



## FINAL REPORT

Project Title: Identification of causative mutations for Ewing sarcoma

Project Number: SFA10-12

1. Date project was initiated: \_April 1, 2010\_\_\_\_\_

2. Period covered by this report:

From \_April 1, 2010\_\_ To\_\_March 31, 2011\_\_\_\_\_

3. Publications, Abstracts, and Presentations:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry must include the author(s), article title, journal [book, editors(s), publisher, volume number, page number(s), and date.]

(1) Lay Press: N/A

(2) Peer-Reviewed Scientific Journals: N/A

(3) Invited Articles: N/A

(4) Abstracts: N/A

b. List presentations made during the last year (international, national, local societies, etc.). Use an asterisk (\*) if presentation produced a manuscript.

### **INTERNATIONAL CONFERENCE:**

(\*): We are preparing manuscripts from all of the following presentations.

1) (\*)

Conference: 2010 9<sup>TH</sup> INTERNATIONAL CONFERENCE ON ZEBRAFISH DEVELOPMENT AND GENETICS

Authors: Mizuki Azuma, Hikmat Al-hashimi and Thilakavathy Subramanian

Date: June 16-20, 2010

Location: University of Wisconsin (Madison)

Sponsoring Organization: ZFIN

Venue: annual conference

2) (\*)

Conference: ZEBRAFISH DISEASE MODELING III: CANCER, BLOOD, DEVELOPMENT & IMMUNE RESPONSES

Authors: Mizuki Azuma, Hikmat Al-hashimi and Thilakavathy Subramanian

Date: June 21-24, 2010

Location: Boston, Massachusetts

Sponsoring Organization: Harvard University

Venue: annual conference

### **LOCAL SYMPOSIUM:**

i) (\*)

Conference: 13<sup>th</sup> Annual KU Honors Program Undergraduate Research Symposium

Authors: Kayla Nelson

Date: April 24, 2010

Location: KU, Lawrence

Sponsoring Organization: University of Kansas

Venue: annual conference

ii) (\*)

Conference: KU-Lawrence Kansas IDeA Network of Biomedical Research Excellence (KINBRE)

Scholar Meeting

Authors: Kayla Nelson

Date: July 12, 2010

Location: KU, Lawrence

Sponsoring Organization: University of Kansas

Venue: annual conference

iii) (\*)

Conference: KU Summer Undergraduate Poster Symposium, Poster Presenter

Authors: Kayla Nelson

Date: July 29, 2010

Location: KU, Lawrence

Sponsoring Organization: University of Kansas

Venue: annual conference

iv) (\*)

Conference: UNIVERSITY OF KANSAS CANCER CENTER SYMPOSIUM

Authors: Kayla Nelson, Brittany Belford, Mizuki Azuma

Date: November 04, 2010

Location: Kansas City: KUMC

Sponsoring Organization: University of Kansas Cancer Center

Venue: annual conference

v) (\*)

Conference: UNIVERSITY OF KANSAS CANCER CENTER SYMPOSIUM

Authors: Hikmat Al-Hasimi, Mizuki Azuma

Date: November 04, 2010

Location: Kansas City: KUMC

Sponsoring Organization: University of Kansas Cancer Center

Venue: annual conference

4. Provide a brief list of keywords: (limit to 20 words)

Mitosis, aneuploidy, multinucleated cells

5. Summarize the progress during the period of this report and its impact on your plans for the remainder of the project. Include a summary of the progress toward the achievement of the originally stated aims and list the significant results:

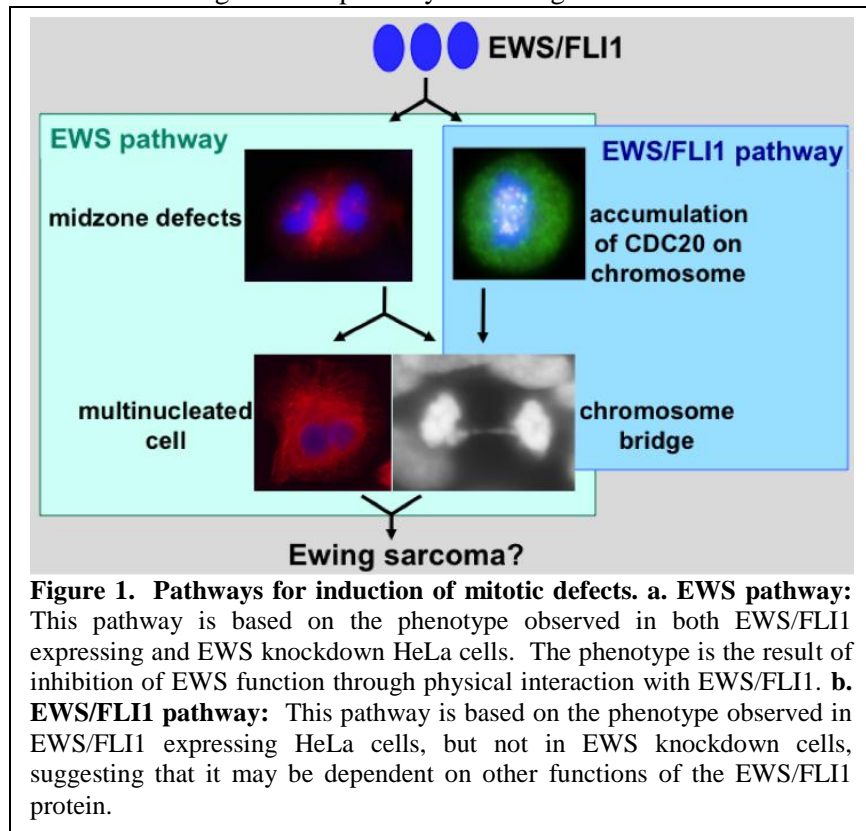
### Accomplishments:

In the grant application, we proposed to establish a transgenic zebrafish line for EWS/FLI1 and test whether the zebrafish develops Ewing sarcoma or chromosome instability. We established the transgenic line at F3 level using CRE lox system. We are currently testing whether the cells in transgenic zebrafish develops higher incidence of aneuploidy compared to wildtypes. In addition, we are in the process of observing whether it develops Ewing sarcoma.

In addition, we have been conducting molecular analysis in cell cultured system to identify the molecules that are involved in EWS/FLI1 dependent mitotic defects. We have discovered two independent pathways for mitotic defect induction in *EWS/FLI1* and *EWS*-siRNA transfected HeLa cells; one involving loss of EWS function, (either through siRNA knockdown or through interaction with EWS/FLI1), and the other involving EWS/FLI1, through an EWS-independent pathway (Figure 1). We will further investigate these pathways in Ewing sarcoma cell lines and in patient cells.

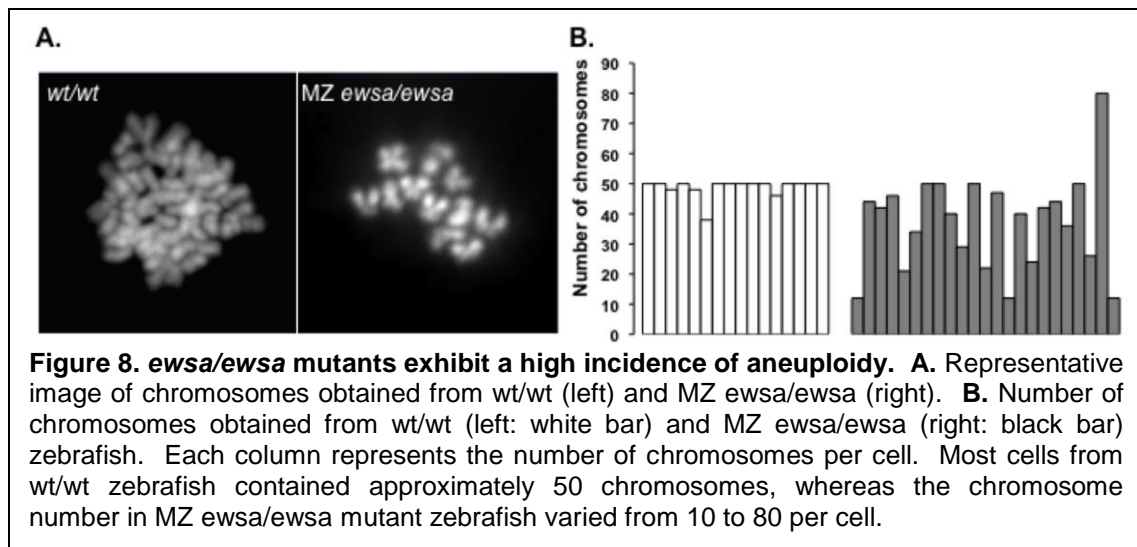
**EWS pathway:** In our studies, we demonstrated that both *EWS/FLI1* and *EWS*-siRNA transfected HeLa cells display incomplete midzone formation, and multinucleated cells (Figures 2 and 3). The resemblance between the phenotypes of *EWS/FLI1* and *EWS*-siRNA transfected HeLa cells implies that the function of EWS is required for midzone formation and for preventing the formation of multinucleated cells.

**EWS/FLI1 pathway:** A high incidence of *EWS/FLI1* transfected HeLa cells exhibited an accumulation of CDC20



proteins on chromosomes during metaphase. Interestingly, the localization of CDC20 proteins in *EWS*-siRNA transfected HeLa cells was normal (data not shown). *EWS*/*FLI1* is a transcription factor, leading to the possibility that misregulation of target genes may contribute to the mitotic defects.

Consistent with *ews*a knockdown experiments using morpholino injections, the Maternal-Zygotic (MZ) *ews*a/*ews*a homozygous mutants exhibit a higher incidence of mitotic defects than wildtype (wt/wt: 11%, MZ *ews*a/*ews*a: 18%; numbers represent scoring of 24 hour post fertilization (hpf) zebrafish embryos (n=40/group) by immunohistochemistry using anti  $\alpha$ -tubulin antibody (n=>440 mitosis). To test whether the mitotic defects we observed result in chromosome instability, chromosome numbers were examined in the MZ *ews*a/*ews*a mutant using metaphase spreads (Figure 2A). The MZ *ews*a/*ews*a mutants displayed higher percentages of cells with aneuploidy, while the majority of wildtype zebrafish cells contained fifty chromosomes (Figure 2B). These results suggest that the *ews*a is essential for chromosome stability. The result supports our hypothesis that the inhibition of *Ews* function contributes to the induction of aneuploidy.



- In layperson's terms, summarize the progress during the period of this report. Explain any medical significance or implications of your results to date:

Because *EWS*/*FLI1* induces mitotic defects through inhibition of endogenous *EWS* function, understanding the function of the wildtype *EWS* protein is crucial to characterizing the mechanism of transformation in Ewing sarcoma. The innovation of this study is that elucidating the function of endogenous *EWS* may not only provide an explanation for Ewing sarcoma, but for other *EWS*-associated sarcomas as well. In addition to *ETS* transcription factors in Ewing sarcoma, *EWS* is fused to a number of different genes in other sarcomas. For example, *EWS* is fused to *ATF-1* in clear cell sarcoma, to *WT-1* in desmoplastic small round cell tumor, to *NR4A3* in extraskeletal myxoid chondrosarcoma, and to *DDIT3* in myxoid liposarcoma. In almost all cases, the amino terminal domain of *EWS* is preserved in the fusion protein. The *EWS* knockout mouse results in B-cell maturation defects, and senescence, suggesting a role for *EWS* in the DNA recombination/repair system. It was also shown that *EWS* protein binds to RNA polymerase II and RNA splicing factors *in vitro*, suggesting additional roles in various aspects of RNA metabolism. Our previous report indicates that *EWS* function is required during mitosis. Given that *EWS* appears to have varying functions within the cell and is involved in several different chromosomal translocations associated with different sarcomas, understanding the cellular function of *EWS* is critical to understanding the transformation process.

It is possible that a common mechanism for chromosome instability in all types of *EWS*-fusion sarcomas is interference of endogenous *EWS* function through the fusion protein. This is supported by our preliminary data showing that the *ews* zebrafish mutant displays a high incidence of aneuploidy. One possibility for *EWS*-fusion related sarcoma formation is that it requires two processes: 1) knockdown of *EWS* function, leading to aneuploidy, and 2) aberrant transcription of

target genes by EWS-fusion proteins, which may induce the specific type of sarcoma. Therefore, elucidating the function of EWS may contribute to the understanding of numerous sarcomas. Accomplishing these aims by analyzing *EWS/FLI1* and *EWS*-siRNA transfected HeLa cells, Ewing sarcoma cell line A673, as well as the zebrafish transgenic *ews/fli1* line and *ews* null mutant line, will provide new insights into the transformation process that occurs in Ewing sarcoma, and will identify key factors useful for patient therapy, as described below.

- Phenotypic analysis during cancer formation, performed in a stage-specific manner, will supply biomarkers that can be used for early detection, or for classification of cancer stages.
- The zebrafish model is a valuable tool for screening drugs; therefore, this model will provide a means to analyze the phenotypes, and to screen therapeutic drugs for tumor suppressor function. In addition, signaling molecules that affect EWS or EWS/FLI1 and trigger transformation may be targets for drug therapy. (A well-known example of drug targeting of a signaling molecule is the tyrosine kinase ABL inhibitor imatinib, which is widely used to treat leukemia patients.)
- If transformation requires additional mutations, the *ews* mutant zebrafish lines can be utilized to screen for second hit mutations.

Mizuki Azuma



Principal Investigator (signature)

August 10, 2011

Date

Mark Richter



Department Chair (signature)

August 11<sup>th</sup> 2011

Date

