PRAS40 Is a Functionally Critical Target for EWS Repression in Ewing Sarcoma

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Abstract

Ewing sarcoma family tumors (ESFT) are highly aggressive and highly metastatic tumors caused by a chromosomal fusion between the Ewing sarcoma protein (EWS) with the transcription factor FLI-1. However, expression of the EWS/FLI-1 chimeric oncogene by itself is insufficient for carcinogenesis, suggesting that additional events are required. Here, we report the identification of the Akt substrate PRAS40 as an EWS target gene. EWS negatively regulates PRAS40 expression by binding the 3’ untranslated region in PRAS40 mRNA. ESFT cell proliferation was suppressed by treatment with an Akt inhibitor, and ESFT cell proliferation and metastatic growth were suppressed by siRNA-mediated PRAS40 knockdown. Furthermore, PRAS40 knockdown was sufficient to reverse an increased cell proliferation elicited by EWS knockdown. In support of a pathologic role for PRAS40 elevation in ESFT, we documented inverse protein levels of EWS and PRAS40 in ESFT cells. Together, our findings suggest that PRAS40 promotes the development of ESFT and might therefore represent a novel therapeutic target in this aggressive disease. Cancer Res; 72(5); 1260–9. ©2012 AACR.

Introduction

Ewing sarcoma is an aggressive and highly metastatic malignancy predominantly afflicting children and young adults. EWS encodes an RNA-binding protein in which function remains largely unknown, whereas the chromosomal translocations, which fuse the N-terminal domain (NTD) of EWS to the DNA-binding domain of the ETS family transcription factors including FLI-1 and other transcription factors such as ATF-1 and WT1, are thought to be responsible for causing Ewing sarcoma family tumors (ESFT). The (11;22)(q24;q12) chromosomal translocation producing the EWS/FLI-1 fusion protein accounts for approximately 85% of ESFT. However, the ectopic expression of EWS/FLI-1 results in growth arrest or cell death rather than the promotion of cellular transformation in cells (1) and in mice (2). Also, EWS/FLI-1 alone was not sufficient to confer sarcomatous change in a transgenic mouse model (3). These results indicated cellular context to be critical to the oncogenic potential of EWS/FLI-1 and additional events to be required for transformation to occur. These additional events may include deregulated p53 pathway (4) and loss of the p16 pathway (1). Moreover, EWS is absent or expressed as 2 splicing variants in ESFT (5), suggesting EWS itself or its targets to play important roles in the development of ESFT.

EWS belongs to the TET family of RNA-binding proteins, which include TLS (also known as FUS), EWS, and TAF15 (also known as TAFII68). TET proteins are involved in fusions with a variety of transcription factors by chromosomal translocation in human cancers. Our limited knowledge about the function of native EWS derives mainly from studies of protein interaction. The NTD of EWS associates with the basal transcription factor TFIIID (6) and with certain subunits of RNA polymerase II (6, 7). In addition, CREB-binding and p300 transcriptional activator proteins (8, 9) have been shown to bind EWS. However, the role of EWS in basal transcription has yet to be shown. A role of EWS in splicing was suggested from its interactions with the splicing factor U1C (10), TASR-1/TASR-2 (11), and Y-box protein-1 (12). Thus, EWS is involved in alternative splicing by regulating the activities of these splicing factors (10–14).

EWS mostly localizes to the nucleus in human embryonic kidney (HEK) 293T cells but is capable of nuclear-cytoplasmic shuttling, which raises the possibility that EWS is involved in the nuclear export of mRNA (15). EWS shows a cytoplasmic expression in secretory cell types, and under oxidative stress EWS was found to localize to stress granules in which untranslated mRNAs are thought to accumulate (16). Accordingly, we examined the role of EWS in the regulation of post-transcriptional gene expression by binding to mRNAs. We found that EWS significantly inhibited protein production from a reporter mRNA. We then determined its mRNA targets and showed that one of the mRNA targets, PRAS40 (also known as AKTIS1), is regulated by EWS through its 3’ untranslated region (UTR). Knockdown of PRAS40 leads to inhibition of the proliferation, migration, and invasion of ESFT cells. Therefore, PRAS40 may be involved in the development or malignancy of ESFT, and...
targeting PRAS40 might provide an effective novel approach for ESFT treatment.

Materials and Methods

Cell lines and cell culture

The human cervical cancer cell line HeLa S3, tested for 8 short tandem repeat (STR) loci and the amelogenin gene in November 2011, was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum. The HEK293 cell line was purchased from RIKEN Cell Bank (Japan). The ESFT cell lines A673, RD-ES, and SK-N-MC were purchased from American Type Culture Collection, and MHH-ES-1 and TC-71 were purchased from DSMZ (Germany). These cells were tested by the cell banks for 8 STR loci and the amelogenin gene. HEK293 and A673 were grown in DMEM supplemented with 10% FBS, RD-ES was grown in RPMI-1640 supplemented with 15% FBS, SK-N-MC was grown in Minimum Essential Medium Alpha supplemented with 10% FBS, MHH-ES-1 was grown in RPMI-1640 supplemented with 10% FBS, and TC-71 was grown in Iscove’s MDM supplemented with 10% FBS. All of the above cell lines were incubated at 37°C with 5% CO₂.

Results

Overexpression of EWS represses reporter expression in a tethering assay

We have isolated Xenopus EWS in a yeast 2-hybrid screening with an RNA-binding protein xCIRP2 as a bait (K. Aoki and K. Matsumoto; unpublished data). To investigate which step of
posttranscriptional gene expression is regulated by EWS, we tethered human EWS to firefly luciferase (FLuc) mRNA through specific interaction between the MS2 coat protein and its binding sites (Fig. 1A). Reporter vectors contained an intron in the 5′UTR with or without 12 copies of MS2-binding sites in the 3′UTR. Expression of MS2–EWS fusion protein in HeLa S3 cells decreased the FLuc activity of the reporter with MS2-binding sites (FLuc-MS2BS) to 17% compared with expression of MS2 alone, but did not decrease that of the control reporter without MS2-binding sites (FLuc). MS2–EWS expression decreased the luciferase activity of FLuc-MS2BS in a dose-dependent manner (Fig. 1B). In addition, the amounts and sizes of the FLuc-MS2BS and FLuc reporter mRNAs were not affected by the expression of MS2–EWS compared with that of MS2, indicating that MS2–EWS had no effect on the stability or splicing of the reporter mRNAs (Fig. 1C). We repeated a similar set of experiments in HEK293 cells. Expression of MS2–EWS decreased the luciferase activity of FLuc-MS2BS to 32% compared with MS2 expression but did not decrease that of FLuc without affecting either the level or the size of FLuc-MS2BS and FLuc reporter mRNAs (Fig. 1D and E). Taken together, the results shown in Fig. 1 suggest that EWS suppresses a later stage(s) of posttranscriptional gene expression, that is, nuclear export or translation of a reporter mRNA when tethered to it.

**PRAS40 mRNA associates with EWS**

The results above raise the possibility that EWS suppresses protein production from the mRNA with which it associates. We then searched for mRNAs that bind EWS protein in cells by

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**Figure 2.** Interaction between EWS protein and PRAS40 mRNA. A and B, the mRNA expression levels in lysates (a) and immunoprecipitates with anti-FLAG M2 antibody (b) were quantified by real-time PCR and were normalized to those of β-actin. Bars, SD. A, β-actin; D, GAR1; F, CFL1; G, ACYP1; H, SAP30; I, RAB18; K, RAD51C; L, ILKAP; M, CDK7; N, RAB27A; P, AKT1S1 (A). A, β-actin; C, FBXO22; D, ARHGDIB; E, H2AFV; F, BAG1; H, BIK; I, PHLD1A1; J, CCNK; K, RABL3; L, FAIM; M, EXOSC3; N, FOSL1; O, DNAJC19 (B). C and D, purified EWS or EWS-FLI-1 (E/F) protein was incubated with 20 fmol of 32P-labeled PRAS40 3′UTR RNA probe. The mixtures were then irradiated with UV. E, Western blotting of EWS and EWS-FLI-1 (E/F) proteins was carried out with anti-FLAG antibody. F, schematic representation of FLuc and FLuc-3UTR competitor RNAs. G, 20 or 60 femtomoles of FLuc or FLuc-3UTR competitor RNA was added to the binding reactions of EWS and the PRAS40 3′UTR probe. H, the quantitative analysis of (G). WB, Western blot.
RNA immunoprecipitation (RIP)-Chip analysis. We introduced FLAG-EWS expression vector into HeLa S3 cells and subjected the cell lysate to immunoprecipitation with anti-FLAG antibody. RNA extracted from the lysate and the immunoprecipitate of FLAG-EWS-expressing cells was hybridized with DNA microarrays. mRNA which emerged more in the immunoprecipitate than in the lysate (false discovery rate < 0.05) was considered to bind the EWS protein (Supplementary Table S1 and National Center for Biotechnology Information Gene Expression Omnibus Series ID GSE29313). We investigated the nature of the EWS target mRNAs identified by RIP-Chip and selected overrepresented Gene Ontology biologic processes. We found enrichment in the biologic processes of protein folding, spliceosome assembly, and DNA repair (Supplementary Table S2).

To verify the results obtained by RIP-Chip, we carried out a real-time PCR analysis with the RNAs from the lysates and the immunoprecipitates of FLAG-EWS-expressing cells. For this analysis, we chose mRNAs that belong to the top 10 in the list (Supplementary Table S1) and, in addition, those implicated in tumorigenesis and/or cell-cycle control. Figure 2A and B show that the mRNAs, which we identified to bind EWS in RIP-Chip, were indeed enriched in the immunoprecipitates. The real-time PCR analysis also confirmed that mRNAs, which were less represented in RIP-Chip (data not shown), were not enriched in the immunoprecipitates (Supplementary Fig. S1). Among the mRNAs bound to the EWS protein, one was AKT1S1 (Akt1 substrate 1), whose protein is named PRAS40 (proline-rich Akt substrate of 40 kDa). For simplicity, we hereafter use PRAS40 for both the mRNA and protein. PRAS40 was originally identified as an Akt substrate which is also a 14-3-3 binding protein (18). PRAS40 associates with the mTORC1 complex. PRAS40 is not only a substrate of Akt1 and mTORC1 but also a functional

Figure 3. Treatment with phosphoinositide 3-kinase (PI3K)/Akt inhibitors of ESFT cells. A-D, A673 (A), SK-N-MC (B), MHH-ES-1(C), and TC-71(D) cells were treated with the indicated amounts of inhibitors. After 48 hours, cell proliferation was examined with SF reagent. Relative absorbance is shown. -, DMSO; API, API-2; LY, LY294002; Wort, Wortmannin. The results of 3 independent experiments are shown. Bars, SD. *, P < 0.01; **, P < 0.05; Student t test. E, the IC50 values were determined for each inhibitor.
regulator of mTORC1 (19–24). We have chosen PRAS40 mRNA for further study in the subsequent part of this work because ESFT cells treated with an Akt inhibitor showed a significant decline in cell proliferation (see below).

We examined whether the EWS protein bound PRAS40 mRNA directly in vitro. The EWS protein has been shown to bind polyanionic acids [poly(G)] and polyuridylic acids [poly(U)] (25). Recently, it was shown that G-rich single-stranded DNA and RNA folded in a G-quadruplex specially bind EWS in vitro (26). A region close to the 3′ terminus of PRAS40 3′UTR is G-rich, yet it is not known whether this region can be folded into a G-quadruplex (Supplementary Fig. S2). Therefore, we tested whether purified EWS binds PRAS40 3′UTR by UV cross-linking analysis (Fig. 2C–H). EWS bound to the 32P-labeled PRAS40 3′UTR RNA probe dependent on the irradiation with UV (Fig. 2C). This shows that the EWS protein associates with PRAS40 3′UTR directly. A 75-kDa band detected by UV cross-linking is likely a degradation product of EWS because the purified EWS contained a similarly migrating protein that was detected by 2 distinct anti-EWS antibodies (Fig. 2E and data not shown). As a control, purified EWS/FLI-1 was also subjected to UV cross-linking, and no binding to the probe was observed, which is probably because EWS/FLI-1 lacks the C-terminal RNA-binding domains of EWS (Fig. 2D). When a 3-fold excess of FLuc-3′UTR mRNA, in which PRAS40 3′UTR was cloned downstream of the FLuc coding region (Fig. 2F), was added as a competitor, the amount of EWS bound to the PRAS40 3′UTR probe was decreased to 32%. The amount bound to the probe was decreased only to 67% by the addition of FLuc mRNA, indicating the specificity of the interaction between the EWS protein and the PRAS40 3′UTR (Fig. 2G and H).

PI3K/Akt inhibitors suppress the growth of ESFT cells

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is constitutively activated in Ewing sarcoma cells (27, 28), and PI3K inhibitors suppress their proliferation (28, 29). PI3K inhibitors augment apoptosis in ESFT cells induced by drugs now clinically used to treat Ewing sarcoma: actinomycin D (30) and doxorubicin (31). To clarify whether the PI3K/Akt inhibitors influence the proliferation of ESFT cells, we treated A673 cells with a PI3K inhibitor, LY294002 or Wortmannin, or an Akt inhibitor, API-2 (triciribine). Cells treated with API-2 showed a
significant decline in proliferation compared with the control 
(DMSO), as did those treated with LY294002 or Wortmannin. 
All of the inhibitory effects were dose dependent (Fig. 3A).

We also treated 3 other ESFT cell lines, SK-N-MC, MHH-ES-1, and TC-71 (Fig. 3B–D), with LY294002, Wortmannin, or API-2. Similar to A673 cells, these cell lines exhibited a significant reduction in cell proliferation on API-2 treatment compared with DMSO treatment, as with 
LY294002 or Wortmannin (Fig. 3E). API-2 inhibits the 
phosphorylation of Akt (32), thus downstream of Akt would be 
important to the proliferation of ESFT cells. This prompted us to elucidate the role of PRAS40 in ESFT cells.

**Expression of PRAS40 is repressed by EWS**

To investigate whether EWS affects PRAS40 protein expression, we overexpressed FLAG-EWS in HeLa S3 cells. The level of the PRAS40 protein upon transfection of the FLAG-EWS expression vector was reduced compared with that on transfection of the empty vector (Fig. 4A). To verify the effect of EWS on the protein expression of PRAS40 and to investigate the biologic consequences, we overexpressed FLAG-EWS in 
HEK293 cells and carried out an anchorage-independent 
proliferation assay (Fig. 4B). The HEK293 cells transfected with 
the FLAG-EWS expression vector showed an obvious decline in cell 
proliferation to 70% (P < 0.05). Consistent with the results in 
HeLa S3 cells (Fig. 4A), the level of the PRAS40 protein was 
markedly decreased upon the overexpression of FLAG-EWS 
(Fig. 4C).

We next examined whether the downregulation of PRAS40 
protein expression caused by the overexpression of EWS is 
attributable to the binding and repression of PRAS40 3’UTR. 
Transfection with an increasing amount of the FLAG-EWS 
expression vector suppressed the luciferase activity of the 
FLuc-3UTR reporter compared with transfection with an 
empty vector, but did not suppress that of the FLuc reporter 
(Fig. 4D). In contrast, overexpression of FLAG-EWS/FLI-1 did 
not show any difference between the luciferase activities of the 
FLuc-3UTR and FLuc reporters. Furthermore, overexpression of 
EWS did not show any effect on the amount of size and of either 
FLuc-3UTR or FLuc reporter mRNA (Fig. 4E).

To confirm the repression of PRAS40 expression by EWS 
further, we next knocked down the expression of EWS with 
siRNAs. The levels of EWS were significantly reduced in HeLa 
S3 cells transfected with EWS siRNA (S1 and S2), but not in 
those transfected with control RNA (C1 and C2). Upon the 
knockdown, the levels of PRAS40 were increased (Fig. 4F). The 
luciferase activity of the FLuc-3UTR reporter was augmented 
about 2- to 2.7-fold by the knockdown of EWS compared with 
that in the absence of siRNA transfection (Fig. 4G) without any effect 
on the level of either FLuc-3UTR or FLuc reporter mRNA 
(Fig. 4H). The results of both the overexpression and knockdown 
experiments indicate that EWS suppresses the production of 
PRAS40 protein.

**EWS protein expression is downregulated in some ESFT cells**

In ESFT cells, as at least 1 EWS allele is fused to tran-
scription factor genes by chromosome translocation, the

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**Figure 5.** Expression of EWS and PRAS40 in ESFT cell lines. The expression of EWS, PRAS40, β-actin, and EWS/FLI-1 in HUVEC, HeLa 
S3, and ESFT (A673, RD-ES, MHH-ES-1, TC-71, and SK-N-MC) cell lines 
was analyzed by Western blotting with antibodies against EWS, PRAS40, 
β-actin, and FLI-1 (top). Relative amounts of EWS and PRAS40 
normalized to that of β-actin are shown (bottom). HUVEC, human 
umbilical vein endothelial cells; WB, Western blot.

**PRAS40 knockdown suppresses the proliferation and 
metastatic potential of ESFT cells**

API-2 was shown to reduce the phosphorylation of PRAS40, 
and PRAS40 promotes the tumorigenesis and chemoresistance 
of melanoma cells (32). We also found that API-2 inhibited the 
proliferation of ESFT cells (Fig. 3). Therefore, we next examined 
the role of PRAS40 in the proliferation of ESFT cells. To do this, 
we knocked down the expression of PRAS40 with siRNAs in 
A673 cells, in which a lower level of EWS and a higher level of 
PRAS40 were expressed than in HUVEC (Fig. 5). The protein 
levels of PRAS40 were significantly reduced in A673 cells 
transfected with 2 PRAS40 siRNAs (#1 and #2), but not in those 
transfected with scrambled siRNA (Fig. 6A). Simultaneously, we 
found that the activated form of caspase 3, cleaved caspase 3, 
was markedly increased in the PRAS40-knockdown cells. These 
results suggest the pathologic consequences of the abnormal expression of EWS and 
PRAS40 in ESFT cells.
with PRAS40 siRNA exhibited a remarkable decline to 26% to 44% in anchorage-dependent cell proliferation compared with no siRNA transfection, whereas those transfected with scrambled siRNA did not show any significant change (Fig. 6B). The anchorage-independent cell proliferation was as low as about 10% of no siRNA control, whereas the proliferation of cells transfected with scrambled siRNA was about 80% of that without siRNA transfection (Fig. 6C). Similar results were obtained in the 2 other ESFT cells (MHH-ES-1 and SK-N-MC cells; Supplementary Fig. S3A–S3C and data not shown).

Ewing sarcoma is highly metastatic, and about 25% of patients exhibit metastatic disease at diagnosis (34). To examine whether PRAS40 plays a role in the metastasis of ESFT cells, we subjected the A673 and MHH-ES-1 cells transfected with siRNAs to a cell migration assay (Fig. 6D and Supplementary Fig. S3D) and a cell invasion assay (Fig. 6E and Supplementary Fig. S3E). The migrating or invading A673 cells transfected with PRAS40 siRNA significantly decreased (20%–25% or 23%, respectively), compared with no siRNA transfection, whereas those transfected with scrambled siRNA did not show any remarkable change. Thus, the data showed that knockdown of PRAS40 repressed the proliferation and metastatic potential of ESFT cells.

Figure 6. Suppression of cell proliferation and metastatic potential of ESFT cells by PRAS40 knockdown. A673 cells were transfected without (-) or with scrambled (S), or PRAS40 (#1, #2) siRNAs. A, after 48 hours, the expression of PRAS40, cleaved caspase 3, and β-actin was analyzed by Western blotting. B, anchorage-dependent proliferation was analyzed at the indicated times. The results of 3 independent experiments are shown. Bars, SD. C, anchorage-independent proliferation was analyzed by soft agar assay. The results of 3 independent experiments are shown. Bars, SD. D and E, cell migration (D) and invasion (E) assays. The quantitative results of 3 independent experiments which are normalized by proliferation are shown on the left. Bars, SD. Representative micrographs are shown on the right. Scale bars, 50 μm. WB, Western blot.
Chromosome translocation PRAS40 expression. depleted cells may be partially mediated by the increase in 7D). Therefore, the stimulated cell proliferation in the EWS-abrogated by the simultaneous knockdown of PRAS40 (Fig. 7A and C and data (Si1 and Si2) target the middle region of EWS so that only native upon EWS knockdown (Fig. 7A). The EWS siRNAs used here was analyzed by soft agar assay. The results of 3 independent experiments are shown. Bars, SD (B). C and D, A673 cells were was analyzed by Western blotting (A). Anchorage-independent proliferation was analyzed as above. E, schematic model. WB, Western blot.

Figure 7. Augmentation of cell proliferation by EWS knockdown. A and B, A673 cells were transfected with control (C1 and C2) or EWS (Si1 and Si2) siRNAs. After 48 hours, the expression of EWS, PRAS40, and β-actin was analyzed by Western blotting (A). Anchorage-independent proliferation was analyzed by soft agar assay. The results of 3 independent experiments are shown. Bars, SD (B). C and D, A673 cells were transfected with control (C2) or EWS (Si2) siRNA. After 4 hours, a sequential transfection with scrambled (S) or PRAS40 (#1 and #2) siRNA was carried out. Protein expression and anchorage-independent proliferation were analyzed as above. E, schematic model. WB, Western blot.

We then examined the effect of EWS knockdown on the cell proliferation. A673 cells depleted of EWS exhibited a significant increase in anchorage-independent cell proliferation compared with control siRNA-transfected cells (Fig. 7A and B). As was seen in HeLa S3 cells, PRAS40 expression was increased upon EWS knockdown (Fig. 7A). The EWS siRNAs used here (Si1 and Si2) target the middle region of EWS so that only native EWS but not EWS/FLI1 was depleted (Fig. 7A and C and data not shown). Interestingly, the increased cell proliferation was abrogated by the simultaneous knockdown of PRAS40 (Fig. 7D). Therefore, the stimulated cell proliferation in the EWS-depleted cells may be partially mediated by the increase in PRAS40 expression.

Discussion

Ewing sarcoma is characterized as a highly malignant tumor. With conventional treatment, the 5-year disease-free survival rate for patients with localized Ewing sarcoma is only 60% to 70% and that for individuals with metastases drops to less than 20% (35). Therefore, novel treatments are needed urgently. Insulin-like growth factor I receptor inhibitors exhibit remarkable activity toward Ewing sarcoma (36, 37), but many patients develop resistance to the therapy and disease recurrence within several months (38). A small molecule, YK-4-279, blocking the interaction of EWS/FLI-1 with RNA helicase A inhibits the growth of Ewing sarcoma cells and orthotopic xenografts (39). Targeting of EWS/FLI-1 by RNA interference results in growth inhibition in Ewing sarcoma cells (40, 41). However, the specificity, toxicity, and clinical utility of YK-4-279 and the siRNAs remain to be clarified.

Recently, increasing attention has been given to the role of RNA-binding proteins in tumorigenesis. To find a novel approach to the treatment of Ewing sarcoma, here we explored the function of native EWS. EWS showed a significant repression of protein production in a tethering assay (Fig. 1), which suggests that MS2–EWS suppressed either mRNA nuclear export or translation, or both. Our preliminary data from in situ hybridization combined with immunofluorescence for MS2–EWS revealed that FLuc–MS2BS mRNA localized to the nucleus of cells expressing MS2–EWS. This finding needs to be studied further. In any case, we hypothesized that EWS binds to a subset of mRNAs and suppresses protein production from them. A number of mRNAs that bound to EWS participate in DNA repair and mRNA splicing (Supplementary Tables S1 and S2). A study with EWS knockout mice showed impaired meiosis and premature senescence of embryonic fibroblasts, suggesting a role for EWS in DNA recombination repair (42). Therefore, further study of the role of the mRNA targets related to DNA repair would help verify the mechanism by which EWS contributes to DNA repair. EWS has already been implicated in mRNA splicing as it interacts with several splicing factors and regulates alternative splicing through these protein–protein interactions (10–14). If we assume that EWS regulates the protein production from mRNAs encoding splicing factors, our data, together with these previous reports, suggest multiple layers of control of mRNA splicing by EWS.

We showed the pharmacologic inhibition of ESFT cell proliferation by an Akt inhibitor (API-2) as well as by PI3K inhibitors (LY294002 and Wortmannin; Fig. 3) consistent with previous reports (28, 29), which indicate the PI3K/Akt pathway to be critical to the proliferation or survival of ESFT cells. The inhibitory action of API-2 toward all the Akts results in systemic toxicity (43, 44). Therefore, more downstream targets would be preferable. Given this, among the targets of EWS we focused on the PRAS40 mRNA, in which product is an Akt substrate. Treatment with API-2 has been shown to suppress the phosphorylation of PRAS40 (32). We showed the specific binding of the 3'UTR of PRAS40 mRNA to the EWS protein (Fig. 2). More importantly, the protein production from a reporter carrying PRAS40 3'UTR was negatively regulated by EWS (Fig. 4). Furthermore, knockdown of EWS resulted in an increase in the level of endogenous PRAS40 protein and the stimulation of anchorage-independent proliferation (Fig. 4F and 7). Taken together, these results are consistent with a model in which EWS suppresses PRAS40 protein expression by

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binding to the 3'UTR. In good agreement with this, whereas EWS was expressed at a lower level, PRAS40 was expressed at a higher level in ESFT cell lines (Fig. 5), which implied a pathologic role for EWS and PRAS40 in ESFT.

We showed that silencing PRAS40 reduced greatly the oncogenic potential including proliferation, migration, and invasion of ESFT cells (Figs. 6 and 7 and Supplementary Fig. S3), which indicates that PRAS40 acts as an oncogene in ESFT in which the level of EWS is decreased possibly by haploinsufficiency (Fig. 7E). Silencing of PRAS40 has been shown to result in impaired TORC1 signaling and cell death (19, 45). PRAS40 also plays an important role in cell survival among different species (20). PRAS40 promotes the tumorigenesis of melanoma by deregulating apoptosis (30). Our results are consistent with these observations. Based on this, we consider that PRAS40 is a candidate target for ESFT treatment. The phosphorylation of PRAS40 results in its release from mTORC1 (22). The release of PRAS40 seems to lead to mTORC1 kinase activity toward 4EBP1 and S6K1 (22, 23). Therefore, functions of PRAS40 in cell proliferation and survival in mTOR-dependent and/or mTOR-independent pathways should be clarified. The present results warrant further study on the molecular mechanisms by which PRAS40 is involved in the proliferation and metastatic potential of ESFT cells. We will investigate the role of other mRNAs bound to EWS, the protein expression of which is regulated by EWS. Elucidation of the dysregulation of the entire mRNA target network by poorly expressed native EWS in ESFT may provide new insights into the molecular mechanisms and the treatment of ESFT.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed by the authors.

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PRAS40 Is a Functionally Critical Target for EWS Repression in Ewing Sarcoma

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