Ewing Sarcoma Fusion Protein EWSR1/FLI1 Interacts with EWSR1 Leading to Mitotic Defects in Zebrafish Embryos and Human Cell Lines

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Abstract

The mechanism whereby the fusion of EWSR1 with the ETS transcription factor FLI1 contributes to malignant transformation in Ewing sarcoma remains unclear. We show that injection of human or zebrafish EWSR1/FLI1 mRNA into developing zebrafish embryos leads to mitotic defects with multipolar and disorganized mitotic spindles. Expression of human EWSR1/FLI1 in HeLa cells also results in mitotic defects, along with mislocalization of Aurora kinase B, a key regulator of mitotic progression. Because these mitotic abnormalities mimic those observed with the knockdown of EWSR1 in zebrafish embryos and HeLa cells, we investigated whether EWSR1/FLI1 interacts with EWSR1 and interferes with its function. EWSR1 coimmunoprecipitates with EWSR1/FLI1, and overexpression of EWSR1 rescues the mitotic defects in EWSR1/FLI1-transfected HeLa cells. This interaction between EWSR1/FLI1 and EWSR1 in Ewing sarcoma may induce mitotic defects leading to genomic instability and subsequent malignant transformation. [Cancer Res 2009;69(10):4363–71]

Introduction

Ewing sarcoma, a malignant bone and soft tissue tumor arising in children and young adults, is characterized by a chromosomal translocation fusing the EWSR1 (Ewing sarcoma breakpoint region 1) gene (also known as EWS) to the DNA-binding domain of an ETS transcription factor gene (1). Although one of five different ETS factors fuses to EWSR1 in Ewing sarcoma, FLI1 is the most frequent partner, occurring in ~95% of Ewing sarcoma cases (2).

Expression of the EWSR1/FLI1 fusion is important for malignant transformation in Ewing sarcoma. Selected strains of NIH3T3 cells expressing EWSR1/FLI1 form colonies in soft agar and form tumors when transplanted into immunodeficient mice (3, 4). Mutants containing a deletion of either the NH2-terminal EWSR1 activation domain or the DNA-binding domain of FLI1 are nontransforming (5). EWSR1/FLI1 seems to be toxic in other cell lines, such as RAT-1 fibroblasts, NCM-1 neural progenitors, and CTR rhabdomysarcoma cells (6, 7), suggesting the importance of the cellular context of EWSR1/FLI1 expression.

Materials and Methods

Construction of zebrafish ewsra/fli1a. The NH2 terminus of ewsra and COOH terminus of fli1a were amplified independently from cdNA synthesized from 24-h postfertilization (hpf) embryos. PCR amplification (94°C for 4 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; 72°C for 7 min) was performed using 2.5 units of high fidelity Taq polymerase (Roche) and 240 nmol/L each of primers (ewsra-F: 5′-TCAGGTTACCATGGCACACGAAATGGC-3′ and ewsra-R: 5′-ATACGGTGGTTGTTGTTGAT-3′) or fli1a-F: 5′-CCGTCTTATGATGCTG-3′ and fli1a-R: 5′-GTGACCGGCGCTTAGATGTTAATCACTACCG3′). The breakpoint was designed as an EWSR1/FLI1 type I fusion gene based upon homology between the human and zebrafish EWSR1 and FLI1 genes.

There is evidence that EWSR1/FLI1 functions through aberrant transcriptional regulation (8–12). Wild-type EWSR1 is a member of the TET family (along with FUS and TAF15; ref. 11), whose members have been shown to interact with components of the general transcriptional complex. The EWSR1 domain, which contains glutamine, serine, and tyrosine residues commonly found in transcriptional activation domains, interacts with the RNA polymerase II enzyme POLR2G (hsRPB7). EWSR1 also associates with specific subunits of the transcription factor IID complex involved in general transcriptional initiation (12). Although a number of target genes have been identified for the fusion protein, the transcriptional deregulation of these genes by EWSR1/FLI1 does not fully explain malignant transformation in Ewing sarcoma.

Genomic instability occurs commonly in tumors, including Ewing sarcoma. It is a generally accepted concept that mitotic defects lead to genomic instability because daughter cells fail to inherit a full complement of chromosomes (13). Cancer cells containing aberrant chromosomes are known to bypass cell cycle checkpoints and escape apoptosis. Despite evidence for transcriptional deregulation by EWSR1/FLI1, it is unknown how Ewing sarcoma cells develop genomic instability.

In this study, we show that the interaction between EWSR1/FLI1 and wild-type EWSR1 leads to mitotic defects. These mitotic defects may contribute to the genomic instability observed in Ewing sarcoma, thereby providing a novel mechanism for EWSR1/FLI1 in malignant transformation.
deletion constructs were generated by PCR amplification using primers delEWSR1/FLI1 (II)-F: 5' - GCATGAATTCATGGCGTCCACGGATTACAG - 3' and delEWSR1/FLI1 (II)-R: 5' - GCATCCCGGGCTCAGTGGGCCTTGTTTCATC - 3'; delEWSR1/FLI1 (III)-F: 5' - GCATGAATTCTCATGGCGTCCACGGATTACAG - 3' and delEWSR1/FLI1 (III)-R: 5' - GCATCCCGGGACTCTGCTGCCCGTAGCTGC - 3'; and delEWSR1/FLI1 (IV)-F: 5'-GCATGAATCTAGTTACCCACCCCAAAC-3' and delEWSR1/FLI1 (IV)-R: 5' - GCATCCCGGGGTAGTAGCTGCCTAAGTGTG - 3'. PCR products were cloned into pCRII-TOPO (Invitrogen). The pCRII-EWSR1/FLI1 constructs were digested with SmaI and EcoRI and cloned into pSG5 digested with HindIII (blunted with Klenow) and EcoRI.

**Embryo collection.** Zebrafish were maintained in accordance with guidelines approved by the NIH Institutional Animal Care and Use Committee. Embryos were collected and maintained in medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L CaCl2, 0.33 mmol/L MgSO4) at 28.5°C.

Live images of 24 hpf embryos anesthetized with Tricaine (16 mg/100 mL medium) were captured using a Zeiss Stemi SV11Apo stereomicroscope.

**mRNA synthesis and injection.** Capped mRNA was generated using the mMessage mMachine kit following the manufacturer's protocol (Ambion). mRNA was diluted 1:1 in 200 mmol/L KCl/0.5% phenol red. Two nanoliters of either 100 ng/μL human EWSR1/FLI1 mRNA or 10 ng/μL zebrafish ewsr1a/fli1a mRNA were injected into one-cell stage embryos.

**In vivo expression of human and zebrafish fusion proteins.** At 24 h after human EWSR1/FLI1 mRNA or zebrafish ewsr1a/fli1a mRNA injection, yolks were removed and 75 embryos were suspended in 100 μL of 1× SDS buffer [50 mmol/L Tris-Cl (pH 6.8), 2% SDS, 10% glycerol] with 5% (v/v) 2-mercaptoethanol and incubated at 95°C for 5 min (14). Ten microliters were subjected to Western blotting using anti-FLI1 antibody (1:1,000 dilution), followed by horseradish peroxidase (HRP)–linked secondary antibody (1:15,000 dilution), and visualized using Super Signal West Femto Substrate (Pierce Biotechnology).

**TUNEL assay.** TUNEL assay on zebrafish embryos was performed as previously described (15).

**Immunohistochemistry.** Embryonic zebrafish mitotic spindles were visualized by immunohistochemistry, as previously described, with minor modifications (16). Mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich; 1:4,000 dilution) and Alexa Fluor 594 goat anti-mouse IgG antibody (Invitrogen; 1:250 dilution) were used. After mounting in 4',6-diamidino-2-phenylindole (DAPI)/Vectashield (Vector Laboratories) to stain DNA,
images were captured as 0.25-μm stack sections on a Zeiss Axioplan2 Imaging System.

**Immunocytochemistry.** HeLa cells were grown on coverslips and then transfected using FuGENE HD transfection reagent (Roche) with a total of 2 μg of pSG5 plasmid (Stratagene) with or without FLAG-tagged human EWSR1/FLI1 fusion. At 18 h after transfection, cells were fixed with 4% paraformaldehyde for 20 min, washed in PBS, and permeabilized with methanol for 10 min at −20°C. Cells were washed in PBS, incubated in blocking solution (1% fetal bovine serum in PBS) for 1 h, followed by 1-h incubation with primary antibodies: anti-α-tubulin (1:4,000 dilution) for visualization of spindles, anti–Aurora B (Sigma; 1:1,000 dilution) for Aurora B localization, rabbit polyclonal anti-FLI1, and anti-EWSR1 5C10 (Affinity BioReagents; 1:1,000 dilution). After three 10-min washes in PBS, cells were incubated in secondary antibody (Alexa Fluor 594 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG) for 1 h. After PBS washes, coverslips were mounted with DAPI/Vectashield and visualized at 1,000× magnification on an Olympus AX70 microscope and images were captured using SPOT imaging software.

Rescue experiments were performed by cotransfection of HeLa cells with 1.9 μg of pSG5-EWSR1/FLI1 fusion gene along with 0.1 μg of pSG5-EWSR1 vector or empty vector. After 16 h of culture, cells were subjected to immunocytochemistry using anti-α-tubulin antibody, as described above, and spindles were scored.

**Coimmunoprecipitation experiments.** HeLa cells were transfected with 14.8 μg of pSG5-EWSR1/FLI1 or empty vector using FuGENE HD. After PBS washes, cells were harvested and lysed with 1 mL of buffer [15 mmol/L Tris-Cl (pH 8.0), 100 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100] at 4°C for 30 min, and supernatants were collected after centrifugation (10,000 × g for 2 min).

In Fig. 4B, supernatants were immunoprecipitated by incubation with 10 μg of FLAG M2 antibody (Stratagene) or mouse IgG at 4°C for 1 h. Protein A-DYNA beads (8 μL; Invitrogen) were added, and samples were incubated at 4°C for 1 h. Beads were washed thrice [15 mmol/L Tris-Cl (pH 8.0), 100 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 0.1% Tween 20] at 4°C for 30 min and then boiled in 1× SDS buffer. The immunoprecipitated samples were subjected to Western blotting using a 1:1,000 dilution of anti-EWSR1 antibody 5C10 followed by HRP-linked secondary antibody (1:10,000 dilution).

Lysates of Ewing sarcoma cell lines A673, SK-N-MC, and RD-ES in Fig. 4D were immunoprecipitated with mouse IgG or anti-EWSR1 antibody C9 (Santa Cruz Biotechnology). Immunoprecipitated samples were subjected to Western blotting using anti-FLI1 antibody (1:1,000 dilution) followed by secondary antibody.

In Fig. 6C, EWSR1/FLI1 deletion constructs were transfected into HeLa cells. Lysates were immunoprecipitated with 5 μg of either mouse IgG or anti-EWSR1 antibody C9. Immunoprecipitated samples were subjected to Western blotting, as described above.

Figure 2. Expression of human EWSR1/FLI1 or zebrafish ewsr1a/fl1a in zebrafish embryos results in apoptotic cell death in the CNS. Embryos were injected with mRNA for the human or zebrafish EWSR1/FLI1 fusion and screened for apoptotic cell death by TUNEL staining. Lateral views of zebrafish embryos at shield stage (6 hpf), bud stage (~10 hpf), 15 somite (~17 hpf), and 24 hpf. A, un.injected embryos; B, water-injected embryos; C, human EWSR1/FLI1 mRNA-injected embryos; D, zebrafish ewsr1a/fl1a mRNA-injected embryos. No changes are noted at the shield stage, but increased apoptosis is detected in the CNS of human and zebrafish EWSR1/FLI1 mRNA-injected embryos (compare images C2-C4 and D2-D4 with images A2-A4 and B2-B4). Development in EWSR1/FLI1-injected embryos is slightly delayed (note less pigment in eyes).
Results

Injection of human EWSR1/FLI1 or zebrafish ewsraa/fliaa mRNA into zebrafish embryos results in morphologic defects and apoptotic cell death in the central nervous system. To study the effect of EWSR1/FLI1 expression during early embryonic development, we injected human EWSR1/FLI1 type IV fusion (17) and zebrafish ewsraa/fliaa type I fusion mRNA (Materials and Methods) into one-cell stage zebrafish embryos (Fig. 1A). Both the human and zebrafish constructs express protein of the predicted size when mRNA is injected into zebrafish embryos (Fig. 1B). The phenotypes that resulted from injection of either construct ranged from mild to severe neurologic defects, with trunk and tail defects occurring with the most severe brain defects (Fig. 1C). The brains of fusion gene-injected zebrafish embryos displayed abnormal neural keels with smaller fourth ventricles and indistinct midbrain–hindbrain boundaries (Fig. 1C, c–h) compared with control embryos (Fig. 1C, a and b) at 24 hpf. Phenotype scoring is shown (Supplementary Fig. S1). mRNA-injected embryos with moderate to severe phenotypes died by 12 days postfertilization.

To determine whether the central nervous system (CNS) abnormalities in embryos injected with the EWSR1 fusions were caused by apoptosis, TUNEL assays were performed. No differences in the level of apoptosis were present at the shield stage of development (6 hpf; Fig. 2A–D). However, increased levels of apoptosis were present in fusion-injected embryos by the bud stage of development (~10 hpf; Fig. 2, compare human and zebrafish EWSR1/FLI1 mRNA-injected embryos in images C2 and D2, with control images A2 and B2). Markedly increased levels of apoptosis were found in the CNS of human EWSR1/FLI1 and zebrafish ewsraa/fliaa-injected embryos at the 15 somite stage (~17 hpf; Fig. 2, C3 and D3 compared with controls A3 and B3). These changes were also seen in mRNA-injected embryos at 24 hpf (Fig. 2, mRNA-injected embryos C4 and D4 compared with controls A4 and B4).
The number of TUNEL-positive cells visible in photographic images of five representative embryos from each of the sample groups was counted. Markedly higher numbers of apoptotic cells were found in the human EWSR1/FLI1 and zebrafish ewsr1a/fli1a-injected embryos compared with controls (Supplementary Fig. S2). These results indicate that expression of EWSR1/FLI1 leads to apoptosis in the CNS and that apoptosis precedes the morphologic defects, which were not observed at the bud stage.

**Human and zebrafish EWSR1/FLI1 lead to mitotic defects in zebrafish embryos.** Apoptosis frequently occurs due to mitotic defects; therefore, we analyzed the mitotic spindles in zebrafish embryos injected with the EWSR1 fusion genes. These studies used immunohistochemistry with anti-α-tubulin antibody to label mitotic spindles and DAPI to stain DNA. Multipolar mitotic spindles, disorganized spindle fibers, and other defects were observed in human EWSR1/FLI1 and zebrafish ewsr1a/fli1a mRNA-injected embryos (Fig. 3A). Comparison of spindles from the shield and 12 somite stages in human EWSR1/FLI1 mRNA-injected embryos (Fig. 3A, c and d) and zebrafish ewsr1a/fli1a mRNA-injected embryos (Fig. 3A, e and f) with uninjected (Fig. 3A, a) and control-injected (Fig. 3A, b) embryos showed a marked increase in the numbers of abnormal spindles in mRNA-injected embryos at both stages.

To verify that the phenotype observed after mRNA injection was specific for the gene being delivered, we injected mRNA for the human fusion TEL/AML1. Low dose (20 pg) and high dose (200 pg) of TEL/AML1 mRNA-injected zebrafish embryos were morphologically normal compared with controls (data not shown). To investigate whether TEL/AML1 mRNA-injected embryos exhibited mitotic defects, the embryos were subjected to immunohistochemistry using anti-α-tubulin antibody. Approximately, 150 to 225 mitotic cells (from five embryos) were scored from each sample group. TEL/AML1 mRNA-injected zebrafish embryos exhibited equivalent levels of abnormal spindles compared with controls: 5% in uninjected, 1% in water-injected, 2% in low-dose TEL/AML1 mRNA-injected, and 4% in high-dose TEL/AML1 mRNA-injected embryos. These results indicate that the mitotic defects caused by human EWSR1/FLI1 and zebrafish ewsr1a/fli1a injection are specific to EWSR1/FLI1 expression, not due to injection stress.

**EWSR1/FLI1 leads to mitotic defects in HeLa cells.** To determine whether the effect of fusion gene expression was conserved between zebrafish and human cells, we transfected HeLa cells with an expression vector containing human EWSR1/FLI1. Increased levels of mitotic defects, including multipolar and disorganized spindles, were observed in HeLa cells transfected with the human EWSR1/FLI1 fusion compared with untransfected and empty vector–transfected control cells (Fig. 3B). These results, generated in four independent experiments, indicate that EWSR1/FLI1 expression leads to increased levels of mitotic defects in both human and zebrafish cells.

**HeLa cells transfected with EWSR1/FLI1 display mislocalization of Aurora kinase B.** To investigate the relationship between mitotic defects and EWSR1/FLI1, the localization of Aurora kinase B (a key regulator of mitosis) was assayed in the presence of EWSR1/FLI1. Aurora B has critical functions during mitosis, including chromosome alignment, spindle checkpoint activation, and cytokinesis. Furthermore, the localization of Aurora B is tightly regulated (reviewed in refs. 18 and 19). Aurora B localizes on the chromosomes during early mitosis, on the central spindle during anaphase, and on the midzone during cytokinesis.
HeLa cells were transfected with vector containing EWSR1/FLI1, and EWSR1 expression when compared with control cells (Fig. 4A). Western blots on HeLa cells transfected with EWSR1/FLI1, however, did not indicate any decrease in EWSR1 expression when compared with control cells (Fig. 4A). Therefore, we tested whether EWSR1/FLI1 coimmunoprecipitates with EWSR1. HeLa cells were transfected with a FLAG-tagged vector containing the EWSR1/FLI1 fusion (or empty vector), and cell lysates were immunoprecipitated with anti-FLAG antibody or IgG control. Western blotting with an antibody recognizing a COOH terminal epitope of EWSR1 showed coimmunoprecipitation of EWSR1 with EWSR1/FLI1, indicating interaction between these two proteins (Fig. 4B).

To determine whether EWSR1/FLI1 interacts with EWSR1 in Ewing sarcoma cell lines, lysates from the Ewing sarcoma cell lines A673, SK-N-MC, and RD-ES were analyzed initially for expression of EWSR1/FLI1 and EWSR1. All three cell lines showed endogenous expression of both the fusion protein and EWSR1 (Fig. 4C). Next, we performed coimmunoprecipitation studies with these cell lines. Lysates were immunoprecipitated with IgG control or an anti-EWSR1 antibody, the epitope of which is located in the COOH terminus of EWSR1 so that it precipitates only EWSR1 protein, not EWSR1/FLI1 protein. Western blotting with anti-FLI1 antibody showed coimmunoprecipitation of EWSR1/FLI1 with EWSR1 in Ewing sarcoma cell lines (Fig. 4D).

**Mitotic defects in HeLa cells expressing EWSR1/FLI1 are rescued by overexpression of EWSR1.** To determine whether the mitotic defects observed with EWSR1/FLI1 expression could be reversed with EWSR1, we performed rescue experiments using EWSR1. HeLa cells were cotransfected with expression constructs containing empty vector, human EWSR1/FLI1, EWSR1, or both EWSR1/FLI1 and EWSR1. The percentages of cells with mitotic defects are shown in Fig. 5. The EWSR1/FLI1-transfected cells exhibited a higher incidence of mitotic defects compared with untransfected, empty vector–transfected, and EWSR1-transfected cells. In contrast, HeLa cells cotransfected with both EWSR1/FLI1 and EWSR1 exhibited a level of mitotic defects similar to that of controls (Fig. 5). These results indicate that EWSR1 rescues the mitotic defects induced by EWSR1/FLI1, suggesting that the mitotic defects resulting from EWSR1/FLI1 are mediated through interference with endogenous EWSR1.

**Recognition of EWSR1 in HeLa cells expressing EWSR1/FLI1 is altered.** The phenotypic similarity between EWSR1/FLI1 expression and EWSR1 knockdown in both zebrafish embryos and transfected HeLa cells, along with the observation that EWSR1/FLI1 associates with endogenous EWSR1 in transfected HeLa cells, suggest that EWSR1/FLI1 might interact with EWSR1 and interfere with its function. Moreover, this interaction might lead to conformational changes in EWSR1, which could be detected by loss of epitopes recognized by antibodies directed against EWSR1. To test this hypothesis, immunocytochemistry was performed on EWSR1/FLI1-transfected HeLa cells with two different commercially available anti-EWSR1 antibodies. Both anti-EWSR1 antibodies recognize COOH terminal epitopes present only in endogenous EWSR1, not in the fusion protein. The antibody designated 5C10 was generated against amino acids 358 to 454 of human EWSR1. The antibody designated C9 was generated against amino acids 431 to 490 of EWSR1 (Supplementary Fig. S4). Untransfected and empty vector–transfected HeLa cells displayed similar staining patterns with both antibodies (Supplementary Fig. S5, a–f and j–o). In EWSR1/FLI1-transfected cells, no difference in staining pattern was observed between cells positive or negative for EWSR1/FLI1 (as indicated by FLI1 staining) using the anti-EWSR1 antibody C9 (Supplementary Fig. S5, boxed cells in a and j). However, the staining pattern using the anti-EWSR1 antibody 5C10 was markedly decreased in cells expressing EWSR1/FLI1 (Supplementary Fig. S5, boxed cells in q and r). These results suggest that EWSR1/FLI1 alters epitopes in endogenous EWSR1, thus providing potential insight into the region of EWSR1 affected by its interaction with EWSR1/FLI1.

**Mapping of domains in EWSR1/FLI1 required for interaction with EWSR1 and the induction of mitotic defects.** To determine the domains of EWSR1/FLI1 required for interaction with EWSR1, a series of FLAG-tagged deletion mutants of EWSR1/FLI1 [DEL(EWSR1/FLI1 [1–IV])] were constructed (Fig. 6A). These deletion constructs were transfected into HeLa cells, and Western blotting was performed using an anti-FLAG antibody. Expression of the EWSR1/FLI1 deletion mutants was confirmed for each construct (Fig. 6B). To identify the domains of EWSR1/FLI1 required for interaction with EWSR1, coimmunoprecipitation assays were performed. EWSR1 was immunoprecipitated with an
anti-EWSR1 antibody that recognizes only endogenous EWSR1 and not the region of EWSR1 contained in the EWSR1/FLI1 fusion protein (Fig. 6C). The immunoprecipitated proteins were blotted using an anti-FLAG antibody. Only the deletion mutant delEWSR1/FLI1 (I) immunoprecipitated with EWSR1 (Fig. 6C). This result indicates that both the NH2 terminal EWSR1 domain of EWSR1/FLI1 and the entire FLI1 region of EWSR1/FLI1 are required for interaction with EWSR1.

To identify the domain of EWSR1/FLI1 required for mitotic defects, deletion mutants of EWSR1/FLI1 were transfected into HeLa cells and the percentages of cells exhibiting mitotic defects were scored for each sample group (Fig. 6D). Controls exhibited low levels of mitotic defects, whereas cells transfected with full-length EWSR1/FLI1 exhibited a higher incidence of mitotic defects. The delEWSR1/FLI1 (II), (III), and (IV)–transfected cells exhibited levels of mitotic defects similar to controls. In contrast, delEWSR1/FLI1 (I)–transfected cells exhibited a higher incidence of defects than controls and a higher incidence than the other deletion mutants. These results indicate that both the NH2 terminal EWSR1 domain of EWSR1/FLI1 and the entire region of FLI1 contained in the EWSR1/FLI1 fusion are required to cause mitotic defects. Taken together, these results indicate that EWSR1/FLI1 interacts with EWSR1 and interferes with EWSR1 function through a dominant-negative mechanism.

Discussion

The Ewing sarcoma phenotype has not been recapitulated in animal models, a finding most likely due to early embryonic lethality. To investigate the effect of EWSR1/FLI1 on early embryonic development, we expressed zebrafish and human EWSR1/FLI1 in developing zebrafish embryos. EWSR1/FLI1 leads to mitotic defects followed by morphologic changes and apoptosis in the CNS in zebrafish embryos. These mitotic defects were duplicated in HeLa cells transfected with EWSR1/FLI1 and were accompanied by mislocalization of the chromosomal passenger complex protein, Aurora kinase B. Because the results in both zebrafish embryos and HeLa cells resembled those observed with small interfering RNA knockdown of EWSR1, we investigated the potential interaction of EWSR1/FLI1 with EWSR1. EWSR1 coimmunoprecipitates with EWSR1/FLI1 in transfected HeLa cells and Ewing cell lines. Transfection of HeLa cells with EWSR1/FLI1 also results in altered binding of an anti-EWSR1 antibody directed against the RNA recognition motif of EWSR1, suggesting that EWSR1/FLI1 interaction with endogenous EWSR1 could alter EWSR1 function at this epitope. These observations suggest a novel mechanism whereby the induction of mitotic defects by the action of EWSR1/FLI1 on EWSR1 contributes to genomic instability and subsequent malignant transformation in Ewing sarcoma.

Wild-type EWSR1 is ubiquitously expressed in both normal tissues and tumor cells, including Daudi (Burkitt’s lymphoma) cells and K562 leukemia cells (21). In Ewing sarcoma, one allele of EWSR1 is disrupted due to the translocation t(11;22). When Ewing sarcoma cell lines were analyzed by Northern blotting, EWSR1 was ubiquitously expressed. However, an informative case of Ewing sarcoma was described, in which EWSR1 expression was lost, leading to the speculation that EWSR1 was not required for growth of the tumor (22). An alternative explanation for this observation is that loss of EWSR1 in Ewing sarcoma might contribute to transformation because, as we previously reported, EWSR1 silencing leads to mitotic defects (20). In previous studies, Spahn and colleagues showed, using fluorescence resonance energy transfer and mammalian two-hybrid assay, that EWSR1/FLI1 binds to EWSR1 in vitro (23). Here, we show a direct association between EWSR1/FLI1 and EWSR1 in transfected HeLa cells and Ewing sarcoma cell lines. In addition, overexpression of EWSR1 rescues...
the mitotic defects caused by EWSR1/FLI1. Our domain analysis indicates that both the NH2 terminal EWSR1/FLI1 and the entire region of FLI1 within EWSR1/FLI1 are required for interaction with EWSR1 and induction of mitotic defects. These results support the hypothesis that EWSR1/FLI1 inhibits EWSR1 through a dominant-negative mechanism.

Understanding the function of wild-type EWSR1 protein during mitosis should provide insight into the role of the fusion protein in Ewing sarcoma. We showed previously that knockdown of EWSR1 in HeLa cells leads to mislocalization of Aurora kinase B during anaphase. Because Aurora B plays a major role in regulating mitosis, mislocalization has the potential to allow cells to escape checkpoint control and proliferate despite genomic defects (24, 25). Defects in the mitotic spindle have been associated with genomic instability and aneuploidy in mammalian cells, which in turn have been associated with malignant transformation and tumor progression. Cells with spindle abnormalities can escape mitotic arrest and reenter G1 of the cell cycle without completing progression. Cells with spindle abnormalities can escape mitotic defects resulting in chromosomal segregation defects. Daughter cells may not inherit a full complement of chromosomes and these cells may be able to bypass apoptotic pathways as a result of loss of checkpoint control. This report describing EWSR1/FLI1 interaction with EWSR1 and the induction of mitotic defects implicates EWSR1/FLI1 in genomic instability, and it provides a potential pathway for EWSR1/FLI1 in the development of malignant transformation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References


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