



**SU2C-CrUK Pediatric Cancer New Discoveries Challenge:**  
**“Targeting R-loop Stability in Ewing Sarcoma”**



[This abstract was provided by the scientists when their application was accepted.]

Ewing sarcoma (EwS) is a pediatric cancer driven by the EWSR1-FLI1 translocation. Despite decades of work, this cancer is still an enigma, with poorly understood biology and no targeted treatment. Our recent work published in Nature demonstrated a previously overlooked consequence of EWSR1-FLI1, that this fusion causes hyper-phosphorylated RNA polymerase II (pRNAPII) due to loss of EWSR1 inhibition of CDK7 and CDK9. We observed high levels of transcription and accumulation of R-loops. We therefore started to ask the consequences of these R-loops and noted that EwS is known to display altered RNA splicing. Interestingly, R-loops can be associated with splicing, and splicing defects, specifically mutations in SF3B1 and SRSF2, cause R-loop accumulation. Further, Dr. Hiom reported that the splicing machinery inhibits DHX9 (aka RNA helicase A; RHA) from causing accumulation of toxic R-loops. Of note, EWSR1-FLI1 interacts with and impairs DHX9 activity. With a genomic RNAi viability screen we determined that EwS cells are acutely sensitive to a loss of SF3B1 and SRSF2, more so than mesenchymal stem cells (a putative cell of origin for EwS). Further, SF3B1 and SRSF2 bind pRNAPII. Given the high levels of pRNAPII in EwS, it is possible that SF3B1 and SRSF2 may be sequestered by pRNAPII providing a basis of the R-loop increase. However, with EWSR1-FLI1 interacting with and inhibiting DHX9, accumulation of toxic R-loops may be prevented. In fact, a compound that inhibits EWSR1-FLI1:DHX9 interaction, YK-4-279, significantly decreases EwS viability. Based on these observations, we reevaluated available R-loop data and defined two types of R-loop increases: Type I – new R-loops where none existed previously, and Type II – expansion of existing R-loops. In EwS, or following inhibition of SF3B1, we observed a substantial increase in Type II R-loops. In other work we found induction of a Type I R-loop was associated with genome instability, which is not seen in EwS. These observations led us to the hypothesis that EwS accumulates high levels of physiological R-loops due to increased SF3B1, SRSF2 and pRNAPII interactions but prevents accumulation of pathological R-loops due to interaction of DHX9 and EWSR1-FLI1. If we are correct, then targeting either of these complexes may offer a novel therapeutic opportunity by increasing toxic pathological R-loops in EwS. Two aims test our hypothesis.

**In Aim 1**, we will examine the interactions of SF3B1, SRSF2 and pRNAPII in EwS versus mesenchymal stem cells (immunoprecipitation, immunofluorescence and proximity ligation assay). We will use high-density CRISPR scanning of these genes to identify amino acids required for EwS viability. Identified mutations will be evaluated for impact on interactions, viability, gene expression, splicing and R-loops as compared to shRNA depletions. We will then test if pharmacological inhibition of SF3B1 confers the same phenotypes.

**Aim 2** will focus on the role of DHX9 and its interaction with EWSR1-FLI1 using much of the same strategy as Aim 1 but using DHX9 overexpression and YK-4-279 to disrupt interaction. We will compare





## Scientific Abstract

changes in R-loops and viability between the aims with the goal of laying a foundation for future preclinical work.