS-DDIT3 transforms a mesenchymal stem cell and further commits it to the adipocytic lineage with subsequent blockage of terminal differentiation.

The transformation event caused by FUS-DDIT3 is poorly characterized at the molecular level while the blockage of differentiation is mapped in more detail. The normal DDIT3 protein is known to form heterodimers with C/EBP members[*,76] and this ability is retained by the oncogenic FUS-DDIT3 protein[77]. C/EBP proteins play a prominent rule in adipogenesis during which these transcription factors are expressed in a cascade. Induction of C/EBPβ and C/EBPδ occurs in the first stages of adipogenesis and further trigger the expression of C/EBPα and PPARγ, the master regulator of fat differentiation. A positive feedback loop between C/EBPα and PPARγ leads to the expression of mature adipocyte markers such as ap2, adiponectin and adipsin. FUS-DDIT3 is able to block terminal differentiation of

* The CCAAT-enhancer-binding protein family of transcription factors consists of six members that are involved numerous cellular processes including differentiation, proliferation, inflammation/immune response, apoptosis and control of metabolism. The members share substantial sequence similarity in the C-terminal that contains a DNA-binding region enriched in basic amino acids followed by a leucine zipper dimerization domain [75].

preadipocytes by sequestration of C/EBPβ which inhibits transcriptional activation of *CEBPA* and *PPARG*. In addition, FUS-DDIT3 is proposed to upregulate early translation factors eIF4E and eIF2 through promoter activation, which could alter the distribution of C/EBP isoforms and invoke a negative effect on adipogenesis[73].

3.2 DDIT3

The *DDIT3* (*CHOP*, *GADD153*) gene was first identified as being transcriptionally induced in response to growth arrest and DNA damage[78], and later in response to glucose deprivation, inflammation, oxidative and endoplasmatic reticulum (ER) stress[79-82]. It is mapped to human chromosome 12 (12q13.1-q13.2) and consists of 4 exons[83]. The encoded DDIT3 protein (see Figure 2) belongs to the C/EBP family[75] of transcription factors and forms heterodimers with the other family members. DDIT3 differs from other C/EBP proteins in that it contains two proline residues in the DNA-binding basic region that disrupts its α-helical structure, while retaining an intact leucine zipper needed for dimerization. As a result, DDIT3 heterodimers are unable to bind canonical C/EBP recognition sites and consequently act as dominant-negative regulators of transcription by sequestering C/EBP members[76]. However, DDIT3-C/EBP heterodimers have been shown to activate gene transcription under certain conditions by binding alternative DNA sequences[84]. Moreover, DDIT3 is able to regulate transcription by forming dimers with other transcription factor families containing basic-leucine zipper regions[85,86]. DDIT3 was recently identified as an intrinsically disordered protein able to form homooligomers through its N-terminal region[87]. This oligomerization state was further suggested to be central for both inhibition of Wnt/Tcf signaling[88] and activation of c-Jun and sucrase-isomaltase promoters[87]. The transactivating ability of DDIT3 is enhanced in response to stress[89] and the protein inhibits adipogenesis in response to metabolic stress[90]. DDIT3 was also shown to induce growth arrest at the G1/S transition, which required an intact basic region as well as the leucine zipper domain[77]. Finally, the protein mediates apoptosis induced by ER stress[91,92] and is therefore implicated in diseases with ER stress-dependent cell death, such as neurodegenerative disorders and diabetes[93,94].

3.3 FUS, EWS and TAF15

FUS (TLS), EWS and TAF15 (RBP56, TAF2N) are three structurally similar proteins that belong to the FET family (previously TET family) of RNA-binding proteins. The proteins share a number of highly conserved regions including an N-terminal serine-tyrosine-glycine-glutamine (SYGQ) rich region, a central RNA-recognition motif (RRM), a cysteine$_2$/cysteine$_2$-zinc finger and numerous C-terminal arginine-glycine-glycine (RGG) repeats (see Figure 3). The FET proteins are shown to bind DNA, RNA and protein by these unique structural features[95].

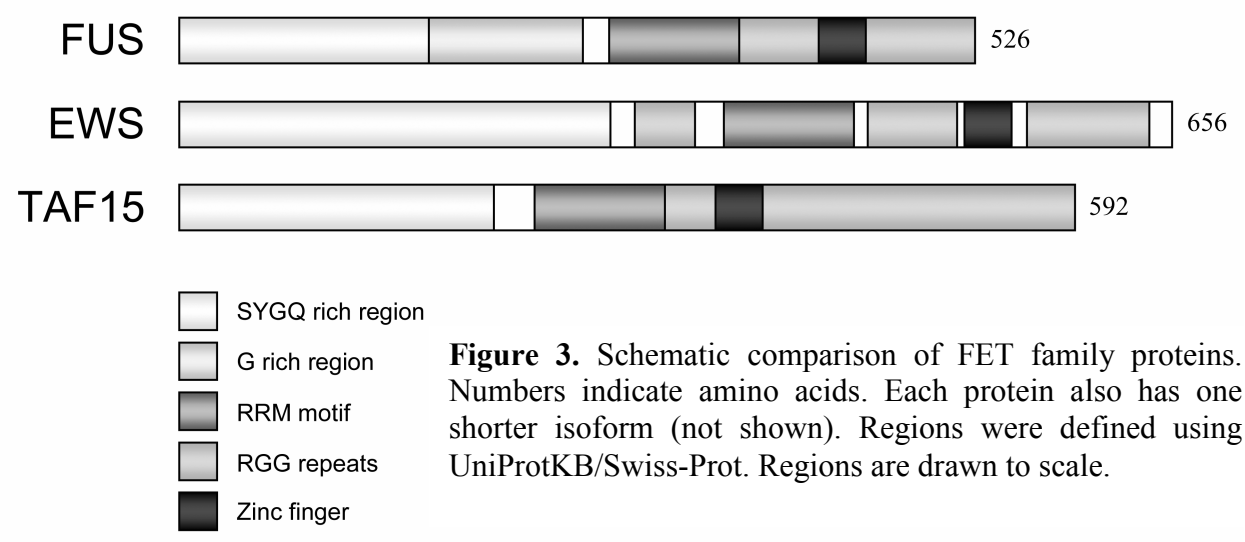

Figure 3. Schematic comparison of FET family proteins. Numbers indicate amino acids. Each protein also has one shorter isoform (not shown). Regions were defined using UniProtKB/Swiss-Prot. Regions are drawn to scale.

FUS, EWS and TAF15 are involved in a wide range of cellular processes. The proteins bind both eukaryotic RNA polymerase II and transcription factor II D (TFIID) complex proteins and are thus implicated in early transcriptional events[95-99]. In addition, the FET proteins are involved in RNA splicing[64,99,100-107] and may furthermore function in primary microRNA* processing as they have been shown to associate with the large Drosha microprocessor complex[109]. The FET proteins shuttle between different cellular compartments in complexes consisting of heterogeneous nuclear ribonucleoproteins (hnRNPs) and mRNA, which implicates these proteins in RNA transport[66,110-116]. Moreover, the FET proteins are reported targets for tyrosine kinase phosphorylation[113,117-120] and implied in additional signaling pathways[121]. Bertrand *et al*. previously reported cell cycle-dependent homologous DNA pairing activity for FUS[122] and such activity was subsequently also reported for EWS and TAF15[123]. *FUS* and *EWS* knockout mice are sensitive to ionizing radiation and show defects in meiosis and B-lymphocyte development. The *FUS* and *EWS* genes were additionally required for genomic stability in these mice[124-126]. Recently, FUS was shown to be a target for ATM phosphorylation following experimentally induced DNA double-strand breaks[127]. Furthermore, FUS was demonstrated to inhibit transcription of the *CCND1* gene by interfering with p300 acetyltransferase activity. This inhibitory effect was induced by ionizing radiation and further mediated by the elevated expression of noncoding RNAs (ncRNAs) expressed from the *CCND1* promoter. Remarkably, these ncRNAs recruited and allosterically modified FUS, which lead to a transcriptional inhibition acting *in cis* on the *CCND1* gene. Similarily, EWS and TAF15 showed inhibitory effects on p300 acetyltransferase activity in these experiments. These findings demonstrated a novel ncRNA/RNA-binding protein-based mechanism of

* MicroRNAs are small, non-coding RNAs with regulatory functions. These may play an important role in many human diseases, including cancer, and several studies have shown that these RNA species can act as both oncogenes and tumor suppressor genes [108].

transcriptional regulation[128]. Collectively, these results attribute roles for the FET proteins in maintaining genomic integrity by acting as mediators of DNA repair through transcriptional regulation and homologous DNA pairing and recombination.

The *FUS*, *EWSR1* and *TAF15* genes reside on chromosomes 16, 22 and 17 respectively, and are ubiquitously expressed in human adult and fetal tissues[129-132]. Though, how their expression is regulated remains poorly understood. The 5' flanking sequences contain multiple transcription start sites, lacks TATA boxes and has a high incidence of C and G nucleotides, features associated with promoter regions of many housekeeping genes[129,131,132]. The FET genes are rearranged in numerous tumor type-specific chromosomal translocations found in human malignancies[36] (see Figure 4). The resulting fusion genes consist of parts of FET genes in 5' and different transcription factor coding genes in their 3'.

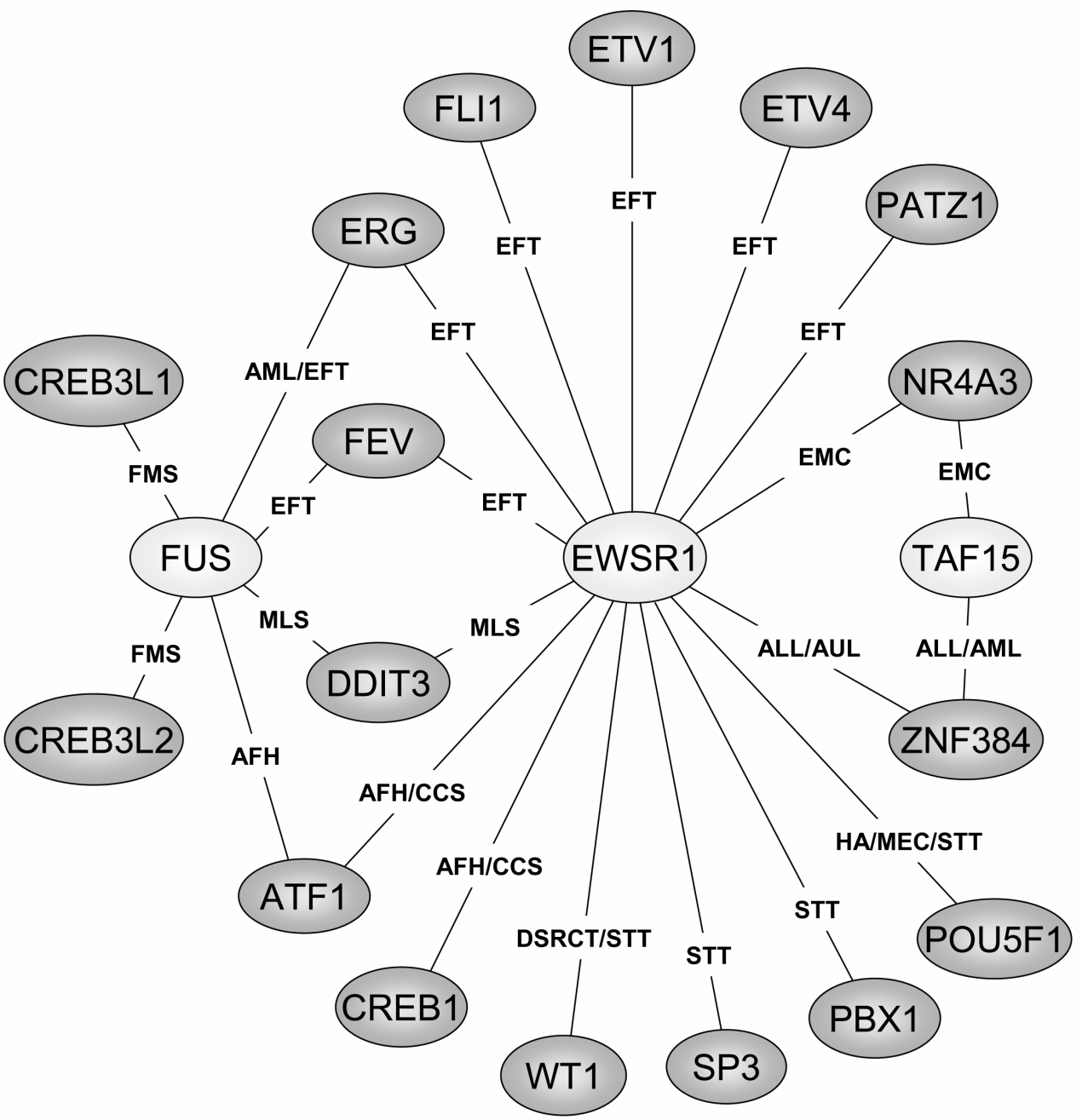

Figure 4. Fusion oncogenes involving FET family members. Nodes depict genes while connectors show associated tumor diseases. AFH - angiomatoid fibrous histiocytoma; ALL – acute lymphocytic leukemia; AML – acute myeloid leukemia; AUL – acute undifferentiated leukemia; CCS – clear cell sarcoma; DSRCT – desmoplastic small round cell tumor; EMC – extraskeletal myxoid chondrosarcoma; EFT – Ewing family of tumors including Ewing sarcoma and primitive neuroectodermal tumors; FMS – fibromyxoid sarcoma; HA – hidradenoma of the skin; MEC – mucoepidermoid carcinoma of the salivary glands; MLS – myxoid/round-cell liposarcoma; STT – soft tissue tumor, special type. The network is based on data contained in the Mitelman Database of Chromosome Aberrations in Cancer[36].

4. Myxoid liposarcoma

Myxoid liposarcoma is the second most common subtype of liposarcoma and constitutes about 10% of all adult soft tissue sarcomas. The tumors occur predominantly in the deep soft tissues of the extremities and arise in more than two-thirds of cases in the musculature of the thigh. MLS has a peak incidence between 40 and 60 years of age with no gender predilection. It is prone to recur locally and one-third of the patients develop distant metastases. MLS tends to metastasize to unusual soft tissue sites (i.e. retroperitoneum, opposite extremity, axilla) or bone (preferentially to spine) before spreading to lung. In a significant number of cases, patients present clinically with synchronous or metachronous multifocal disease[53].

MLS tumors are composed of uniform round to oval shaped primitive nonlipogenic mesenchymal cells and a variable number of small signet-ring lipoblasts, contained in a prominent myxoid stroma with a rich branching vascular pattern. A subset of these tumors shows histological progression to hypercellular or round-cell morphology characterized by closely packed primitive round cells with no intervening myxoid stroma, a high nuclear to cytoplasmic ratio and clearly visible nucleoli. The presence of areas with gradual transition from myxoid to round-cell morphology in some tumors provides evidence that RC liposarcoma represents a histological continuum of MLS[53].

Fine needle aspiration is routinely used for morphologic diagnosis and the further presence of *FUS-DDIT3* or *EWSR1-DDIT3* transcripts, expressed as a result of cytogenetically detectable translocations, is a specific indicator of myxoid liposarcoma[53]. Other cytogenetic abnormalities are rarely seen in these tumors. High histological grade (≥ 5% RC component), presence of necrosis and p53 overexpression are associated with a significantly poorer prognosis[51].

The main treatment regime is surgery which is combined with chemotherapy (ifosfamide, doxorubicin) for advanced or unresectable disease[133]. Several studies have shown that preoperative radiation therapy results in improved local control and significant tumor volume reduction[134-137]. Conflicting clinical responses were obtained in two studies using PPARγ agonists for treatment of MLS/RCLS[138,139]. Recently, antitumor activity of the DNA minor groove-binding alkaloid trabectedin was shown in patients with advanced MLS/RCLS[140]. The 5-year survival of myxoid liposarcoma is approximately 70% while the 10-year survival of patients that present with a localized disease showing >5% RC component decline to 30%[51].

Aims of the thesis

The objectives of this thesis were to analyze the expression of the FET family proteins (FUS, EWS, TAF15) in cells of normal tissues and to clarify the molecular mechanisms behind FUS-DDIT3-induced tumorigenesis.

The overall aim of our cancer research group is to search for shared mechanisms of transformation in tumors carrying *FUS*, *EWS* and *TAF15* fusion oncogenes.

The global goal of cancer research is to define the molecular defects giving rise to cancer and to turn these discoveries into effective treatment and prevention regimens.

The main aims of the papers included in this thesis were the following:

Paper I – To characterize the expression patterns of FUS, EWS and TAF15 in human tissues and cells.

Paper II – To elucidate the molecular mechanisms behind opposing *IL8* transcription in FUS-DDIT3 and DDIT3 expressing cells.

Paper III – To investigate the significance of the putative FUS-DDIT3 target gene *FLT1* in myxoid liposarcoma etiology.

Paper IV – To study the role of FUS-DDIT3 in the abnormal expression of cell cycle regulators seen in myxoid liposarcomas.

Materials and methods

Formalin-fixed paraffin-embedded tumor tissue from seven cases diagnosed with myxoid/round-cell liposarcoma was used for immunohistochemical analysis. In addition, MLS/RCLS tumor tissue from SCID mice xenografts, human normal organ and various cancers tissue arrays (Super Bio Chips) and angiosarcoma tissue were analyzed by immunohistochemistry. Total RNA was extracted from three fresh-frozen MLS/RCLS tumors as well as from adipose tissue and isolated adipocytes of healthy individuals, and used for quantitative real-time PCR. The MLS/RCLS-derived cell lines 402-91, 1765-92 and 2645-94[63,141] as well as HT-1080 human fibrosarcoma cells[142] and stably transfected subclones of this cell line[70] were used in molecular studies. In addition, HeLa cells, SH-SY5Y neuroblastoma cells[143], U1242MG glioblastoma cells[144], F470 primary human fibroblasts[145] and human embryonic stem cell lines SA121 (Cellartis AB), HUES1[146] and HUES3[146] were used for quantitative real-time PCR and immunofluorescence studies.

The results presented in this thesis are based on multiple molecular and cell biological methods such as: human cell culture, experimental cell assays, recombinant DNA techniques, stable and transient transfections, RNA interference, colocalization studies, luciferase reporter assays, chromatin- and co-immunoprecipitations, immunohistochemistry/immunofluorescence, fluorescence microscopy, laser scanning confocal microscopy, western blot analysis, quantitative real-time PCR, microarray analysis and liquid chromatography-mass spectrometry.

Statistical tests included independent samples t-test and Spearman correlations.

See the individual papers (I-IV) for detailed descriptions of the materials and methods used.

Results and discussion

Paper I. Cell type-specific expression of FET family proteins

Organ homeostasis in the multicellular organism is governed by temporal expression of tissue-specific transcription factors[147] and deregulation of this timely expression can lead to cancer development through perturbation of normal differentiation programmes[5].

The presence of translocated FET genes in tumors arising in a wide range of organs and the consistent association of these genes with tumor type-specific transcription factor coding fusion partners (see Figure 4), prompted us to characterize normal FET protein expression in human tissues and cells. In this work, we performed immunohistochemical analyses of human tissue microarrays with FET-targeted antibodies.

Our results showed that the three proteins are ubiquitously expressed throughout human tissues and only a few cell types lack FET expression. The FET proteins were expressed in the nuclei of all positive cells and FUS and TAF15 were expressed in the cytoplasm of most cells types. EWS was normally restricted to the nucleus and the few cell types wherein EWS showed cytoplasmic expression were generally secretory cell types, suggesting functions for EWS in the expression of secreted proteins. In addition, individual cells in tissues showed variations in FET expression levels, even within the same cell type. Such divergence was absent in cultured cells, which showed similar expression levels of the three proteins. This could indicate roles for the FET proteins in regulation of specialized functions. The nature of these cell type-specific functions is yet to be clarified but they are likely connected with regulation of cell type-specific transcription[147] and splicing[148] during tissue development and homeostasis. Roles for the FET proteins at different stages of maturation is further supported by our findings of downregulated FET protein expression in neuroblastoma cells experimentally induced to differentiate, the attenuated FET gene expression seen in spontaneously differentiating human embryonic stem cells and the absence of FET expression in some terminally differentiated cell types. Previous studies have also shown alterations in FUS and EWS expression during maturation of specific cell types[118,149-151]. In terms of mechanisms for FET protein regulation, one study reported that FUS expression is in part controlled by proteaosome-mediated degradation regulated by PKCβII-dependent phosphorylation, c-Jun expression and possibly hnRNPA1 ubiquitination[152]. The stability of the EWS protein has been linked with arginine methylation in its C-terminal region[153].

In cultured cells, the three FET proteins localized to stress granules* upon heat and oxidative stress. The FET proteins therefore appear to be part of the normal cellular response to stress. Perturbation of cellular stress responses in cancer cells leads to avoidance of growth inhibition and thereby maintained proliferation of damaged cells. Moreover, FUS and TAF15 localized to early spreading initiation centers, structures that contain RNA and RNA-binding proteins, involved in cell spreading and adhesion. Disturbance of such processes could promote characteristics associated with invasion and metastasis.

In conclusion, this work suggests cell type-specific activities for the FET family proteins and involvement in stress response and cell spreading. Proteins with multiple functions are vulnerable targets for cancer-causing mutations as such mutations could induce a simultaneous deregulation of multiple cellular control systems. This may explain why few additional genetic abnormalities are detected in tumors expressing FET fusion oncogenes. Furthermore, fusion of FET genes to transcription factor coding genes leads to an unscheduled expression of aberrant oncogenic transcription factors governed by FET family expression. As expression of the FET family is attenuated upon differentiation, this work suggests that agents which are able to induce differentiation of cells may reduce expression of chimeric FET oncoproteins.

* Stress granules are dense cytoplasmic aggregates that are composed of stalled translation pre-initiation complexes, mRNAs, microRNAs and RNA-binding proteins. These particles appear in cells exposed to a range of cellular stresses [154].

Paper II. Mechanisms of *IL8* regulation by FUS-DDIT3

Transcription factors are proteins that bind specific regulatory DNA sequences of target genes and hereby control the transfer (i.e. transcription) of genetic information from DNA to RNA[155]. A transcription factor can perform this function alone or in complex with other proteins and the result is either an activation or repression of gene transcription.

We have previously reported differential regulation of interleukin 8 (*IL8*) transcription in cells stably expressing DDIT3 and FUS-DDIT3 proteins[156]. In these cells, the DDIT3 protein seemingly acts as a strong repressor of *IL8* expression, while FUS-DDIT3 instead activates expression of *IL8*. In the present work, we investigate the molecular mechanism behind this opposing effect on *IL8* activity.

The *IL8* promoter contains a C/EBP–NF-κB composite site and the gene is reportedly regulated by C/EBP and nuclear factor kappa B* (NF-κB) transcription factors[158]. We explored the functional relevance of this site for *IL8* expression in our system by using plasmid vectors carrying mutant *IL8* promoters in frame with the coding regions of firefly luciferase. These reporter constructs were transfected into cells having stable DDIT3 or FUS-DDIT3 expression and the transcriptional activity of the different promoters was estimated by measurements of the luciferase enzymatic activity. In control cells (expressing neither DDIT3 nor FUS-DDIT3), the C/EBP site was judged to be important for *IL8* transcriptional regulation as mutation of this region resulted in a strong reduction of the *IL8* transcriptional activity. In contrast, the NF-κB part of the composite site was involved in negative regulation of *IL8* as mutation here lead to an increased transcription. Stable and transient expression of DDIT3 caused downregulation of *IL8* mRNA and ectopically expressed DDIT3 lacking the leucine zipper domain retained the ability to suppress *IL8* expression. These findings suggest that DDIT3 exerts its inhibitory action on *IL8* independently of C/EBP proteins. A recent study showed that DDIT3 lacking the C-terminal region can indeed regulate *IL8* transcription by binding an upstream responsive element in the *IL8* promoter[159]. However, the results obtained from our model indicate that the inhibitory effect seen by DDIT3 on the *IL8* promoter is mediated by a factor binding to the NF-κB promoter site, as mutation of this region strongly counteracted the repressing effects imposed by DDIT3 on *IL8*. Expression of FUS-DDIT3 on the other hand caused an increased *IL8* transcription that was maintained in the absence of the C/EBP site. The prominent *IL8* expression was instead dependent on the NF-κB site and mutation of this site led to a dramatic reduction of *IL8* transcription in FUS-DDIT3 expressing cells. Chromatin immunoprecipitation assays further confirmed that FUS-DDIT3

* The NF-κB system is composed of the NF-κB/Rel and IκB families and involved in immunity, oncogenesis and development [157].

associates with the *IL8* promoter in myxoid liposarcoma cells. Moreover, FUS-DDIT3 expressing cells were resistant to the dominant-negative effects induced by DDIT3 on *IL8* transcription. These results imply a tight association between FUS-DDIT3 and the *IL8* promoter. However, the analyses could not reveal whether this association was through a direct binding of the *IL8* promoter or mediated by an NF-κB site binding protein. As FUS-DDIT3 has not been reported to bind NF-κB sites of gene promoters, we hypothesized that FUS-DDIT3 might interact with an NF-κB family member and hereby induce *IL8* expression. We performed systematic colocalization studies between FUS-DDIT3 and NF-κB family members and identified NFKBIZ as a putative nuclear interaction partner of FUS-DDIT3. By transient transfections, we demonstrated that ectopically expressed FUS-DDIT3 colocalizes with NFKBIZ in nuclear speckles. Furthermore, FUS-DDIT3 was able to translocate a cytoplasmically localized NFKBIZ mutant to the nucleus. Truncated forms of FUS-DDIT3 showed reduced ability to associate with NFKBIZ, which could explain the opposing effects seen between FUS-DDIT3 and DDIT3 on *IL8* expression. The interaction between FUS-DDIT3 and NFKBIZ was confirmed by co-immunoprecipitation experiments in which we could show that FUS-DDIT3 binds the C-terminal of NFKBIZ. Our results imply that FUS-DDIT3 and NFKBIZ bind directly or are present in a common complex and hereby regulate *IL8* transcription. To further strengthen this hypothesis, we showed that ectopically expressed NFKBIZ was on its own able to induce expression of *IL8*, *IL6* and *LCN2* in our experimental system, genes which are highly transcribed in FUS-DDIT3 expressing cell lines.

Based on these results, we here propose a novel mechanism of FUS-DDIT3-mediated gene regulation in which the fusion oncoprotein augments expression of NF-κB target genes through a cooperative action with the NF-κB protein NFKBIZ. This model is further supported by our previous findings of FUS-DDIT3-driven expression of the NF-κB-controlled *IL6* gene[156]. Elevated serum levels of IL6 and IL8 are demonstrated as independent prognostic factors of soft tissue sarcomas and associated with a significantly shortened overall survival of patients[160]. These factors participate in inflammation-related carcinogenesis through stimulation of cell proliferation, angiogenesis, invasion, metastasis and inhibition of apoptosis[161,162]. Thus, the functional interaction between FUS-DDIT3 and the NF-κB system, which plays a central role during inflammation[163], suggests a role for inflammation-related processes during myxoid liposarcoma tumorigenesis.

Paper III. FLT1 and PGF expression in *FUS-DDIT3* carrying cells

Normal cells receive stimulatory signals from their surroundings that are processed and integrated into a complex network of pathways within the cell. This signaling subsequently leads to a decision of whether the cell should divide or remain quiescent. Such regulation is essential to preserve cellular homeostasis in tissues of multicellular organisms. Many stimulatory signals are conveyed by growth factors* released by certain cells and these factors bind to growth factor receptors on the surface other cells. Deregulation of growth factor and growth factor receptor expression is associated with multiple forms of human cancer[164].

In this paper, we have studied the expression of the growth factor receptor FLT1 (VEGFR1) in *FUS-DDIT3* carrying cells. FLT1 belongs to the VEGFR family of receptor tyrosine kinases and is a receptor for PGF, VEGFA and VEGFB. Its best known role is in regulation of angiogenesis but it has also been associated with tumor growth and invasiveness of cancer cells[165]. Previously collected microarray data indicated that FLT1 is upregulated in FUS-DDIT3 expressing cells[70]. We investigated this finding further by quantitative real-time PCR analysis of HT1080 human fibrosarcoma cells stably expressing FUS-DDIT3, DDIT3 or the N-terminal of FUS present in the type II fusion. Cells expressing ectopic FUS-DDIT3 showed more than 20 times higher *FLT1* expression than untransfected HT1080 cells and DDIT3 expressing cells also showed minor but significant upregulation of *FLT1* expression. In contrast, cells expressing the 5' part of the *FUS* gene showed a slight downregulation of *FLT1* mRNA. Three cell lines derived from MLS/RCLS expressed high levels of *FLT1* transcripts, which further demonstrated that *FLT1* is expressed in *FUS-DDIT3* carrying cells. However, when HT1080 cells were transiently transfected with plasmid vectors expressing the above proteins, no changes in *FLT1* expression were detected during a 48 hour observation period. Therefore, the upregulated *FLT1* expression seen in cell lines with stable FUS-DDIT3 expression may not be directly attributed to a transcriptional activation by FUS-DDIT3. The FUS-DDIT3 protein has the capacity to induce adipocytic characteristics in HT1080 cells and the upregulation of *FLT1* expression could hence be a part of this process. This hypothesis is supported by measurements showing high *FLT1* expression in isolated normal adipocytes.

Immunohistochemical analysis of tumor tissue from patients diagnosed with myxoid liposarcoma and an MLS cell line xenografted in SCID mice showed a strong, predominantly nuclear expression of the FLT1 protein in tumor cells. Similar results were obtained from immunofluorescence analysis of cultured MLS-derived cells. The nuclear localization was surprising since FLT1 is commonly

* Relatively small soluble proteins capable of stimulating growth, proliferation and differentiation.

reported in the plasma membrane or cytoplasm[166]. Thus, we prepared nuclear and cytoplasmic fractions from the MLS/RCLS-derived cell line MLS 402-91 and analyzed these by western blot using an FLT1 specific antibody. This analysis confirmed the nuclear expression of the protein seen with immunostaining. We further analyzed whether FLT1 shows a nuclear localization in other tumor types and normal tissues. For this purpose, we used immunohistochemical analysis of human tissue arrays containing 59 normal and 60 tumor-derived tissues. In tumors, we observed strong nuclear staining of FLT1 in pancreatic carcinoma and ovary carcinoma. Mesenchymal cells present in several normal tissues also showed nuclear localization of FLT1 and most notably FLT1 antibodies stained the nuclei of adipocytes, suggesting that nuclear FLT1 is a normal feature of this cell type. In addition, fibroblasts in culture showed prominent FLT1 staining in the nucleus but had a stronger cytoplasmic staining compared with cultured MLS cells. These observations imply functions for FLT1 in the nuclear compartment of these cells and such functions have been reported for other receptor tyrosine kinases[167,168].

Microarray data from FUS-DDIT3 expressing HT1080 cells further suggested that *PGF*, encoding placental growth factor (PGF), could be transcribed in MLS cells. *PGF* expression is increased in several human cancers and serum levels of the encoded PGF correlate with clinicopathological features of multiple human malignancies[165]. Hence, we analyzed the expression of *PGF* in cultured MLS cells and MLS tumor tissue by quantitative real-time PCR. Results showed that *PGF* was indeed expressed in cultured MLS cells and to a much higher degree than in normal adipocytes. We also detected PGF expression in cultured MLS 402-91 cells by immunofluorescence. In contrast, *VEGFA* and *VEGFB*, encoding the other ligands of FLT1, were expressed to considerably lower levels in MLS cells than in adipocytes. These results suggest the existence of an autocrine circuit involving PGF and FLT1 operating in cultured MLS cells. However, analysis of MLS tumor tissue showed a more heterogeneous expression of the ligand coding genes, which could result from differences of *in vitro* and *in vivo* ligand expressions.

We assayed the presence of an autocrine loop in MLS cells by incubating the cells with an FLT1 blocking antibody reported to inhibit growth of tumor cells dependent on FLT1 signaling[169]. Treatment with this antibody failed however to affect the growth of MLS cells. The nuclear localization of FLT1 seen in MLS cells could be the reason for this as receptor-targeted antibodies would be ineffective if the receptor and ligand interact inside the plasma membrane. Such intracrine growth stimulatory circuits involving FLT1 were recently reported in mammary carcinoma cells[170]. To test for the presence of an intracrine circuit in MLS cells, we performed knockdown experiments using short interfering RNAs (siRNAs) targeting *FLT1* and *PGF* mRNA. However, we were not able to achieve efficient knockdown of transcripts in these cells and could not determine the existence and importance of such an intracrine signaling loop in MLS.

We conclude that the upregulation of *FLT1* seen in MLS is an indirect downstream effect of FUS-DDIT3 expression, which can induce a liposarcoma-like phenotype. The FLT1 protein is expressed as a nuclear protein in MLS tumors, in additional nonmesenchymal tumors as well as in normal mesenchymal cell types. This implies functions for FLT1 in the nuclear compartment of certain cells. The expression of the FLT1 ligand gene *PGF* in MLS cells suggests the existence of auto- or intracrine loops operating in these cells and such circuits could be of therapeutic relevance in treatment of myxoid liposarcomas.

Paper IV. DDIT3 binding of cyclin-dependent kinase 2

The cellular decision to divide or remain quiescent is ultimately governed by the cell cycle. This signal processing unit receives stimuli of both extracellular and intracellular origin and integrates these into a complex molecular control circuitry. Roughly, the cell cycle is divided into four phases* that comprise sequential phosphorylations of the pRb tumor suppressor protein by different cyclin/cyclin-dependent kinase (CDK) complexes. Completion of the entire cell cycle leads to DNA replication and cell division[171].

We have previously reported that MLS/RCLS cells show abnormal expression of the cell cycle regulators cyclin D and E as well as their associated cyclin-dependent kinases CDK4 and CDK2[172]. In this work, we searched for putative interaction partners of FUS-DDIT3 among G1 cyclins and CDKs, as these might provide insights into the molecular mechanisms by which the chimeric oncoprotein induces malignancy.

By transiently transfecting HT1080 cells with a *FUS-DDIT*-GFP construct, we could show that, in cells expressing the encoded recombinant protein, endogenous expression patterns of cyclin E and CDK2 were altered and that these proteins showed overlap with FUS-DDIT3 in the nuclear speckles previously reported for the fusion oncoprotein[145]. We thus speculate that translocation of cyclin/CDK complexes to such foci may result in changed phosphorylation patterns and substrate specificity for these complexes. To further confirm and interaction between FUS-DDIT3 and cyclin E/CDK2, we performed co-immunoprecipitation experiments using ectopically expressed variants of the fusion protein tagged with GFP. The experiments demonstrated that the DDIT3 part of the fusion binds to CDK2, while cyclin E was not found in immunoprecipitates. These results displayed similarities with previous reports showing that the DDIT3-related C/EBPα can disrupt cyclin/CDK complexes and thereby inhibit their activity[173]. However, we can not rule out that the absence of cyclin E in immunoprecipitates was due to the low levels of endogenous cyclin E detected in the cells used for these experiments. Further studies, using a mutant *FUS-DDIT3* construct lacking the sequences encoding the leucine zipper motif, showed that this domain was redundant for CDK2 binding. This suggests that DDIT3 binds CDK2 independently of heterodimerization with other C/EBP family members. When we performed

* During the S phase a cell synthesizes a replica of its genetic material DNA. In the M phase a cell undergoes mitosis and partitions all cellular components into two daughter cells. These phases are separated by the two tightly regulated "gap" phases G1 and G2, where the cell prepares for successful completion of the S and M phases. Cells that have ceased to proliferate, either by receiving anti-mitogenic signals, or by the lack of sufficient mitogenic stimuli, enter a nondividing, quiescent state termed G0.

sequence alignments between C/EBPα (which contains a region shown to bind and inhibit CDK2[173]) and DDIT3, we were unable to find a similar region in the DDIT3 protein, which implies that DDIT3 binds CDK2 in a different manner than C/EBPα.

In order to examine the biochemical relevance of a binding between DDIT3/FUS-DDIT3 and CDK2, we analyzed expression levels, half-life and the status of activating or inhibiting phosphorylations of CDK2 proteins present in FUS-DDIT3 expressing cells. Though, we did not detect significant differences in these parameters between FUS-DDIT3 expressing cells and control cells. To further analyze the functional effects of the DDIT3/FUS-DDIT3 and CDK2 interaction, we immunoprecipitated CDK2 from cells transfected with *DDIT3, FUS-DDIT3* and GFP constructs. Mass spectrometric analysis of the precipitates obtained from these experiments revealed an enhanced binding of CDK2 to components of the cytoskeleton in DDIT3 and FUS-DDIT3 expressing cells. Such an alteration in binding affinity could result in a change of the overall cytoskeleton structure, possibly leading to deregulation of motility.

To summarize, we demonstrate that endogenous CDK2 is translocated to nuclear structures characteristic for FUS-DDIT3 and that it binds to the DDIT3 part of the fusion oncoprotein. Cyclin E is also recruited to such nuclear structures but can not be found in DDIT3/FUS-DDIT3 immunoprecipitates. The interaction between DDIT3 and CDK2 appears to alter the protein binding affinity of CDK2, possibly leading to changed phosphorylation patterns and deregulation of cytoskeletal proteins in DDIT3 and FUS-DDIT3 expressing cells. In contrast to FUS-DDIT3, DDIT3 has been found to induce growth arrest as well as block adipogenesis under certain conditions[77,90] and phosphorylation of the DDIT3-binding C/EBPβ by CDK2 is required for adipogenesis[174]. In light of these findings, our results suggest that inhibition of proliferation and differentiation by DDIT3 could be mediated through an interaction with CDK2. Deregulation of proliferation, differentiation and cell motility are traits associated with the cancer phenotype. Hence, the significance of binding between FUS-DDIT3 and CDK2 in the context of myxoid liposarcoma development remains to be investigated.

Conclusions

The main findings of this thesis are concluded as follows:

- The FET proteins show heterogeneous cell type-specific expression patterns in human tissues and cells. Their expression levels are altered upon differentiation and they localize to stress granules in response to cellular stress. Furthermore, FUS and TAF15 localize to spreading initiation centers upon early cell spreading. Our results point to cell type-specific functions for the FET family proteins in regulation of gene expression during both normal and stress conditions (Paper I).

- FUS-DDIT3 deregulates expression of the NF-κB target gene *IL8* through a cooperative action with the NF-κB protein NFKBIZ. Together with previous findings of elevated *IL6* expression in MLS/RCLS cells, these results suggest a mechanism of FUS-DDIT3-driven tumorigenesis through deregulation of of NF-κB target genes. Our results further propose a role for inflammation-related processes in MLS/RCLS development (Paper II).

- The upregulation of FLT1 seen in FUS-DDIT3 expressing cells is likely a downstream result of a FUS-DDIT3-induced liposarcoma-like phenotype. Nevertheless, the nuclear expression of FLT1 and the expression of its ligand PGF in myxoid liposarcoma cells suggest the existence of an intracrine signaling loop in these cells (Paper III).

- FUS-DDIT3 binds the cell cycle regulator CDK2 through its DDIT3 part and this interaction alters the binding specificity of CDK2 to an increased affinity for cytoskeletal proteins. Our results imply that the inhibition of proliferation and differentiation caused by DDIT3 could be mediated by an interaction with CDK2. However, the significance of a binding between FUS-DDIT3 and CDK2 in the context of myxoid liposarcoma development remains to be clarified (Paper IV).

The multifunctionality of the normal FET proteins may explain why their oncogenic counterparts are often the sole detectable abnormalities in their associated tumors. Specifically, the FUS-DDIT3 protein studied in this thesis may promote several of the physical characteristics known as the hallmarks of cancer and thereby drive malignancy. Obviously, a direct targeting of FUS-DDIT3 would be optimal in the clinical setting. However, this work suggests that agents which induce cellular differentiation, inhibit inflammatory processes (in particular the NF-κB system) or block FLT1/PGF signaling may aid current treatments and thereby improve survival of patients afflicted with myxoid liposarcoma.

Future perspectives

With the advent of next generation DNA sequencing, whole-genome sequencing is now possible at a reasonable cost and timeframe[175]. We are now able to identify genetic changes at single base level in cytogenetically normal cells. Recently, such methods were successfully applied to analyze cells obtained from a patient suffering from acute myeloid leukemia, identifying eight previously unknown mutations believed to contribute to the progression of this disease[176]. These new techniques can hopefully soon be used to discover cancer-promoting mutations also in genomically unstable cells. The next big challenge will be to functionally characterize such mutations in an effort to determine which should be targeted therapeutically. Though, in addition to the genetic element of cancer, our conception of this multifaceted disease is further confounded by the existence of nongenetic factors with roles in tumor progression. For instance, chronic mitogenic stimuli have been linked to tumor development, mainly by promoting increased cell proliferation[177]. Moreover, heterotypic interactions between tumor cells and their microenvironment are recognized with an increased importance to cancer progression[178] and inflammatory processes seemingly serve a central role in the development of many human cancers[161]. The discovery of cancer stem cells[179,180] has dramatically changed our views of multistep tumorigenesis as these self-renewing cells, rather than the bulk population of cancer cells, may be the principal objects of genetic alteration and clonal selection that must be targeted in the clinic. However, even though the existence of pathological nongenetic factors further complicates our understanding of human cancer, they may at the same time provide new avenues for treatment of this devastating disease.

Populärvetenskaplig sammanfattning

Cancer är den näst vanligaste dödsorsaken i västvärlden och i Sverige upptäckts ungefär 50 000 nya fall per år. Cancer är egentligen en grupp av cirka 200 olika sjukdomar som främst drabbar äldre personer.

Det är när något går snett i kontrollen över kroppens egna celler som cancer kan uppstå. I de flesta cancerformer uppkommer en tumör, en samling förändrade celler med förmågan att föröka sig okontrollerat. Ibland kan celler från en sådan tumör sprida sig i kroppen och sätta dödliga dottertumörer (metastaser). Man vet idag att cancer beror på skador (mutationer) i arvsmassan (DNA). I arvsmassan finns över 20 000 olika gener som styr de otaliga processer som hela tiden pågår i en cell. Det är framförallt gener som har med cellförökning att göra som är muterade i cancer. Det räcker dock inte med att endast en gen blir muterad för att cancer skall uppstå och man har visat att ett flertal mutationer i olika gener krävs för canceruppkomst.

I denna avhandling har vi undersökt en familj av gener (FET familjen) som är muterade i över tio olika cancerformer. Dessa gener förekommer i en speciell form i sjukdomarna och hittas som s.k. fusionsgener. En fusionsgen uppkommer när två olika gener smälts samman och bildar en ny förändrad gen. Den nya genen består nu av delar från båda de två ursprungliga generna (se Figure 1b, sidan 15). De fusionsgener där FET gener ingår har skapats genom att två olika kromosomer brutits av och felaktigt fogats samman. Specifika FET fusionsgener har endast hittats i en eller ett fåtal tumörtyper och man tror att de förändrade generna bidrar stort till att just dessa tumörer uppkommer (se Figure 4, sidan 21). Gener utgör dock i de flesta fall endast mallar för proteiner, som är de verkliga aktörerna i cellen. Det är dessa molekyler som utför de olika livsnödvändiga funktionerna i cellen. När FET familjens fusionsgener uppkommer skapas abnorma proteiner som tros påverka ett flertal olika processer på cellnivå.

I det första arbetet har vi undersökt de vanliga, oförändrade FET proteinerna (FUS, EWS och TAF15) för att förstå vad som kan gå fel när dessa förändras i tumörceller. Vi har analyserat FET proteinernas förekomst (uttryck) och lokalisering i celler från en mängd olika humana vävnader. Från dessa försök drar vi slutsatsen att FET proteinerna visar stor variation i uttryck och lokalisering mellan olika celltyper. Detta tros innebära att de har specialiserade funktioner i olika celltyper och är involverade i cellers utmognad. Genom experimentella försök med odlade celler såg vi även att FET proteinerna verkar vara inblandade dels i cellens svarsreaktioner mot inre och yttre stress och dels när celler sprider ut sig på ytor. Förändringar i sådana processer är vanligt förekommande hos cancerceller.

I det andra arbetet har vi specifikt studerat fusionsproteinet FUS-DDIT3. Detta protein hittas endast i tumörformen myxoid liposarkom, en fettcellstumör som uppstår i kroppens mjukdelar. Vi har undersökt de molekylära mekanismer varmed FUS-DDIT3 påverkar ett flertal av cellens gener. Från dessa försök drar vi slutsatsen att FUS-DDIT3 kan felaktigt reglera en viss typ av gener genom att samarbeta med NF-κB proteinet NFKBIZ. NF-κB systemet är inblandat i inflammationsrelaterade processer och vi drar därför slutsatsen att sådana processer kan vara av betydelse för uppkomsten av myxoid liposarkom.

I det tredje arbetet undersöker vi ett protein, FLT1, som fungerar som en mottagare för signaler som härstammar från cellens utsida. Sådana signaler kan reglera cellförökning och används av kroppen för att hålla antalet celler i en vävnad i balans. I många cancerformer är dock dessa signalleringssystem satta ur spel och cellerna förökar sig oavsett vilka signaler som kommer utifrån. Oftast uttrycker en viss celltyp signalsubstansen och en annan celltyp mottagarproteinet. När vi studerade tumörceller som innehåller FUS-DDIT3 proteinet såg vi att dessa uttrycker både mottagarproteinet FLT1 och dess signalmolekyl PGF. Vi tror därför att dessa celler på så sätt kan stimulera sig själva att bli fler. Blockering av denna signalväg skulle därför kunna vara en möjlig väg till behandling av myxoid liposarkom.

I det sista arbetet har vi studerat om FUS-DDIT3 proteinet direkt kan påverka proteiner som verkar för cellförökning. Vi har funnit att FUS-DDIT3 binder till proteinet CDK2, ett protein som är centralt vid cellförökning. Denna inbindning verkar påverka CDK2s förmåga att koppla till sig andra proteiner. I nuläget vet vi dock inte vilken relevans interaktionen mellan FUS-DDIT3 och CDK2 har för tumörutveckling och detta kräver ytterligare studier.

Vi drar slutsatsen att FET fusionsproteinerna, och specifikt FUS-DDIT3, verkar kunna påverka en mängd olika kontrollsystem i cellen vilket kan leda till cancer. Detta tros vara anledningen till att man inte hittar så många ytterligare genförändringar i tumörer med FET fusioner. Vi föreslår även att behandling av myxoid liposarkom skulle kunna förbättras genom att använda medel som stimulerar cellutmognad, inflammationshämmande läkemedel eller mediciner som blockerar FLT1/PGF signalering.

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