Fusion protein EWS-FLI1 partners with EWSR1 in a protein granule in Ewing sarcoma

Nassha Ahmed and Jacob Schwartz
Department of Molecular and Cellular Biology, The University of Arizona, Tucson, AZ 85719

Abstract
Chromosomal translocation events drive many types of pediatric cancers. Ewing sarcoma is a pediatric bone cancer driven by a chromosomal translocation where the N-terminal low-complexity domain of EWSR1 fuses to the DNA-binding domain of the ETS transcription factor FLI1. This fusion creates the aberrant transcription factor EWS-FLI1, which drives tumor formation by altering the transcriptional landscape. Interestingly, constitutive expression of EWSR1 is retained in Ewing sarcoma cells. The low-complexity domain found in EWSR1 and EWS-FLI1 is intrinsically-disordered and can mediate protein-protein interactions with itself and other protein partners. Moreover, this domain has the propensity to phase separate. Despite the common domain between the two proteins, very little is known about the effect of EWSR1 on EWS-FLI1 activity. We performed RNA-sequencing to reveal that EWSR1 and EWS-FLI1 coregulate a large pool of genes in Ewing sarcoma cells. Loss of EWSR1 in inhibits anchorage-independent growth, suggesting that EWSR1 plays a role in cancerous properties of Ewing sarcoma cells. We have shown EWSR1 and EWS-FLI1 bind each other directly in vitro and in vivo. Finally, we have found the existence of EWSR1, EWS-FLI1, and RNA Pol II in a novel granular body in Ewing sarcoma cell lines. This direct interaction of EWSR1 and EWS-FLI1 presents a straightforward model that merges wild-type and fusion properties to alter transcriptional output and drive Ewing sarcoma biology.

Introduction
In 85-90% of Ewing sarcoma cases, the translocation between the genes EWSR1 and FLI1 to generate the novel oncogenic fusion transcription factor EWS-FLI1. The LC domain of EWSR1 contributes to transcriptional regulation, while the FLI1 DNA-binding domain directs the fusion proteins towards target genes. The LC domain donated by EWS is capable of mediating protein-protein interactions. The LC domain binds to the CTD of RNA Pol II, and is also considered intrinsically disordered, allowing it to oligomerize.

Figure 2. A large number of transcripts had similar responses after a loss of EWS-FLI1 or EWSR1 in Ewing sarcoma. A. Schematic of siRNA targets and western blot of confirming knockdown using siRNAs targeted against EWS-FLI1 (siEF), EWSR1 (siEWSR1) or both EWSR1 and EWS-FLI1 (si-EF). EWSR1 and both EWSR1 and EWS-FLI1 (si-EF-EF) in A673 cells. B. A comparison of fold changes revealed 388 transcripts (green) increased or decreased >2-fold in the same direction after either EWS or EWS-FLI1 knockdown, and 39 transcripts (yellow) were changed in opposing directions.

Figure 3. Loss of EWSR1 cells anchorage-independent growth in Ewing sarcoma cells. A. Soft agar assays in A673 cells following knockdown of EWS-FLI1, EWSR1, or scramble control. B. Bar graph quantifies relative colony count in A673 cells. Error bars represent standard error. (n=3). C. Soft agar assays in HEK293 cells with control and knockdown treatments. D. Bar graph quantifies relative colony count in HEK293 cells. Error bars represent standard error. (n=4). Images shown are representative of three biological replicates. Student t-test, * p<0.05, *** p<0.001; n.s. = p>0.05.

Figure 4. Expression of EWS-FLI1 in HEK293 cells recapitulates loss of EWSR1 phenotype. A. Western blot of HEK293 cells co-transfected with V5-tagged EWS-FLI1 (V5-EWS-FLI1) and siRNA targeting EWSR1 or scramble control. B. Soft agar assays of HEK293 cells co-transfected with V5-EWS-FLI1 and siEWSR1 or VS-EWS-FLI1 and control siRNA, siSCR. C. Quantification of relative colony number from soft agar assays. Error bars represent standard error. D. Western blot of co-immunoprecipitation of EWSR1 from HEK293 cells transfected with V5-tagged EWS-FLI1. Mouse IgG serves as the negative control. Student t-test, n.s. = p>0.05. (n=6).

Figure 5. EWS-FLI1, EWSR1, and RNA Pol II coimmunoprecipitate in a crosslinked protein granule. A. Crosslinked immunoprecipitation assay of HEK293 cells transfected with EWS-FLI1, EWS-FLI1 immunoprecipitated with endogenous EWSR1 and RNA Pol II. Bars represent standard error (n=3). B. Crosslinked immunoprecipitation assay of A673 cells. Endogenous EWS-FLI1 immunoprecipitated with EWSR1 and RNA Pol II. Bars represent standard error (n=4). C. Crosslinked immunoprecipitation assay of HEK293T cells transfected with EWSR1, EWSR1 and RNA Pol II immunoprecipitated with EWS-FLI1. D. Transmission electron microscopy of RNA Pol II and EWS-FLI1 granules from HEK293T cells transfected with wild-type or mutant EWS-FLI1. Scale bar = 50nm (top), 80nm (bottom). For A-C, bars only served as the negative control.

Figure 6. FET protein granules regulate transcription. Our model is that FET-fusion proteins redirect FET proteins to generate aberrant granules that ignite a new transcription program driving cells toward tumor formation.

Acknowledgments
This work is supported by The American Cancer Society and NIH grants R01CA186376-03, R21CA238499-01 and T32-GM008659. I would also like to thank the Schwartz lab in the development of this project.

Figure 1. EWS-FLI1 fusion drives Ewing sarcoma tumor development. A. Shown here is wildtype FLI1 and EWSR1. EWS-FLI1 is derived from a translocation between chromosomes 22 and 11, which keeps the expression of EWSR1 and the FLI1 promoter remains silent. B. Both EWS-FLI1 and EWSR1 immunoprecipitate with RNA Pol II in Ewing sarcoma cells and EWSR1 immunoprecipitates in HEK293T cells, suggesting transcriptional activity of EWSR1 in normal cells and in the disease state.