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Myxoid liposarcoma *FUS-DDIT3* fusion oncogene induces C/EBP β-mediated interleukin 6 expression

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The myxoid/round cell liposarcoma oncogene *FUS-DDIT3* is the result of a translocation derived gene fusion between the splicing factor *FUS* and *DDIT3*. In order to investigate the downstream targets of DDIT3, and the transforming effects of the FUS-DDIT3 fusion protein, we have introduced *DDIT3-GFP* and *FUS-DDIT3-GFP* constructs into a human fibrosarcoma cell line. The gene expression profiles of stable transfectants were compared to the original fibrosarcoma cell line by microarray analysis. We here report that the NFκB and C/EBP β controlled gene *IL6* is upregulated in DDIT3- and FUS-DDIT3-expressing fibrosarcoma cell lines and in myxoid liposarcoma cell lines. Strong expression of the tumor associated multifunctional cytokine interleukin 6 was confirmed both at mRNA and protein level. Knockdown experiments using siRNA against *CEBPB* transcripts showed that the effect of FUS-DDIT3 on *IL6* expression is C/EBP β dependent. Chromatin immunoprecipitation revealed direct interaction between the *IL6* promoter and the C/EBP β protein. In addition, the effect of DDIT3 and FUS-DDIT3 on the expression of other acute phase genes was examined using real-time PCR. We demonstrate for the first time that DDIT3 and FUS-DDIT3 show opposite transcriptional regulation of *IL8* and suggest that FUS-DDIT3 may affect the synergistic activation of promoters regulated by C/EBP β and NFκB.

Key words: MLS/RCLS; FUS; DDIT3; CEBP β; NFκB

Myxoid/round cell liposarcoma (MLS/RCLS) is one of the most common soft tissue sarcomas in adults. The characteristic translocation t(12;16) (q13;p11) is found in more than 90% of the cases[1] and results in a gene fusion between *FUS* (previously called *TLS*) and *DDIT3* (previously called *CHOP*). The expressed fusion protein consists of the N-terminal part of the splicing factor FUS fused with the entire DNA-damage inducible transcript 3 (DDIT3).[2,3] DDIT3 is a bZIP transcription factor known to form heterodimers with C/EBP-family members[4] and other bZIP subfamilies such as the AP-1 and ATF3 families.[5,6] Thus, DDIT3 and its oncogenic variant FUS-DDIT3 may act by binding to bZIP proteins and modulate their activity and/or specificity. DDIT3 was initially reported to act as a dominant negative modulator in the C/EBP system.[4] However, as DDIT3 harbors a basic DNA binding domain and binds DNA in a sequence specific manner, it was later proposed to act as a more broad transcriptional regulator.[7] Previous studies indicate that expression of the FUS-DDIT3 fusion protein is required for the development of MLS/RCLS and sufficient for initiation of tumors resembling MLS/RCLS in transgenic mice.[8] In order to understand the transformation process leading to this tumor type, it is crucial to identify downstream target genes of the FUS-DDIT3 fusion protein. We have introduced *DDIT3-GFP* and *FUS-DDIT3-GFP* fusion constructs into a human fibrosarcoma cell line and compared the gene expression profiles in stable transfectants with the original fibrosarcoma cell line by microarray analysis. The *IL6* gene was found to be upregulated both at mRNA and protein level by the forced expression of *DDIT3* or *FUS-DDIT3*. We show that upregulation at the *IL6* promoter by DDIT3 and FUS-DDIT3 is C/EBP β dependent using siRNA knockdown and Chromatin Immuno Precipitation (ChIP) analysis. Further, DDIT3 and FUS-DDIT3 are found to have opposite effect on the expression of the inflammatory cytokine IL8.

Material and methods

Plasmid construction

The full length coding regions of *DDIT3* and *FUS-DDIT3* cDNA type II[9] were cloned into the pEGFP-N1 and/or pDsRed1-N1 vectors (ClonTech, Laboratories, Inc., Palo Alto, CA) in frame with the GFP or DsRED sequence, as previously described.[10] Similarly, the full length *CEBPB* cDNA and a shorter variant corresponding to the 20 kDa isoform were PCR amplified from human adipose tissue cDNA using the CEBPB-XhoI-U (5′GTTCTCGAGATGCAACGC CTGGTGGCCTG3′), CEBPB20-XhoI-U (5′ATACTCGAGATGG CGGCGGGCTTCCCGTA3′) CEBPB-BamHI-L (5′ATAGGATCC GGGCAGTGGCCGGAGGAGGCGA3′) primer pairs, and subsequently cloned into the pEGFP-N1 vector in frame with the GFP sequence. All constructs were confirmed by sequencing.

Cell lines

The myxoid liposarcoma (MLS) cell lines MLS 402-91, MLS 1765-92 and MLS 2645-94,[1,11] all of which express the FUS-DDIT3 fusion protein, and human fibrosarcoma cell lines HT1080, HT1080 DDIT3-GFP and HT1080 FUS-DDIT3-GFP were kept frozen in liquid nitrogen or cultured at 37°C and 4.8% CO_2 in RPMI 1640 medium with HEPES buffer supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 8% FCS (Invitrogen, La Jolla, CA). Cell lines HT1080 DDIT3-GFP and HT1080 FUS-DDIT3-GFP were generated by plasmid transfection using FuGENE™ 6 transfection reagent (Roche Diagnostics Scandinavia, Bromma, Sweden) as previously described[10] followed by selection in 500 μg/ml of geniticine (G418) (Invitrogen) and limiting dilution cloning. Geniticine (200 μg/ml) was constantly added to cell lines HT1080 DDIT3-GFP and HT1080 FUS-DDIT3-GFP to ensure stable expression of GFP constructs in the cell population.

Microarray analysis

Duplicates of total RNA were isolated from independent cultures of human fibrosarcoma cell lines HT1080, HT1080 DDIT3-GFP and HT1080 FUS-DDIT3-GFP using TRIzol reagent (Invitrogen) followed by RNeasy Mini Kit (Qiagen, Chatsworth, CA). Labeled cRNA was produced and hybridized onto the Affymetrix GeneChip Human Genome U133 set according to Affymetrix standard protocols. Expression data was analyzed using Microarray Suite 5.0.

Transient transfection

Transfections were performed using the FuGENE™ 6 transfection reagent (Roche). In brief, cell line HT1080 was subcultured 1 day prior to transfection. Fifty μl FuGENE™ 6 was added

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to 2 ml of serum free medium. The mixture was transferred to a tube containing 16 μg plasmid DNA and incubated for 15 min at RT before it was added to approximately 3×10^6 subconfluent cells. Transfection efficiency was monitored by fluorescence microscopy and cells were harvested 24 hr after transfection.

RT-PCR analysis

Cells were washed twice in cold phosphate-buffered saline 0.01 M, pH = 7.2 (PBS), and total RNA was isolated using QIAshredder and RNeasy Mini Kit (Qiagen). cDNA was generated from 5 μg total RNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen). RT-PCR amplification was carried out using a MJ Research thermal cycler PTC-200. The expression of *IL6 and ACTB* was monitored using the following primers: IL6U (5′CAGCCCTGAGAAAGGAGACAT3′), IL6L (5′AATCTGAGGTGCCCATGCTAC3′), ACTBU (5′TCATGATCATGAAGTGTGTGACGTTGACATCCGT3′) and ACTBL (5′CCTAGAAGCAATTTGCGGTGCACGATG3′).

Real-time PCR analysis

Total RNA was isolated and cDNA was generated as described in the RT-PCR analysis section. Real-time PCR was measured in the LightCycler (Roche) as described.[12] Formation of expected PCR product was confirmed by agarose gel electrophoresis and melt curve analysis. Gene expression data was normalized against *ACTB* and *GAPD* by geometric averaging.[13] The expression of *IL6, ACTB, GAPD, IL8,* and *IL1B* was monitored using the following primer pairs (5′-3′): IL6U, IL6L, ACTBU, ACTBL (see RT-PCR section above); GAPDU (GTGAAGGTCGGAGTCAACG), GAPDL (GGTGAAGACGCCAGTGGACTC); IL8U (TACTCCAAACCTTTCCACCCC), IL8L (CCTTGGCCTCAATTTTGCTAT) and IL1BU (AGCACCTTCTTTCCCTTCATCTT), IL1BL (TCGCTTTTCCATCTTCTTCTTTG).

IL6 Bioassay

Samples of *in vitro* secreted IL6 were taken from 3 ml low serum (2% FCS) cell culture medium conditioned by 5×10^6 monolayer cells for 18 hr. *In vivo* serum samples of human IL6 were collected from xenografted SCID mice (see below). The concentration of bioactive IL6 protein was determined as in a previously described bioassay using the B9 subclone of the cell line B13.29.[14]

Animal experiments

SCID mice were maintained under pathogen-free conditions. At the age of 5 weeks, 4 SCID mice were injected subcutaneously in the flank region with 1×10^7 cells of the MLS 2645-94 cell line. After 8 days, these mice developed solid tumors of approximately 1 cm in diameter and were sacrificed. Blood samples were collected from each of the inoculated SCID mice and from a control SCID mouse. The serum levels of secreted bioactive human IL6 protein were measured by an IL6 bioassay.

Promoter data analyses

Five hundred basepairs upstream of initiation of the IL6, IL8 and IL1B genes were searched for DDIT3, NFκB and C/EBP β motifs using MATCH™ at http://www.gene-regulation.de.

ChIP assay

Chromatin Immuno Precipitation (ChIP) was performed as described in the Acetyl-Histone H4 Immunoprecipitation Assay Kit (Upstate Biotechnology, Inc., Lake Placid, NY; 17-229). Briefly, MLS 402-91, HT1080 FUS-DDIT3-GFP and HT1080 transiently transfected with *CEBPB-GFP* were grown in monolayer cultures. Approximately 3×10^6cells were cross-linked with 1% formaldehyde for 30 min in 37°C. Cells were washed twice in PBS and then collected in PBS using a rubber policeman and sonicated at maximum power in 3 pulses of 10 sec each. Immunoprecipitation was performed according to kit protocol with either 10 μl anti-acetyl-Histone H4 antibody (Upstate 06-866) or 10 μl of anti-GFP antibody (ClonTech 8367-2). Immunoprecipitated DNA was analyzed by PCR using primers specific for the *IL6* and *IL8* promoters: IL6promU (5′AGCACTGGCAGCACAAGGCAAAC3′), IL6promL (5′CAAGCCTGGGATTATGAAGAAGG3′) and IL8promU (5′AAATTACCTCCCCAATAAAATGA3′) IL8promL (5′CCCTACTAGAGAACTTATGCACC3′), respectively.

siRNA knock down experiment

C/EBP β siRNA target region was chosen according to the AA(N19)dTdT, (~50% GC) format.[15] The designed C/EBP β duplex (5′GAAGACCGUGGACAAGCACdTT3′), 5′GUGCUUGUCCACGGUCUUCdTT3′) and rhodamine coupled nonsilencing control siRNA (target sequence: 5′AATTCTCCGAACGTGTCA CGT3′) was purchased from Qiagen and transfected using the RNAifect transfection reagent (Qiagen). In brief, cell line HT1080 FUS-DDIT3-GFP was subcultured in 12-well plates 1 day prior to transfection. Three microgram of siRNA was diluted in EC-R buffer (Qiagen) to a final volume of 100 μl before addition of 18 μl RNAifect transfection reagent. After 15 min RT incubation, the siRNA-RNAifect transfection mixture was added dropwise to 1×10^5 subconfluent cells in 600 μl serum medium. Cells were incubated for 6 hr at 37°C before the RNA-transfection mixture was removed and the cells were cultured under standard conditions. Transfection efficiency was monitored by fluorescence microscopy. RNA and protein samples were taken at 24, 48 and 72 hr after transfection.

Western blot analysis

Cells were lysed in modified RIPA buffer containing 50 mM TRIS-HCL, pH 7.4, 1% NP-40, 0.25% deoxycholic acid, 50 mM NaCl 0.1 mM EDTA, 1 mM NaF, 1 mM sodiumorthovanadate and 10% protease inhibitor cocktail (Roche 1836153). The lysates were centrifuged and the supernatants were mixed with Western sample buffer (Invitrogen). Protein samples were separated on a 4–12% SDS-polyacrylamide gel (Invitrogen), blotted onto an Immobilon-P PDVF membrane (Millipore Corp., Bedford, MA) and detected using antibodies directed against C/EBP β (C-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by alkaline phosphatase conjugated secondary antibodies (Dako Co., Carpinteria, CA) and CDP-Star (Applied Biosystems, Foster City, CA) as substrate. The chemiluminescent signal was detected by ECL film (Kodak, Rochester, NY). Subsequently, the PDVF membrane was stripped for 1 hr at 60°C in stripping solution (60 mM TRIS pH 6.8, 2% SDS, 100 mM mercaptoethanol) and reprobed with antibodies directed against Actin (C-2) (Santa Cruz Biotechnology, Inc.).

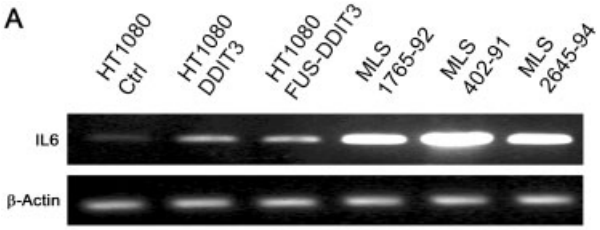
Coimmunoprecipitation

Human fibrosarcoma HT1080 cells transiently transfected with *CEBPB38-GFP* or *CEBPB20-GFP* in concert with FUS-DDIT3-DsRed were harvested 24 hr after transfection and lysed in lysis buffer [50 mM TRIS-HCL, pH 7.4, 1% NP-40, 50 mM NaCl, 0.1 mM EDTA and 10% protease inhibitor cocktail (Roche 1836153)]. The lysates were incubated overnight with 4 μg anti-GFP peptide antibody (Clontech 8367-2) and precipitated using 50 μl protein A agarose beads (Upstate). The precipitates were washed 3 times in lysis buffer and eluated in Western sample buffer (Invitrogen) containing 10% protease inhibitor cocktail. Immunoprecipitated material was visualized on Western blots using antibodies directed against GFP (Clontech 8367-2), DDIT3 (GADD153 R20) or C/EBP β (C-19) (Santa Cruz Biotechnology, Inc.).

Results

DDIT3 and FUS-DDIT3 upregulate IL6 *mRNA expression and IL6 protein secretion*

The mRNA expression profiles of human fibrosarcoma cell line HT1080, HT1080 DDIT3-GFP and HT1080 FUS-DDIT3-GFP were compared by microarray analyses using the Affymetrix



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HT1080 Ctrl	HT1080 DDIT3	HT1080 FUS-DDIT3	MLS 1765-92	MLS 402-91	MLS 2645-94
990	1 300	28 000	1 700	270 000	160 000

C

SCID Ctrl	SCID 1	SCID 2	SCID 3	SCID 4
38	58	110	110	19000

FIGURE 1 – Upregulation of IL6 by DDIT3 and FUS-DDIT3 in HT1080 stable transfectants and MLS cell lines as measured by the following: (*a*) RT-PCR or (*b*) levels of *in vitro* secreted IL6 protein (pg/ml) (representative values from duplicate experiments). (*c*) Xenografted SCID mice carrying tumors originating from the MLS 2645-94 cell line all show detectable or high *in vivo* serum levels (pg/ml) of IL6 protein compared to the control SCID mouse.

Quantitative real time PCR and microarray analysis

	HT1080 Ctrl	HT1080 DDIT3	Micro-array change	HT1080 FUS-DDIT3	Micro-array change	MLS 1765-92	MLS 402-91	MLS 2645-94
IL6	1.0	4.4	U	6.5	U	18	490	42
IL8	1.0	-27	D	2.5	NC	12	230	39
IL1B	1.0	-3.3	MD	-1.1	MD	-3.1	-2.7	-5.3

Note. QPCR values represent fold change compared to the HT1080 Ctrl

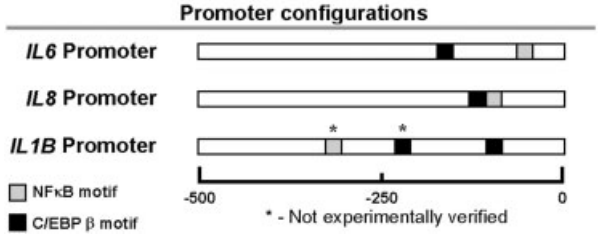


FIGURE 2 – Real time PCR verification of Affymetrix genechip data shows that the downstream effects of DDIT3 and FUS-DDIT3 may differ based on the promoter architecture of target genes. In contrast to IL6 and IL8, the IL1B promoter has no NFκB site at the immediate promoter. Microarray data is presented as upregulated (U), downregulated (D) minor downregulated (MD) or no change (NC) compared to the original HT1080 control cell line. Similarly, real time PCR values represent fold induction (+) or fold reduction (−) compared to the original HT1080 control.

HG-U133 set. The *IL6* gene was found to be upregulated in DDIT3 and FUS-DDIT3 expressing cell lines and this finding was confirmed by RT-PCR and real-time PCR (Figs. 1*a* and 2). FUS-DDIT3 appeared to be a stronger activator than DDIT3 on the expression of *IL6*. In addition, all 3 examined myxoid liposarcoma cell lines were found to express the *IL6* mRNA at very high levels (Fig. 1*a* and 2). IL6 protein quantification using an IL6 bioassay showed that conditioned media from 3 out of 4 FUS-DDIT3-expressing cell lines contained very high levels of secreted bioactive IL6 (Fig. 1*b*).

High serum levels of secreted IL6 protein in MLS 2645-94 inoculated mice

The high levels of FUS-DDIT3-induced IL6 expression prompted us to investigate the possibility of measuring the serum levels of secreted IL6 protein in the bloodstream of animals carrying FUS-DDIT3 expressing tumors. Blood samples were collected from SCID mice carrying tumors originating from the MLS 2645-94 cell line. The serum levels of bioactive human IL6 were measured by an IL6 bioassay and compared to the serum level of a control mouse (Fig. 1*c*). Although variable, the serum IL6 protein levels in MLS 2645-94 inoculated mice were all higher than in the control mouse, suggesting that the secreted IL6 protein reach the bloodstream in detectable levels. Serum levels of IL6 are currently regarded as a diagnostic marker for tumor progression and prognosis in various forms of cancer.[16,17] However, it remains to be established if IL6 can be used as a marker to monitor progression of MLS/RCLS.

C/EBP β but not FUS-DDIT3 binds to the IL6 *promoter*

Previous studies have shown that IL6, at least in part, is regulated by C/EBP β (also called nuclear factor for IL6 expression), which binds to a consensus site ACATTGCACAATCT at the *IL6* promoter.[18] Interestingly, the C/EBP β consensus site at the *IL6* promoter overlaps with a potential DDIT3 site located on the minus strand. We used Chromatin Immuno Precipitation (ChIP) to investigate if FUS-DDIT3, C/EBP β or heterodimers of FUS-DDIT3 and C/EBP β bound directly to the *IL6* promoter. Cell lysates from HT1080 fibrosarcoma cells transiently transfected with the *CEBPB-GFP* plasmid and stable *FUS-DDIT3-GFP* expressing HT1080 cells were subjected to ChIP using antibodies directed against GFP. The immunoprecipitated DNA was then analyzed by PCR with primers specific for the IL6 gene promoter. An anti-acetyl-histone H4 antibody was used together with the MLS 402-91 cell line as a positive control to validate the performance of the ChIP protocol. The results show that C/EBP β binds directly to the *IL6* promoter, but FUS-DDIT3 could not be detected bound to the *IL6* promoter (Fig. 3).

IL6 induction by FUS-DDIT3 is C/EBP β dependent

We used the siRNA knockdown technique to investigate if the strong effect of FUS-DDIT3 on IL6 expression is dependent on the presence of C/EBP β. The efficiency of RNA interference was monitored both at mRNA and protein level at 24, 48 and 72 hours after transfection. The 8.6-fold reduction of the *C/EBP* β mRNA level and the subsequent effect on the C/EBP β protein level observed after 48 hr reduced the effect of FUS-DDIT3 on the *IL6* mRNA level (Fig. 4*a*,*b*). We therefore conclude that C/EBP β is an important mediator of the FUS-DDIT3 induced IL6 expression.

FUS-DDIT3 interacts with both large and small isoforms of C/EBP β

Several C/EBP β isoforms with distinct functional properties arise by differential initiation of translation.[19] The major products are the 38 and 20 kDa isoforms. DDIT3 was recently reported to be able to function as an activator by binding and inhibiting the negatively acting C/EBP β 20 kDa isoform.[20] We used coimmunoprecipitation to investigate the interaction between FUS-DDIT3 and the different 38 and 20 kDa C/EBP β isoforms. Immunoprecipitates of coexpressed GFP tagged C/EBP β 38 or 20 kDa isoforms and FUS-DDIT3-DsRED in HT1080 showed no difference between C/EBP β isoforms in their ability to coprecipitate FUS-DDIT3 (Fig. 5). Similar results were obtained in MLS cell line 402-91, which constitutively expresses the native FUS-DDIT3 fusion oncoprotein (data not shown).

DDIT3 and FUS-DDIT3 have opposite effect on expression of IL8

Apart from the evident effect on the expression of *IL6*, C/EBP β is also an important regulator of other interleukines involved in the acute phase response, such as the *IL8* and *IL1B* genes. Comparison of mRNA levels in HT1080 DDIT3-GFP and HT1080

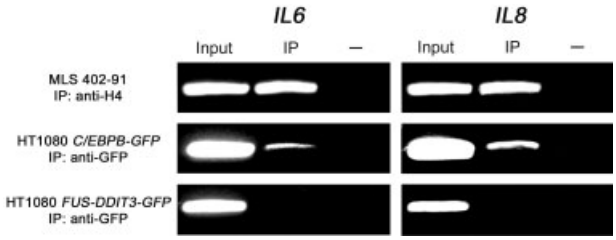
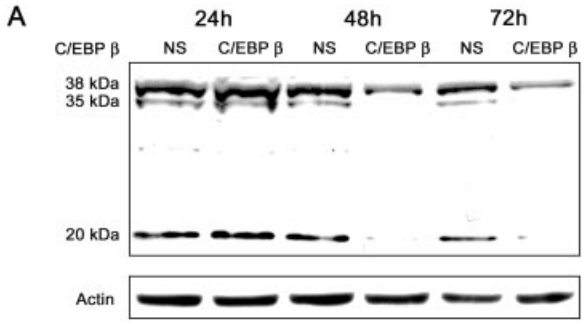


Figure 3 – ChIP analysis showing that C/EBP β binds directly to the IL6 and IL8 promoters and that FUS-DDIT3 could not be detected at these promoters. The IL6 and IL8 promoters are highly acetylated in the 402-91 myxoid liposarcoma cells. Lysates from myxoid liposarcoma cell line 402-91, HT1080 cells transiently transfected with CEBPB-GFP and HT1080 cells stably expressing FUS-DDIT3-GFP were immunoprecipitated using anti-acetyl-histone H4 or anti-GFP antibodies. Precipitated DNA was analyzed by IL6 and IL8 promoter specific PCR. Input (lane 1) represents input material and is used as positive control. Ip (lane 2) represents immunoprecipitated material. Negative control: (Lane 3) represents material that was immunoprecipitated using no antibody and is included as a negative control.



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	24h		48h		72h	
	NS	C/EBP β	NS	C/EBP β	NS	C/EBP β
C/EBP β	1.0	1.0	1.0	−8.6 ± 0.9	1.0	−2.0
IL6	1.0	−1.2	1.0	−2.4 ± 0.3	1.0	−2.3

Figure 4 – C/EBPB siRNA knockdown counteracts the FUS-DDIT3 induced IL6 expression. The protein and mRNA levels of C/EBP β and IL6 were investigated by (*a*) Western blot and (*b*) Real time PCR in HT1080 FUS-DDIT3-GFP cells subjected to nonsilencing (NS) or C/EBPB siRNA (C/EBP β) for 24, 48 (duplicate experiments) and 72 hr. Values represent fold reduction (−) compared to the nonsilencing siRNA control.

FUS-DDIT3-GFP cell lines by real-time PCR showed that DDIT3 and FUS-DDIT3 expressing cell lines all have a slightly lower expression of *IL1B* than the HT1080 control. In contrast, the effect on the expression of *IL8* shows substantial variation between DDIT3 and FUS-DDIT3 expressing cell lines (Fig. 2). Similar to the *IL6* promoter, the promoter of *IL8* contains a C/EBP consensus site were C/EBP β but not FUS-DDIT3 could be detected by ChIP analysis (Fig. 3). Based on these observations we conclude that the downstream effects may differ between DDIT3 and FUS-DDIT3, suggesting that the N-terminal portion of FUS-DDIT3 may influence the formation of a transcriptionally active complex at the *IL8* promoter without actual binding of FUS-DDIT3 to the C/EBP consensus site.

Discussion

We have found that expression of the *IL6* gene is upregulated in FUS-DDIT3 carrying cell lines. The high mRNA level of *IL6* is accompanied by very high levels of secreted bioactive IL6 protein, which probably also give systemic effects as it reached the blood-

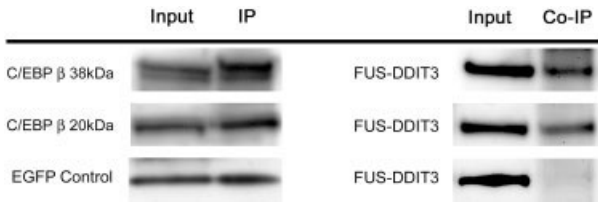

Figure 5 – FUS-DDIT3 is coimmunoprecipitated with both large and small isoforms of C/EBP β. Protein extracts from HT1080 cells transiently transfected with CEBPB38-GFP (large isoform), CEBPB20-GFP (small isoform) or GFP (control) together with FUS-DDIT3-DsRED were immunoprecipitated using a GFP antibody. Precipitated and coprecipitated proteins were visualized by Western blot using antibodies directed against C/EBP β, GFP or DDIT3, respectively.

stream in the MLS xenografted mice. IL6 is a multifunctional cytokine that has been shown to act as an autocrine growth factor in human prostate cancer cells,[21] and in the case of multiple myeloma, the existence of an IL6 autocrine loop has been implicated in the oncogenesis process.[22] In addition, it was recently shown that IL6 plays a pivotal role for proliferation and invasion of malignant fibrous histocytoma (MFH).[23] It is possible that IL6 may have similar functions in MLS/RCLS. The oncogenic potential of IL6 makes it important to determine if this factor is a downstream target of FUS-DDIT3 in MLS.

C/EBP β, which is the preferred dimerization partner of DDIT3[4], and also binds to FUS-DDIT3, has been reported as an important regulator of IL6 expression. As shown by the RNA interference experiment, the presence of C/EBP β is essential for expression of IL6 in FUS-DDIT3 carrying cells. We confirmed by ChIP analysis that C/EBP β binds the *IL6* promoter in FUS-DDIT3 carrying cells but found no direct binding of FUS-DDIT3 to this promoter. Thus, the effect of FUS-DDIT3 on IL6 expression must be indirect, possibly by interaction with C/EBP β.

C/EBP β is translationally regulated and expressed in multiple isoforms of various lengths. The major isoforms, 38 and 20 kDa, retain their leucine zipper and DNA binding regions but are reported to have opposing effects on C/EBP β controlled gene expression.[19] DDIT3 was recently reported to bind the small inhibitory 20 kDa C/EBP β isoform and thereby blocking its effect.[20] However, an earlier investigation concluded that DDIT3 interacts with both major C/EBP β isoforms.[24] We compared the binding of FUS-DDIT3 to the 2 major isoforms of C/EBP β but found no preferential binding to the small inhibitory 20 kDa C/EBP β isoform since they both were able to coprecipitate FUS-DDIT3 in comparable amounts. Thus, specific binding and blocking of the small, inhibitory C/EBP β isoform can not explain the strong expression of IL6 in FUS-DDIT3 carrying cells. FUS-DDIT3 appeared to be a stronger activator of IL6 than DDIT3, indicating that the FUS-derived part of the fusion protein interacts with IL6-regulation. The importance of the FUS N-terminal part for full oncogenic capacity of the FUS-DDIT3 fusion protein has previously been shown by NIH-3T3-based transformation assays[25] and in transgenic mice.[26]

Interestingly, both the normal FUS protein and C/EBP β have been shown to interact physically with the NFκB complex.[27,28] This indicates that the other major factor for IL6 expression, the NFκB system, may be involved in the upregulated expression of IL6 in FUS-DDIT3 carrying cells.

Similar to *IL6*, the *IL8* promoter contains adjacent C/EBP β and NFκB binding sites,[29,30] and it has been reported that C/EBP β and NFκB may act synergistically to regulate transcription at the 2 promoters.[31]

We found that the expression of *IL8* was upregulated more than 2-fold in FUS-DDIT3-carrying cell lines. In contrast, DDIT3 expressing cell lines showed 27-fold downregulation of IL8 expression. To our knowledge, *IL8* is the first example of a gene that shows opposite transcriptional regulation by DDIT3 and FUS-

DDIT3. This finding confirms the hypothesis that the FUS-N-terminal may change the transcription factor activity of DDIT3.[32]

C/EBP β is also an important regulator of the *IL1B* gene, which is involved in the acute phase response together with *IL6* and *IL8*. In contrast to *IL6* and *IL8*, the *IL1B* promoter has no NFκB site at the immediate promoter. Our real-time PCR based expression analysis showed minor downregulation of *IL1B* by DDIT3 and FUS-DDIT3.

The difference in promoter architecture between the *IL1B, IL6* and *IL8* genes may explain the divergent regulation of these genes by DDIT3 and FUS-DDIT3. The configuration of close C/EBP β and NFκB binding sites (71 bp apart) at the immediate promoter of *IL6* and direct proximity of these sites at the *IL8* promoter may enable enhanced transcriptional activation by FUS-DDIT3. It is tempting to speculate that interaction between the N-terminal part of FUS and NFκB may modulate gene expression by physically inhibit or facilitate binding of other factors to this complex. Since FUS-DDIT3 is capable of binding to C/EBP β through its leucine zipper domain and to members of the NFκB system through the N-terminal of FUS, it may act as a mediator by coupling these factors. The lack of a NFκB site in the immediate promoter of *IL1B* would allow only for C/EBP β interaction mediated regulation of transcription in *FUS-DDIT3* and *DDIT3* transfected cells.

Further research that addresses interactions between FUS-DDIT3 and members of the NFκB and IκB families is required to investigate the importance of this fusion protein in NFκB controlled gene expression.

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Microarray analysis was conducted in collaboration with SWEGENE MARC.

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