PRAS40 is a functionally critical target for EWS repression in Ewing's sarcoma

Lin Huang¹, Yuji Nakai², Iku Kuwahara¹, and Ken Matsumoto¹,³,⁴

¹Molecular Entomology Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; ²Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; ³PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Running title: Expression of PRAS40 is regulated by EWS

Key words: EWS/ PRAS40/ RNA binding protein

Financial support: This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, by PRESTO program from Japan Science and Technology Agency, and by RIKEN Foreign Postdoctoral Researchers Program. L.H. is a Foreign Postdoctoral Researcher of RIKEN.

Conflict of interest: The authors declare no conflicts of interest.

Word count (excluding references): 4,966

Total number of figures and tables: 7

⁴Corresponding author: Ken Matsumoto
Molecular Entomology Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Tel: 81-48-467-9764; Fax: 81-48-462-4678; E-mail: matsumok@riken.jp
Abstract

Ewing's sarcoma family tumors (ESFT) are highly aggressive and highly metastatic tumors caused by a chromosomal fusion between the Ewing's 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' UTR 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. Further, PRAS40 knockdown was sufficient to reverse an increase cell proliferation elicited by EWS knockdown. In support of a pathological role for PRAS40 elevation in EFST, 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.
Introduction

Ewing’s sarcoma is an aggressive and highly metastatic malignancy predominantly afflicting children and young adults. EWS (Ewing’s sarcoma) encodes an RNA-binding protein whose 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’s 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 two 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 TFIID (6) and with certain subunits of RNA polymerase II (6, 7). In
addition, CREB-binding (CBP) 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 demonstrated. 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 HEK293T 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 hypothesized that EWS plays a role in the regulation of the nuclear export or translation of mRNA. We found that EWS significantly inhibited protein production from a reporter mRNA. We then determined its mRNA targets and demonstrated that one of the mRNA targets, PRAS40 (also known as AKT1S1), is regulated by EWS through its 3’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 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 human embryonic kidney (HEK) 293 cell line was purchased from RIKEN Cell Bank (Japan). The ESFT cell lines A673, RD-ES and SK-N-MC were purchased from ATCC, 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% fetal bovine serum (FBS), RD-ES was grown in RPMI-1640 supplemented with 15% FBS, SK-N-MC was grown in MEM 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. The FreeStyle™ 293-F cell line, which had been screened for viruses, mycoplasma, and sterility, was purchased from Invitrogen and cultured in FreeStyle™ 293 Expression Medium at 37°C with 8% CO₂. Cell lines purchased were passaged less than 6 months after resuscitation.

MS2 tethering assay

HeLa S3 or HEK293 cells inoculated in 96-well plates were transfected in triplicate with 20 ng of pClneo-Luc or pClneo-Luc-12xMS2 and indicated amounts of expression vectors of bacteriophage MS2 coat protein (pMS2-3xFLAG) and MS2-EWS fusion protein (pMS2-EWS-3xFLAG) using Lipofectamine 2000 (Invitrogen). Two nanograms of phRL-SV40 (Promega), was co-transfected to serve as an internal control. The total
amounts of plasmids used were kept constant at 62 ng with pMS2-3xFLAG. Luciferase activities were measured 48 h later.

Results

Overexpression of EWS represses reporter expression in a tethering assay

We have isolated Xenopus EWS in a yeast two-hybrid screening with an RNA-binding protein xCIRP2 as a bait (unpublished data, but see (17)). To investigate which step of post-transcriptional 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 (Figure 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 firefly luciferase 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 (Figure 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 to that of MS2, indicating that MS2-EWS had no effect on the stability or splicing of the reporter mRNAs (Figure 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 (Figure 1D and 1E). Taken together, the results in Figure 1 suggest that EWS suppresses a later stage(s) of post-transcriptional gene expression, i.e., 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 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 (Table S1 and NCBI GEO Series ID GSE29313). We investigated the nature of the EWS target mRNAs identified by RIP-Chip and selected over-represented Gene Ontology biological processes. We found enrichment in the biological processes of protein folding, spliceosome assembly and DNA repair (Table S2).

To verify the results obtained by RIP-Chip, we performed a real-time PCR analysis using the RNAs from the lysates and the immunoprecipitates of FLAG-EWS-expressing cells. For this analysis, we chose mRNAs that belong to the top ten in the list (Table S1) and, in addition, those implicated in tumorigenesis and/or cell cycle control. Figure 2A and 2B demonstrate 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 (Figure 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 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 \textit{in vitro}. The EWS protein has been shown to bind poly (G) and poly (U) RNA (25). Recently, it was shown that G-rich single-stranded DNA and RNA folded in a G-quadruplex specially bind EWS \textit{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 (Figure S2). Therefore, we tested if purified EWS binds PRAS40 3’UTR by UV crosslinking analysis (Figure 2C-H). EWS bound to the \textsuperscript{32}P-labeled PRAS40 3’UTR RNA probe dependent on the irradiation with UV (Figure 2C). This demonstrates that the EWS protein associates with PRAS40 3’UTR directly. A 75 kDa band detected by UV crosslinking is likely a degradation product of EWS, because the purified EWS contained a similarly migrating protein that was detected by two distinct anti-EWS antibodies (Figure 2E and data not shown). As a control, purified EWS/FLI-1 was also subjected to UV crosslinking, and no binding to the probe was observed, which is probably because EWS/FLI-1 lacks the C-terminal RNA binding domains of EWS (Figure 2D). When a 3-fold excess of FLuc-3UTR mRNA, in which PRAS40 3’UTR was cloned downstream of the FLuc coding region (Figure 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 PRAS40 3’UTR mRNA (Figure 2G and 2H).

**PI3K/Akt inhibitors suppress the growth of ESFT cells**

The PI3K/Akt signaling pathway is constitutively activated in Ewing’s 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’s 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 (Figure 3A).

We also treated three other ESFT cell lines, SK-N-MC, MHH-ES-1 and TC-71 (Figure 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 (Figure 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 (Figure 4A). To verify the effect of EWS on the protein expression of PRAS40 and to investigate the biological consequences, we overexpressed FLAG-EWS in HEK293 cells and performed an anchorage-independent proliferation assay (Figure 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 (Figure 4A), the level of the PRAS40 protein was markedly decreased upon the overexpression of FLAG-EWS (Figure 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 (Figure 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. Further, overexpression of EWS did not show any effect on the amount and size of either FLuc-3UTR or FLuc reporter mRNA (Figure 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 (Si1 and Si2), but not in those transfected with control RNA (C1 and C2). Upon the knockdown, the levels of PRAS40 were increased (Figure 4F). The luciferase activity of the FLuc-3UTR reporter was augmented about 2-2.7 folds by the knockdown of EWS compared with that in the absence of siRNA transfection (Figure 4G) without any effect on the level of
either FLuc-3UTR or FLuc reporter mRNA (Figure 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, since at least one EWS allele is fused to transcription factor genes by chromosome translocation, the native EWS protein is produced from the remaining allele. We examined the protein levels of EWS, EWS/FLI-1 and PRAS40 in ESFT cell lines. Human umbilical vein endothelial cells (HUVEC) were used as a control because there is a high degree of similarity in gene expression between ESFT cells and HUVEC (33). The results suggest that EWS is expressed at lower levels in 4 out of 5 ESFT cells that express EWS/FLI-1 than in HUVEC. Significantly, in ESFT cell lines except RD-ES cells, the PRAS40 protein level is higher than that in HUVEC (Figure 5). These results suggest the pathological consequences of the abnormal expression of EWS and PRAS40 in ESFT cells.

**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 (Figure 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 (Figure 5). The protein levels
of PRAS40 were significantly reduced in A673 cells transfected with two PRAS40 siRNAs (#1 and #2), but not in those transfected with scrambled siRNA (Figure 6A). Simultaneously, we found that the activated form of caspase 3, cleaved caspase 3, was markedly increased in the PRAS40-knockdown cells. These results provide an interpretation for the previous finding that PI3K inhibitors augment the apoptosis in ESFT cells induced by actinomycin D (30) and doxorubicin (31). A673 cells transfected with PRAS40 siRNA exhibited a remarkable decline to 26-44% in anchorage-dependent cell proliferation compared with no siRNA transfection, whereas those transfected with scrambled siRNA did not show any significant change (Figure 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 (Figure 6C). Similar results were obtained in the two other ESFT cells (MHH-ES-1 and SK-N-MC cells) (Figure S3A-C and data not shown).

Ewing’s sarcoma is highly metastatic, and about 25% of patients exhibit metastatic disease at diagnosis (34). In addition, a PI3K inhibitor suppressed the migration of Ewing’s sarcoma cells (28). 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 (Figures 6D and S3D) and a cell invasion assay (Figures 6E and 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.

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 (Figure 7A and B). As was seen in HeLa S3 cells, PRAS40 expression was increased upon EWS knockdown (Figure 7A). The EWS siRNAs used here (Si1 and Si2) targets the middle region of EWS so that only native EWS but not EWS/FLI1 was depleted (Figure 7A and C and data not shown). Interestingly, the increased cell proliferation was abrogated by the simultaneous knockdown of PRAS40 (Figure 7D). Therefore the stimulated cell proliferation in the EWS depleted cells may be partially mediated by the increase in PRAS40 expression.
**Discussion**

Ewing’s sarcoma is characterized as a highly malignant tumor. With conventional treatment, the 5-year disease-free survival rate for patients with localized Ewing’s sarcoma is only 60-70% and that for individuals with metastases drops to less than 20% (35). Therefore, novel treatments are needed urgently. Insulin-like growth factor 1 receptor (IGF1R) inhibitors exhibit remarkable activity toward Ewing’s 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’s sarcoma cells and orthotopic xenografts (39). Targeting of EWS/FLI-1 by RNA interference results in growth inhibition in Ewing’s 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’s sarcoma, here we explored the function of native EWS. EWS showed a significant repression of protein production in a tethering assay (Figure 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 (Tables S1 and S2). A study with EWS knock-out mice demonstrated 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 demonstrated the pharmacological inhibition of ESFT cell proliferation by an Akt inhibitor (API-2) as well as by PI3K inhibitors (LY294002 and Wortmannin) (Figure 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 towards 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, whose 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 (Figure 2). More importantly, the protein production from a reporter carrying PRAS40 3’UTR was negatively regulated by EWS (Figures 4). Furthermore, knockdown of EWS resulted in an increase in the level of endogenous PRAS40 protein and the stimulation of anchorage-independent proliferation (Figure 4F and 7). Taken together, these results are consistent with a model in which EWS suppresses PRAS40 protein expression by binding to the 3’UTR. In good agreement with this, while EWS was expressed at a lower level, PRAS40 was expressed at a higher level in ESFT cell lines (Figure 5), which implied a pathological 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 (Figures 6, 7 and S3), which indicates that PRAS40 acts as an oncogene in ESFT in which the level of EWS is decreased possibly by haploinsufficiency (Figure 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 –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.
Acknowledgements

We thank Dr. Kazuma Aoki for the initial isolation of EWS by yeast two-hybrid screening, Drs. Stephen L. Lessnick and Yasufumi Minami for providing plasmids and the Support Unit for Bio-material Analysis, RIKEN BSI Research Resources Center, for help with microarray analysis. We are grateful to Drs. Masafumi Tsujimoto and Shogo Matsumoto for their continuous support during this work.
References


34. Terrier P, Llombart-Bosch A, Contesso G. Small round blue cell tumors in bone: prognostic factors correlated to Ewing's sarcoma and neuroectodermal tumors.


Figure Legends

**Figure 1. EWS repressed reporter expression in the MS2 tethering assay**

(A) Schematic representation of the MS2 tethering assay. (B-E) HeLa S3 cells (B and C) and HEK293 cells (D and E) were co-transfected with 20 ng of FLuc or FLuc-MS2BS reporter vectors and various amounts of MS2 or MS2-EWS expression vectors together with 2 ng of the Renilla luciferase expression vector phRL-SV40. Firefly luciferase activities were measured and normalized to Renilla luciferase activities. The results of three independent experiments are shown. Bars, SD (B and D).

Lysates were subjected to Western blotting and MS2-EWS was detected with anti-FLAG antibody. RNAs were electrophoresed in an agarose gel, stained with ethidium bromide and analyzed by Northern blotting with a FLuc probe (C and E).

**Figure 2. Interaction between EWS protein and PRAS40 mRNA**

(A-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, PHLDA1; J, CCNK; K, RABL3; L, FAIM; M, EXOSC3; N, FOSL1; O, DNAJC19 (B). (C-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 performed with anti-FLAG antibody. (F) Schematic representation of FLuc and FLuc-3UTR competitor RNAs. (G) Twenty or sixty 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).

Figure 3. Treatment with 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 h, cell proliferation was examined using SF reagent. Relative absorbance is shown. -: DMSO; LY: LY294002; Wort: Wortmannin; API: API-2. The results of three independent experiments are shown. Bars, SD. *: p<0.01; **: p<0.05, Student’s t-test. (E) The IC_{50} values were determined for each inhibitor.

Figure 4. PRAS40 expression negatively regulated by EWS

(A) Left panels: HeLa S3 cells were transfected with an empty vector (-) or the FLAG-EWS (EWS) expression vector. After 48 h, cell lysates were subjected to Western blotting with antibodies against FLAG, EWS, PRAS40, and β-actin. β-actin served as a loading control. Right panels: The quantitative analysis of Western blotting (n=3) is shown. (B-C) HEK 293 cells were transfected with an empty vector (-) or the FLAG-EWS (EWS) expression vector. The anchorage-independent growth was analyzed by soft agar assay. The results of three independent experiments are shown. Bars, SD. **: p<0.05, Student’s t-test (B). At 48 h after transfection, cell lysates were analyzed by Western blotting (C). (D) HeLa S3 cells were transfected with the FLuc or FLuc-3UTR reporter vector and the indicated amounts of the FLAG-EWS or FLAG-EWS/FLI-1 (E/F) expression vector together with phRL-SV40. Firefly luciferase activities were measured and normalized to Renilla luciferase activities. The results of three independent experiments are shown. Bars, SD. *, p<0.01, Student’s t-test. (E)
HeLa S3 cells were transfected with the FLuc or FLuc-3UTR reporter vector and 60 ng of empty vector (-) or the FLAG-EWS (EWS) expression vector. After 48 h, total RNA was recovered and subjected to Northern blotting with a DIG-labeled FLuc probe. (F-H) HeLa cells were transfected with phRL-SV40, and the FLuc or FLuc-3UTR expression vector without (-) or with control (C1 and C2) or EWS (Si1 and Si2) siRNAs. After 48 h, the expression of EWS, PRAS40 and β-actin was analyzed by Western blotting (F). Firefly luciferase activities were measured and normalized to Renilla luciferase activities, Bars, SD. (G) Total RNA was recovered and subjected to Northern blotting with a FLuc probe (H).

**Figure 5. Expression of EWS and PRAS40 in Ewing's sarcoma 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 (Upper panels). Relative amounts of EWS and PRAS40 normalized to that of β-actin are shown in lower panels.

**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 h, 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 three independent experiments are shown. Bars, SD. (C) Anchorage-independent proliferation was analyzed by soft agar assay. The
results of three independent experiments are shown. Bars, SD. (D-E) Cell migration (D) and invasion (E) assays. The quantitative results of three independent experiments which are normalized by proliferation are shown in the left panel. Bars, SD. Representative micrographs are shown in the right panels. Scale bars, 50μm.

**Figure 7. Augmentation of cell proliferation by EWS knockdown**

(A-B) A673 cells were transfected with control (C1 and C2) or EWS (Si1 and Si2) siRNAs. After 48 h, 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 three independent experiments are shown. Bars, SD (B). (C-D) A673 cells were transfected with control (C2) or EWS (Si2) siRNA. After 4 h, a sequential transfection with scrambled (S) or PRAS40 (#1 and #2) siRNA was performed. Protein expression and anchorage-independent proliferation were analyzed as above. (E) Schematic model.
Huang et al. Figure 1
Huang et al. Figure 2
Huang et al. Figure 3

<table>
<thead>
<tr>
<th>IC50 (µM)</th>
<th>A673</th>
<th>SK-N-MC</th>
<th>MHH-ES-1</th>
<th>TC-71</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY</td>
<td>24.2</td>
<td>15.3</td>
<td>11.4</td>
<td>8.6</td>
</tr>
<tr>
<td>Wort</td>
<td>26.0</td>
<td>10.1</td>
<td>6.4</td>
<td>28.8</td>
</tr>
<tr>
<td>API</td>
<td>4.2</td>
<td>42.3</td>
<td>0.78</td>
<td>5.4</td>
</tr>
</tbody>
</table>
A

HeLa S3

WB:
- EWS

FLAG
EWS
PRAS40
β-actin

FLAG-EWS
Endogenous EWS

PRAS40

1.5

B

HEK 293

WB:
- EWS

FLAG
EWS
PRAS40
β-actin

1

2

C

PRAS40

EWS

Absorbance (490nm)

1

2

2.5

D

HeLa S3

Relative luciferase activity

EWS (ng)

0

20

40

60

E/F (ng)

0

20

40

60

Vector (ng)

0

60

2

3

3.5

E

HeLa S3

FLuc FLuc-3UTR

NB

Total RNA

F

WB:
- C1 C2 Si1 Si2

EWS
PRAS40
β-actin

G

Relative luciferase activity

FLuc FLuc-3UTR

H

FLuc FLuc-3UTR

NB

Total RNA

Huang et al. Figure 4
Huang et al. Figure 5
A

WB:

- S #1 #2

PRAS40

β-actin

Cleaved caspase3

B

1.5

Absorbance (490nm)

1

0.5

0

0 1 2 3

day

Absorbance (490nm)

C

1

0

0.5

0

- S #1 #2

D

Number of migrating cells (normalized by proliferation)

600

400

200

0

- S #1 #2

E

Number of invading cells (normalized by proliferation)

200

100

0

- S #1 #2

Huang et al. Figure 6

Downloaded from cancerres.aacrjournals.org on April 12, 2017. © 2012 American Association for Cancer Research.
**Huang et al. Figure 7**

**A**

**WB:**
- EWS
- PRAS40
- β-actin

**B**

- **Absorbance (490nm)**
  - C1: 0.05 ± 0.01
  - C2: 0.15 ± 0.03
  - Si1: 0.28 ± 0.02
  - Si2: 0.35 ± 0.04

**C**

**WB:**
- EWS
- PRAS40
- β-actin

**D**

- **Absorbance (490nm)**
  - C2: 0.01 ± 0.002
  - Si2: 0.95 ± 0.04

**E**

- **Chromosome translocation**
- **Haploinsufficiency**
- **EWS/FLI-1 (or other EWS/ETS) fusion protein**
- **PRAS40**
- **Caspase 3 activity**
- **Regulation of Transcription and/or splicing**
- **Ewing’s sarcoma**
PRAS40 is a functionally critical target for EWS repression in Ewing's sarcoma

Lin Huang, Yuji Nakai, Iku Kuwahara, et al.

Cancer Res  Published OnlineFirst January 12, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-2254

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/01/12/0008-5472.CAN-11-2254.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.