MITOTIC DYSREGULATION IN EWS FUSION EXPRESSING SARCOMAS

BY

Shivani J.M

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Chairperson: Dr. Mizuki Azuma

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Dr. Stuart J Macdonald

_____

Dr. Kristi Neufeld

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The thesis committee for Shivani J.M
certifies that this is the approved version of the following thesis:

Mitotic dysregulation in EWS fusion expressing sarcomas

Chairperson: Dr. Mizuki Azuma

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ABSTRACT:

Chromosomal instability (CIN), an unstable genome induced by mitotic dysfunction and unfaithful chromosome segregation, plays a pivotal role in cancer initiation and progression. However, the various upstream events that lead to mitotic dysfunction and how they ultimately result in tumorigenesis are still poorly understood.

Prior publications and studies from the laboratory focused on Ewing sarcoma, a pediatric malignant bone cancer caused by a chromosomal translocation which leads to the expression of an aberrant fusion protein (EWS-FLI1). In addition to the well-studied role of EWS-FLI1 as an aberrant transcription factor, EWS-FL1 induces defects in midzone formation during mitosis. The midzone is a midline structure formed between segregating chromosomes during anaphase, and it consists of the central spindle along with associated midzone proteins. Among these midzone proteins, Aurora-B (kinase) was found to be an integral player in phosphorylating various checkpoint molecules involved in cell cycle regulation thus ensuring proper chromosome segregation. The inability of Aurora-B to locate to the midzone in a timely manner during anaphase has been known to cause failure during the process of cytokinesis and results in induction of aneuploidy. Previous studies in the lab had demonstrated that EWS-FLI1 interacted with wildtype EWS and inhibited its function in a dominant negative manner. Wildtype EWS is known to interact with Aurora-B and recruit it to the midzone; therefore, inhibiting its function could result in mitotic defects. Our studies provided insight into the mechanism by which EWS-FLI1 regulates mitosis, which is of importance as mitotic defects can lead to chromosomal instability, aneuploidy and ultimately tumorigenesis.

Interestingly, EWS is also fused to a number of different transcription factors and is thought to induce various types of sarcoma. For example, EWS-ATF1 is expressed in clear cell sarcoma, EWS-WT1 is expressed in desmoplastic round cell sarcoma and EWS-SP3 is expressed in undifferentiated round cell sarcoma. Each of these fusions retain the transactivation domain of EWS and are fused to the DNA binding domain of the transcription factor. Since all of the EWS fusion proteins retain the same N terminus region of EWS there could be some mechanisms that are conserved between the fusion proteins.

My data suggest that all three EWS fusion proteins (EWS-ATF1*,* EWS-WT1 and EWS-SP3*)* induce mis-localization of Aurora-B at the midzone during anaphase. This could lead to uneven distribution of chromosomes in the daughter cells, and result in high incidence of aneuploidy, a hallmark of cancer. Since Aurora-B is known to localize to the chromosome during early stages of mitosis the EWS- fusion proteins may interact with Aurora-B and inhibit its function during early stages of mitosis. To obtain a better mechanistic understanding behind the mis-localization of Aurora-B, the localization patterns of the EWS fusion proteins were analyzed during early stages of mitosis by immunocytochemistry. As a result, all three EWS fusion proteins localized to the chromosome with varying degrees of chromosomal localization; EWS-ATF1 and EWS-FLI1 displayed high level of chromosomal localization, whereas EWS-WT1 and EWS-SP3 displayed low level of chromosomal localization. Despite variation in the degree of the chromosomal localization of EWS fusion proteins, all proteins induced mitotic dysregulation, characterized by mis-localization of Aurora-B during anaphase. Taken together, my study results support a novel mechanism that the molecular pathogenesis of the EWS fusion expressing sarcomas are mediated by a common mechanism of mitotic dysfunction.

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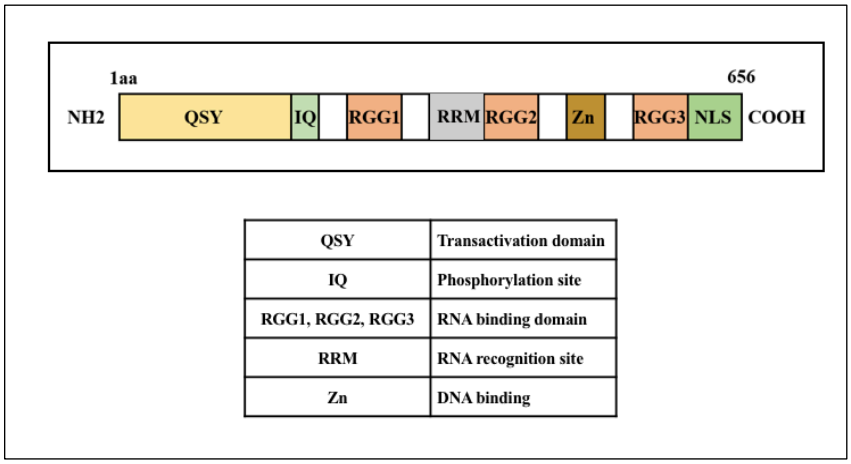
INTRODUCTION:

EWS – A key protein involved in regulating mitosis

The protein, Ewing Sarcoma protein (EWS), regulates different cellular events and has multiple domains (Figure 1) which enable it to perform different functions such as mitosis, cellular differentiation, splicing and transcription[1]. EWS also plays a pivotal role as a bridging molecule between splicing and transcription by interacting with serine/arginine rich splicing factor through its C-terminal and RNA polymerase II through its N-terminal domain [2].



QSY	Transactivation domain
IQ	Phosphorylation site
RGG1, RGG2, RGG3	RNA binding domain
RRM	RNA recognition site
Zn	DNA binding

FIGURE 1: EWS Structural Organization.
EWS has multiple domains attributed to it that enables it to perform different functions

Our previous data indicated that EWS is important for maintaining mitotic integrity in zebrafish embryos and in human cell lines (HeLa)[3]. EWS regulates mitosis by interacting with Aurora-B and recruiting it to the midzone[4] (a midline structure between segregating chromosomes), this interaction was further confirmed by immunoprecipitation. Further, a domain analysis indicated that a particular residue R565 in the RGG3 domain of EWS was necessary for recruiting Aurora-B to the midzone. Site directed mutagenesis of this residue to R565A compromised Aurora B localization [4].

Aurora- B is a kinase that is a part of the chromosomal passenger complex (CPC) along with three other components, Borealin, Survivin and INCENP [5]. The CPC is a key factor driving a faithful mitosis. One example of the activity of CPC is to activate the various checkpoint molecules involved in cell cycle regulation like the Cyclins and CDK. The CPC phosphorylates substrate molecules ensuring proper chromosome segregation during mitosis[5]. During metaphase, Aurora-B localizes to the inner centromere. During onset of anaphase, the CPC is relocated to the central spindle by interacting with MKLP2, a kinesin involved in relocating Aurora-B and other CPC components to the central spindle [4]. Prior publications from the lab established that EWS was crucial in recruiting Aurora-B to the central spindle suggesting that EWS is essential for regulating midzone formation.

Further in vivo studies in zebrafish suggested that EWS regulates spindle formation and chromosomal stability in zebrafish. To examine the function of EWS in an in-vivo model, a zebrafish mutant line was established to knockout EWS (ewsa) in the maternal zygotic embryo by viral insertion which generated a premature stop at the ewsa nineteenth base pair[6]. The studies concluded that the maternal zygotic (MZ) ewsa $^{m/m/}$ displayed higher incidence of disorganized spindles than ewsa $^{w/w}$ upon visualization of these embryos post fertilization through imaging and microscopy[6]. It was further noted through metaphase spread of chromosomes that ewsa $^{w/w}$ exhibited a normal chromosome number however the ewsa$^{m/m}$ displayed an aberrant number of chromosomes. These studies suggested that EWS is crucial for regulating spindle formation and loss of EWS causes chromosomal instability which is a hallmark of cancer.

Pathogenesis in Ewing sarcoma

EWS was originally discovered as a part of *EWS-FLI1* aberrant fusion gene, generated by chromosomal translocation (11:22) in Ewing Sarcoma. EWS-FLI1 contains the N-terminal region of EWS fused to the DNA binding domain of the transcription factor FLI1 (Friend Leukemia Integration site). The DNA binding domain of FLI1 exerts aberrant transcriptional control by binding to specific target genes (*NKX2.2, MYC, CYCLIN D1, TGFBRII* etc.) and mis-regulating their expression thus potentially leading to sarcoma progression [7]. However previous studies indicated that EWS-FLI1 was able to mediate transformation independent of the DNA binding domain of the transcription factor. EWS-FLI1 with point mutations or entire deletion of the DNA binding domain were able to accelerate transformation in NIH3T3 cells, an immortalized embryonic fibroblast cell line. When these transformed NIH3T3 cells were injected into SCID mice they were able to form tumor [8].

This suggests that there is an additional mechanism of EWS-FLI1 mediating oncogenesis, and it is not entirely dependent on the DNA binding domain of the transcription factor. One possibility for the DNA binding independent mechanism of EWS-FLI1 is the induction of mitotic defects leading to the induction of unfaithful chromosome segregation, and of aneuploidy. As expected, we observed that EWS-FLI1 did affect mitosis adversely. Overexpression of EWS-FLI1 in HeLa cells indicated that the fusion protein was capable of inducing mitotic defects as characterized by mis-localization of Aurora-B (a major mitotic regulator). Another study from our lab indicated that haplo-insufficiency which occurs mainly because of EWS-FLI1 losing one wildtype EWS allele

and this event during the formation of EWS-FLI1 could also contribute and cause mitotic defects and tumorigenesis[6].

Further, in vivo studies in which EWS-FLI1 was overexpressed in transgenic mice indicated that the fusion protein alone did not cause tumor, but the combination between the fusion gene and the deletion of Tp53 (tumor suppressor protein that controls cell proliferation, DNA repair, apoptosis and senescence) accelerated sarcoma formation [7]. This suggests that EWS-FLI1 induces sarcoma when there is an additional mutation in Tp53. Haplo-insufficiency or loss of Ewsa (homologue of human EWS) promotes tumorigenesis in homozygous and heterozygous ewsa mutant (ewsa$^{m/w}$, ewsa $^{m/m}$) zebrafish in a tp53 $^{m/w}$ background [6]. Thereby loss of EWS allele may also contribute to pathogenesis of Ewing sarcoma. These results suggest that the pathogenesis of Ewing Sarcoma may require both loss of an EWS allele and overexpression of the fusion gene. Previous literature on EWS-FLI1 provided a number of mechanisms by which the fusion protein can exert its influence and can cause sarcoma progression [9].

EWS fusion expressing sarcomas

The N-terminus of EWS fuses to a number of different transcription factors and mediates different sarcomas as listed in (Table 1). The EWS fusion expressing sarcomas of interest in my study include- EWS-ATF1, EWS-WT1 and EWS-SP3. Each transcription factor (FLI1, ATF1, SP3, WT1) has its own activation domain and recognizes specific target sequences present on genes.

Type of EWS Fusion	Sarcoma
1) EWS-CHOP	Myxoid liposarcoma
2) EWS-TEC	Extra skeletal myxoid chondrosarcoma
3) EWS- FLI1	Ewing Sarcoma
4) EWS-ATF1	Clear cell sarcoma
5) EWS-SP3	Undifferentiated round cell sarcoma
6) EWS-WT1	Desmoplastic round cell sarcoma
7) EWS-CIZI	Acute leukemia

Table 1: EWS fusion expressing sarcomas. The N terminus of EWS fuses to the DNA binding domain of the transcription factor mediating different sarcomas

However, when the EWS fusion protein is generated, each of the transcription factors loses its respective activation domains and gains the EWS-derived transactivation domain.

Pathogenesis of EWS fusion mediated sarcomas is a culmination of number of mechanisms. We suspect that some of these mechanisms may be independent of DNA binding and maybe conserved across the fusion proteins [8]. It is possible that one of these non-DNA binding mechanisms are mediated by biochemical interaction of the fusion protein with wildtype EWS. The fusion protein would then exert a dominant negative effect on EWS, which is known to play an important role to ensure proper mitotic progression.

Additionally, there is loss of one wildtype EWS allele during the formation of the EWS fusion protein and previous studies from our lab indicated that the functional loss of an allele could also promote tumorigenesis and CIN [6]. The studies so far suggest that both loss of an EWS wildtype allele and overexpression of the fusion protein may contribute to the pathogenesis of Ewing Sarcoma.

Eventhough there are many EWS-fusion proteins, EWS-ATF1, EWS-WT1 and EWS-SP3 fusion proteins are of because they exhibit interesting and unique properties. EWS-WT1 and EWS-SP3 were chosen for the study because the transcription factors WT1 and SP3 are known to function as repressors of genes but when fused to the N-terminus of EWS they function as activators [9]. This suggests that fusion of the transcription factors WT1 and SP3 to the N- terminus of EWS may alter target specificity and may activate genes it once repressed. EWS-ATF1 was also included as a part of the study as it contains an additional RGG domain from EWS when fused to ATF1 as compared

to other EWS fusions that just retain the transactivation domain of EWS[10]. Though these fusion proteins have unique properties, we want to evaluate if they could potentially share a common mechanism through the mechanism of altering mitosis. Each fusion protein has its own distinct properties and function as listed below-

EWS-ATF1-

Clear-cell sarcoma mainly develops in soft tissues and the dermis and is caused by the formation of an aberrant fusion protein EWS-ATF1. EWS-ATF1 is formed by a chromosomal translocation (12:22) that results in the fusion of the N-terminal transactivation domain of EWS to the bZIP DNA binding domain of ATF1 (Figure 2). ATF1 mediates activation of cAMP- responsive genes by binding to a conserved cAMP responsive element (CRE). The activity of ATF1 is regulated through phosphorylation of its kinase inducible domain mediated by protein kinase A. However, the EWS-ATF1 fusion protein no longer has the kinase inducible domain because it is replaced by the N-terminal transactivation domain (EAD) of EWS[10]. The EWS-ATF1 fusion protein still binds to CRE via because it retains the ATF1-derved bZIP domain[10].

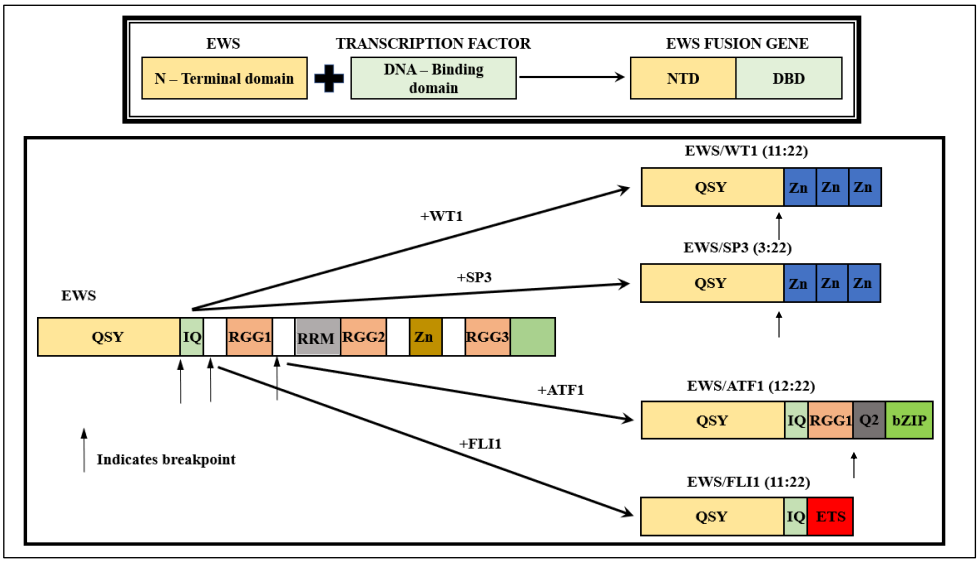


FIGURE 2: **Structural constructs of EWS fusion proteins.** The N terminal transactivation domain of EWS is fused to the DNA binding domain of the transcription factor.

Further in vivo studies indicated that EWS-ATF1 independently, without any mutation in the tumor suppressor P53 has an activity to induce tumor in mice. Specifically, when. the EWS-ATF1 inducible mice were fed with doxycycline in the drinking water, they developed soft tissue tumors within 4 weeks[11].

EWS-WT1- This is a fusion protein formed out of a chromosomal translocation (11:22) and it causes desmoplastic small round cell tumor. Desmoplastic small round cell tumor typically manifests itself in different regions of the body including lymph node, lining of abdomen, spleen and liver. The fusion protein consists of the N-terminal domain of EWS fusing to the two to four zinc finger domains of WT1, excluding the first zinc finger of WT1 in the process [12]. The transcription factor WT1 is expressed in renal blastemal stem cells and is important for the normal development of kidney. WT1 normally functions as a repressor of transcription by binding to GC and TC rich promoters [12].

There are two alternatively spliced isoforms of EWS-WT1 proteins; the EWS-WT1 (+KTS) has extra three amino acids Lys, Thr and Ser, derived from the linker region located between zinc fingers 3 and 4, and EWS-WT1 (-KTS) lacks the three amino acids. EWS-WT1 (+KTS) is known to abrogate binding of WT1 to its target sequence but its exact function remains elusive [12]. EWS-WT1 (-KTS) isoform has the ability to promote tumorigenesis and can trigger apoptosis, cell cycle arrest and activate a number of downstream targets [12]. Expression of this isoform transcriptionally triggered expression of PDGFA, a potent mitogen and chemo-attractant for fibroblasts. However, PDGFA could be activated only by EWS-WT1 and not by wildtype WT1 indicating that there is a

difference in target specificity between the transcription factor and its oncogenic derivative[13]. Transcriptional activation of Platelet derived growth factor (PDGFA) by the fusion protein relied on GC and TC rich sequences flanking the transcriptional start site. Other downstream targets activated by the EWS-WT1 (- KTS) isoform include IGF1 receptor, IL2 receptor, BAIAP3, MLF1.

Also, prior literature revealed that neither isoform of EWS-WT1 are not sufficient to transform murine embryonic fibroblasts, which requires an additional deletion of p53 to unveil its oncogenic properties[14].

EWS-SP3- Another EWS fusion protein that is of interest in this thesis work is EWS-SP3. The fusion protein is derived from chromosomal translocation (2:22) which results in the fusion of the N-terminal transactivation domain of EWS to the DNA binding domain of the transcription factor SP3. SP3 is a member of the mammalian SP transcription factor family and consists of three zinc finger domains at the C-terminus along with a glutamine rich activation domain at the N-terminus [15]. The mechanism of action and the specific region it targets in the body still remains elusive.

My main goal and experiments were catered towards unraveling the mechanism of pathogenesis of EWS fusion based sarcomas. The studies I performed aimed at narrowing down on a candidate that is responsible for equal chromosome segregation and studying whether any of the EWS fusion adversely affects its localization and function. Since EWS/FLI1 was already known to affect localization of Aurora-B, my studies shed light on whether this mechanism was conserved across three other EWS fusion proteins of interest in this proposa

MATERIALS AND METHODS:

Cell culture and induction of EWS fusion proteins using Doxycycline

The DLD1 cells were cultured and maintained in McCoy's 5 A medium with 10% vol Tet system approved FBS at 37 degree Celsius. All EWS fusion proteins were induced with 1ug/ml of Doxycycline for 17 hours. All cells used in this study were passaged for less than 20 times.

Immunocytochemistry

The cells were plated on fibronectin coated coverslips and were subjected to the immunocytochemistry. The cells were fixed and permeabilized with methanol for 3 minutes at -20 degree Celsius, washed with PIPES buffer (PIPES free acid powder, 10N NaOH and water), buffer known to stabilize microtubules and subjected to blocking thereafter. The cells were incubated in blocking solution (1% fetal bovine serum in PIPES) for 1 hour followed by incubation with primary antibodies mouse anti Aurora- B (1:300 dilution) (BD Biosciences, #611082) and rat m-cherry (1:500 dilution). After three 5 min washes with PIPES buffer, the cells were incubated in Alexa Fluor 488 goat-anti- rabbit IgG (1:500 dilution) (Invitrogen, Cat: A11034) and Alexa Fluor 594 goat anti-mouse IgG (1:500 dilution) (Invitrogen, Cat: A11032) for one hour. After the PBS washes, the coverslips were mounted with DAPI/ Prolong gold and visualized at 60x magnification on a Nikon Ti Eclipse microscope. Meta-morph software was used to process the images captured by the camera.

Cell synchronization for cell fractionation experiments:

The cells were synchronized in mitosis using a Thymidine/ Nocodazole cell cycle protocol [16]. The cells were first seeded and upon reaching 40 % confluency 2mM thymidine was added and incubated for 18 hours. Following this step, the cells were released from the thymidine block by

washing three times with PBS. After the washes, the medium containing 10% FBS was replaced and the cells were incubated for 6 hours. Then the cells were treated with 100ng/ml of Nocodazole for 6 hours. After this step the mitotic cells were collected using a mitotic shake off and then washed three times with PBS and finally McCoy's 5A 1X L- glutamine media was added. The mitotic cells were re-released for 30 min and then subjected to cell fractionation studies.

Cell fractionation

Cells were first subjected to synchronization and then lysed using lysis buffer (250mM Sucrose, 20mM HEPES pH 7.8, 100mM NaCl, 1.5mM MgCl2, 1mM EDTA pH8.0, 1mM EGTA pH7.5, 0.2% TritonX-100, 10ug/ml of protease inhibitor-Leupeptin, pepstatin, chymostatin, 1mM DTT, 1mM PMSF) and incubated on ice for 5 minutes. 10% of the lysate obtained from this step served as the whole cell lysate fraction. The remaining lysate was subjected to centrifugation (500 X g for 5 min at 4 degree Celsius, the supernatant obtained from this step was used as the cytoplasmic fraction. The remaining lysate was resuspended in lysis buffer and layered over a 40% glycerol cushion and subjected to centrifugation at 10,000 rcf for 5 min at 4 degree Celsius. The pellet obtained from this step was subjected to the same process again and the final pellet obtained from this step was used as the chromosome fraction.

Western blotting:

The cell lysates obtained from EWS-ATF1 from Dox untreated and treated samples were subjected to SDS PAGE gel electrophoresis on a gradient gel (10-20%) and transferred to a PVDF membrane. The membrane was then probed with rabbit- mcherry (1:1000) followed by 1:10.000 dilution of goat-anti rabbit 680. The whole cell lysates and cell fraction samples of EWS-SP3 obtained from Dox- and Dox+ treated samples of *EWS-SP3* were subjected to western blot using mouse anti- EWSR1 antibody C9 (1:1000) (Santa Cruz, #sc-48404) to detect presence of

endogenous EWS and EWS-SP3 as the antibody detects n terminus of EWS. This was followed by treatment with 1:10,000 dilution of IRDye 680RD donkey anti-mouse IgG secondary antibody (LI-COR, #926-68072). The western blot of the loading controls was probed with (1:1000) dilution of mouse beta actin and (1:1000) dilution of mouse anti- Histone 3. The western blot images were captured using LI-COR odyssey imaging system.

Statistical analysis:

Statistical analysis was carried out using the Paired T test and the p value and level of significance were determined using GraphPad prism 8 software.

RESULTS:

Establishing a stable cell line with EWS fusion constructs integrated into the safe harbor locus in DLD1 cells.

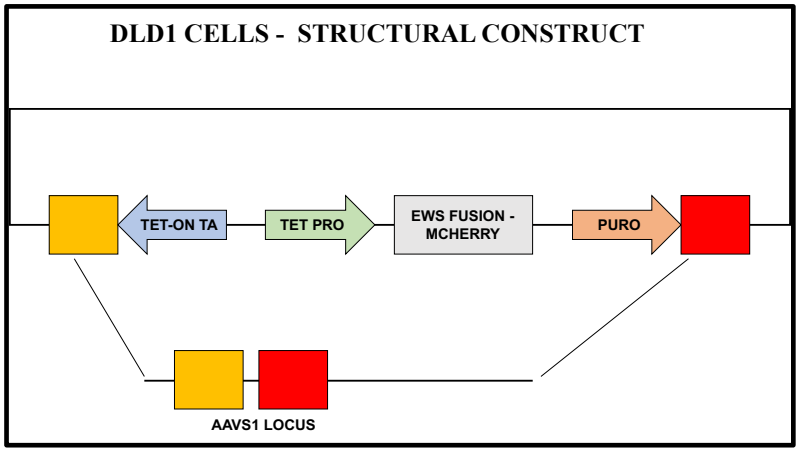


FIGURE 3: DLD1 structural construct: All the fusions proteins were integrated into the safe harbor locus of DLD1 cells to ensure stable and consistent expression of the protein. Upon addition of Dox the Tet on trans-activator was expressed and binds to Tet promoter region causing expression of the EWS-fusion protein. EWS- fusion protein expression was visualized through microscopy as it was tagged to m-cherry.

The DNA construct of each of the EWS fusion genes was integrated into the safe harbor locus (*AAVS*) locus of DLD1 cells using CRISPR/ CAS9 technology. Integrating the fusion gene into the safe harbor locus will ensure consistent and stable expression of the protein of interest. The DLD1 cell line will be used for our study since it is euploid and doesn't display CIN [16].

The DLD1 cells are engineered to express trans-activator protein which bind to the PTRE3G promoter containing the TET - operator sequences only in the presence of doxycycline (Figure 3). Once the protein

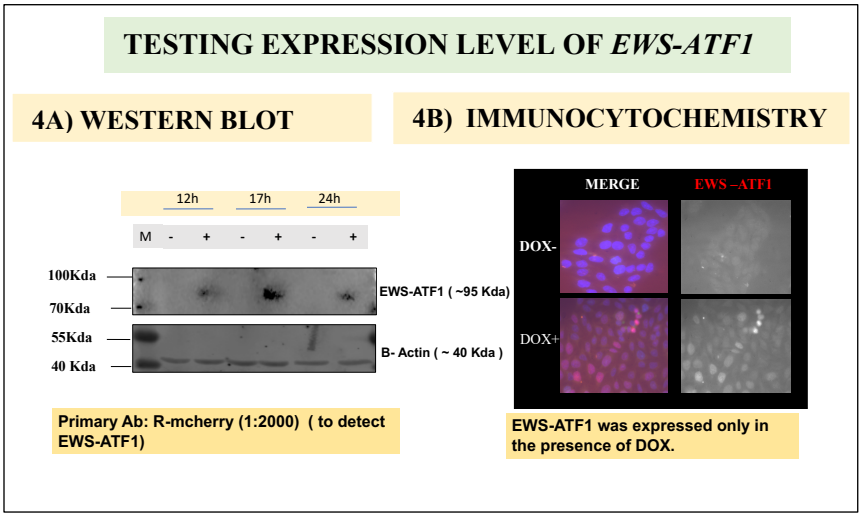


FIGURE 4: Testing expression levels of EWS-ATF1: a) A western blot was first carried out under different time points of dox induction. EWS-ATF1 expression was detected at all timepoints and enhanced at 17 hours of dox induction. B) The immunocytochemistry study also indicated presence of EWS-ATF1 which was tagged with m-cherry in presence of Dox

binds to the promoter it can mediate transcription of the gene (EWS fusion gene tagged to m-cherry) which is placed downstream to it. The fusion protein is tagged to m-cherry and hence we can visualize its expression upon addition of Dox. The Dox- sample would serve as the control for the study.

My preliminary studies to test expression of the EWS fusion constructs in DLD1 cells were carried out by overexpressing EWS-ATF1 in DLD1 cell lines using doxycycline. Initially, a western blot was carried out to test expression levels of EWS-ATF1 at different time points of induction. The western blot indicated that EWS-ATF1 was expressed at all time points, but the level of the expression was highest seventeen hours after Dox treatment (Figure 4a).
Further, the expression of EWS-ATF1 in DLD1 cells was validated using an immunocytochemistry study (Figure 4b). Since the western blot indicated that EWS-ATF1 expression level was enhanced at 17 hours, DLD1 cells were treated with Dox for 17 hours. Upon Dox induction, EWS-ATF1 was expressed and could be visualized under the microscope as it was tagged with m-cherry. The Dox- sample served as a control for the study and EWS-ATF1 wasn't expressed as indicated in (Figure 4b) .

The expression level of all other EWS fusion proteins in this study were visualized and studied after 17 hours of Dox induction. This is primarily because of the data drawn from the western blot (Figure 4a). Induction with Dox for 12 hours might not be sufficient for the fusion protein to be optimally expressed. The 24-hour timepoint for Dox induction was also ruled out because expression of the fusion protein for long periods of time might be lethal and lead to increased cell death.

All EWS fusion proteins cause mis-localization of Aurora-B at the midzone and induce mitotic defects

Aurora-B is a key kinase that phosphorylates various checkpoint molecules involved in cell cycle regulation like outer KNL1/Mis12 complex/Ndc80 complex (KMN) network, the key player in kinetochore-microtubule attachment to ensure proper chromosome segregation.

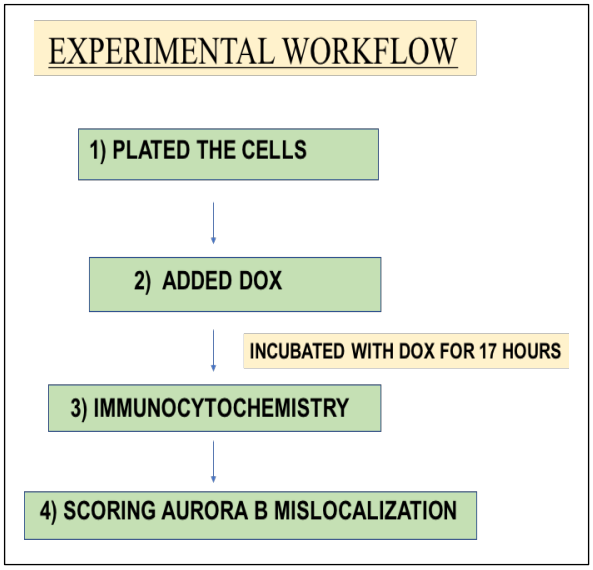


FIGURE 5: Experimental workflow : Workflow indicating steps followed to study effect of EWS-ATF1 on Aurora-B localization through immunocytochemistry.

Aurora-B has to be localized in a timely manner along with other CPC proteins to the midzone to ensure proper chromosome segregation[5]. Previous studies in the lab had established that EWS is crucial for recruiting Aurora-B to the midzone[4]. An siRNA mediated knockdown of EWS compromised Aurora-B localization to the midzone. Similar studies in A673 cell line (a cell line that expresses EWS-FLI1 endogenously) also indicated mis-localized patterns of Aurora-B at the midzone [4]. Aurora-B is one of the key mitotic regulators. If its function is compromised, it results in chromosomal instability which is a hallmark of cancer.

I aimed to determine whether the mechanism of Aurora-B mis-localization is common across all the EWS fusion proteins. In this study, we aimed to determine the activity of the EWS fusion protein on Aurora- B localization. To determine whether the EWS-ATF1 induced mitotic defects

in DLD1 cells, immunocytochemistry was carried out as indicated in the experimental workflow (Figure 5). The antibodies m- Aurora-B and rat-m cherry used in this study were targeted to recognize Aurora-B and the EWS fusion protein.

Once the experiment was carried out, cells were scored and quantified as normal or abnormal based on Aurora-B localization. The normal cells were characterized by complete lines or dots aligning the midzone without any missing gaps in between (as seen in the Dox- sample- Figure 6). Contrary to this, the abnormal cells had missing lines or dots at the midzone indicating abnormal Aurora-B localization. The results suggested that a greater number of abnormal cells (cells with mis-localized Aurora-B signal) was observed when the cells were induced with expression of the fusion protein

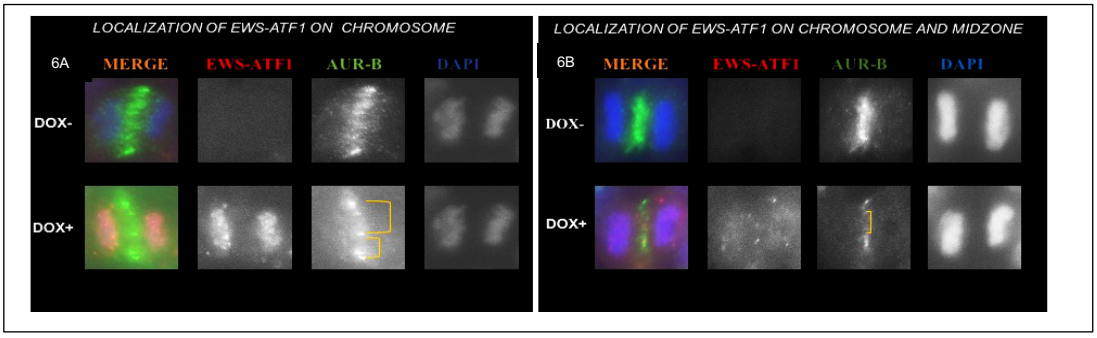


FIGURE 6: Localization pattern of EWS-ATF1 in DLD1 cells: *EWS-ATF1* either localized on the chromosome (Figure 6A left panel) or chromosome and midzone (Figure 6B right panel) and mediated abnormal localization of Aurora-B (characterized by missing spaces or gaps in the Dox+ sample)

The fusion protein either localized on the chromosome or the chromosome and midzone and mediated midzone defects. When EWS-ATF1 localized on the chromosome as indicated in the left panel in Figure 6A there were really strong EWS-ATF1 puncta present on the chromosome

upon Dox induction and there was no presence of EWS-ATF1 signal in the midline between segregating chromosomes.

The other localization pattern that was observed in EWS-ATF1 was localization on the chromosome and midzone as indicated in the right panel in Figure 6B upon Dox induction. In this localization pattern, EWS-ATF1 localized on the chromosome but also displayed diffused tiny puncta in the midzone region between segregating chromosomes

To further quantify the percentage of cells exhibiting each localization pattern, 50 cells were scored for Dox- and Dox+. The overall study of localization pattern of EWS-ATF1 in DLD1 cells indicated that the majority of the cells (39.68 %) expressing EWS-ATF1 displayed localization on the chromosome and midzone . About 23.8% of the cells exhibited localization exclusively on the chromosome. Also, some of the cell population (36.52%) did not express EWS-ATF1 suggesting that expression of EWS ATF1 in DLD1 cells was mosaic (Figure 7).

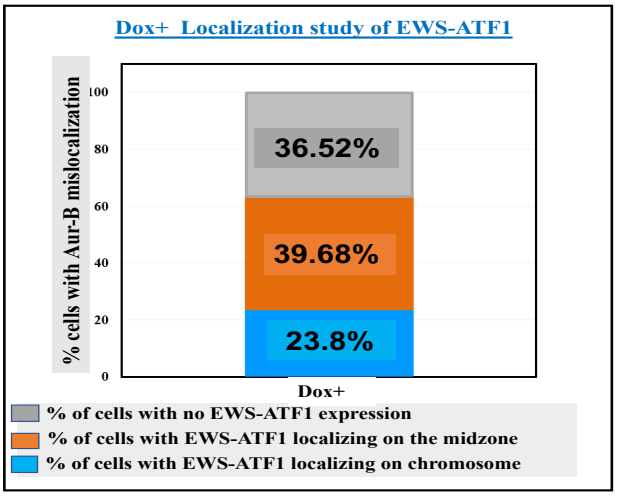


FIGURE 7: Statistical analysis of localization pattern of *EWS-ATF1* in DLD1 cells: Majority of the cells expressing EWS-ATF1 localized on the chromosome and midzone. However there was mosaic expression of EWS-ATF1in DLD1 cells.

To study effect of these localization pattern of EWS-ATF1 on Aurora-B, 40 cells were scored for the Dox- and Dox+ sample. In the Dox+ sample, the abnormal cells (cells that exhibited Aurora-B mis-localization) were scored.

The total number of cells were further sub-classified based on localization trend of EWS-ATF1.

It was noted that when EWS-ATF1 localized on the chromosome and midzone the number of cells that exhibited Aurora-B mis-localization was significantly higher (76%) higher than when EWS-ATF1 localized on the chromosome (20%) (Figure 8).

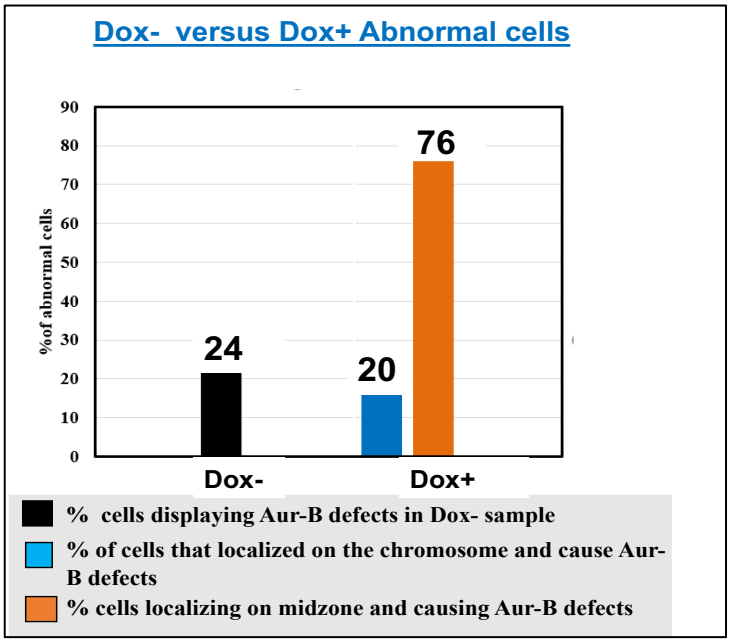


FIGURE 8: More mis-localization of Aurora-B was observed when EWS-ATF1 localized on the midzone. The data indicated that though EWS-ATF1 localized on the chromosome, it did not cause much mis-localization of Aur-B (only 20% cells were abnormal). However localization of EWS-ATF1 on midzone caused more AurB- defects

Overall, it was observed that when EWS-ATF1 was induced with Dox in DLD1 cells, the percentage of cells exhibiting Aurora-B mis-localization was significantly higher compared to the Dox- sample (control for the study)

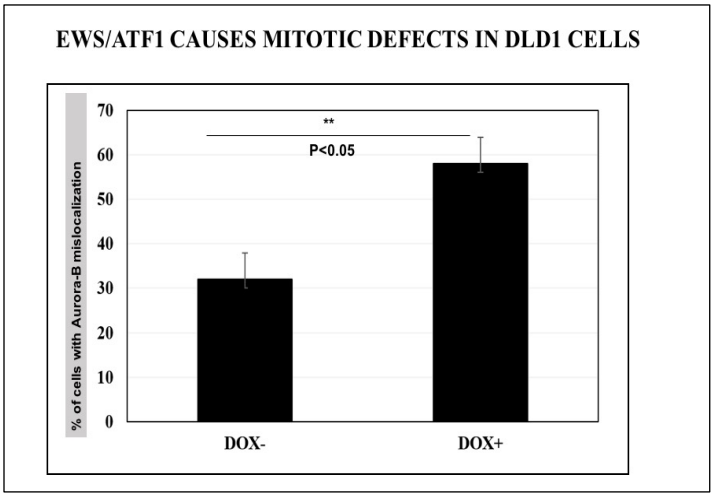


FIGURE 9: EWS/ATF1 induces mislocalization of Aurora-B. Overexpression of EWS/ATF1 caused mitotic defects in DLD1 cells. The study was repeated three times and mitotic defects were mediated more in the DOX + sample (upon expression of the fusion protein). The difference between DOX+ and DOX- sample was found to be statistically significant

(Figure -9). This result was consistent and significant across three trials.

To further test if this mechanism is conserved across other EWS fusion proteins, I initiated my study in EWS-WT1. EWS-WT1 was overexpressed in DLD1 cells using Dox and localization of Aurora-b was studied and scored in Dox- versus Dox+ condition.

The observations from this study indicated that EWS-WT1 also induced mis-localization of Aurora-B at the midzone. (Figure 10). As compared to the EWS-ATF1 which displayed high incidence of chromosomal localization, EWS-WT1 displayed minimal chromosome localization and more cytoplasmic localization.

Furthermore, when the same experiment was conducted in EWS-FLI1 and EWS-SP*3*, both EWS fusion proteins caused similar mis-localization of Aurora-B and mediated midzone defects (Figure 11 and 12). The mis-localization of Aurora-B was characterized by missing chunks of Aurora-B between the two segregating chromosomes, as indicated by the bracketed region in the Aurora-B panel marked in green (Figure 11 and 12).

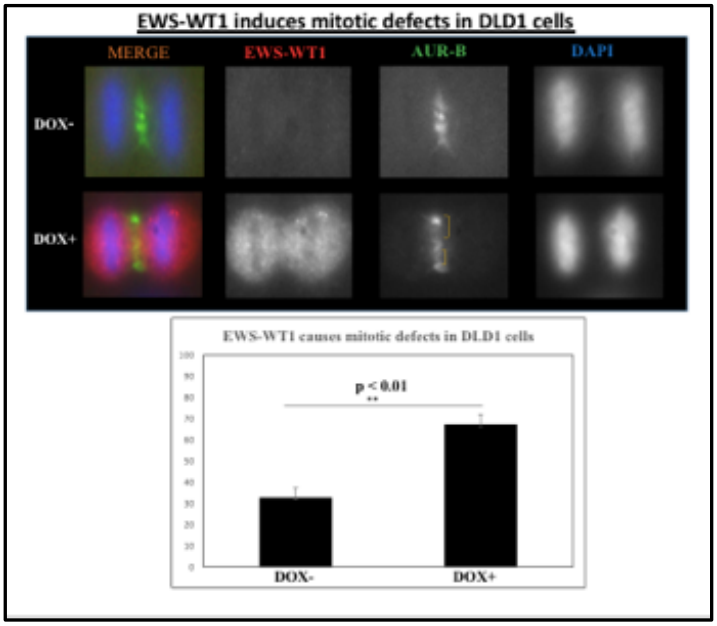


FIGURE 10: EWS-WT1 induces mis-localization of Aurora-B. Overexpression of EWS/FLI1 caused mitotic defects in DLD1 cells. The study was repeated three times and mitotic defects were mediated more in the DOX + sample (upon expression of the fusion protein). The difference between DOX+ and DOX- sample was found to be statistically significant

The Dox- samples served as a control for the study and no mis-localization of Aurora-B indicated in this condition.

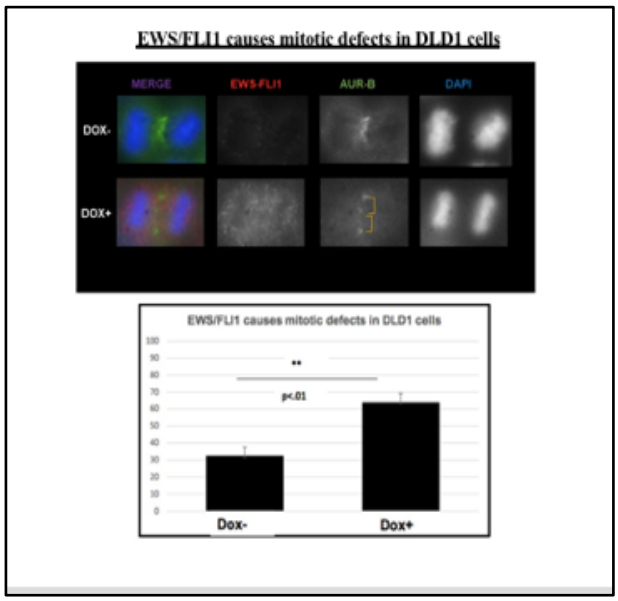


FIGURE 11: EWS-FLI1 induces mis-localization of Aurora-B. Overexpression of EWS/FLI1 caused mitotic defects in DLD1 cells. The results between Dox-and Dox+ sample were found to be statistically significant.

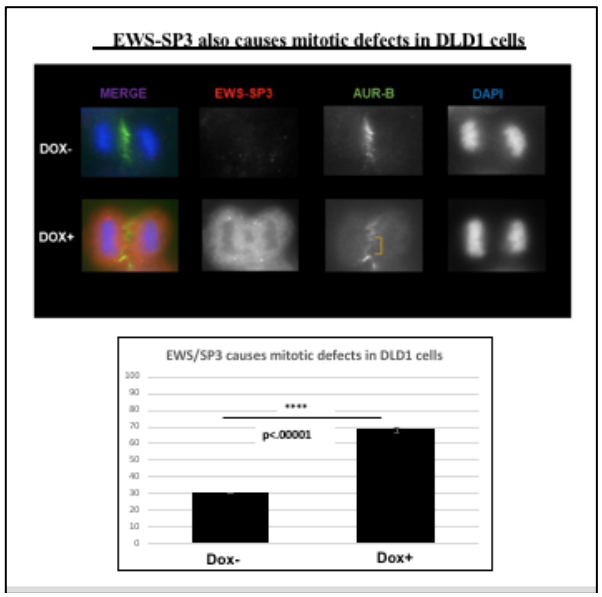


FIGURE 12: EWS-SP3 induces mis-localization of Aurora-B. Overexpression of EWS/FLI1 caused mitotic defects in DLD1 cells. The results between Dox-and Dox+ sample were found to be statistically significant.

The overall study across all EWS fusion proteins indicated that all the EWS fusion proteins induce mis-localization of Aurora-B. (Figure 13). Also, the EWS fusion proteins display different localization pattern, EWS-FLI1 and EWS-ATF1 exhibit more chromosomal localization than EWS-WT1 and EWS-SP3 demonstrated abundant cytoplasmic localization.

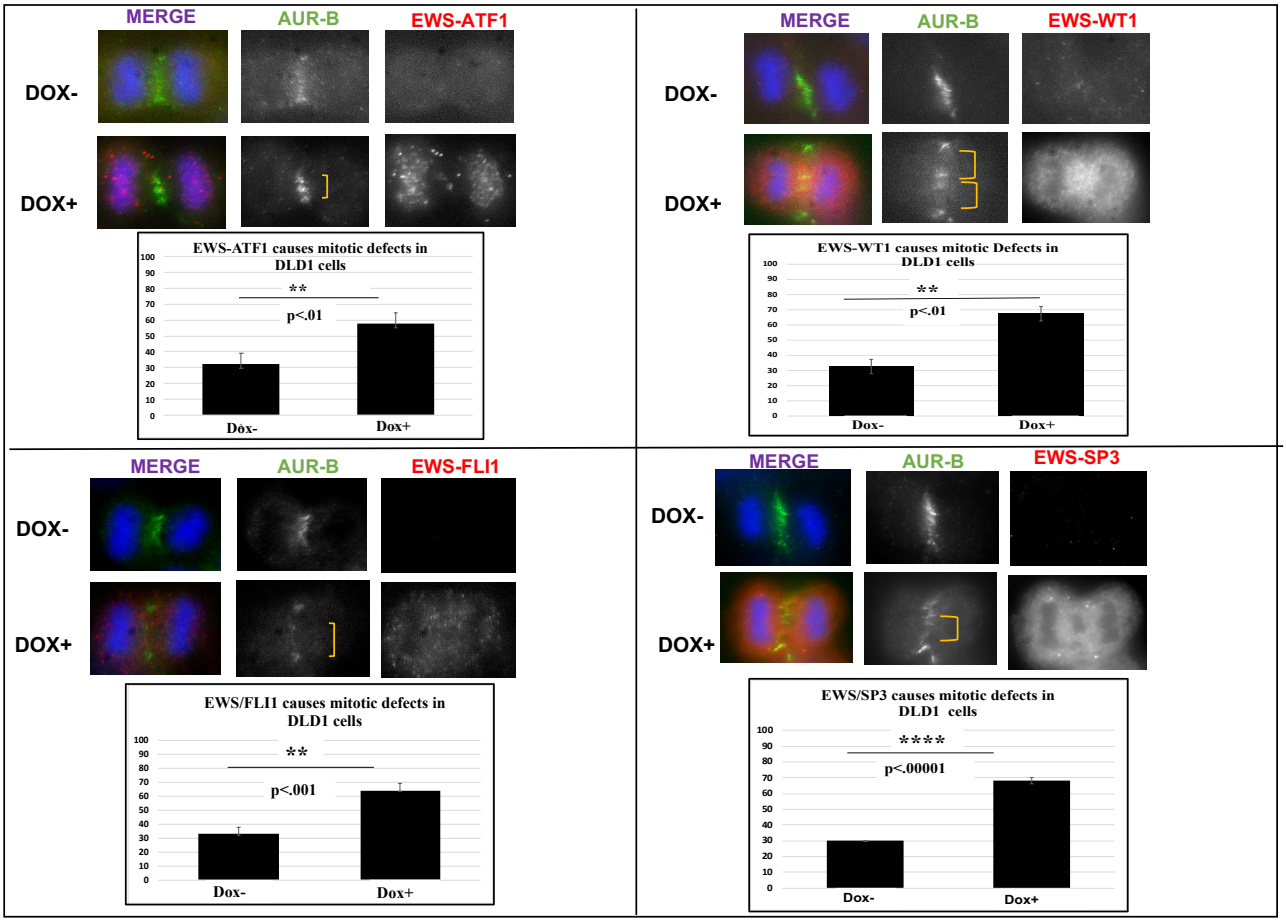


FIGURE 13: All the EWS fusions proteins cause mis-localization of Aurora-B – Upon Dox induction, all the EWS fusion proteins were expressed and induced mis-localization of Aurora-B at the midzone. The Dox- control sample served as the control for the study. During anaphase, EWS-ATF1 and EWS-FLI1 exhibited localization on the chromosome and midzone. However, EWS-SP3 and EWS-WT1 exhibited more cytoplasmic localization with few foci observed on the chromosome

Sequence analysis of the EWS fusion proteins:

The EWS fusion constructs were made in our lab by fusing the 3xFLAG tag to the N terminus of EWS, fused to the DNA binding domain of each transcription factor. These plasmids were transfected into the safe harbor locus of the DLD1 cells using CRISPR/Cas9 system.

A sequence analysis of these constructs (Figure 14) confirmed that all the EWS fusion proteins shared the same N terminus region which includes the transactivation domain and partial phosphorylation domain. All of these constructs share some portion of the N terminus of EWS and is fused to each of the DNA binding domains of various transcription factors

FIGURE 14: Sequence analysis of EWS fusions proteins: All the EWS fusion proteins include the same N terminus region of EWS compromising the transactivation domain and partial phosphorylation domain. The N terminus region of EWS is fused to the DNA binding domain of the different transcription factors.

Localization pattern of EWS fusion proteins in DLD1 cells:

We discovered that there is a variation of the localization pattern of the EWS fusion proteins during early mitosis (prophase, metaphase and anaphase). The EWS-ATF1 and EWS-FLI1 exhibited higher incidence of chromosome localization, while EWS-WT1 and EWS-SP3 displayed lower incidence of chromosome localization with higher incidence of cytoplasmic localization (Figure 15).

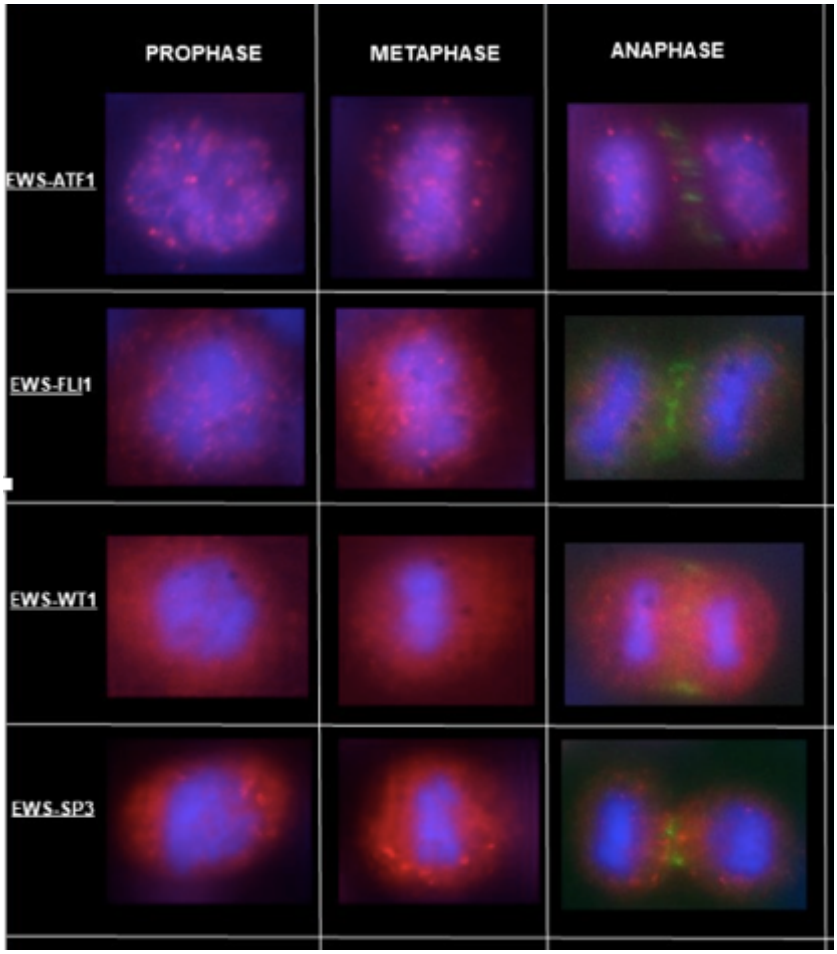


FIGURE 15: Localization trend of EWS fusion proteins during mitosis. Through immunocytochemistry studies it was observed that EWS-ATF1 and EWS-FLI1 had more chromosomal localization during prophase, metaphase and anaphase. EWS-WT1 and EWS-SP3 on the other hand displayed more abundant cytoplasmic localization.

In Figure 15, nuclei were stained with DAPI (blue), the fusions proteins appeared red and were identified by probing with rat-mcherry, Aurora-B appeared green and was probed with mouse-AuroraB.

For this study, 20 cells from each stage of mitosis (prophase, metaphase and anaphase) were scored. If the fusion protein displayed more than 5 foci on the chromosome then the cells were classified as cells exhibiting higher incidence of chromosome localization.

Some of the cells expressing the fusion protein displayed really faint, 0-5 puncta on the chromosome and these cells were deemed as cells exhibiting lower incidence of chromosome localization.

To further confirm the results obtained from the analysis of immunocytochemistry, the chromosome fraction of each of the EWS fusion expressing cells were isolated and subjected to western blot.

The EWS-SP3 cell was treated without and with Dox (Dox- and Dox+) to induce the expression of the transgene. Concurrently, the cells were also subjected to a thymidine-nocodazole treatment (indicated in the experimental workflow) to synchronize the cells at early mitosis [17] (Figure 16).

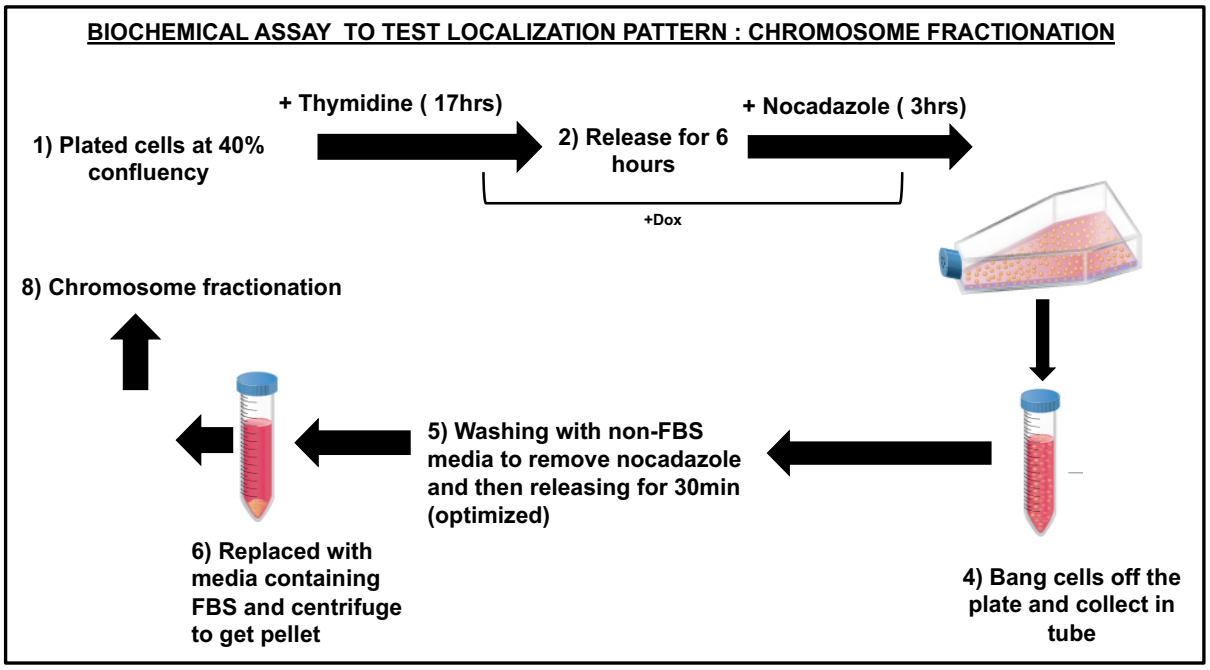


FIGURE 16: Experimental workflow of procedure followed for cell synchronization and chromosome fractionation. Cells were synchronized using a thymidine and nocadazole block to enrich the number of mitotic cells. The mitotic cells were then collected and subjected to fractionation.

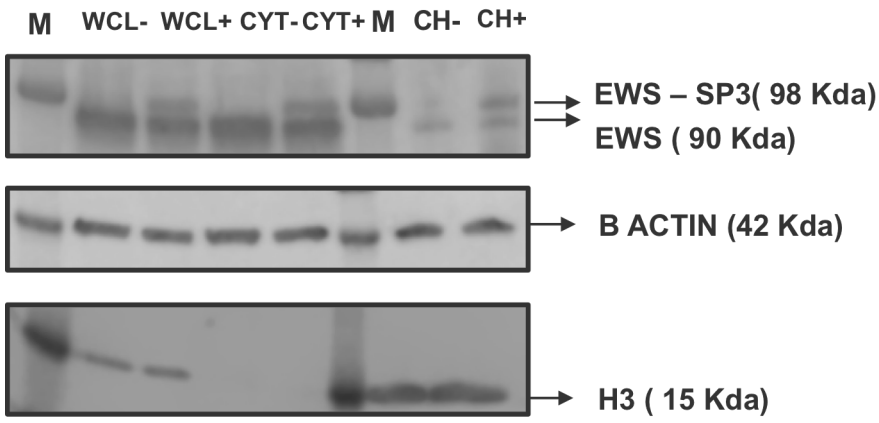


FIGURE 17: Biochemical fractionation studies followed by western blot to detect presence of EWS-SP3 in different cell fractions. Expression of *EWS-SP3* was probed for in the whole cell lysate, cytoplasmic and chromosome fraction. EWS-SP3 was detected in all fractions at 98 Kda along with endogenous EWS at 90 Kda when probed with antibody C-9.

The Dox- and Dox+ EWS-SP3 cells were utilized to isolate whole cell lysate, cytoplasmic and chromosomal fraction. The lysates were subjected to a western blot using anti-EWS C9, beta-actin and Histone H3 antibodies. Beta- actin is a protein that is known to be abundantly present in the whole cell lysate and cytoplasm and served as an appropriate control for these fractions. However, since Beta-actin was also detected in the chromosome fraction, this might not be the best control for the study. Repeating the experiment and using a cytoplasmic marker like tubulin might be a better candidate for the control. Also, more stringent fractionation techniques to completely separate chromosome fraction may prevent cytoplasmic contamination from occurring. This will help to ascertain if EWS-SP3 actually localizes on the chromosome as visualized through the western blot in Figure 17. H3 was used as a control for the chromosome fraction. EWS-SP3 was detected in the whole cell lysate and cytoplasmic fraction as expected.

Microscopic analysis to visualize the EWS-SP3 foci on the chromosome has been challenging due to its nature with a weak signal and non-distinct foci. For this reason, the western blot analysis helped ascertain the localization of EWS-SP3 on the chromosome.

Taken together, my results indicate that all the EWS fusion proteins cause mis-localization of Aurora -B at the midzone. However, there was a variation of the localization patterns of the EWS fusion proteins in DLD1 cells. EWS-ATF1 and EWS-FLI1 exhibit more chromosomal localization as visualized by distinct foci on the chromosome. EWS-WT1 and EWS-SP3 display more abundant cytoplasmic localization with minimal chromosomal localization (as displayed by very tiny foci through microscopy).

Further, extending this biochemical study to all the EWS fusion proteins of interest in this study will help delineate the localization and putative mechanism of the pathogenesis of the EWS-fusion expressing sarcomas.

DISCUSSION:

Previous literature on EWS fusion mediated sarcoma has suggested that all the fusions exert their influence by mis-regulating expression of their target genes [9]. This is primarily mediated through the DNA binding domain of the transcription factor that EWS is fused to and causes sarcoma progression. However, pathogenesis of EWS fusion mediated sarcomas is a culmination of number of mechanisms. We suspected that some of these mechanisms are independent of the DNA binding domain and are conserved among the EWS fusion proteins[8].

My results suggest that the EWS-fusion dependent induction of abnormal mitosis may also play an important role in the pathogenesis of sarcomas. All EWS fusions of interest induces mis-localization of Aurora-B (one of the important mitotic kinases that ensure proper chromosome segregation) at the midzone. The mis-localization of Aurora-B could potentially induce chromosomal instability, a hallmark of cancer [16]. Also, some of the EWS fusions including EWS-ATF1 and EWS-FLI1 display more chromosomal localization as compared to EWS-SP3 and EWS-WT1 which display more cytoplasmic localization and minimal chromosomal localization. However, studies so far through biochemical fractionation studies in the past and my own study have indicated that EWS-FLI1 and EWS-SP3 localize on the chromosome.

The results from the chromosome fractionation and western blot study also indicate that EWS levels in the whole cell lysate and cytoplasmic fractions increased compared to EWS-SP3. However, in the level of the EWS-SP3 protein in the chromosome fraction is higher than that of EWS. This suggests that the stoichiometric ratios of EWS and EWS-SP3 might be changing

dynamically in these different extracts. This data also helps to delineate some of the putative mechanisms by which EWS-SP3 could cause pathogenesis.

Localization pattern of the EWS fusion proteins on the chromosome might be one of the key factors for mediating chromosomal instability. EWS-SP3 and other fusion proteins on the chromosome may interact with other important candidates that govern the process of mitosis and affect its function. One of the major speculated candidates is Aurora-B, the EWS fusion proteins may interact with Aurora-B during early stages of mitosis and affect its localization and function. Aurora-B is known to localize to the inner centromere during metaphase along with the chromosome passenger complex (CPC) which includes INCENP, Survivin and Borealin to ensure proper attachment of spindle to kinetochore [5]. The EWS fusion may also colocalize with Aurora-B at this region and inhibit its function. Another candidate for the EWS fusion interacting protein is EWS because it also localizes on the chromosome and is known to recruit Aurora-B to the midzone. Ewsa, a zebrafish homologue of EWS, has also been shown to ensure proper spindle formation [3]. Thus, if the fusion protein inhibits the function of EWS, this may indirectly affect localization of Aurora-B onto the chromosome. Identifying interaction partners of these fusion proteins might help in deciphering if there could be important mitotic regulators that could interact and adversely be affected by the fusion protein. This could unravel important targets and also delineate a pathway for the mechanism of pathogenesis for EWS fusion expressing sarcomas.

Also, previous studies in the lab have indicated that EWS-FLII1 localization on the chromosome is mediated through phosphorylation at the residue threonine79 (T79) present on the N terminus

of EWS [18]. Modification of this residue to threonine79A through site directed mutagenesis, did not enable EWS-FLI1 to localize on the chromosome and to cause Aurora-B mis-localization. Chromosome spreads also indicated that the normal number of chromosomes were maintained and there was no aneuploidy in the T79A condition, when EWS-FLI1 did not localize on the chromosome[18].

The study concluded that the residue Threonine79 (Thr79) residue mediates Aurora -B mis-localization and causes aneuploidy. Since all the EWS fusions share the same N-terminus and the threonine 79 residue is conserved across all the EWS fusion proteins, it would be interesting to test if this mechanism is conserved across all the EWS fusions. If all the EWS fusions localize on the chromosome and this localization on the chromosome is mediated by T79, mutating this residue to ensure that the EWS fusion protein doesn't localize on the chromosome thereby not causing Aurora-B mis-localization and aneuploidy could lead to therapeutic avenues in the field.

Previous studies suggested that EWS-ATF1 induces tumorigenesis in mice without mutating the tumor suppressor P53 [11], thus making it a strong oncogene. Since EWS-ATF1 has the most chromosomal localization as indicated through microscopy it would be interesting to know if mutating the T79 residue might actually cause complete reversal of its localization and its effects on progression on pathogenesis of clear cell sarcoma. Though each fusion protein affects different regions of the body and has different DNA binding domains contributed by the transcription factor the transactivation domain of EWS is conserved across the fusion proteins. It might be possible that there are common mechanisms shared by the EWS fusion proteins in mediating the pathogenesis of sarcomas.

Future studies aimed at addressing these goals would provide a comprehensive test of another mechanism independent of DNA binding mediated through mitosis that might be required to mediate pathogenic states of the sarcomas. Studies in this direction will also indicate if this mechanism is conserved across the EWS fusions. It would also give us a deeper understanding of how mitotic defects are induced in EWS fusion sarcomas and would enable us to unravel and establish a link between mitotic defects and tumorigenesis.

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