**EWS/FLI1 Oncogene Activates Caspase 3 Transcription and Triggers Apoptosis In vivo**

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**Abstract**

EWS/FLI1 is a fusion gene product generated by a chromosomal translocation t(11;22)(q24;q12) found in Ewing sarcoma. EWS/FLI1 encodes an aberrant transcription factor with oncogenic properties in vitro. Paradoxically, expression of EWS/FLI1 in nontransformed primary cells results in apoptosis, but the exact mechanism remains unclear. In primary mouse embryonic fibroblasts derived from conditional EWS/FLI1 knock-in embryos, expression of EWS/FLI1 resulted in apoptosis with concomitant increase in the endogenous Caspase 3 (Casp3) mRNA. EWS/FLI1 directly bound and activated the CASP3 promoter, whereas small interfering RNA–mediated knockdown of EWS/FLI1 led to a marked decrease in CASP3 transcripts in Ewing sarcoma cell lines. Ectopic expression of EWS/FLI1 resulted in an increased expression of CASP3 protein in heterologous cell lines. Importantly, expression of EWS/FLI1 in the mouse triggered an early onset of apoptosis in kidneys and acute lethality. These findings suggest that EWS/FLI1 induces apoptosis, at least partially, through the activation of CASP3 and show the cell context–dependent roles of EWS/FLI1 in apoptosis and tumorigenesis.

**Introduction**

Ewing sarcoma is a cancer found in bone and soft tissues (1). About 85% of Ewing sarcoma cases are characterized by the presence of EWS/FLI1 generated by a balanced chromosomal translocation t(11;22)(q24;q12), resulting in the fusion of EWS to FLI1 (2, 3). The remaining cases of Ewing sarcoma contain chromosomal translocations of EWS with other members of erythroblast transformation–specific (ETS) family genes (4). EWS/FLI1 translocation is also found in related tumors, primitive neuroectodermal tumor, and Askins tumor (5). Chromosomal rearrangements involving EWS with other transcription factors are also observed in desmoplastic small round cell tumor [DSRCT; fused with Wilms’ tumor gene (WT1); ref. 6], myoid liposarcoma (fused with CHOP; ref. 7), extrachondrosarcoma (fused with CHN; ref. 8), and clear cell sarcoma (fused with ATF1; ref. 9).

The oncogenic activity of EWS/FLI1 has been shown by its ability to transform NIH3T3 cells (10–12) and primary bone marrow–derived mesenchymal stem cells of human (13) and mouse origin (14, 15), all of which formed tumors in immunodeficient mice. The DNA binding domain of FLI1 and EWS/FLI1 recognizes the same GGAA/T core motif (16, 17). Interestingly, recent reports have shown that GGAA-containing microsatellites may function as the EWS/FLI1-responsive element (18, 19). A large number of EWS/FLI1 target genes have been identified, which contribute to cell proliferation and tumorigenesis (20, 21).

Although the oncogenic mechanisms of EWS/FLI1 have been studied extensively, expression of EWS/FLI1 has also been shown to result in apoptosis and growth arrest in primary cells. EWS/FLI1 expression in primary mouse embryonic fibroblasts (MEF) resulted in rapid cell death and growth arrest, which were abolished by the inactivation of p16, p19ARF, or p53 pathway (22). In human primary fibroblasts, expression of EWS/FLI1 causes growth arrest via p53-dependent pathway but not apoptosis (23), suggesting that the effects of EWS/FLI1 may be cell context dependent. Although it is clear that EWS/FLI1 causes apoptosis in murine primary fibroblasts, the mechanism has not been fully elucidated. We now report the identification of Caspase 3 (Casp3) as a new target of EWS/FLI1.

**Materials and Methods**

**Cell lines and antibodies.** TC71, A4573, CHP100, U2OS, and JN-DSRCT-1 cells were grown in standard culture condition. The following antibodies were used: rabbit polyclonal α-EWS (24), α-Casp3 (Upstate), α-actin (Sigma), α-FLI1 (C19,
Santa Cruz Biotechnology), α–FLAG (M2, Sigma), or α–green fluorescent protein (GFP; Clontech).

**Generation of Ews/FLI1Stop conditional knock-in mouse.** The targeting vectors were generated by inserting a loxP-flanked transcriptional Stop signal (25) in either sense or antisense direction into Ews intron 6–containing genomic DNA (Supplementary Fig. S1). A human FLI1 cDNA (exons 5–9) with a FLAG epitope at the COOH terminus was PCR amplified and fused to Ews exon 7. The targeting vectors were electroporated into mouse embryonic stem (ES) cells and used to generate Ews/FLI1Stop (EFStop) knock-in mouse (see Supplementary Materials and Methods for detail). A transgenic mouse constitutively expressing the CreER allele [B6.Cg-Tg(CAG-cre/Esrt)5Amc/j] was purchased from The Jackson Laboratory. EFStop heterozygous mice were crossed with CreER to generate EFStop;CreER− and EFStop;CreER+ mice. For administration of tamoxifen, mice (4–8 wk) were injected i.p. with tamoxifen at 3 mg/40 g body weight for 3 consecutive days. Animals were handled following the NIH Animal Research Advisory Committee guidelines.

**Generation of Ews/FLI1Stop;CreER+ and Ews/FLI1Stop;CreER− MEFs.** Ews/FLI1Stop or Ews/WT1Stop (24) heterozygotes were crossed with CreER transgenic mice, and E12.5 embryos were used to generate MEFs. For cell growth analysis, MEFs were plated at 5 × 10⁴ cells, and after addition of 1 μmol/L 4-hydroxytamoxifen (4-HT), cell number was determined daily. Caspase activity was measured using a Homogenous Caspases Assay (Roche Molecular Biochemicals).

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChiP) assay was performed as previously described (26). Chromatin was immunoprecipitated with either α-FLI1 antibody or rabbit IgG antibody and amplified by PCR (see Supplementary Materials and Methods for primers).

**Cloning of the human CASP3 promoter and luciferase reporter assay.** Human CASP3 promoter was amplified by PCR (see Supplementary Materials and Methods for primers) and inserted in the pGL3-Basic promoterless luciferase vector (Promega). CASP3 promoter constructs (P1–P4) were transfected into TC71, CHP100, A4573, and JN-DSRCT-1 cells along with Renilla luciferase reporter plasmid, and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega).

**Small interfering RNA knockdown.** TC71, CHP100, and A4573 cells were transfected with 50 nmoL of FLI1 small interfering RNA (siRNA), 5′-GUACCCUUUGACACUGUCdCdTdT-3′ (MWG Biotech; ref. 27), or scrambled siRNA using Lipofectamine 2000 (Invitrogen). Total RNAs were reverse transcribed, and the expression levels of CASP3 and GAPDH were analyzed by quantitative reverse transcription-PCR (qRT-PCR) using Taqman probes (Applied Biosystems). Data were analyzed by comparative Cₘ method using GAPDH as an endogenous control.

**Electrophoretic mobility shift assay.** Annealed wild-type or M5 oligonucleotides were end labeled with [γ-32P]ATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs). pcDNA3-EWS/FLI1 or pcDNA3 empty vector was transfected and translated in vitro using T7 TNT Quick Coupled Transcription/Translation System (Promega) and incubated with 32P-labeled oligonucleotides (25,000 cpm) in the binding buffer (4 mmol/L HEPES, 50 mmol/L NaCl, 0.5 mmol/L DTT, 0.5 mmol/L EDTA, 1 mmol/L MgCl₂) containing 1 μg of poly(dehydroinosinic-deoxythymidylic acid), 0.2 μg bovine serum albumin, 12% glycerol, and either with or without α-FLI1 or α-WT1 antibodies. For the competition assay, unlabeled wild-type or M5 probes were added at 25–, 50–, or 100-fold excess to the binding reaction.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining.** Tamoxifen-injected Ews/FLI1Stop;CreER+ and WTCreER+ kidneys (4–8 wk old) were fixed, embedded in paraffin, and sectioned. For terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining of sectioned kidneys, TdT-FragEL Fragmentation Detection kit (Calbiochem) and ApopTag Red In Situ Apoptosis Detection kit (Millipore) were used. Representative images of TUNEL-stained slides were captured with Leica DMLB microscope (Leica Microsystems) or with a confocal LSM 5 Live/Axio Observer.Z1 microscope using 20× objective lens (Carl Zeiss Microlmaging, Inc.).

**Results**

**Generation of Ews/FLI1 knock-in mouse.** To study the in vivo functions of EWS/FLI1, we used a knock-in strategy to direct the expression of Ews/FLI1 under the native Ews transcriptional elements. To accomplish this, we amplified the human FLI1 cDNA (exons 5–9) with a FLAG epitope and fused it with the mouse genomic DNA containing the Ews exon 7 breakpoint (Fig. 1). When we screened for the mouse ES cells harboring the correctly targeted Ews/FLI1 (mEF) knock-in allele, we failed to obtain any positive clones, indicating that the constitutive expression of Ews/FLI1 is incompatible with ES cells.

To circumvent this, we inserted a transcriptional termination signal (Stop) flanked by loxP sequences (25) in the Ews intron 6 of the targeting vector (Supplementary Fig. S1). This allows for mEF expression to be regulated by a Cre recombinase. As a control, we also inserted the Stop in the antisense direction (Supplementary Fig. S1). The sense Stop-containing targeting vector was then used to screen ES cells for homologous recombination, but we again failed to obtain any positive clones (Supplementary Fig. S1). This is probably due to the leaky expression of mEF even in the presence of Stop. Surprisingly, when we used the antisense Stop-containing targeting vector, we obtained 8 correctly targeted ES clones out of 170 screened (Supplementary Fig. S1). However, the targeted ES cells expressed a read-through transcript, which was slightly larger than the predicted Ews/FLI1 transcript as shown by RT-PCR analysis (data not shown). Sequencing of the read-through transcript revealed that it contained an additional 147 nucleotides derived from the antisense Stop sequence, resulting in an in-frame insertion of 49 amino acids between the Ews exons 6 and 7 (Supplementary Fig. S2), likely due to the presence of cryptic splice acceptor and donor sites in the antisense Stop. We could not, however, detect a protein product generated from the read-through transcripts by Western blotting using polyclonal antibodies against the NH₂-terminal region of Ews (data not shown; ref. 24). However, we cannot

Published OnlineFirst January 26, 2010; DOI: 10.1158/0008-5472.CAN-09-1993
exclude the possibility that a very low level of mEF containing the 49 amino acids might be expressed in these ES cells. Subsequently, we used the targeted ES cells to generate Ews/FLI1 knock-in mice harboring the conditional knock-in allele (EFStop) in their germline.

**Expression of Ews/FLI1 causes apoptosis in MEFs.** We generated MEFs expressing Ews/FLI1 (mEF) by crossing EFStop heterozygotes with a transgenic mouse constitutively expressing Cre recombinase fused to a mutant estrogen receptor ligand binding domain (CreER). CreER can be activated by a synthetic ligand 4-HT but not by the endogenous estrogen (28). As a control, we also generated MEFs expressing Ews/WT1(+KTS) knock-in heterozygotes (EWStop; ref. 24) with CreER mouse.

Addition of 4-HT to the cultures of EFStop;CreER+ or EWStop;CreER+ MEFs led to the expression of mEF and mEW, respectively, as early as 4 hours (Fig. 1B). After 24 hours, cells expressing mEF (designated EF;CreER+) started to die in culture, and by 48 to 72 hours, most cells were undergoing cell death (Fig. 1C). In contrast, 4-HT treatment had no effect on the cells expressing mEW (designated EW/CreER+) or wild-type MEFs expressing CreER (Wt;CreER+; Fig. 1C). To further show that EF;CreER+ cells were undergoing apoptosis, we measured caspase activity following the 4-HT treatment. We observed a 3-fold higher caspase activity in EF;CreER+ cells as compared with the control cells at 24 hours after the 4-HT treatment, and by 48 to 72 hours, the caspase activity increased dramatically (Fig. 1D). Taken together, our data showed that expression of mEF causes apoptosis in primary MEFs.

**Casp3 mRNA is elevated following expression of Ews/FLI1.** To determine the transcriptional changes induced by mEF that might contribute to apoptosis, we examined the expression levels of 84 apoptosis-related genes in EF;CreER+ MEFs using a Mouse Apoptosis RT2 Profiler PCR Array (SA Biosciences). We analyzed the expression levels of each gene in EF;CreER+ cells at 12 and 24 hours after the 4-HT treatment in comparison with the levels observed without the treatment. Following the expression of mEF, several proapoptotic gene transcripts were increased compared with the uninduced cells [e.g., death-associated protein kinase 1 (Dapk1), Casp3, LIM homeobox protein 4 (Lhx4), and transformation-related protein 53 binding protein 2 (Trp53bp2)], whereas several antiapoptotic genes showed decreased expression (Supplementary Table S1). We decided to further...
characterize Casp3 because it is directly involved in apoptosis and showed significant expression changes ($P < 0.05$) at both time points (Supplementary Table S1). We confirmed the induction of Casp3 following mEF expression in EF; CreER+ MEFs using a qRT-PCR analysis (Taqman; data not shown) as well as by immunoblotting (Fig. 2A). We also observed the cleavage of Casp3 and poly(ADP-ribose) polymerase (PARP) following mEF expression.

**EWS/FLI1 activates Casp3 transcription.** To determine whether mEF-mediated induction of Casp3 also occurs in a relevant cell line, we examined the CASP3 transcript level in two Ewing sarcoma cell lines: A4573 and CHP100. As controls, we used U2OS, an osteosarcoma cell line, and JN-DSRCT-1, a cell line derived from DSRCT-expressing EWS/WT1 (29). As compared with U2OS or JN-DSRCT-1 cells, CASP3 transcript levels were at least 2-fold or higher in both Ewing sarcoma cells (Supplementary Fig. S3A). We then examined CASP3 at the protein level in three Ewing sarcoma cell lines: TC71 containing the type 1 EWS/FLI1 translocation (EWS exon 7 fused to FLI1 exon 6), CHP100 containing the type II fusion (EWS exon 7 fused to FLI1 exon 5, same as in EF;CreER+ MEFs), and A4573 harboring the infrequent type III fusion (EWS exon 10 fused to FLI1 exon 6). Western blot analysis with an anti-FLI1 antibody showed the expression of different EWS/FLI1 fusion products in these cells (Fig. 2B) but not the endogenous FLI1 (Supplementary Fig. S3B). Expression of EWS/WT1 in JN-DSRCT-1 cells was detected using rabbit polyclonal anti-EWS antibodies (24). In all three Ewing sarcoma cell lines, expression of Pro-CASP3 was much higher than U2OS or JN-DSRCT-1 cells (Fig. 2A). We note that the cleaved (activated) CASP3 was not observed in any of the cell lines (data not shown).

We next used siRNA to deplete EWS/FLI1 in Ewing sarcoma cells and measured CASP3 transcript levels. We used the siRNA against the FLI1 DNA binding region (FLI-siRNA) that had been previously shown to silence EWS/FLI1 (27). Transfection of FLI-siRNA into CHP100 and A4573 cells reduced the expression of EWS/FLI1 efficiently but not in TC71 cell (Fig. 2C, top). The reason for this is not clear, but the inefficient knockdown in TC71 cells served as a negative control.

![Figure 2. EWS/FLI1 induces CASP3 expression. A, MEF cells were cultured with or without 1 μmol/L 4-HT and cell lysates were immunoblotted with α-CASP3, α-PARP, and α-actin antibodies. B, cell lysates were analyzed by immunoblotting with α-EWS, α-FLI1, α-CASP3, and α-actin antibodies. C, cells transfected with si-FLI1 or scrambled siRNA were analyzed by immunoblotting with α-FLI1 and α-actin antibodies (top) or total RNAs were analyzed for CASP3 by qRT-PCR (Taqman). Data represent the mean ± SEM from three independent experiments. D, induction of Pro-CASP3 by EWS-FLI1. Empty vector (pcDNA3), pcDNA3-EWS/FLI1, or pcDNA3-EWS/WT1(△KTS) along with CMV-EGFP plasmids were transfected into NIH3T3 or PC3 cells and immunoblotted as in B.](cancerres.aacrjournals.org)
Following the knockdown of EWS/FLI1, we observed approximately 30% to 40% reduction of CASP3 mRNA in A4573 and CHP100 cells but not in TC71 cells (Fig. 2C, bottom). To further show that EWS/FLI1 can induce CASP3 expression, we ectopically expressed EWS/FLI1 in mouse NIH3T3 or human prostate cancer cell line PC3. Compared with the empty vector–transfected, EWS/WT1(+KTS)–transfected, or FLI1–transfected cells, EWS/FLI1 expression resulted in an increased expression of Pro-CASP3 in both NIH3T3 and PC3 cells (Fig. 2D). Western blotting of cotransfected GFP showed similar transfection efficiency.

**EWS/FLI1 activates the CASP3 promoter.** Analysis of the human CASP3 proximal promoter region (~1.5 kb upstream of the transcription start, +1) revealed the presence of several ETS binding sites: one putative EBS (ETS binding site) motif (AGAATCCCGC; ref. 30) and 10 GGAA/T motifs (Fig. 3A; ref. 17). To determine whether EWS/FLI1 can activate the CASP3 promoter, we subcloned a 1.3-kb fragment (from −1561 to −245) of the human CASP3 promoter (P1) into the promoterless luciferase plasmid, pGL3. We also generated a series of CASP3 promoter deletion constructs, P2, P3, and P4 (Fig. 3A). A promoter-reporter analysis of different CASP3 promoter constructs in Ewing sarcoma cell lines as compared with the JN-DSRCT-1 cells (control) revealed that the P4 promoter (−472 to −245) contained the minimal EWS/FLI1-responsive elements (Fig. 3B). The P4 promoter contains a single EBS and two GGAA sites as the potential EWS/FLI1-responsive elements (Fig. 3C). To determine whether these sites are involved, we mutated each site by site-directed mutagenesis to generate M4, M5, and M6 promoter constructs (Fig. 3C). Introduction of these constructs in Ewing sarcoma cells showed that the mutation at the M5 site resulted in a significant reduction of luciferase activity compared with the P4 (wild-type) promoter (Fig. 3D). Mutations in the M4 or M6 site had no effect on the promoter activity.

**EWS/FLI1 binds to the GGAA site on the CASP3 promoter.** To determine whether EWS/FLI1 can bind directly to the M5 site on the P4 promoter, we synthesized EWS/FLI1 in vitro using a rabbit reticulocyte lysate and performed electrophoretic mobility shift assay (EMSA) with 32P-labeled oligonucleotides containing the wild-type GGAA or the mutated TTA site (M5; Fig. 4A). Incubation of EWS/FLI1–programmed lysate with the wild-type probe did not reveal any specific band as compared with the empty vector–programmed reticulolysate (Fig. 4A, lanes 2 and 3). However, when the antibody against FLI1 was added to the binding reaction, specific complexes appeared in the EMSA assay (Fig. 4A, arrows, lane 6). The specific bands were not observed when a control antibody (anti-WT1, lanes 7–9) or the mutated M5 probe was used (lanes 10–18), suggesting that the specific

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**Figure 3.** EWS/FLI1 activates CASP3 promoter. A, P1 to P4 CASP3 promoters. Putative ETS binding sites (GGAA and EBS motif) are indicated. The numbers are relative to the transcription start (+1). B, pGL3–Basic or P1 to P4 promoter constructs were cotransfected with Renilla luciferase, and luciferase activities were measured and expressed as relative to the luciferase activities of pGL3–Basic (set to 1). Data represent the mean ± SEM from three independent experiments. C, P4 and the mutated M4 to M6 CASP3 promoters. Mutations in the EBS and the GGAA sites are indicated. D, P4 or the mutated promoters (M4–M6) were introduced along with Renilla luciferase and luciferase activities were measured as in B. Data represent the mean ± SEM from three independent experiments. *, P < 0.01; **, P < 0.05.
complexes were due to the stable interaction of EWS/FLI1, the wild-type probe, and the anti-FLI1 antibody. This was further confirmed by the addition of 25-fold excess cold wild-type probe to the binding reaction, which effectively reduced the formation of specific complexes (Fig. 4B, arrows, lane 4). Addition of 50× or 100× unlabeled wild-type probe (Fig. 4B, lanes 5 and 6), but not the mutant (M5) probe (Fig. 4B, lanes 7–9), completely abolished the formation of specific complexes. The reason for the absence of specific complexes when EWS/FLI1 was incubated with the wild-type probe could be due to a weak binding of EWS/FLI1 to a single GGAA site, which might be stabilized by the presence of anti-FLI1 antibody. In support of this view, recent reports showed that EWS/FLI1 did not bind well to a single GGAA site but the binding increased dramatically when multiple GGAA sites were present (18, 19).

To determine whether EWS/FLI1 is present on the human CASP3 promoter in vivo, we performed ChIP experiment using the anti-FLI1 antibody (C-19), which has been previously used for ChIP assay (18, 19). In both CHP100 and A4573 cells, the ChIP analysis showed that EWS/FLI1 is consistently recruited to the P4 region of the CASP3 promoter (Fig. 4C). We also observed the recruitment of EWS/FLI1 to the P1 (−1102 to −1561) and the P3 regions (−721 to −472) of the CASP3 promoter in CHP100 cells but not in A4573 cells. Recruitment of EWS/FLI1 to the CASP3 P4 promoter region in CHP100 and A4573 cells was further confirmed by ChIP assay performed with the NH2-terminal EWS-specific antibody (24) but not

Figure 4. EWS/FLI1 binds to the GGAA. A, sequences of the wild-type and the mutated (M5) probes are shown. 32P-labeled wild-type (lanes 1–9) or M5 probes (lanes 10–18) were incubated with the indicated reticulocyte lysates containing either α-FLI1 (C19) or α-WT1 (C19, Santa Cruz Biotechnology) antibodies. The arrows indicate specific complexes. F, free probe; V, empty vector; EF, EWS/FLI1; NS, nonspecific band. B, competitive EMSA assay was performed as in A with either 25-, 50-, 100-fold molar excess of unlabeled wild-type (lanes 4–6) or M5 probe (lanes 7–9). C, ChIP assay. Chromatin from CHP100 and A4573 cells immunoprecipitated with either rabbit IgG or α-FLI1 (C19) was amplified by PCR with the indicated primers.
with the COOH-terminal EWS-specific antibody (Supplementary Fig. S3C). Together, these results suggest that EWS/FLI1 binds directly to the P4 region of CASP3 promoter.

**EWS/FLI1 is efficiently expressed on tamoxifen treatment in vivo.** We next examined the effect of mEF expression in the mouse. To detect the expression of mEF in vivo, we administered three daily injections (ip.) of tamoxifen to 4- to 8-week-old EF Stop;CreER + and EF Stop;CreER – mice. Following the last injection, tissues were harvested and genomic DNAs were analyzed for the Cre-mediated recombination by PCR (Fig. 5A). In most tissues examined, three daily administration of tamoxifen resulted in the Cre-mediated excision of Stop (Fig. 5A). However, the recombination efficiency varied in different tissues: kidney, heart, lung, and pancreas showed the highest level of recombination, whereas the recombination was less efficient in the other tissues.

To detect the expression of mEF transcripts in different tissues, we performed RT-PCR analysis (Fig. 5B). As positive controls, we used total RNAs isolated from TC71 and CHP100 cells expressing either the type I or the type II EWS/FLI1, respectively. Total RNA from JN-DSRCT-1 cells was used as a negative control. In the kidneys of tamoxifen-treated EF;CreER+ mice, EF;CreER+ mouse, only the larger mEF/Stop transcript was detected (Fig. 5B, lanes 6 and 7). No product was amplified in the WT;CreER− kidneys (Fig. 5B, lanes 8 and 9). To show the expression of mEF at the protein level, we performed immunoprecipitation of kidney lysates from the tamoxifen-treated EF;CreER+ mouse with the anti-FLI1 antibody followed by immunoblotting with either anti-FLAG or anti-FLI1 antibody. The results showed that mEF is expressed in the kidneys of EF;CreER+ mice (Fig. 5C, right), showing that a protein can be synthesized from both the EF/Cre+ allele before and after Cre recombination. The arrows indicate the three primers used in the genomic PCR analysis. Bottom, genomic DNAs from tamoxifen-treated EF;CreER+ (EF/C) and CREER− (EF), and CreER tissues were analyzed by RT-PCR. C, mEF expression analysis. Left, tamoxifen-treated tissues were analyzed by PCR. Lu, lung; Li, liver; B, brain; P, pancreas; S, spleen; H, heart; K, kidney. B, total RNAs from the kidneys of tamoxifen-treated EF;CreER+ (EF/C) and CREER− (EF), or CreER+ (Cre) mice were analyzed by RT-PCR. Gapdh was amplified as a control. The arrows indicate the primers used in RT-PCR. C, mEF expression analysis. Left, tamoxifen-treated EF;CreER+ kidney lysate was immunoprecipitated with α-FLI (C19) and immunoblotted with a-FLAG or α-FLI1 antibodies; right, in vitro translated mEF and mEF/Stop cDNAs were immunoblotted with α-FLI1. D, U2OS cells cotransfected with pCMV-SPORT6-mEF or pCMV-SPORT6-mEF/Stop along with CMV-EGFP vector and treated with cycloheximide (CHX: 10 μg/mL) or cycloheximide plus MG132 (10 μmol/L/mL) were immunoblotted with α-FLI1.

**The read-through transcript containing the Stop produces an unstable protein.** Despite the presence of the mEF/Stop transcripts in the EF Stop;CreER− mouse (Fig. 5B, lanes 6 and 7), we could not detect a protein product generated from the read-through transcripts in the MEFs or in the kidneys of these mice (data not shown), indicating that the protein generated from the mEF/Stop transcript might be unstable. To test this, we cloned the full-length mEF/Stop and mEF cDNAs by RT-PCR. Using an in vitro transcription-translation system, mEF/Stop produced a higher-migrating protein compared with the mEF (Fig. 5C, right), showing that a protein can be synthesized from mEF/Stop transcript. However, when transiently transfected into U2OS cells, expression of mEF/Stop was markedly less than mEF (Supplementary Fig. S4B), consistent with the notion that mEF/Stop encoded an unstable protein.
Next, we transfected mEF/Stop or mEF cDNAs together with a small amount of CMV-GFP into U2OS cells and examined the protein half-life. We used cycloheximide to block de novo protein synthesis and examined the steady-state levels of mEF/Stop and mEF. The results showed that mEF/Stop encoded an unstable protein with a half-life of 30 to 60 minutes, whereas the half-life of mEF was >240 minutes (Fig. 5D). The addition of a proteasome inhibitor, MG132, prolonged the half-life of mEF/Stop in U2OS (Fig. 5D), suggesting that mEF/Stop might be degraded via proteasome pathway.

To determine whether a short-lived mEF/Stop might still retain its transcriptional activity, we performed a promoter-reporter assay. Cotransfection of mEF along with the CASP3 P4 promoter resulted in a 2.5-fold activation of the promoter (Supplementary Fig. S4C), but mEF/Stop failed to activate the CASP3 P4 promoter. A similar 2-fold activation of the CASP3 promoter was observed when the human EWS/FLI1 type II cDNA (hEF) was used. Similar results were obtained with the c-MYC promoter (Supplementary Fig. S4C), which is regulated by EWS/FLI1 (31).

**Induction of Ews/FLI1 expression in EF mouse leads to rapid death.** Two studies have recently shown lethal effects of EWS/FLI1 expression in the mouse (32, 33). Consistent with these reports, we observed a similar lethal effect of mEF expression in the tamoxifen-injected EF;CreER+ mice. On tamoxifen injection, all EF;CreER+ mice quickly became moribund and died within 4 to 6 days. The lethality was dose dependent, as the animals injected with lower doses of tamoxifen survived longer, but eventually, all EF;CreER+ animals died even at the lowest dose of tamoxifen (0.25 mg/40 g

**Figure 6.** mEF triggers apoptosis in the kidney. A, kidneys of tamoxifen-treated Wt/CreER+ and EF;CreER+ were examined for TUNEL assays. Bright-field images (IHC) were taken under Leica DMLB microscope. Scale bar, 100 μm. Immunofluorescent images (IF) were captured by confocal microscopy (n = 3 for each genotype). Scale bar, 50 μm. B, total RNAs from tissues of tamoxifen-treated EFStop;CreER− and EF;CreER+ mice were analyzed for Casp3 and Gapdh levels by qRT-PCR (Taqman). The Casp3 levels in the EFStop;CreER− tissues were used as a control (n = 3 for each genotype).
body weight; data not shown). Tamoxifen had no discernible effects on EF<sup>CreER</sup>− mouse, which showed no gross abnormalities, except in the kidneys, where the dying cells were readily observed in the epithelia of renal tubules as confirmed by TUNEL staining (Fig. 6A).

To determine whether mEF was activating Casp3 expression in EF<sup>CreER</sup>− mouse, we isolated various tissues following tamoxifen treatment (3 mg/40 g body weight) and examined the expression of Casp3 by qRT-PCR analysis. Of the tissues examined, only the kidneys showed 3-fold higher level of Casp3 mRNA compared with the control mice (EF<sup>CreER</sup>; Fig. 6B).

**Discussion**

The apoptotic effects of EWS/FLI1 in primary cells have been well documented in the literature (15, 22). However, the molecular mechanism(s) underlying the cellular toxicity has not been fully elucidated. A previous study has shown that loss of p16<sup>INK4A</sup>, p19<sup>ARF</sup>, or p53 pathway is sufficient to attenuate the toxic effects of EWS/FLI1 (22), but how these pathways are involved remains unknown. In the present study, we showed that EWS/FLI1 directly activates Casp3 transcription and causes apoptosis in the MEFs and in the kidneys, which is the likely cause of death in the EF<sup>CreER</sup>− mouse. Casp3 is one of the central caspases essential for the execution of apoptosis (34), and overexpression of ProCASP3 alone was sufficient to trigger apoptosis in primary MEFs (Supplementary Fig. S5A and B). Thus, our findings suggest that the induction of Casp3 might be one of the mechanisms by which EWS/FLI1 triggers apoptosis. Interestingly, expression of EWS/FLI1 in a transgenic mouse also resulted in apoptosis in liver and spleen and an increase in Casp3 transcripts (35). At the cellular level, the induction of Casp3 by EWS/FLI1 was also observed in human mesenchymal progenitor cells following EWS/FLI1 expression (35). Collectively, these results provide strong evidence for Casp3 as a bona fide target of EWS/FLI1.

Examination of the expression profiling data from Khan and colleagues (36) as well as the data from Kauer and colleagues (37) revealed that Casp3 expression is moderately elevated in primary Ewing sarcoma (data not shown), whereas no evidence of elevated Casp3 expression was found in other data sets (38, 39). We have thus analyzed Casp3 expression in nine primary Ewing sarcomas and nine osteosarcomas (control), and the results showed moderate but consistent increase in Casp3 transcripts in Ewing sarcoma as compared with the control (Supplementary Fig. S6). In addition to Casp3, expression of other genes involved in apoptosis was altered following mEF expression (Supplementary Table S1), which could also contribute to apoptosis. Among them, Dapk1 and Lhx4 were highly induced by mEF. Interestingly, induction of Dapk1 was also documented following EWS/FLI1 expression in human mesenchymal stem cells (13) as well as in primary Ewing sarcoma (37−39). However, it remains to be determined whether these genes are directly regulated by EWS/FLI1. Increased expression of Casp3 and other proapoptotic genes by EWS/FLI1 raises an interesting hypothesis about the tumor cell of origin. Because the only cell type that tolerates EWS/FLI1 expression is the mesenchymal stem cells (13−15), this suggests that the mesenchymal stem cells have a mechanism to oppose EWS/FLI1-mediated apoptosis. Thus, identifying the antiapoptotic mechanism(s) of the mesenchymal stem cells could have therapeutic implications for Ewing sarcoma.

Generation of EWS/FLI1 transgenic mouse has been difficult due to its toxic effects. Previously, a conditional EWS/ERG knock-in mouse was generated using an inverter approach (40), which caused T-cell leukemia (41). Recently, a conditional EWS/FLI1 transgenic mouse was used to express EWS/FLI1 in the hematopoietic compartment, which resulted in a rapid onset of myeloid leukemia (33). A second transgenic mouse with EWS/FLI1 expression in the mesenchymal cells of the limbs caused limb deformities but no tumors (32). However, with a concomitant loss of p53, EWS/FLI1 expression led to the development of poorly differentiated sarcoma (32). Thus, our conditional EWS/FLI1 knock-in mouse, which allows the expression of the fusion product from the native Ews locus, represents an important first step toward generating an animal model of Ewing sarcoma. The obvious challenge will be to circumvent the lethality of EWS/FLI1. One approach might be to use a transgenic mouse expressing CreER under a tissue-specific promoter, in which EWS/FLI1 expression is tolerated. In this regard, both human and mouse mesenchymal stem cells from the bone marrow have been shown to not only tolerate EWS/FLI1 expression but also form tumors in nude mice (13−15). Thus, limiting the expression of EWS/FLI1 to the mesenchymal stem cell lineage in our Ews/FLI1 knock-in mouse will be the next step toward the generation of Ewing sarcoma mouse model.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Jun Nishio (Fukuoka University, Fukuoka, Japan) and Crystal Mackall (National Cancer Institute, NIH) for providing the cell lines, Culing Li and Chuxia Deng (National Institute of Diabetes and Digestive and Kidney Diseases Knockout Mouse Core) for the generation of Ews/FLI1 knock-in mouse, Su Young Kim (National Cancer Institute, NIH) for providing primary Ewing sarcoma and osteosarcoma samples, and Yun-Ping Wu (National Institute of Diabetes and Digestive and Kidney Diseases, NIH) for the confocal microscopy.

**Grant Support**

Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases (S.B. Lee).

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Received 6/3/09; revised 10/21/09; accepted 11/16/09; published OnlineFirst 1/26/10.
EWS/FLI1 Oncogene Activates Caspase 3 Transcription and Triggers Apoptosis *In vivo*

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*Cancer Res* 2010;70:1154-1163. Published OnlineFirst January 26, 2010.