The Role of Fusion Oncogenes and Cancer Stem Cells in Myxoid Liposarcoma

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Cover illustration: Micrograph of MCM6 protein expression and nuclei staining of myxoid liposarcoma 402-91 cell line by Soheila Dolatabadi.

*This thesis is dedicated to
my family*

"*The important thing is not to stop
questioning. Curiosity has its own
reason for existence. One cannot
help but be in awe when he
contemplates the mysteries of
eternity, of life, of the marvellous
structure of reality. It is enough if
one tries merely to comprehend a
little of this mystery each day.*"

-Albert Einstein 1955

ABSTRACT

Myxoid liposarcoma (MLS) is characterised by the *FUS-DDIT3*, or the less common *EWSR1-DDIT3* fusion oncogene and is the second most common type of liposarcoma. The fusion oncogenes encode chimeric transcription factors that are causal factors in tumourigenesis however, their functions are poorly known. Notwithstanding continuous progress in treating MLS patients, existing therapies suffer from a major flaw as they do not target the cancer stem cells (CSCs). Unique features of CSCs include self-renewal, tumour initiating capacity and increased resistance to radiotherapy- and chemotherapy-induced cell death. Thus, CSCs are crucial targets for successful therapy. The aims of this project were to define the role of fusion oncogenes in tumourigenesis and to define signalling pathways controlling CSC features in MLS. Here, we demonstrated that MLS has an intact TP53 system that may explain why this tumour entity is genetically stable. We investigated the regulatory mechanisms, expression levels and effects of *FUS-DDIT3* in detail, and showed that *FUS-DDIT3* was uniquely regulated at both transcriptional and post-translational level. We also screened 70 well-characterised kinase inhibitors and determined their effects on cell proliferation and *FUS-DDIT3* expression at mRNA and protein levels. To facilitate these studies, we developed a novel direct lysis approach that enables us to quantify, cell proliferation, mRNA and protein expression in the same sample. This method allowed us to identify a number of previously unknown signalling pathways that regulated the expression of *FUS-DDIT3*. To study cell division and growth in detail, we applied single-cell analysis on unsynchronized cells at different cell cycle phases and cell sizes. We found that the total transcript level per cell and the expression of most individual genes correlated with progression of the cell cycle, but not with cell size. Detailed studies of cell cycle predictive genes revealed a previously unknown G1 subpopulation. Finally, we showed that MLS contains cells with CSC features and that JAK-STAT signalling controls their numbers. Leukaemia inhibitory factor stimuli increased the number of CSCs, while JAK inhibition depleted the CSC pool. Inhibition of JAK-STAT also showed synergistic effects when combined with chemotherapy *in vitro*. Our findings concerning *FUS-DDIT3* function and CSCs have increased our molecular understanding of tumour development and therapy resistance in MLS that will facilitate development of specific treatment strategies.

Sammanfattning på svenska

Myxoid liposarkom (MLS) är en cancertyp som oftast utvecklas i kroppens mjukdelar, främst i muskelvävnad. MLS karaktäriseras molekylärt av en genetisk förändring där två kromosomer felaktigt sammanfogas och bildar en ny gen, en så kallad fusionsgen. Den vanligaste sjukdomsdrivande fusionsonkogenen i MLS är *FUS-DDIT3*, men även *EWSR1-DDIT3* förekommer. Utöver fusionsonkogenen har MLS, till skillnad från många andra tumörtyper, inte många genetiska förändringar. Dessa fusionsonkogener ger upphov till abnormala proteiner som kan styra uttrycket och funktionen av andra gener och är på så sätt viktiga för tumörbildningen. Trots denna centrala roll i MLS så är fusionsonkogenernas funktion dåligt kartlagd.

Tumörer innehåller flera olika typer av cancerceller och man tror att det finns en ovanlig typ, cancerstamcellerna, som är extra viktiga för tumörens utveckling och behandlingsresistens. Trots ständig utveckling av behandlingsmetoder för MLS så finns det inga som är direkt riktade mot dessa potentiellt mycket farliga cancerstamceller.

Syftet med detta arbete var att definiera fusionsonkogenernas roll i tumörbildning samt att definiera de signalvägar som kontrollerar cancerstamcellernas egenskaper i MLS. Först visade vi att MLS oftast har ett välfungerande TP53-system vilket skyddar cancercellerna från att få fler mutationer och därmed gör dem genetiskt stabila. Vi har också kartlagt de mekanismer som styr mängden av fusionsonkogenen *FUS-DDIT3* på både transkript- och proteinnivå. Vidare så utvecklades även en metod för att kunna analysera uttryck av transkript, protein samt celltillväxt i ett och samma prov. Denna metod använde vi för att identifiera signalvägar som påverkade både celltillväxt och uttryck av *FUS-DDIT3* i MLS via behandling med olika droger.

Cellcykeln är ofta påverkad i cancer och vi studerade detta genom att analysera enskilda MLS-celler i olika cellcykelfaser. Vi visade att den totala nivån av transkript i cellerna ökade när cellerna förflyttade sig genom cellcykeln. Vi upptäckte även att det fanns två olika typer av celler i den första cellcykelfasen. Slutligen så kunde också identifiera att en signalväg kallad JAK-STAT reglerade mängden cancerstamceller i MLS. Genom att blockera denna signalväg med en specifik drog kunde vi minska antalet cancerstamceller i MLS, speciellt i kombination med en i MLS vanligt förekommande cellgiftsbehandling.

Våra upptäckter gällande funktionen av *FUS-DDIT3* och cancerstamceller i MLS ökar kunskapen gällande tumörutveckling och behandlingsresistens i denna sjukdom vilket möjliggör att mer effektiva och specifika behandlingsstrategier kan utvecklas.

LIST OF PAPERS

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

I. Ståhlberg A, Kåbjörn Gustafsson C, Engtröm K, Thomsen C, **Dolatabadi S**, Jonasson E, Li CY, Ruff D, Chen SH, Åman P. Normal and Functional TP53 in Genetically Stable Myxoid/Round Cell Liposarcoma. *PLoS ONE*, 2014.

II. Åman P, **Dolatabadi S**, Svec D, Jonasson E, Safavi S, Andersson D, Grundevik P, Thomsen C, Ståhlberg A. Regulatory mechanisms, expression levels and proliferation effects of the FUS-DDIT3 fusion oncogene in liposarcoma. *J Pathol*, 2016.

III. **Dolatabadi S**, Candia J, Akrap N, Vannas C, Tomic T, Losert W, Landberg G, Åman P, Ståhlberg A. Cell cycle and cell size dependent gene expression reveals distinct subpopulation at single-cell level. *Frontiers in Genetics*, 2017.

IV. Svec D*, **Dolatabadi S***, Thomsen C, Cordes N, Shannon M, Fitzpatrick P, Landberg G, Åman P, Ståhlberg A. Identification of inhibitors regulating cell proliferation and FUS-DDIT3 expression in myxoid liposarcoma using combined DNA, mRNA and protein analyses. *Manuscript*.

*These authors contributed equally

V. **Dolatabadi S**, Jonasson E, Lindén M, Fereydouni B, Bäcksten K, Nilsson M, Martner A, Åman P, Ståhlberg A. JAK-STAT signalling controls cancer stem cell properties in myxoid liposarcoma. *Manuscript*.

Papers not included in the thesis

I. Safavi S, Jarnum S, Vannas C, Udhane S, Jonasson E, Tomic T.T, Grundevik P, Fagman H, Hansson M, Kalender Z, Jauhiainen A, **Dolatabadi S**, Stratford E.W, Myklebost O, Eriksson M, Stenman G, Stock R.S, Ståhlberg A, and Åman P. HSP90 inhibition blocks ERBB3 and RET phosphorylation in myxoid/round cell liposarcoma and causes massive cell death in vitro and in vivo. *Oncotarget,* 2016.

II. Kroneis T, Jonasson E, Andersson D**, Dolatabadi S**, and Ståhlberg A. Global preamplification simplifies targeted mRNA quantification. *Scientific Reports,* 2017.

Contents

Abbreviations

ABC	ATP-binding cassette
ALDH	Aldehyde dehyrogenase
ATP	Adenosine triphosphate
CDC6	Cell division cycle 6
Cdk	Cyclin-dependent kinase
CDT1	Cdc10 dependent transcript 1
CEBP	CCAAT-enhancer-binding protein
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
CSC	Cancer stem cell
CTF1	Cardiotrophin-1
DNA	Deoxyribonucleic acid
hnRNP	Heterogeneous nuclear ribonucleoprot
HP1γ	Heterochromatin protein 1 gamma
IL6	Interleukin 6
IL11	Interleukin 11
JAK	Janus kinase
LIF	Leukaemia inhibitory factor
MCM	Mini chromosome maintenance
MLS	Myxoid liposarcoma
ORC	Origin recognition complex

OSM   Oncostatin M

PIAS   Protein inhibitor of activated STAT

PPARγ2   Proliferator-activated receptor-γ 2

RBL2   Retinoblastoma-like 2

RGG   Arginine-glycine-glycine repeat/motif/box

RRM   RNA recognition motif

SOCS   Suppressor of cytokine signalling

STAT   Signal transducer and activator of transcription

SYGQ   Serine-tyrosine-glycine-glutamine rich

UTR   Untranslated region

ZF   Zinc finger domain

Introduction

Cancer

Cancer is a heterogeneous group of diseases characterised by immortalised and proliferative cells, which are growing in an uncontrolled manner and is a major cause of death worldwide. Its incidence is primarily associated with increased age even though cancer occurs in all age groups, including children and young people [1]. Cancer originates from a single normal cell that has received several mutations and features, termed "hallmarks of cancer" [2]. In 2000 Hanahan and Weinberg proposed six essential hallmarks of cancer: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. In 2011, two new additional hallmarks, reprograming of energy metabolism and evading immune destruction, were proposed to be involved in pathogenesis of cancer [3]. Full transformations of normal cells to a neoplastic state require multistep alterations of all eight stated hallmarks, however, the mechanism, order and time scale of these changes varies between individual malignancies. The term tumour and cancer are widely used in many publications without stating that the tumours are not invasive or metastatic. Hence, the forms of cancer that invade into surrounding tissues and metastasise to distant sites in the body are referred as malignant tumours [4]. In contrast, the term benign tumour (non-cancerous tumour) refers to abnormal cells that do not invade or metastasise to the surrounding area of the body and, in general, grow slowly [5]. Thus, the proposed hallmarks of cancer with the exception of "invasion and metastasis", are also characteristics of benign tumours.

Malignant tumours are categorized by the type of cells that the tumours originate from. Tumours that arise from epithelial tissues are classified as carcinoma. The remaining tumours arising from non-epithelial cells are categorized into: lymphoma and leukaemia which are derived from hematopoietic cells, neuroectodermal tumours derived from the central and peripheral nervous system, and sarcomas derived from mesenchymal cells. Sarcoma is a rare and heterogeneous group of malignant tumours, arising in or from bone and connective tissues such as muscle, fat, peripheral nerves,

fibrous, or other tissues supporting the body [6, 7]. The histopathological spectrum of sarcomas is broad, and based on their cells of origin they are divided into different types such as osteosarcoma (osteoblasts), leiomyosarcoma (smooth muscle cells), fibrosarcoma (fibroblasts) and liposarcoma (adipocytes) [8].

Today, cancer is usually treated with surgery, radiation and chemotherapy. Despite an improved survival of patients standard therapies have major shortcomings as they are unspecific and cause many unwanted side effects. Hence, immune-, endocrine- and targeted therapies have been developed. However, therapy responses are still often temporary. To overcome therapy failures we need to have better understanding of the complex tumours and the mechanisms involved in tumour initiation and development, which eventually will allow the development of tumour-specific treatments with few side effects.

Myxoid liposarcoma

Sarcomas account for less than 1 percent of all adult malignancies and 12 percent of pediatric cancers [9-11]. Liposarcomas, which are categorized in three subtybes; well-differentiated, pleomorphic and round-cell/ myxoid liposarcoma (MLS), account for 15 to 20 percent of all sarcomas, making them the most common type of sarcoma in adults [12]. The Scandinavian Sarcoma Group has reported that liposarcomas constitutes 17.5 percent of all registered sarcomas (5837 patients) from 1987 through 2011 [13]. MLS is the second most common liposarcoma subtype that constitutes about 10 percent of all adult soft tissue sarcomas. MLS grows in the deep soft tissue, including muscle and fat of the extremities, and the majority of cases occur in the thighs. Histologically, MLS tissue is composed of uniform round to oval-shaped mesenchymal cells. MLS is notable for a high abundance of extracellular matrix with myxoid appearance and relatively sparse cellular components, set in a myxoid matrix with a fine piped capillary network (Fig. 1) [14, 15]. In addition to histological criteria, MLS is characterised by its karyotypic hallmark, the chromosomal translocation t(12;16)(q13;p11) that results in a fusion oncogene arrangement between *FUS* and *DDIT3* (90 percent of the cases). In rare cases, an alternative translocation event occurs, t(12;22)(q13;q12), that results in an *EWSR1-DDIT3* fusion oncogene [16]. Approximately, one third of all MLS patients develop distant metastasis with special tendency to recur in extrapulmonary sites such as retroperitoneum (anatomical space in abdominal cavity behind the peritoneum), opposite extremity, axilla, and bone [17]. In a subset of cases, the cellularity increases with a predominance of round cells containing a high nuclear to cytoplasmic ratio and clearly visible nucleoli. MLS with more than 5 percent of cells presenting round cell characteristics is associated with a worse clinical outcome, since they are considered to be aggressive with high risk to develop metastases [18].
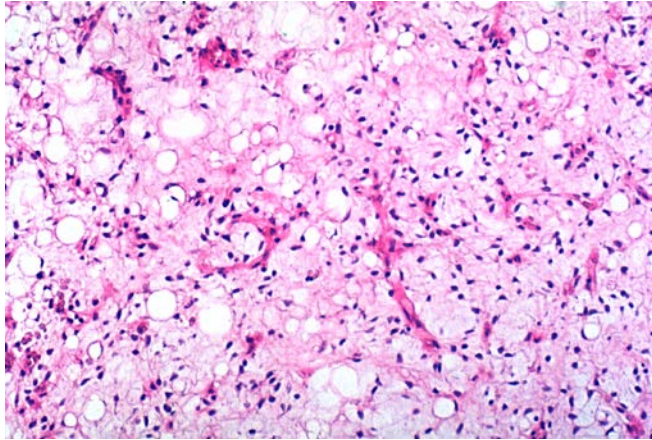
Figure 1. Immunohistochemistry of myxoid liposarcoma. A typical MLS morphology is shown. MLS is characterised by a large myxoid extracellular matrix, low cell density and a fine piped capillary network. The tumour contains several cell types, where most cells display a mesenchymal phenotype, but lipoblasts are also commonly observed.

Surgery is the most common treatment for localized, primary MLS. In patients with advanced or metastatic disease, radiation and cytotoxic chemotherapy are used [19]. The most commonly chemotherapeutic drugs used for treatment of MLS are trabectedin, doxorubicin and a combination of doxorubicin and ifosfamide [20, 21]. The combination of doxorubicin and ifosfamide has resulted in a synergistic response rate of 43 percent in MLS [22]. Despite the success of using chemotherapy to treat MLS, chemotherapy is not beneficial for all patients and is also associated with high toxicity and side effects, such as neutropenia (low concentration of neutrophils in the blood), nausea (sickness of the stomach), and anaemia (low amount of red blood cells) [20, 23]. Hence, new specific treatments are needed that target genuinely malignant subsets of cancer cells.

The genetics of myxoid liposarcoma

Fusion oncogenes

Chromosome abnormalities have an important role in the initiation of human cancer. Chromosomal translocations are considered as primary causes for many cancers, including hematopoietic, lymphoid and solid tumours [24]. An oncogenic chromosomal translocation can broadly have two consequences. One is to generate oncogenic fusion proteins, formed by fusion of two genes with breakpoints located within protein coding regions. An example of this mechanism is the BCR-ABL oncoprotein formed as a result of chromosomal translocation t(9;22)(q34;q11) in chronic myeloid leukaemia [25]. The second mechanism is a juxtaposition of the coding region of a gene near the transcriptionally active promoter or enhancer region of another gene, hence leading to

their altered expression. The overexpression of the proto-oncogene c-*MYC* as a result of the juxtaposition of c-*MYC* to the regulatory element of immunoglobulin heavy chain in Burkitt lymphoma is a classic example of this second mechanism of oncogenic chromosomal translocation [24]. The use of advanced sequencing techniques during the past decade has revealed numerous gene fusions in various cancer types. Gene fusions occur in the majority of lymphomas, over half of leukaemias [26], and in one third of soft tissue tumours [27]. The probability that a tumour is formed as a result of fusion oncogenes depends on several factors, including the fusion oncogene mutation rate, cell type, and accumulation of additional genetic and/or epigenetic changes [28]. The majority of discovered fusion oncogenes encode abnormal transcription factors, while a minority express chimeric proteins that deregulate growth factor signalling [29, 30].

Interestingly, a large number of sarcoma fusion oncogenes have shown to be tumour-type specific. Three models may describe this specificity; (i) a cell type-specific mechanism for chromosomal rearrangements, (ii) cell/tissue-type dependence for survival/oncogenic activity, and (iii) phenotype-instructive activity of the fusion oncogene [31-35]. In some tumours, including MLS, the fusion oncogenes may be the only observed mutation. Several of these fusion oncogenes have also been shown to have the capacity to transform cells in culture and to form tumours in transgenic mice, indicating that the fusion oncogenes have an "instructing master activity" that differentiates the cell towards a specific cell fate [36]. These properties of fusion oncogenes suggest their importance in tumour initiation and development and their importance as diagnostic and prognostic biomarkers. Fusion oncogenes are also considered to be powerful therapeutic targets, supported by data showing that targeting fusion oncogenes cause cell death and decreased proliferation [37, 38].

FET family of fusion oncogenes

The FET group (*FUS*, *EWSR1* and *TAF15*) of fusion oncogenes are found in human sarcomas and certain leukaemias [28]. MLS and Ewing sarcoma are the two most common entities, while the other tumour types are extremely rare. All three genes (*FUS, EWSR1* and *TAF15*) form fusion oncogenes with different transcription factors as a result of chromosomal translocations (Fig. 2). These mutations cause the N-terminal domain of *FUS*, *EWSR1* or *TAF15* to become juxtaposed to the C-terminal parts of various transcription factors, thereby forming abnormal chimeric transcription factors.

The FET family genes encode proteins with comprehensive structural similarities that share a number of highly evolutionarily conserved regions including an N-terminal serine-tyrosine-glycine-glutamine rich sequence, variable number of arginine-glycine-glycine repeats, G-rich regions, a zinc finger domain, and a 87-amino acid RNA recognition motif (Fig. 3) [39]. FET proteins are ubiquitously expressed in all cell types

and are involved in: (a) transcriptional regulation by binding to both eukaryotic RNA polymerase II and transcription factor II [40], (b) microRNA (miRNA) processing by modulating the activity of the Drosha microprocessor complex which is required for miRNA biogenesis [41, 42], (c) pre-mRNA splicing due to interactions with various splicing and transcription factors [43, 44], (d) RNA transport in which FET proteins in complex with heterogeneous nuclear ribonucleoproteins (hnRNPs) shuttle between nucleus and cytoplasm [45], (e) translation [46], and (f) DNA repair, as they promote homologous DNA pairing and DNA D-loop formation [47].
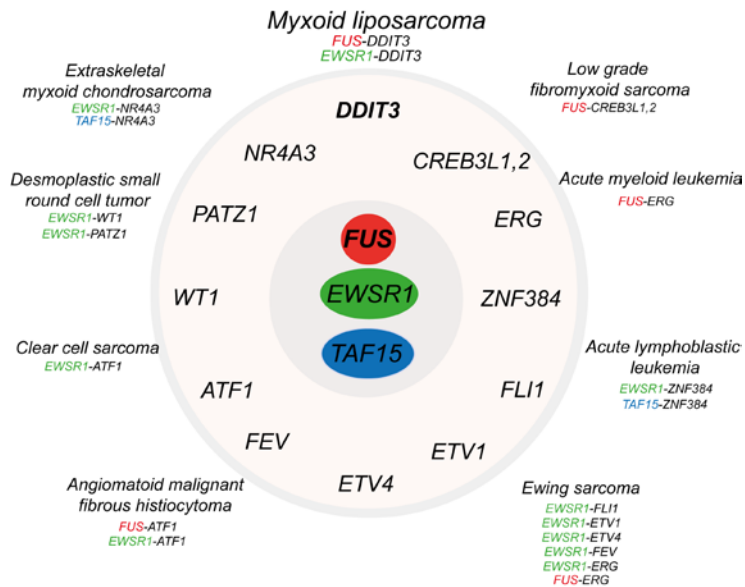

Figure 2. FET sarcomas and leukaemias are characterised by fusion oncogenes. FUS, EWSR1 and TAF15 (inner circle) form fusion oncogenes with specific transcription factor genes (outer circle). The latter determines the tumour entity. The common fusion oncogenes for respective tumour entity are shown.

FUS-DDIT3

Earlier research of chromosome analysis has identified the t(12;16)(q13;p11) translocation, resulting in the *FUS-DDIT3* fusion oncogene in MLS. Depending on the location of the fusion genetic breakpoints, different isoforms of the *FUS-DDIT3* fusion transcript are formed. Type I and II fusion transcripts are the most common isoforms generated by alternative splicing of exon 5 or 7 of the *FUS* gene to exon 2 of the *DDIT3* gene [48]. The *FUS-DDIT3* chimeric gene encodes a protein that functions as an aberrant oncogenic transcription factor (Fig. 3).

The FUS-DDIT3 protein is localised to the nucleus. In most FUS-DDIT3 forms, the central and the C-terminal domains of FUS are lost and replaced by the entire DDIT3 protein. Our group has previously identified an evolutionarily conserved N-terminal motif (FET binding motif, FETBM1) in all FET protein that mediates binding of the full-length FET proteins in homo- and heterocomplexes (Fig. 3B) [49]. The binding motif is retained in all FET fusion oncoproteins, mediating the binding of the fusion oncoprotein to all three normal FET proteins. This binding site is believed to play an important role in transcription factor activity of FUS-DDIT3 [49].
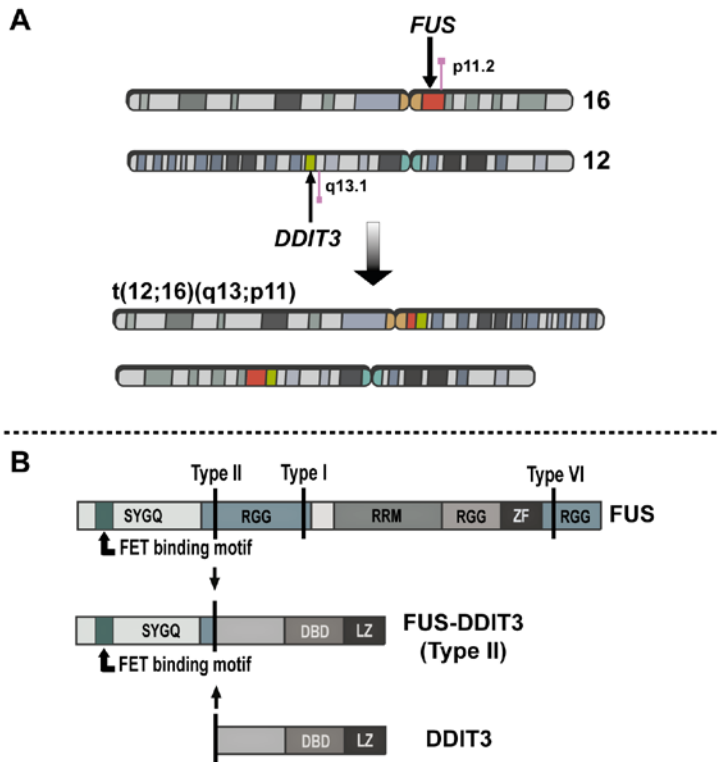
Figure 3. Chromosomal translocation t(12;16)(q13;p11) and FUS-DDIT3 protein domains. *(A) The chromosomal translocation t(12;16)(q13;p11) forms the fusion oncogene FUS-DDIT3. In FUS-DDIT3, the N-terminal part of FUS is fused to the entire DDIT3. (B) The FUS protein domain contains a 26-amino acids long FET binding motif, a serine-tyrosine-glycine-glutamine (SYGQ)-rich region, an argenine-glycine-glycine (RGG) region, an RNA recognition motif (RRM), and a zing finger (ZF) domain. DDIT3 contains DNA-binding domain (DBD) and leucine zipper (LZ) domain structures. All FUS-DDIT3 forms contain the FET binding motif and SYGQ domains of FUS and both DDIT3 domains. The breakpoints for three different FUS-DDIT3 forms (Type I, II and VI) are shown.*

DDIT3 determines the tumour entity, while the N-terminal part of FET proteins may replace each other whilst giving rise to the same tumour type (Fig. 2) [50]. Expression of FUS-DDIT3 in transgenic mice has been shown to develop MLS-like phenotype in adipose tissue only, indicating the sensitivity of certain target cells for the action of FUS-DDIT3 [36]. Furthermore, in another study it has been shown that FUS-DDIT3 has the ability to transform mesenchymal stem cells to MLS-like tumour cells. In contrast to the previous study, the used transgenic mice model required inactivation of TP53 to form tumours [51]. FUS-DDIT3 commits mesenchymal progenitor cells to the adipocyte lineage, followed by blockage of terminal differentiation of preadipocytes [31, 51]. Termination of adipocyte differentiation at the transcriptional level occurs by interaction of FUS-DDIT3 with proliferator-activated receptor-γ 2 (PPARγ2) and CCAAT-enhancer-binding protein-α (C/EBPα), two key players in terminal adipocyte differentiation. It is known that normal DDIT3 forms heterodimers with other CCAAT-enhancer-binding protein members and this capability is preserved by the oncogenic FUS-DDIT3 protein. Therefore, the DDIT3 domain of the fusion oncoprotein is thought to be responsible for blockages of adipocyte differentiation [52-54]. In conclusion, the cell or origin for MLS initiation is believed to be a mesenchymal stem cell, but the exact nature of this tumour initiating cell remains unknown, especially in human.

Cell cycle regulation in myxoid liposarcoma

The cell cycle control system is the regulatory network that controls the order and timing of the cell cycle. Cyclin-dependent kinases (Cdks) which govern distinct cell-cycle events are key components of the cell cycle. Mammalian cells have nine Cdks, of which only four (Cdk1, 2, 4 and 6) are involved in the cell cycle, and their enzymatic activation requires their binding to cyclin subunits. In most cases, full Cdk activation requires phosphorylation of its threonine residues by Cdk-activating kinases. Cyclins are divided into 4 groups, based on the timing of their expression: G1/S (cyclin E), S (cyclin A), and M (cyclin B) cyclins, which are involved in cell-cycle control, and G1 cyclins (cyclin D) which controls cell-cycle entry (Fig. 4). The activity of cyclin-Cdk complexes is regulated by the addition or removal of inhibitory phosphorylation and by the expression level of various Cdk-inhibitory proteins that, by binding to cyclin-Cdk complexes, causes inactivation of cell cycle complexes. Cdk-inhibitory proteins are important for G1 cell-cycle arrest during unfavourable situations or when DNA damage occurs. CDKN1B (P27) and CDKN1A (P21) are two examples, which inhibit both cyclin E-Cdk2 and cyclin A-Cdk2 complexes.

In MLS, a substantial proportion of tumour cells are senescent [55]. Cellular senescence refers to permanent and irreversible cell-cycle arrest without undergoing cell death that otherwise may occur when cells experience oncogenic stress [56]. Senescent MLS cells are

characterised by the expression of certain cytokines and cytokine receptors as well as an increase in the expression of heterochromatin protein 1 gamma (HP1γ) and retinoblastoma-like 2 protein (RBL2). All are involved in cell cycle regulation and maintenance of cell senescence [14]. Low expression of proliferation marker Ki67 also indicates a low cell division rate and mitotic activity of MLS cells [14, 57].
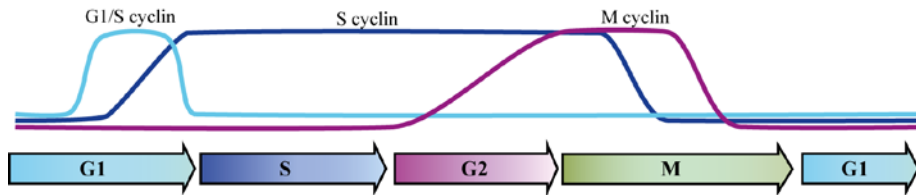
Figure 4. Cell-cycle control system. *The concentration of three main cyclins during the cell-cycle phases are shown.*

Normal DDIT3 causes G1/S arrest in stress conditions and prevents cells from progressing throughout the cell cycle. In contrast, FUS-DDIT3 expression does not mediate growth arrest [58], even if FUS-DDIT3 induction *in vitro* and *in vivo* has been shown to decrease growth rate, increased senescence and even caused cell death [59]. FUS-DDIT3 deregulates the expression of target genes such as growth-controlling genes. Previous studies of cell cycle regulating proteins in MLS revealed abnormal expression patterns of several cell cycle controlling proteins. Cyclins D1 and E together with their associated kinases Cdk4 and Cdk2, which are associated with the G1 cell-cycle phase, are strongly overexpressed in all MLS tumours. At the same time, cyclin A, which is specific for the S and G2 phases of the cell cycle, is lowly expressed in these tumours [57]. Additionally, Cdk inhibitors CDKN2A (P16), CDKN2D (P19) and CDKN1B (P27) are also highly expressed together with G1 cyclins-Cdk complexes. Hence, a deregulated cell-cycle possibly plays an important role in the pathogenesis of MLS. Defining the mechanisms and understanding whether the specific *FUS-DDIT3* fusion oncogene is directly involved in deregulation of the cell cycle requires the analysis of individual cell cycle regulators in more detail.

Cancer stem cells and tumour heterogeneity

Tumours, including MLS, display large inter- and intraheterogeneity with multiple cell types that display different phenotypes and genotypes. Numerus factors, including genetic and epigenetic changes as well as the microenvironment, contribute to tumour cell heterogeneity. The clonal evolution and cancer stem cell (CSC) models are two theories that have been postulated to account for intratumoural heterogeneity. The clonal evolution model is a non-hierarchical tumour development model where genetic

and epigenetic aberrations during tumour development result in a selective growth advantage for specific cells that contribute to tumour development. Conversely, the CSC model suggests a hierarchical manner where a small subset of cells (CSCs), are capable to sustain tumourigenesis and to generate heterogeneity through different degrees of differentiation. The CSC model, at least to some degree, mimics the cell hierarchy observed in normal development (Fig. 5) [60, 61].

CSCs refer to a subset of tumour cells that has the ability to self-renew and generate the distinct cells that constitute the tumour. These cells have been termed CSCs to reflect their 'stem-like' features and tumourgenic capabilities [62]. CSCs are low proliferative cells with the tendency to resist therapy and they are responsible for tumour initiation, progression and metastasis [63]. CSCs have been shown to be associated with aggressive disease, poor prognosis and therapy relapses. Thus, characterisation and elimination of CSCs is crucial in patient treatment [64].
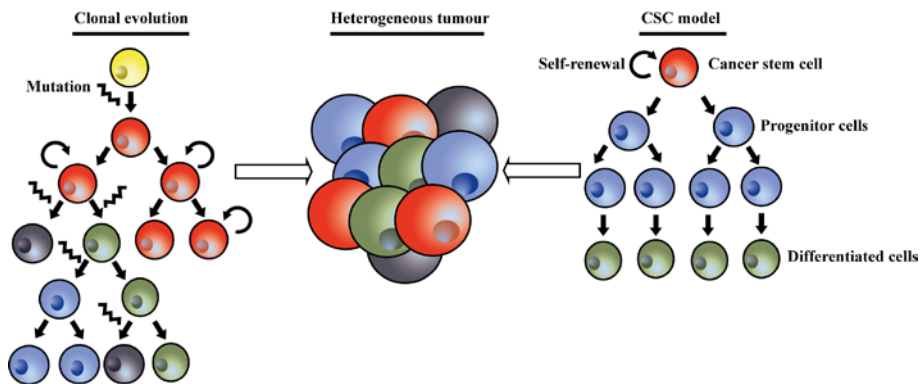
Figure 5. Tumour heterogeneity and theoretical models. *The clonal evolution (left) and the cancer stem cell (right) models are illustrated. A third concept is a combination of these two models.*

Al-Hajj and co-workers were the first to identify CSCs in solid tumours [65]. After that, CSCs have been identified in various tumour entities, such as melanoma [66], glioblastoma [67], prostate cancer [68] and lung cancer [69]. As mentioned earlier, CSCs are quiescent or slowly dividing cells and this feature of CSCs might potentially be one of the mechanisms by which CSCs resist therapy. The currently developed therapeutic agents generally target proliferative cells. Thus, CSCs can survive therapy and remain vital to regenerate the tumour [64]. Another mechanism by which CSCs resist therapy is through the expression of adenosine triphosphate-binding cassette (ABC) transporters. ABC transporters are complex molecular pumps that are able to efflux a wide range of substrates from the cells by the hydrolysis of adenosine triphosphate (ATP) [70]. Elevated expression of ABCB1, ABCG2 and ABCC1 proteins is associated with drug resistance

in several tumour types, such as breast cancer, lung cancer, acute myeloid leukaemia and pediatric sarcoma [71-73]. Furthermore, CSCs have an enhanced DNA repair capacity, which is yet another mechanism of therapy resistance [74]. All these properties of CSCs contribute to therapy resistance. Hence, improved understanding of CSC functions and properties are essential to develop better targeted treatment strategies.

Isolation and characterisation of cancer stem cells

Identification and isolation of CSCs can be performed with different methodologies. CSC enrichment using the expression of specific surface markers is today a widely used approach. One of the first reported strategies was demonstrated in leukaemia, where CSCs were isolated by collecting CD34^{+} and CD38^{-} cells [75]. Since then CSCs have been enriched using different sets of cell surface markers, including CD20, CD24, CD34, CD44, CD90, CD117 and CD133, in various tumour entities [76]. Despite the successful use of CSC surface markers, several studies have shown that most markers are not always expressed by all CSCs of the same tumour type [77, 78]. In addition, the expression of CSC surface markers are affected by various factors [61, 79]. Hence, it is important to consider several biological and technical aspects when using antibodies targeting CSC surface markers, including using other isolation techniques. If possible, multiple isolation techniques may be applied to increase specificity and limit the possibility to miss subpopulations of CSCs.

Cancer stem cells can also be isolated based on their functional properties using the aldehyde dehydrogenase (ALDH) assay, the side population (SP) discrimination assay and the non-adherent sphere formation assay [61]. Previous studies have shown that ALDH plays an important role in stem cell biology and therapy resistance. These studies have shown that cells with high ALDH activity are highly associated with enhanced tumourigenicity and CSC characteristics [80]. However, ALDH activity is problematic to be detected in numerous tissues, ALDH exists in multiple isoforms and chemotherapy may influence their activity. Hence, ALDH activity is not always a universal CSC marker [81, 82].

The SP discrimination assay is an *in vitro* method to identify CSCs based on their ability to efflux Hoechst dye staining via the ABC protein family. In this method, CSCs subsequently efflux the loaded Hoechst DNA binding dye out of the cell membrane using ABC transporters, whereas non-CSCs retain the dye. Identified SP cells can undergo asymmetric division generating both SP and non-SP populations. In addition, SP cells have displayed an increased capacity of self-renewal and tumourigenicity when transplanted into immune-deficient mice [83-85]. Consequently, the existence of a SP phenotype may explain why tumours contain subpopulations that display chemotherapy resistance [70, 86]. However, it is important to note that not all tumours contain SP populations. Therefore, SP cells may define one specific type of CSCs.

The non-adherent condition assay (sphere formation assay) is another *in vitro* assay that has been adapted for the quantification of stem cell activity and self-renewal [87]. Stem and progenitor cells can grow and form spherical structures in serum-free medium in non-adherent culture conditions when seeded as single-cells. These cells are equipped with the unique feature to avoid anoikis in order to overcome apoptosis signalling. Observation of enriched SP fractions, CSC surface markers and expression of pluripotency associated markers in sphere cells compared to non-sphere cells demonstrates that the sphere formation assay can provide a functional *in vitro* tool to investigate pathways involved in stem/progenitor cell survival [88].

The JAK-STAT signalling pathway

The Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway is an intracellular signal transduction cascade linking extracellular cytokines, interleukins and growth factors with nuclear gene transcription [89]. The JAK-STAT pathway is involved in many physiological processes including cell proliferation, differentiation, cell migration, survival, apoptosis, development and inflammation [90, 91]. Activation of JAK-STAT pathway occurs when any from a variety of ligands bind to transmembrane receptors families. Ligand-receptor binding produces conformational changes in their receptors, causing hetero- or homo-dimerization of receptor subunits. This multimerization of receptors brings two JAKs into close proximity allowing them to phosphorylate each other. The activated JAKs subsequently phosphorylate tyrosine residues in the cytoplasmic tails of the receptors, creating binding sites that recruit STATs. Once STATs are bound to the receptors, JAKs can phosphorylate STATs (p-STAT) on tyrosines, causing the STATs to dissociate from the receptor. Activated STATs then forms dimers that translocate to the nucleus and binds to specific enhancer elements [92-94] (Fig. 6). Negative regulators that are divided in three major classes: protein tyrosine phosphatases, protein inhibitors of activated STAT (PIAS) and suppressors of cytokine signalling (SOCS), modulate the activation of the JAK-STAT signalling pathway at multiple levels [92]. Protein tyrosine phosphatases remove phosphate groups from both JAKs and STATs. Protein inhibitors of activated STAT (PIAS) inhibit the DNA binding of STATs, control STAT cellular localisation and assist post-translational modification of STATs [93]. Suppressors of cytokine signalling (SOCS) inhibit the JAK-STAT signalling through three mechanisms: (i) blocking the recruitment of STATs, (ii) inhibiting JAK kinase activity by binding to the phosphorylated JAKs and their receptors and (iii) by acting as ubiquitin ligases and thereby causing degradation of JAK-STAT components with proteasomes [92, 95].

The mammalian JAK family has four members, JAK1-3 and Tyk2. JAK1, JAK2 and Tyk2 are ubiquitously expressed, whereas expression of JAK3 is mainly found in the haematopoietic system [95, 96]. JAKs have unique structures compared to other protein

tyrosine kinases and are composed of seven regions of conserved homology, known as JAK homology domains (JH1-JH7). The mammalian STAT family comprises seven structurally and functionally related proteins: STAT1-4, STAT5A, STAT5B and STAT6. As a result of specific ligand-receptor activation distinct dimers of active STATs can be generated [97, 98]. Various cytokine receptors can act via the JAK-STAT pathway. Interleukin 6 (IL6) family of cytokines compromises IL6, IL11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CTF1) and cardiotrophin-like cytokine (CLC) [99]. The IL6-type cytokines activate the JAK-STAT signalling pathway via signal transducers glycoprotein 130 (GP130, also known as IL6ST or CD130), LIF receptor (LIFR) and OSM receptor (OSMR) [100]. These cytokines strongly active STAT3 and to a less extent other STAT family members (STAT1 and STAT5) [101]. Phosphorylated STAT3 plays a central role in transcriptional regulation of a wide range of genes involved in cell growth, survival, differentiation, cell movement and pluripotency [101, 102].
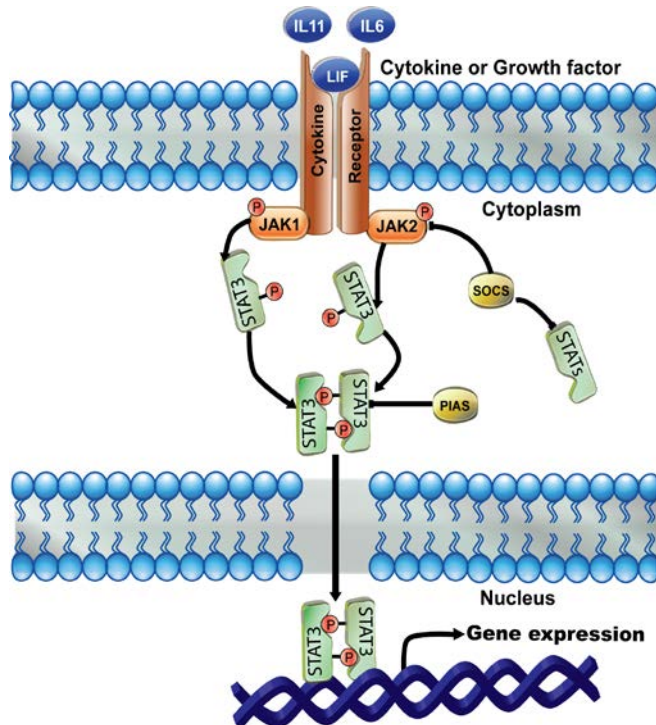
Figure 6. The JAK-STAT signalling pathway. *Schematic and simplified illustration of the canonical JAK-STAT pathway. Janus kinase (JAK), signal transducer and activator of transcription (STAT), protein inhibitors of activated STAT (PIAS) and suppressors of cytokine signalling (SOCS) are shown. Cytokines bind to their receptors, which phosphorylate JAKs, causing activation of STATs. Dimerized phospho-STATs enter the nucleus, resulting in gene regulation.*

Various human malignancies are characterised by abnormal JAK-STAT activation [103]. Somatically acquired JAK2 kinase point mutation (V617F) has been frequently found in haematological malignancies, such as myeloproliferative neoplasms and cancers arising from hematopoietic progenitor cells [104]. In addition, large numbers of solid tumours exhibit activation of the JAK-STAT signalling pathway, including breast cancer [105], prostate cancer [106], lung cancer [107], glioblastoma [108] and malignant melanoma [109]. In contrast to hematopoietic malignancies, solid tumours usually display constant activation of JAK1 and JAK2 as a result of alternative mechanisms including epigenetic silencing of negative regulators of JAKs, protein tyrosine phosphatases and suppressors of cytokine signalling, as well as an abnormal autocrine stimulation of cytokines and growth factors [110-112]. STAT3 has also been observed to be constitutively activated in many tumours with important roles in different aspect of tumourigenesis, such as tumour survival, proliferation, angiogenesis, invasion, metastasis, drug resistance as well as CSCs. Hence, STAT3 is considered to be a suitable target for anti-cancer therapy [113-117].

Aim

The overall aims of this thesis were to investigate the role of the *FUS-DDIT3* fusion oncogene in MLS by defining its regulatory and functional mechanisms, and to determine tumour heterogeneity, with focus on cancer stem cells and cell cycle regulation.

Specific aims:

Paper I: To determine the role and function of TP53 in MLS.

Paper II: To determine the expression levels and regulatory mechanisms that control *FUS-DDIT3* expression at both mRNA and protein level in MLS.

Paper III: To define cell heterogeneity and identify cell subpopulations related to cell growth and cell division at single-cell level.

Paper IV: To develop an approach that enables simultaneous DNA, mRNA and protein analysis of the same samples and to identify signalling pathways that regulate *FUS-DDIT3* and *FUS* at both mRNA and protein level in MLS.

Paper V: To define cancer stem cell features in MLS and how JAK-STAT signalling controls the number of cells with cancer stem cell properties.

Materials and methods

Experimental model system and methods

In this thesis, we used cell lines and formalin-fixed paraffin-embedded tissue material. MLS derived cell lines 2645-94, 1765-92, 402-91 [118, 119] and DL221 [120], mesenchymal stem cells differentiated from an embryonic stem cell line[121], breast cancer cell line MCF7 [122], human fibrosarcoma cell line HT1080 [123] as well as stably transfected subclones of the HT1080 cell line [59] were used. The following methods were used: cell culture, cell proliferation assay, flow cytometry, fluorescence-activated cell sorting, immunofluorescence, immunohistochemistry, immunoprecipitation, mass spectrometry, next generation sequencing, non-adherent condition assay, proximity ligation assay, scratch assay, side population analysis, single-cell analysis, transfections, quantitative real-time PCR and western blot analysis.

Experimental details are outlined in each paper (I-V).

Single-cell gene expression profiling

Cytogenetic characterisations and microsatellite studies have revealed large variability among individual cells within tumours [124]. However, the limited understanding of tumour heterogeneity has been due to the lack of analytical techniques to study individual cells. Gene expression profiling, currently one of the most commonly applied techniques in tumour characterisation, is generally performed on a large pool of tumour cells. Consequently, samples constitute mixes of different cell types present in unknown proportions (Fig. 7). These studies will neither reveal heterogeneity within cell types nor important correlations in gene expression between cells. Cells in a population are in many aspects unique in their characteristics, even in a seemingly homogenous culture or tissue. Single-cell studies on both the protein and mRNA level show large cell-to-cell variation in both resting and stimulated states [125]. This implies that data obtained from large pools of cells does not, and indeed, cannot, accurately reflect the behaviour of the individual cell. The need for single-cell gene expression analysis to understand tumour heterogeneity and the dynamic transition between cell states has been recognized for a long time, but the lack of sensitive analytical techniques to detect and quantify few transcripts has limited such measurements.
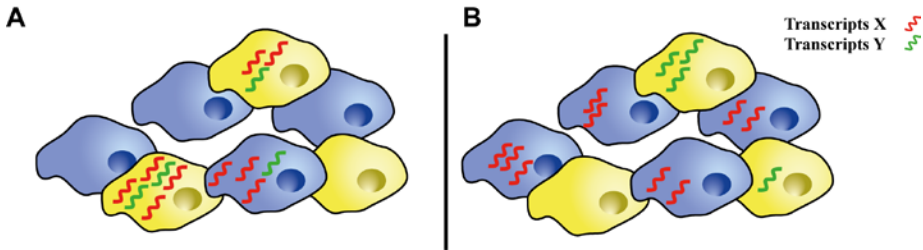
Figure 7. Cell and gene-expression heterogeneity. *Single-cell analysis allows us to study differences between cell types as well as heterogeneity within defined cell types and/or cell states. Cases (A) and (B) show the same expression of X and Y transcripts at population level. However, single-cell analysis enables us to distinguish between the two cases. In case (A) transcripts X and Y are co-expressed in both cell types, while in case (B) X transcripts are only expressed in blue cells and Y transcripts are only expressed in yellow cells.*

In this thesis, we have designed and developed gene expression assays for single-cell analysis. These assays include markers for cell proliferation, cell cycle regulation, TP53 function, stemness, differentiation, cell signalling, and housekeeping functions. The experimental setup of single-cell qPCR is well established and has previously been reported in detail [126, 127]. Briefly, single-cells were collected using fluorescence-activated cell sorting, followed by direct cell lysis, reverse transcription, preamplification and final analysis using quantitative real-time PCR (Fig. 8, paper II and III). In this thesis, single-cell gene expression profiling in combination with proximity ligation assay was used to determine the expression of *FUS-DDIT3* at both mRNA and protein level, as well as studying the effects of cell cycle phase and cell size on gene expression.
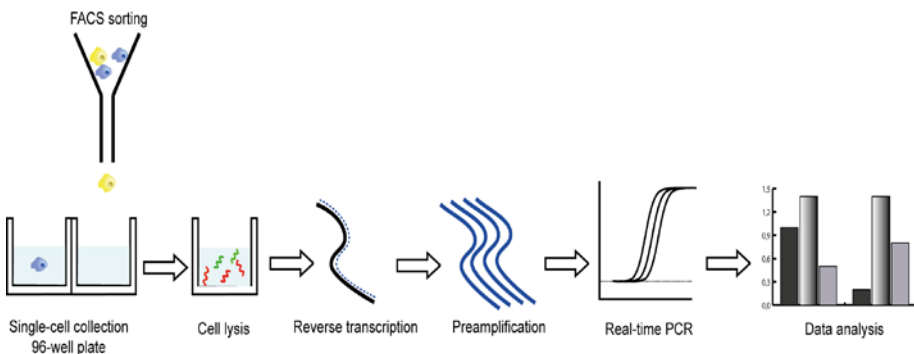
Figure 8. Schematic overview of the single-cell gene expression analysis workflow. *Individual cells are collected by fluorescent-activated cell sorting (FACS). Cells are lysed without extraction or purification and followed by, reverse transcription, preamlification and quantitative real-time PCR. Data analysis is performed with uni- and multivariate statistical tools.*

Results and discussion

Paper I: Normal and Functional TP53 in Genetically Stable Myxoid/Round Cell Liposarcoma

Translocations causing the *FUS-DDIT3* and *EWSR1-DDIT3* fusion oncogenes are the only chromosomal aberrations in the vast majority of MLS tumours. *TERT*-promoter mutations are detected in 70% of the cases and 10-15% are also carrying other mutations in genes such as *PIK3CA* and/or loss of *PTEN* [128]. Except for these mutations, MLS tumours show few other changes and are considered as genetically stable. In some cases the fusion oncogene is the only mutation detected.

Inactivation of the tumour suppressor gene *TP53* is a frequent event in tumourigenesis. The accumulation of TP53 expression as a result of its mutation is an independent marker for poor prognosis in several tumours [129]. Hence, *TP53* mutations have also been studied in MLS, however the results obtained with *TP53* analysis have been inconsistent. Some studies have reported that *TP53* mutation is related with poor prognosis and is associated with progressive disease [130]. In contrast to these studies, our group and others have observed sporadic TP53 expression in MLS, which is not expected when *TP53* is mutated, since TP53 mutations usually result in its overexpression. Instead, these data indicate that MLS has a normal TP53 function [131]. A recent study also showed that the *FUS-DDIT3* fusion oncogene formed MLS-like tumours in *TP53*-deficient mice only [132]. These contradicting observations promoted us to study TP53 expression and function, using MLS cell lines as an experimental model system.

Here, we analysed four MLS-derived cell lines. Ion Torrent AmpliSeq sequencing using a cancer hotspot panel was performed to identify mutations. Data revealed no dysfunctional mutations among the 50 genes included in this panel covering the most common COSMIC (Catalogue of Somatic Mutations in Cancer) mutations, in three of the MLS cell lines. For the DL221 cell line we confirmed a previously known *TP53* mutation and a sequence variant for *PIK3CA* (data not reported). These data indicate an active and functional TP53 system in three out of four cell lines even after twenty years of *in vitro* cell culturing. The TP53 protein expression was analysed in all MLS cell lines by western blot and immunofluorescence showing that all cells expressed TP53 protein. However, different sizes of the protein were observed in western blot data (Fig. 9). This may be explained by post-translational modifications reported for TP53, such as phosphorylation, methylation, ubiquitinylation and sumoylation. Analysis of TP53 function was performed using irradiation experiments with downstream western blot and immunofluorescence analyses. Two of the MLS cell lines (1765-92, 402-91) showed elevated TP53 expression and DL221 showed slightly elevated TP53

expression after irradiation, while MLS 2645-94 showed no or small regulation (Fig. 9). Low regulation of TP53 in MLS 2645-94 cell line may be explained by the fact that this cell line was immortalized using full length SV40 virus, while the MLS 1765-92 and 402-91 cell lines only carry SV40 T-large antigen. Enhanced expression of TP53 after irradiation was also confirmed with immunofluorescence analysis. Functional TP53 in the MLS cell lines was also confirmed by activation of the TP53 target gene CDKN1A (P21) upon irradiation. To study if FUS-DDIT3 expression affected the TP53 expression and its function, the fibroblast cell line HT1080 with and without stably transfected FUS-DDIT3 was analysed. Irradiation induced post-translational modification of TP53 in both HT1080 and HT1080 expressing FUS-DDIT3, indicating that FUS-DDIT3 does not affect TP53 function. Our results support that most MLS cases have a normal and functional TP53 system, which may explain why MLS tumours are sensitive to radiation and chemotherapy [133].
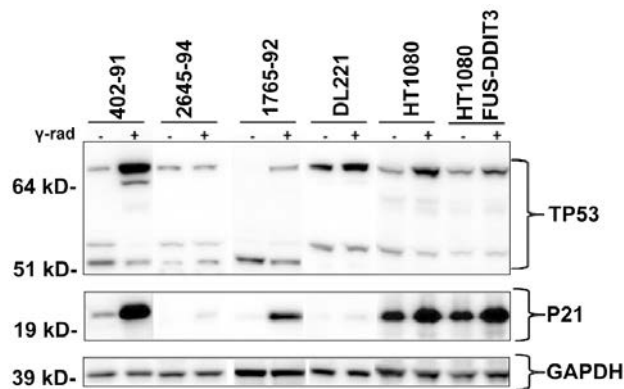
Figure 9. TP53 expression in MLS. TP53 and CDKN1A (P21) expression in control and irradiated cells. The different TP53 bands correspond to different post-translationally modified TP53 forms. GAPDH was used as internal control.

Paper II: Regulatory mechanisms, expression levels and proliferation effects of the *FUS-DDIT3* fusion oncogene in liposarcoma

Myxoid liposarcoma is a unique tumour entity characterised by the *FUS-DDIT3* fusion oncogene that is observed in more than 90% of all cases. MLS rarely contains secondary mutations (paper I). The *FUS-DDIT3* fusion oncogene encodes a chimeric transcription factor that is believed to be involved in tumour initiation and development.

To date, 12 different sequence variants of *FUS-DDIT3* fusion oncogene are known [134]. Type I and II fusion oncoproteins are the most common isoforms caused by

different breakpoint locations of *FUS,* resulting in varying portions of the FUS protein being fused to the entire DDIT3 protein (Figs. 3B and 10A). Previous studies have shown distinct adipogenesis inhibitory activity between type I and II [135, 136]. In addition, a recent study has shown different therapy response between isoforms of the fusion oncoprotein, which suggests a possible role of the FUS-DDIT3 isoforms in predicting a response to therapy [137]. Hence, understanding the role of FUS-DDIT3 and its regulatory mechanism and functions can be used to develop better therapeutic and targeted treatments against the fusion oncoprotein.

Here, we studied the regulatory mechanisms that control the expression level of *FUS-DDIT3* at mRNA and protein level. We analysed four MLS-derived cell lines as a model system. They represent fusion oncogenes with three different breakpoints: Type I (MLS 402-91 and DL221), Type II (MLS 2645-94) and Type VI (MLS 1765-92). Type II has the shortest *FUS* sequence part, while almost the entire *FUS* gene is fused with *DDIT3* in Type VI. We showed that the normal *FUS* is expressed to a higher degree than *FUS-DDIT3* at both mRNA and protein level (Fig. 10B, C). MLS 1765-92 with the longest *FUS* sequence showed the lowest *FUS-DDIT3* mRNA expression. In contrast, FUS-DDIT3 had the highest expression in MLS 1765-92 at protein level compared to the other isoforms. The stability of *FUS-DDIT3* and *FUS* were determined at both mRNA and protein level. We showed that *FUS-DDIT3* has a shorter half-life at both transcriptional and translational level compared to *FUS*. Only minor differences in mRNA half-life were observed between different *FUS-DDIT3* variants, while the Type VI FUS-DDIT3 protein showed longer half-life compared to the other isoforms. The increased protein stability of Type VI FUS-DDIT3 may explain the high level of FUS-DDIT3 protein in MLS 1765-92. We concluded that the *FUS-DDIT3* mRNA stability was dependent on the *DDIT3* sequence. In contrast, we could show that FUS-DDIT3 protein stability was dependent on protein interactions through FUS rather than DDIT3 protein partners. Figure 10D shows that there is no correlation between *FUS-DDIT3* mRNA and protein expression, further indicating the importance of post-transcriptional regulation of FUS-DDIT3 for its protein expression.

DDIT3 expression arrests cells in the G1/S phase, while FUS-DDIT3 expression enables cell proliferation [58]. To determine the correlation between FUS-DDIT3 expression and cell cycle regulation we performed single-cell analysis. Our data showed that cells containing high levels of FUS-DDIT3 protein displayed low expression of transcripts related to cell proliferation (Fig. 10E). In addition, forced overexpression of FUS-DDIT3 resulted in decreased cell proliferation. Others have shown that down-regulation of FUS-DDIT3 also caused decreased cell proliferation [138]. However, we have not been able to validate these findings. Altogether, our data show that the exact level of FUS-DDIT3 protein is important for cell proliferation.
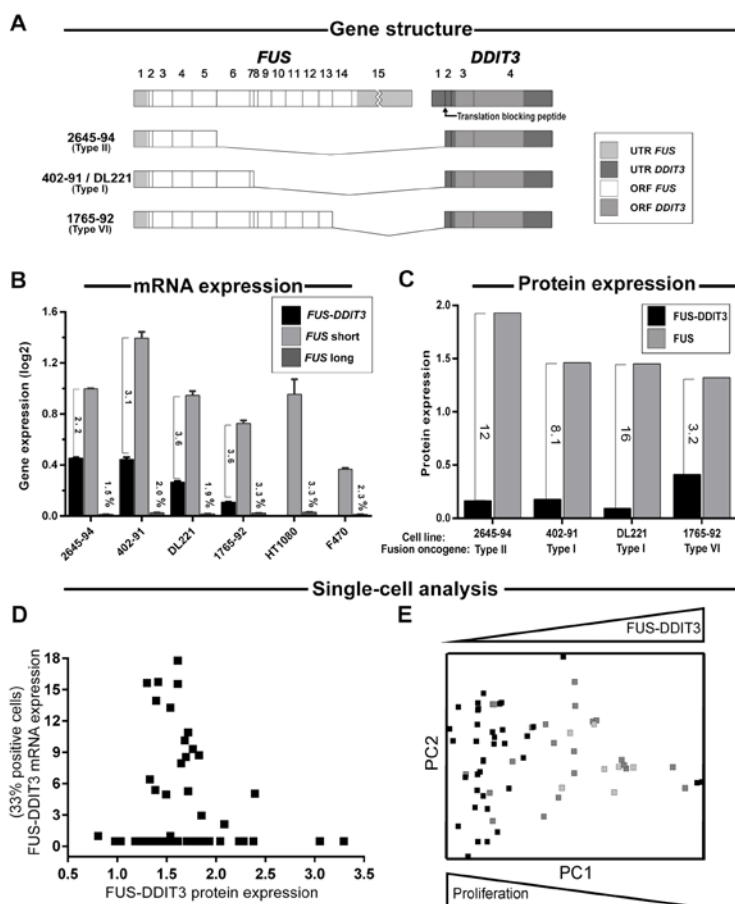
Figure 10. FUS-DDIT3 and FUS expression and regulation. *(A) FUS-DDIT3 gene structure. Different transcript variants of FUS-DDIT3 in MLS 2645-94, 402-91, 1765-92 and DL221 are shown. (B) Absolute quantification of FUS-DDIT3, FUS short (without 3'-untranslated region (UTR)) and FUS long (with 3'-UTR region) mRNA variants showed 2.2-3.6-fold lower expression of FUS-DDIT3 compared to FUS short. (C) Quantification of FUS-DDIT3 protein expression compared to FUS showed 3.2-16 times lower expression. MLS 1765-92 with the longest fusion oncoprotein showed higher expression than the two other isoforms. (D) Single-cell analysis of FUS-DDIT3 at mRNA compared to protein level displayed no correlation. Each dot represents an individual cell. (E) Principal component analysis of individual MLS 1765-92 cells. The FUS–DDIT3 protein and 43 mRNAs of which 33 were proliferation-related transcripts, were analysed. The FUS-DDIT3 protein expression was negatively correlated with cell cycle related transcripts, i.e., cell proliferation. Each dot represents an individual cell.*

Paper III: Cell cycle and cell size dependent gene expression reveals distinct subpopulations at single-cell level

Cell proliferation is one of the most fundamental biological processes, and it involves cell division and cell growth. Single-cell gene expression analysis opens up new possibilities to study and understand cell proliferation in detail.

In this article, single-cell analysis allowed us to assess the total transcript level between individual cells in a way that is not possible. Analysis of single MLS 402-91 cells showed a correlation between total transcript levels and cell cycle phases, but not toward cell sizes (Fig. 11A). Principal component analysis of individual cells from different cell cycle phases displayed partly overlapping clusters. We performed supervised algorithms to classify cells (random forests) and to identify the subset of genes with highest predictive capacity (recursive feature elimination). By applying unsupervised algorithms and only analysing informative genes we were able to identify a subpopulation of cells (named G1´ subpopulation) with differentially expressed genes in MLS 402-91 (Fig. 11B) and MCF7. To visualize the cell cycle progression we calculated a cell cycle index based on gene expression (Fig. 11C). The index correlated with the G1, S and G2/M cell cycle phases. The G1´ subpopulation displayed the lowest cell cycle index, illustrating that these cells belong to the early G1 phase. The G1´ subpopulation in MLS 402-91 displayed a similar gene expression profile as the one identified in MCF7, except for the *MCM6* gene. *MCM6* was highly expressed in the G1´ MLS 402-91 subpopulation, while it was downregulated in MCF7. *MCM6* belongs to the mini-chromosome maintenance (MCM) gene family that are essential DNA replication factors for initiation of DNA synthesis. In early G1, MCM proteins form heterohexamers with origin recognition complex (ORC), cell division cycle 6 (CDC6) and cdc10-dependent transcript 1 (CDT1) proteins to form the pre-replication complex. Interestingly, *CDT1* was the second most upregulated gene in the G1´ MLS 402-91 subpopulation (data not shown), indicating that the MCM complex might have a specific role in MLS. We also confirmed a heterogeneous MCM6 expression at protein level. Previous studies have shown that MCM proteins are overexpressed in multiple cancers and can provide important prognostic information in tumour biology [139, 140]. Further studies are required to determine the role of MCM in MLS.

Figure 11D shows that the gene expression of both *FUS-DDIT3* and *FUS* in MLS 402-91 scaled with cell cycle phase, similarly as the total transcript level and most other genes. Hence, we conclude that *FUS-DDIT3* expression is not abnormally regulated throughout the cell cycle. In contrast, unpublished data indicate that the protein expression of FUS-DDIT3 is upregulated in the G2/M phase. In paper II, we showed that the level of FUS-DDIT3 protein was negatively correlated to the expression of

genes related to cell proliferation. Altogether, these data suggest that FUS-DDIT3 protein, but not *FUS-DDIT3* transcript, may play a direct role in cell proliferation and its regulation. This is further supported by the fact that FUS-DDIT3 interacts with Cdk2 through its DDIT3 part [141]. In addition Cdk2 and Cdk4 with their associated cyclins, cyclin E and cyclin D1, are strongly overexpressed in MLS tumours [57]. Other studies have also shown that the MCM complex and CDT1 are regulated by cyclin-dependent kinases such as Cdk2 [142, 143]. Therefore, we speculate a possible link between FUS-DDIT3 and the MCM complex. However, the mechanisms by which FUS-DDIT3 is controlling cell cycle needs further investigation.
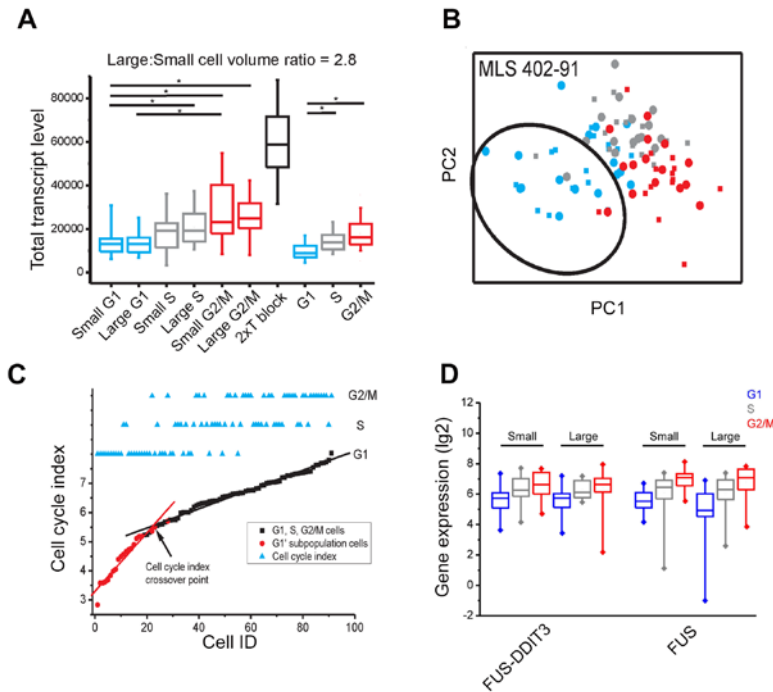
Figure 11. Cell-cycle dependent gene expression in MLS 402-91. *(A)The total transcript level for small and large cells in the G1 (blue), S (gray), and G2/M (red) phases and double thymidine G1/S blocked cells (2xT block, black) are shown. B) An MLS 402-91subpopulation (encircled) was identified using principal component analysis using genes identified by the random forest algorithm only. In the principal component analysis score, individual small (squares) and large (dots) MLS 402-91 cells in G1 (blue), S (gray), and G2/M (red) phase are shown. The position of a cell is defined by its gene expression profile. (C) The cell cycle index of each cell is shown in relation to its cell cycle phase. The G1' subpopulation cells are shown in red. (D) FUS-DDIT3 and FUS expressions in small and large cells in each cell cycle phase.*

Paper IV: Identification of inhibitors regulating cell proliferation and *FUS-DDIT3* expression in myxoid liposarcoma using combined DNA, mRNA and protein analyses

Myxoid liposarcoma is a complex disease despite its simple genomic aberration (paper I). Most of the currently used clinically therapeutic agents such as trabectedin and doxorubicin are not effective for all patients. Persistence of the fusion oncoprotein after treatment indicates that these agents were not capable of eradicating the disease completely [144, 145]. One possible strategy to target MLS in a specific manner is to target the fusion oncoprotein itself. Although the FUS-DDIT3 oncoprotein is present in the vast majority of MLS tumours, screening for drugs that directly or indirectly affect FUS-DDIT3 has never been employed on MLS cell lines to our knowledge.

In paper II we observed differential expression of *FUS-DDIT3* at mRNA and protein level between MLS cell lines. In addition, the results from our single-cell analysis (paper II) together with our cell cycle analysis (paper III) showed association between FUS-DDIT3 protein expression and cell proliferation. Thus, analysing the regulation of *FUS-DDIT3* at both mRNA and protein level as well as cell proliferation facilitate the identification of novel therapeutic targets against the fusion oncoprotein.

To enable fast and simple analyses of cell proliferation, mRNA and protein we developed an extraction-free workflow, allowing specific DNA, RNA and protein to be measured with qPCR, reverse transcription qPCR and proximity ligation assay, respectively, in the same sample (Fig. 12). This approach was based on our experiences of analysing DNA, RNA and protein on direct lysed cells at single-cell level [146-148]. Our data showed that RNA and DNA and protein could be accurately quantified using bovine serum albumin supplied in glycerol as direct lysis buffer. We also showed that both mRNA and protein data were correctly normalized using genomic DNA as reference when using direct lysis.

To identify potential signalling pathways involved in cell proliferation and regulation of *FUS-DDIT3* and *FUS* at both mRNA and protein level, we performed a screen with 70 kinase inhibitors in MLS 402-91 cells. Different ranges of growth arrest could be detected where the majority of the inhibitors reduced cell proliferation (Fig. 13A).
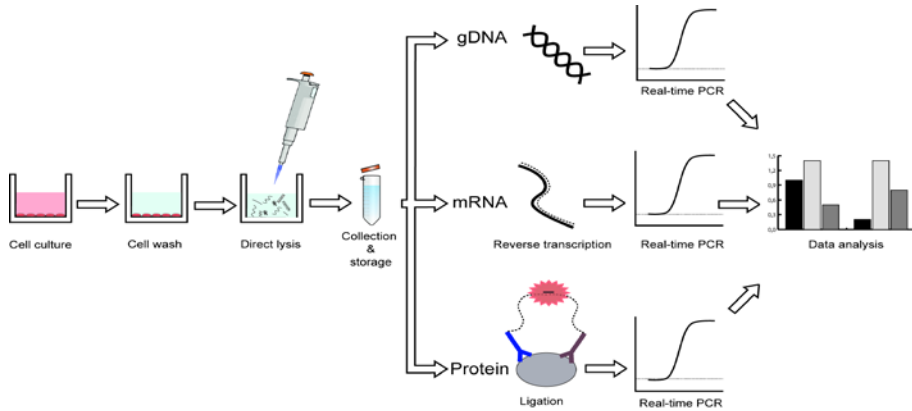
Figure 12. Schematic experimental workflow. *Direct lysis of bulk samples followed by cell proliferation, mRNA and protein analysis.*

FUS-DDIT3 and *FUS* mRNA expression showed strong correlation in all conditions. Based on our data in paper II, the 5' *FUS* promoter rather than the *DDIT3* sequence was important for the observed positive correlation (Fig. 13B) [149]. FUS-DDIT3 protein expression more than FUS correlated more with its respective transcript level (Fig 13B, C). This observation can in part be explained by the fact that FUS protein has longer half-life than FUS-DDIT3 (paper II). Therefore, FUS-DDIT3 protein expression will adjust to altered gene expression faster than FUS, especially when *FUS-DDIT3* is downregulated. Interestingly, FUS-DDIT3 expression showed a positive correlation with cell proliferation, while we in paper II showed that cells with ectopic FUS-DDIT3 expression caused reduced cell proliferation. These data indicate that the exact level of FUS-DDIT3 is an important factor for cell proliferation in MLS cells.

Our kinase screening data demonstrated that the inhibitors that resulted in downregulation of the *FUS-DDIT3* at both mRNA and protein level were mainly targeting JAK and GSK-3 kinases (Fig. 13). Inhibitors targeting tyrosine kinases belonging to the same signalling pathway showed different degree of effect on proliferation rates. For example, inhibitors number 2 (TG101348), 25 (ruxolitinib) and 27 (barinitinib) all target the JAK2 kinase. TG101348 also maintained its proliferative effect even at the lower applied concentration, while the other two inhibitors showed no or minimal cell proliferation effect. The specificity of TG101348 and ruxolitinib have previously been examined using a near-kinome-wide survey [150]. According to this study TG101348 inhibits the activity of more kinases as off-targets compared to ruxolitinib. Therefore, it is likely that the proliferative effect of TG101348 is more related to off-target inhibition than to JAK2 inhibition. It has been suggested that the lack of specificity may not always be a disadvantage in therapy. However, lack of target specificity may result in unknown side effects and is therefore usually an unwanted feature in therapy [151].
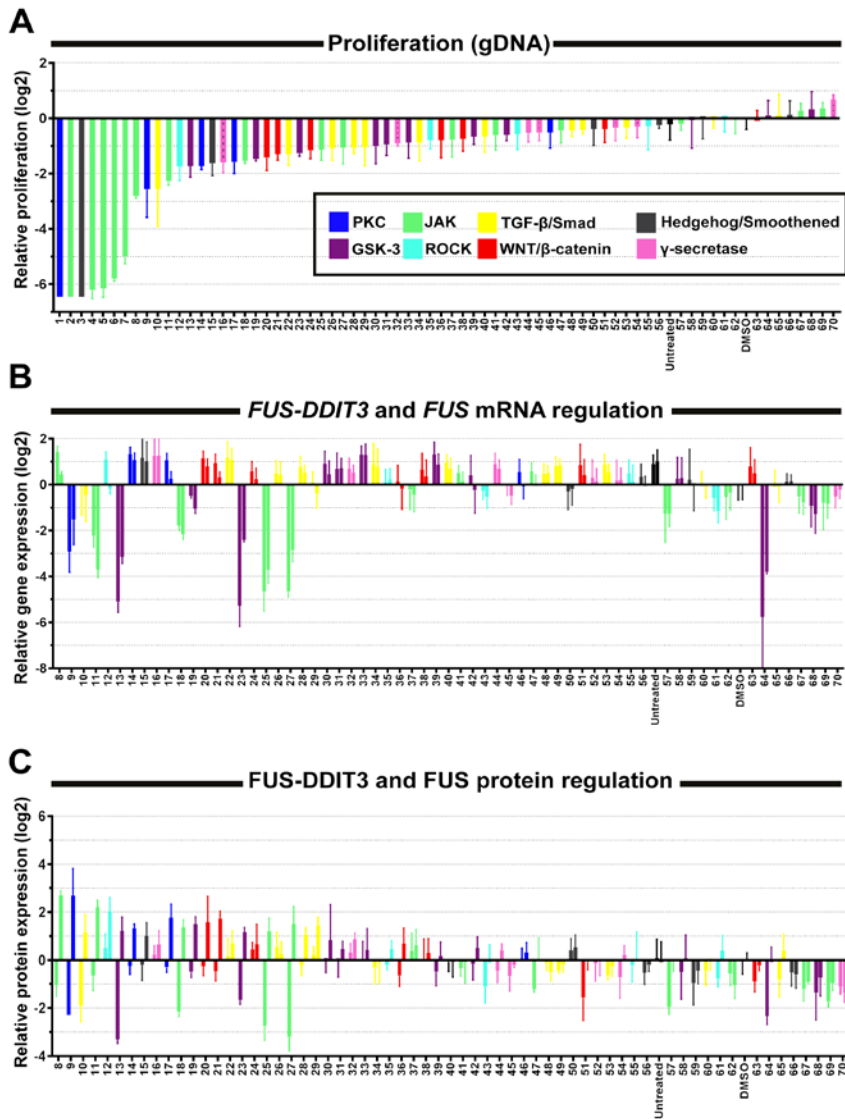
Figure 13. Kinase inhibitor screening. *MLS 402-91 cells were treated with 70 kinase inhibitors compared to treatment control (DMSO) and untreated cells. (A) Proliferation was analysed by measuring gDNA. (B) Pairwise FUS-DDIT3 (left) and FUS (right) mRNA regulation are shown for each inhibitor. Inhibitors 1 to 7 are eliminated due to lack of reliable data, because of cell death. (C) Pairwise FUS-DDIT3 (left) and FUS (right) protein regulation are shown for each inhibitor. Inhibitors 1 to 7 are eliminated due to lack of reliable data, because of cell death. Data are compared to the DMSO controls in each data set. Mean ± SEM is shown, n=4.*

Paper V: JAK-STAT signalling controls cancer stem cell properties in myxoid liposarcoma

In spite of extensive efforts in conventional and targeted chemotherapeutic treatments, therapy relapses occur in clinical oncology. Recent studies suggest that the functional and molecular properties of small subpopulations of cancer cells play an important role in therapy resistance and tumour relapse [64, 152]. These cells usually possess stem-like characteristics and are referred to as CSCs. In this study we studied CSC properties, including chemotherapy resistance, in three MLS cell lines. We showed that the MLS cell lines were capable of forming tumour spheres, *i.e.*, sarcospheres, a feature specific for CSCs (Fig. 14A). Although various surface markers have been used to identify and isolate CSC subpopulations in epithelial tumours, to date no specific and useful surface marker has been discovered in MLS. Instead, we used the functional side population discrimination assay (Hoechst staining) to identify subpopulation of cells that are able to efflux dyes, an additional property associated with CSCs.

The overall response of MLS to single chemotherapy treatment, such as doxorubicin and trabectedin, is about 50% [153-155]. A hypothesis that may explain therapy resistance in patients is the presence of drug resistant CSC subpopulations. Our data displayed a large association between the sarcosphere formation capacity and the IC_{50} value of doxorubicin. Furthermore, we observed increased numbers of sarcospheres and side population fractions in doxorubicin treated cells. Interestingly, the cells that formed the least number of sarcospheres (MLS 402-91) displayed almost no side population cells and were the most sensitive to doxorubicin treatment. Altogether, these data demonstrate the existence of subpopulations with CSC features in MLS. In agreement with previous studies, we showed that doxorubicin mainly kills sensitive and proliferative cells, resulting in the enrichment of therapy resistant cells with CSC properties [156, 157].

Next, we showed that the JAK-STAT signalling controls the number of cells with CSC characteristics in MLS. Dysregulation of the JAK-STAT signalling pathway upon constant activation of STAT3 has been shown in tumour development and CSC maintenance in several tumour entities, including gliobastoma [158], lung cancer [159, 160] and colon cancer [161]. In MLS cell lines, sarcosphere formation was decreased when the activation of STAT3 was blocked using ruxolitinib (JAK1/2 inhibitor) (Fig. 14B). MLS 1765-92, with the highest expression of phosphorylated STAT3 under normal growth conditions generated the largest sarcosphere number reduction, showing that the level of phosphorylated STAT3 correlated to the number of cells with CSC features in respective MLS cell line. In a previous study, we have showed that elevated expression of IL6 is observed in FUS-DDIT3 expressing cell lines [162]. IL6 belongs to the IL6-type cytokine family that mainly act via homo- or hetero-dimerization of GP130, LIF and

IL6 receptors [100, 101]. Protein analysis of LIF, GP130 and IL6 receptors showed strong, intermediate and almost no expression, respectively. Moreover, LIF mediated the induction of phosphorylated STAT3 and increased the number of formed sarcospheres (Fig. 14C). Ruxolitinib inhibited the LIF-induced stimulation, suggesting that the JAK-STAT signalling mediate the maintenance of cells with CSC properties via the LIF/JAK1/2/STAT3 signalling pathway (Fig. 14D).

Finally we evaluated whether JAK-STAT signalling could be a potential therapeutic target that could be used in combination with doxorubicin. The combined treatment of doxorubicin and ruxolitinib resulted in reduction of formed sarcospheres compared to cells treated with doxorubicin only. However, the combined treatment did not eliminate all cells with capacity to form sarcospheres (Fig. 14E). These observations indicate the existence of a subpopulation of cells with CSC features that may resist therapy through another mechanism other than JAK-STAT signalling. In summary, our data indicates that MLS treatment may be improved through a combined doxorubicin and ruxolitinib treatment. Further studies, including *in vivo* experiments, are needed to determine the full potential of applying JAK-STAT inhibition to treat MLS.
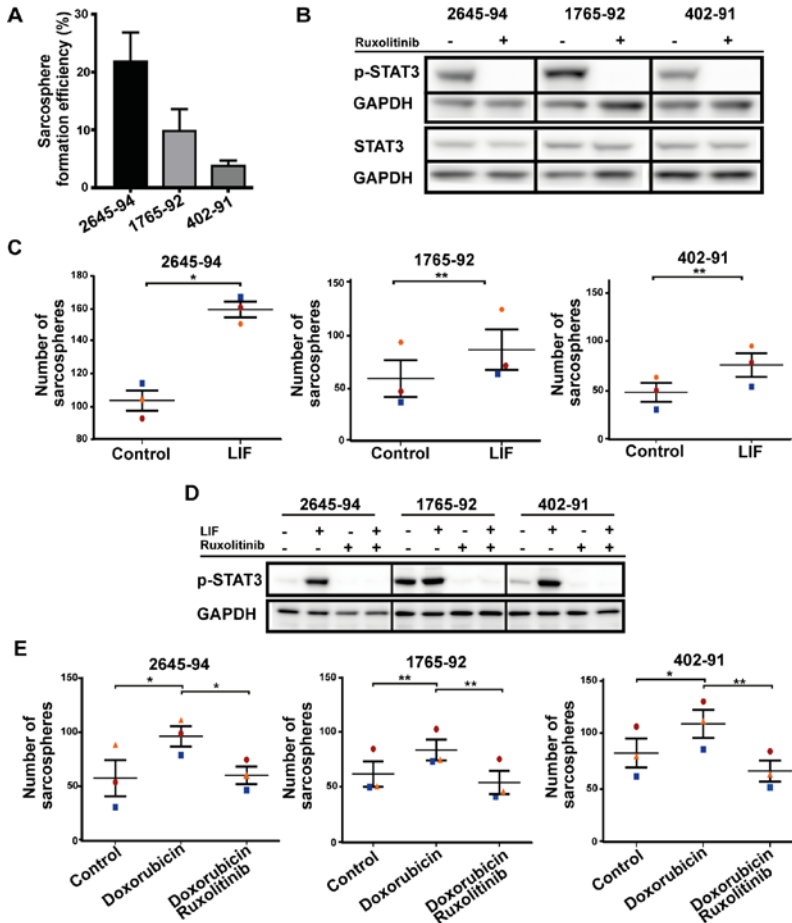
Figure 14. CSC properties and JAK-STAT signalling in MLS. *(A) Sarcosphere formation efficiency of MLS 2645-94, 1765-92 and 402-91cells calculated as the fraction of formed spheres in relation to the total number of seeded cells. Mean ± SEM is shown, n = 3. (B) Ruxolitinib inhibition of JAK1/2. Western blot analysis of phosphorylated STAT3 (p-STAT3) and STAT3 compared to untreated control (DMSO) cells. GAPDH was used as an internal protein loading control. (C) LIF induced cancer stem cell properties in MLS. Sarcosphere formation capacity of MLS cells treated with LIF. Three independent experiments were performed (orange, red and blue). For each individual experiment three technical replicates were performed. Mean ± SEM is shown, paired student t-test, *p< 0.05, ** p< 0.01. (D) LIF acts through JAK1/2. Western blot analysis of p-STAT3 in MLS cells treated with LIF, ruxolitinib, and LIF in combination with ruxolitinib. GAPDH was used as an internal protein loading control. (E) Sarcosphere forming capacity of MLS cells when treated with doxorubicin and ruxolitinib in combination. Three independent experiments (orange, red and blue) were performed. For each individual experiment, three technical replicates were performed. Mean ± SEM is shown, paired student t-test, *p< 0.05, ** p< 0.01.*

Conclusions

Paper I

In conclusion, our sequencing data of long-term cultured MLS cell lines confirms that MLS cells are genetically stable with no additional mutations accumulating even after long-term passaging. Furthermore, our data show that MLS has a functional TP53 system, which is consistent with the low numbers of secondary mutations observed in this tumour entity.

Paper II

In conclusion, we found that the expression of *FUS-DDIT3* is regulated by the *FUS* promotor and its stability was dependent on the *DDIT3* sequence at mRNA level. At protein level, FUS-DDIT3 stability was dependent on protein interactions through the FUS rather than the DDIT3 protein. Furthermore, cell proliferation was negatively correlated to FUS-DDIT3 protein expression. Detailed characterisation of FET fusion oncogenes and precise understanding of their regulation is important when defining the role of *FUS-DDIT3* in tumour initiation and development. In the long-term perspective this knowledge may be used to develop tumour specific treatments targeting the fusion oncoprotein itself or biological processes directly associated with FUS-DDIT3 function.

Paper III

In conclusion, we provide useful experimental approaches and bioinformatics tools to identify informative and predictive genes at the single-cell level, which opens up new means to describe and understand cell proliferation and subpopulation dynamics. By implementing suitable analytical tools, we were able to identify potential biomarkers that are differentially expressed throughout the cell cycle in MLS. This allowed us to identify a G1 subpopulation with a distinct gene expression profile that was specific to MLS. Detailed analysis of individual cells in different proliferative states opens up new possibilities to identify cell cycle abnormalities and the role of FUS-DDIT3 in this biological process that is essential in MLS development. Identification of tumour-specific biomarkers opens up new possibilities to develop targeted therapies.

Paper IV

We developed an extraction-free workflow that enabled DNA, mRNA and protein analysis in the same sample. This approach was applied to study how well-characterised kinase inhibitors regulated cell proliferation and expression of *FUS-DDIT3* and *FUS* at

both mRNA and protein level. We identified a number of previously unknown pathways, including JAK-STAT and GSK signalling pathways that regulated the expression of *FUS-DDIT3* at both transcriptional and translational level. FUS-DDIT3 itself and its functions are potential therapy targets specific for MLS.

Paper V

In conclusion, we found that MLS contained subpopulation of cells with CSC properties, including chemotherapy resistance. We also showed that JAK-STAT signalling controls the number of cells with CSC features. More specifically we show that LIF act through JAK1/2 and STAT3 signalling. Furthermore, our data showed that the inhibition of JAK-STAT signalling in combination with chemotherapy displayed synergistic treatment effects. Better understanding and characterisation of CSCs in MLS will provide new means to identify novel targets and to develop specific therapies that target truly malignant subpopulations of tumour cells.

Future perspectives

Notwithstanding continuous progress in treating MLS, existing therapies suffer from a major flaw; they do not target cells with CSC properties. Unique features of CSCs, and theoretically possible targets for specific therapy, include self-renewal, tumourigenicity, multi-lineage differentiation, and increased resistance to radio- and chemotherapy-induced cell death. Thus, cells with CSC features are critical targets in cancer therapy. Little is known about CSCs and fusion oncoproteins in MLS despite the fact that they are causal factors in tumourigenesis. Current experimental approaches to identify and characterise rare subpopulation of cells are technically challenging. Thus innovative approaches, such as single-cell analysis, are needed to decipher the nature of tumour subpopulations. Furthermore, expression profiling of individual cells opens up new possibilities for precise understanding of fusion oncogenes and their specific functions. By defining CSCs at the molecular level and determining the function of fusion oncogenes, we will be able to develop specific treatments that ultimately will increase the survival and life quality of patients diagnosed with MLS.

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