FET fusion oncoproteins rewire alternative splicing patterns to drive sarcomagenesis

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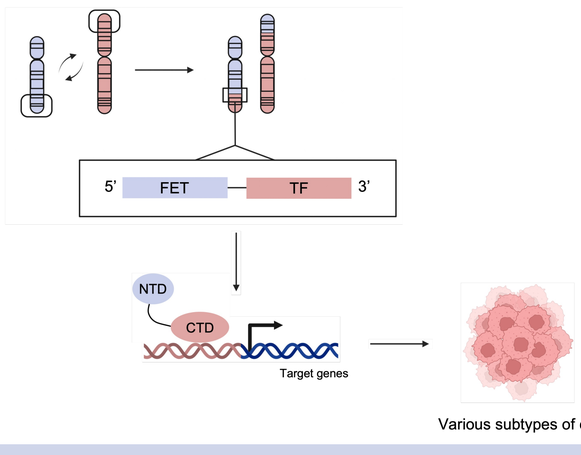
Introduction



FET (*FUS*, *EWSR1*, *TAF15*) genes are regularly involved in chromosomal translocations with diverse transcription factors (TF) genes, leading to gene fusions that drive multiple sarcoma and leukaemia subtypes. As the FET low-complexity domain provides a strong transactivation domain to the DNA-binding domain of the TF-derived moiety, FET fusion oncoproteins were initially considered as aberrant TFs modulating transcription. However, emerging evidence suggest that the oncogenic potential of FET fusions might also be explained by a post-transcriptional function in pre-mRNA splicing, as reported for the EWSR1::FLI1 fusion which drives Ewing sarcoma.

Our aim is to explore whether this post-transcriptional role can be extended to all FET fusions, and to identify a common mechanism underlying FET fusion-mediated sarcomagenesis.

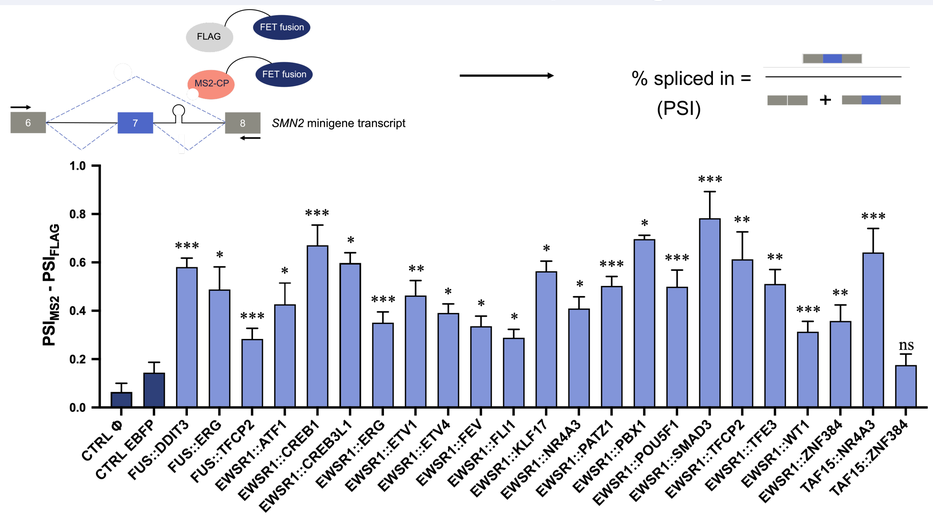
Results

1. FET fusions control the alternative splicing of hundreds of genes

Tumour type	FET fusion	Cell lines	Number of alternatively spliced genes
Clear cell sarcoma (CCS)	EWSR1::ATF1	KAS	**1111**
		MP-CCS-SY	
		SU-CCS-1	
Ewing sarcoma (EWS)	EWSR1::FLI1*	MHH-ES1	**1059**
		A673	
		SK-N-MC	
		TC71	
Desmoplastic small round cell tumour (DSRCT)	EWSR1::WT1**	JN-DSRCT-1	**816**
		BER	
Myxoid liposarcoma (MLPS)	FUS::DDIT3	MLS-1765	**1294**
		DL-221	
		MLS-402	

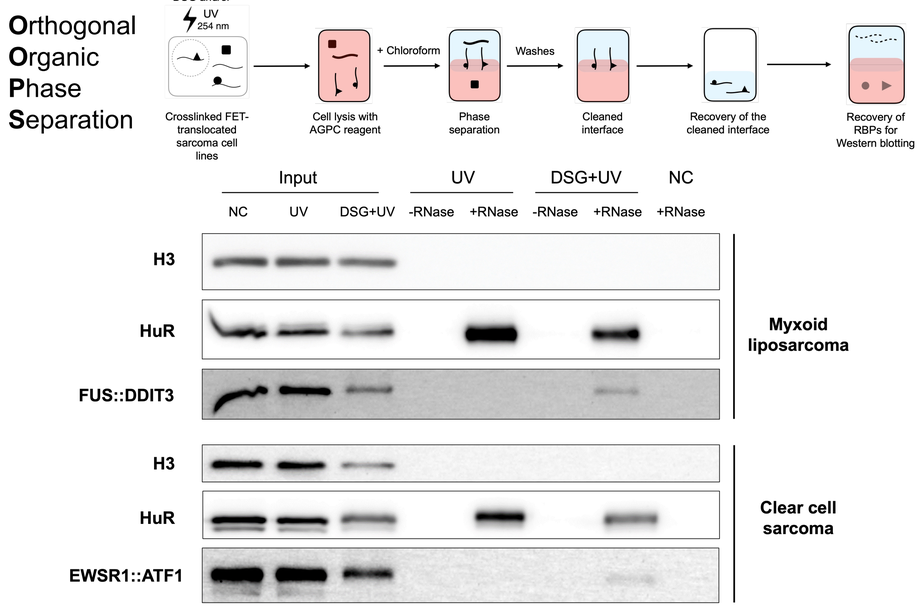
Transcriptomic analysis of sarcoma cell lines following FET fusion knockdown. The values shown represent the genes whose splicing is significatively affected in all cell lines characterized by a specific FET fusion. These results prompted us to test whether the effect of FET fusions on alternative splicing could be direct through a reporter minigene assay. *Buchou *et al*., Cancers, 2022; **Gedminas *et al*., Oncogenesis, 2020.

2. Recruitment of FET fusions on a reporter minigene affects its alternative splicing



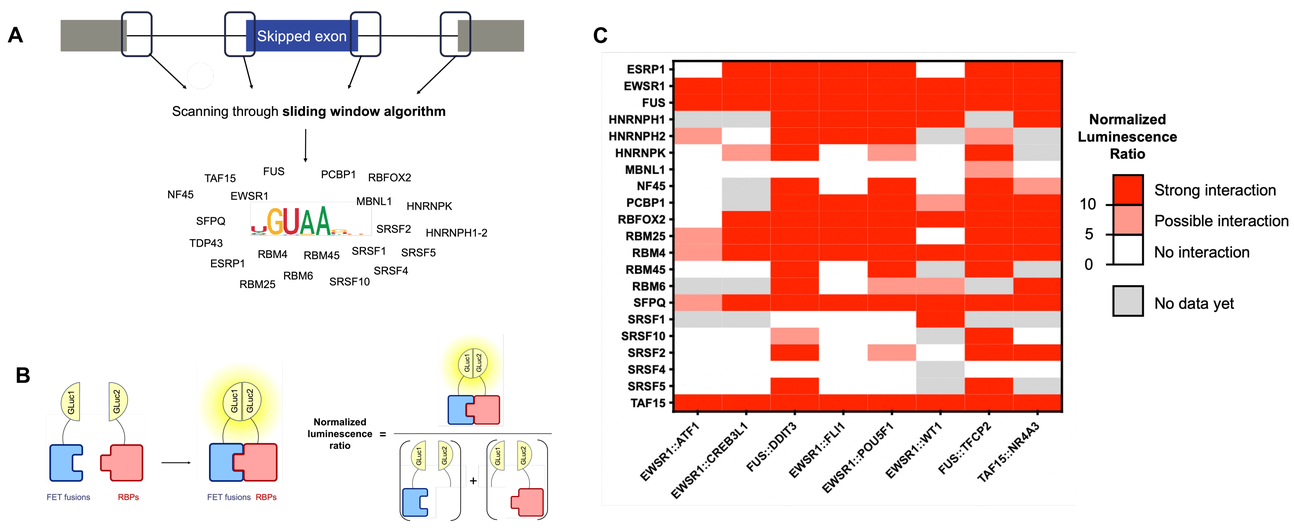
RT-PCR analysis of exon 7 inclusion in the *SMN2* minigene transcript upon FET fusion tethering. The effect of FET fusions on exon inclusion in the *SMN2* minigene transcript is strictly dependent on their recruitment to the transcript, which is surprising as FET fusions lack a canonical RNA-binding domain. We subsequently assessed the presence of FET fusions on RNA using the orthogonal organic phase separation method.

3. FET fusions are indirectly bound to RNA



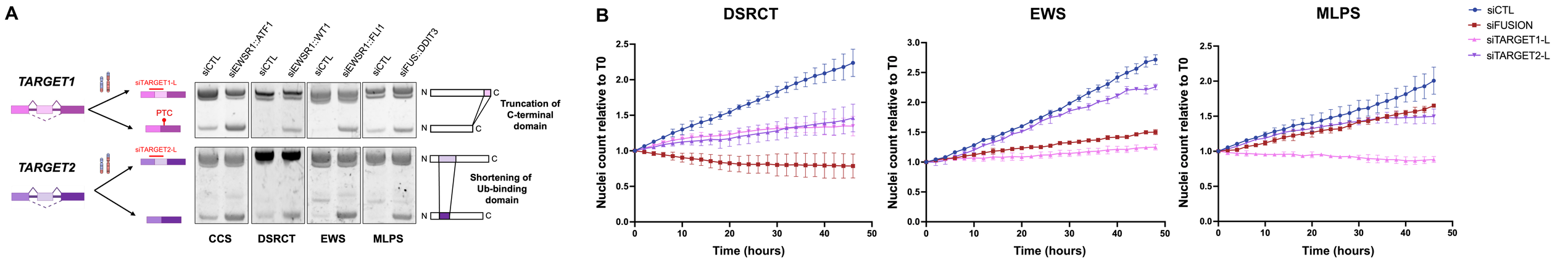
Orthogonal organic phase separation (OOPS) in myxoid liposarcoma and clear cell sarcoma cell lines. This technique described by relies on the differing physicochemical properties of free RNA, free proteins and RNA-protein adducts obtained by crosslinking. Briefly, addition of chloroform to cells lysed with an acid-guanidinium-phenol (AGPC) reagent causes phase separation of the lysate. Free RNA and free proteins migrate to the aqueous and organic phase, respectively. RNA-bound proteins can be purified and detected by western blotting. Ultra-violet (UV) crosslinked proteins directly bound to RNA, and disuccinimidyl glutarate (DSG) was used to stabilize protein-protein interactions. Histone H3 (H3) was used as a negative control, and the RNA-binding protein HuR as a positive control. OOPS was performed using UV crosslinking to highlight direct protein-RNA interactions, and using DSG in addition to UV crosslinking to highlight indirect protein-RNA interactions. We have determined that EWSR1::FLI1 (not shown), FUS::DDIT3, and EWSR1::ATF1 bind RNA indirectly. We therefore sought to identify the intermediary proteins enabling the recruitment of FET fusions to ribonucleoproteic particles.

4. FET fusions interact with numerous splicing factors



Identification of putative protein partners of FET fusions and testing of their interaction. A. Motif enrichment analysis of our transcriptomic data using a sliding window algorithm in regulatory regions flanking significant skipped exons. The motifs of several RNA-binding proteins (RBPs) were highlighted in this analysis, most of which being established splicing factors. They include wild-type FET proteins and RBFOX2, an important player in the splicing-regulatory function of the EWSR1::FLI1 fusion (Saulnier *et al*., NAR, 2021). **B. The *Gaussia* luciferase protein complementation assay (GPCA)** was performed to test the interaction between FET fusions and candidate RBPs identified in A. This assay relies on the reconstitution of luciferase (GLuc) upon interaction of the two candidate proteins, in which case light is emitted in the presence of coelenterazine. For each interaction, the luminescence value is normalized with regards to the sum of two negative control. **C.** We arbitrarily set normalized luminescence ratios for interactions (above 5), and strong interactions (above 10). Each FET fusion interacted with several splicing regulators whose motifs were enriched around FET fusion-regulated exons. Therefore, FET fusions could be recruited to their target transcripts via their interaction with these factors. N ≥ 3 for all.

5. Transcript variants favoured by FET fusions sustain sarcoma cells proliferation



A. Consequences of the alternative splicing of *TARGET1* and *TARGET2* regulated by FET fusions. Given the numerous similarities between FET fusions highlighted hereabove, we aimed to determine whether FET fusions impose a similar splicing pattern to certain target transcripts. The analysis of our transcriptomic data uncovered 13 splicing events regulated by all FET fusions in the same manner. We assessed the outcome of these events on transcript fate and the downstream effects of the splicing event on protein structure and function. We chose to focus our efforts on two candidate transcripts, referred to as *TARGET1* and *TARGET2*. *TARGET1* encodes a transcription factor involved in cell cycle regulation. As shown by RT-PCR, FET fusion knockdown by siRNA (siEWSR1::ATF1, siEWSR1::WT1, siEWSR1::FLI1 or siFUS::DDIT3) results in exon exclusion, which introduces a premature termination codon (PTC) and results in the production of a truncated *TARGET1* transcript encoding a protein with a shortened C-terminal domain, which could be involved in the stability of the protein. The TARGET2 protein is involved in proteosomal degradation. As depicted by the RT-PCR above, an exon encoding a portion of the ubiquitin (Ub) - binding domain of this protein is excluded upon FET fusion depletion, resulting in the shortening of this domain in the final protein. As the isoforms of TARGET1 and TARGET2 produced in the absence of FET fusions are likely to be dysfunctional, we hypothesized that the long isoforms of TARGET1 and TARGET2 favoured by FET fusions (TARGET1-L and TARGET2-L) would be essential to FET-translocated sarcoma cells proliferation and survival. We subsequently designed siRNAs specifically targeting these long isoforms (siTARGET1-L and siTARGET2-L, in red) and assessed the effect of their depletion on DSRCT, EWS and MLPS cells (ongoing experiment for CCS cells). **B. Proliferation analysis of FET-translocated sarcoma cells upon specific knockdown of *TARGET1-L* or *TARGET2-L*.** To assess the impact of TARGET1-L or TARGET2-L depletion on cell fitness, we monitored cell proliferation during 48 hours in representative cell lines for each FET-translocated sarcoma. As previously described, FET fusion-depleted cells proliferated less than control cells. In line with our hypothesis, it appears that the knockdown of TARGET1-L or TARGET2-L affects the proliferation of FET-translocated sarcoma cell lines, highlighting the potential involvement of these isoforms in FET fusion-mediated sarcomagenesis. N = 2.

Conclusion and perspectives

We have gathered pieces of evidence supporting a direct role in alternative splicing for all FET fusions and have paved the way towards a better understanding of the mechanism underlying this function. Indeed, we showed that FET fusions may exert their splicing-regulatory function in association with splicing factors. We have yet to thoroughly characterize their involvement in RBP-RNA interaction networks. Moreover, we have identified two candidate transcripts whose alternative splicing is regulated by FET fusions to promote the production of isoforms sustaining the proliferation of sarcoma cells. We designed splice-switching oligonucleotides forcing the production of the small isoform of these transcripts in the hopes of proposing a new therapeutic avenue for the treatment of FET fusion-associated sarcomas, which still lack efficient care to this day.