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 gene) (1) with its counterparts FLI1 in malignant transformation of Ewing sarcoma remains unclear. We show that the EWSR1 (Ewing sarcoma breakpoint region gene) (1) with its counterpart FLI1 in malignant transformation of 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.

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-hour 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 240nmol/L of each of primers (ewsra-F: 5′-TCAGGTACCAGGCACAGAAATGCC-3′ and ewsra-R: 5′-ATACGTTTTTGTTGGTTATAG-3′ or fli1a-F: 5′-GCGTCAGTTGATGCT-3′ and fli1a-R: 5′-GTAGCGCCGCGCTTAGTTAACTACAAGG-3′). The breakpoint was designed as an EWSR1/FLI1 type I fusion gene based upon homology between the human and zebrafish EWSR1 and FLI1 genes. The ewsra PCR product was digested by Kpn I, and the fli1a PCR product was digested by Not I. BlueScript SKII(+) vector was digested by Kpn I and Not I. Digested PCR products and vector were ligated using T4 ligase (Fermentas). SV40 Poly A was amplified by PCR using Not I linker-attached primers (polyA-F: 5′-TGACGCCGGCCGCTTAGTTAACTACAAGG-3′ and polyA-R: 5′-TGACGCCGGCCGCTTAGTTAACTACAAGG-3′) from pEGFP-C2 plasmid (Clontech). The Poly A product was inserted into the Not I site of BlueScript SKII(+) vector. A series of human EWSR1/FLI1 deletion constructs were designed from pSG5-EWSR1/FLI1 type IV (with NH2 terminal FLAG tag). pSG5-del EWSR1/FLI1 (I) was constructed by digesting pSG5-EWSR1/FLI1 with Spe I, thereby deleting an in-frame fragment of EWSR1. The remaining EWSR1/FLI1

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deletion constructs were generated by PCR amplification using primers delEWSR1/FLI1 (II)-F: 5′-GCATGAATTCATGGCGTCCACGGATTACAG-3′ and delEWSR1/FLI1 (II)-R: 5′-GCATCCCGGGCTCAGTGGGC-TTGTTCCATC-3′; delEWSR1/FLI1 (III)-F: 5′-GCATGAATTCCATGGCGTCCACGGATTACAG-3′ and delEWSR1/FLI1 (III)-R: 5′-GCATCCCGGGACTCTGCTGCCCGTAGCTGC-3′; and delEWSR1/FLI1 (IV)-F: 5′-GCATGAATTCCATGGCGTCCACGGATTACAG-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 Smal and EcoRI and cloned into pSG5 digested with Hind III (blunted with Klenow) and Eco RI.

**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 (generated in rabbit against the COOH terminal sequence PRHPNTHVPSHLGSYYC; 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, microtubules were imaged with a Zeiss LSM 510 confocal microscope (16).

**Figure 1.** Expression of human EWSR1/FLI1 or zebrafish ewsr1a/fli1a in zebrafish embryos results in morphologic defects in the CNS. A, schematic of the human EWSR1/FLI1 type IV fusion construct and zebrafish ewsr1a/fli1a type I fusion construct. B, in vivo expression of EWSR1/FLI1 proteins in zebrafish embryos injected with mRNA for human EWSR1/FLI1 and zebrafish ewsr1a/fli1a. Products were detected by Western blotting using anti-FLI1 antibody. C, lateral views of zebrafish embryos at 24 hpf: uninjected (a), water-injected (b), human EWSR1/FLI1 mRNA-injected (c, e, and g), and zebrafish ewsr1a/fli1a mRNA-injected (d, f, and h). Lines indicate areas of defects. Hu, human; Zf, zebrafish.
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 empty 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.
Results

Injection of human EWSR1/FLI1 or zebrafish ewsr1a/fli1a 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 ewsr1a/fli1a 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, A3–D3 compared with controls A2 and B2). Markedly increased levels of apoptosis were found in the CNS of human EWSR1/FLI1 and zebrafish ewsr1a/fli1a-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, C4 and D4 compared with controls A4 and B4).

Figure 3. Expression of EWSR1/FLI1 results in abnormal mitotic spindles. A, embryos injected with mRNA for human EWSR1/FLI1 or zebrafish ewsr1a/fli1a; spindles visualized by immunohistochemistry using anti-α-tubulin antibody (red) and DAPI to stain DNA (blue). Mitotic spindles from un.injected embryos (a), water-injected embryos (b), human EWSR1/FLI1-injected embryos (c and d), and zebrafish ewsr1a/fli1a-injected embryos (e and f). In four independent experiments, ~10 mitotic cells were scored from each of five embryos per sample and an increased percentage of abnormal spindles were counted in human and zebrafish mRNA-injected embryos. At the shield stage, 34.6 ± 8.9% (n = 197) total mitoses counted from four experiments) in human and 38.3 ± 9.0% (n = 183) in zebrafish mRNA-injected embryos versus 6.0 ± 2.8% (n = 201) in un injected and 4 ± 3.2% (n = 199) in water-injected embryos had abnormal mitotic spindles. At the 12 somite stage (~15 hpf), 35.9 ± 8.3% (n = 206; human mRNA) and 38.0 ± 4.4% (n = 206; zebrafish) of mitotic spindles were abnormal versus 9.0 ± 3.6% (n = 200) in un injected and 8.2 ± 3.3% (n = 206) in water-injected embryos. B and C, HeLa cells transfected with human EWSR1/FLI1 display increased levels of mitotic defects and Aurora B mislocalization. B, ~100 mitotic cells were scored per sample in each of four experiments; mitotic spindles from untransfected, control-transfected, and human EWSR1/FLI1-transfected HeLa cells. Increased numbers of abnormal mitotic spindles were identified in the fusion-transfected cells [30.1 ± 12.0% (n = 514), in contrast to 11.9 ± 4.5% (n = 404) in untransfected and 16.2 ± 7.7% (n = 406) in control cells]. C, Aurora B staining from untransfected (a, d, and g), control-transfected (b, e, and h), and EWSR1/FLI1-transfected (c, f, and i) HeLa cells. Aurora B was visualized using an anti–Aurora B antibody (green) and DAPI was used to stain DNA (blue), counting 100 to 200 mitoses/sample group in each of four experiments. Increased levels of Aurora B mislocalization were identified in the EWSR1/FLI1-transfected cells [45.1 ± 8.8% (n = 594) versus 13.0 ± 3.6% (n = 690) in untransfected and 17.4 ± 3.6% (n = 614) in control cells].
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. 3 A). Comparison of spindles from the shield and 12 somite stages in human EWSR1/FLI1 mRNA-injected embryos (Fig. 3 A, c and d) and zebrafish ewsr1a/fli1a mRNA-injected embryos (Fig. 3 A, e and f) with uninjected (Fig. 3 A, a) and control-injected (Fig. 3 A, 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.

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**Figure 4.** EWSR1/FLI1 fusion protein associates with endogenous EWSR1 in transfected HeLa cells and Ewing sarcoma cell lines. A, Western blot of lysates from empty vector–transfected and pSG3 FLAG-tagged EWSR1/FLI1-transfected cells probed with anti–human FLI1 antibody to verify transfection, anti-EWSR1 antibody to show expression of endogenous protein, and anti–iα-actin antibody control. B, immunoprecipitation of lysates from empty vector–transfected and from EWSR1/FLI1-transfected HeLa cells using IgG control and anti-FLAG M2 antibody. Probing blots with anti-EWSR1 antibody C9 shows that EWSR1 coimmunoprecipitates with EWSR1/FLI1. C, Western blot of cell lysates from Ewing sarcoma cell lines A673, SK-N-MC, and RD-ES using IgG control and the anti-EWSR1 antibody C9 that recognizes an epitope in EWSR1 not present in EWSR1/FLI1. Probing with anti-FLI1 antibody shows association of endogenous EWSR1 with EWSR1/FLI1.
HeLa cells were transfected with vector containing EWSR1/FLI1, and endogenous EWSR1 expression was decreased when compared with control cells (Fig. 4A). 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. S3). These results indicate that the mislocalization of Aurora B preceded the occurrence of abnormal mitotic spindles.

EWSR1/FLI1 interacts with EWSR1 in HeLa cells and Ewing sarcoma cell lines. The EWSR1/FLI1 phenotype of CNS morphologic changes and apoptosis, mitotic defects, and Aurora B mislocalization closely resembles the EWSR1-knockdown phenotype, suggesting that EWSR1/FLI1 might decrease expression of endogenous EWSR1 (20). 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 cells, 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).

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 [delEWSR1/FLI1 (I–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

Figure 6. The NH2-terminal EWSR1 domain of EWSR1/FLI1 and the entire region of FLI1 contained in EWSR1/FLI1 are necessary for interaction with EWSR1 and mitotic defects. A, schematic of EWSR1/FLI1 deletion constructs. B, Western blot of lysates from delEWSR1/FLI1 (I–IV)–transfected HeLa cells using anti-FLAG antibody to show expression of constructs. Bottom panel shows anti-α-actin antibody control. C, immunoprecipitation of lysates from deletion EWSR1/FLI1 (I–IV)–transfected HeLa cells using IgG or anti-EWSR1 antibody (C9). Probing blot with anti-FLAG antibody shows association of delEWSR1/FLI1 (I) and endogenous EWSR1. D, immunocytochemistry using anti-α-tubulin antibody on delEWSR1/FLI1 (I–IV)–transfected HeLa cells. The scoring of mitotic defects shows that delEWSR1/FLI1 (I) retains mitotic defect-inducing activity.
the mitotic defects caused by EWSR1/FLI1. Our domain analysis indicates that both the NH2-terminal EWSR1 domain of 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 anaphase, 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 cytokinesis through “mitotic slippage” (26). Two genetic diseases, Schwachmann-Diamond syndrome and mosaic variegated aneuploidy syndrome, are characterized by mitotic spindle defects and cancer susceptibility (27, 28). The mosaic aneuploidy syndrome results from mutations in the mitotic spindle protein Bub1B, leading to premature chromatid separation and disruption of the spindle checkpoint (29).

The chromosomal changes described in Ewing sarcoma are consistent with extensive genomic instability. Karyotypic analysis in 88 patients with primary Ewing sarcoma showed secondary chromosomal changes in 80% of the tumors at diagnosis (29–32). Secondary chromosomal changes ranged from single chromosomal gains or losses to polyploidy with multiple chromosomal changes. The findings presented here implicate EWSR1/FLI1 in the acquisition of mitotic defects leading to chromosomal segregation errors.

Knowledge of how EWSR1/FLI1 leads to malignant transformation has broad implications because the majority of hematologic malignancies and sarcomas have chromosomal translocations involving transcription factors. A number of studies suggest that EWSR1 fusions play a role in the regulation of gene expression. EWSR1/FLI1 has the same binding specificities as FLI1, and both EWSR1/FLI1 and FLI1 transactivate target genes with ETS binding sites in their promoters (33, 34). However, EWSR1/FLI1 may be expressed in different cells than FLI1 because the fusion protein is expressed from the EWSR1 promoter; thus, a different spectrum of genes may be targeted by the fusion protein (33, 35). Target genes for EWSR1/FLI1 are numerous and have been reviewed recently (36, 37). Several of the genes transcriptionally deregulated by EWSR1/FLI1 have the potential to contribute to malignant transformation, including the cell cycle regulators CCND1 (cyclin D1; refs. 38, 39), CDKN1C (p57, KIP2), and CDK4 (ref. 40) and the cyclin-dependent kinase inhibitor CDKN1A (p21; ref. 41). Misexpression of cell cycle genes in EWSR1/FLI1-expressing cells may allow the cells to bypass the G1 checkpoint.

Here, we report a novel function of EWSR1/FLI1 that leads to mitotic defects resulting in chromosomal segregation defects. Daughter cells may not inherit a full complement of chromosomes due to the mitotic defects resulting from EWSR1/FLI1 expression, 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


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

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