In extraskeletal myxoid chondrosarcoma, chromosomal translocation creates a gene fusion between EWS and the orphan nuclear receptor NOR1. The resulting fusion gene product, EWS/NOR1, has been believed to lead to malignant transformation by functioning as a transcriptional activator, but an alternative mechanism may also be involved. Here, using a newly developed functional complementation screening in yeast, we found that EWS/NOR1, but not EWS or NOR1, complemented the loss of function of the small nuclear ribonucleoprotein Smu23p, an essential factor for pre-mRNA splicing in yeast. To verify the potential function of EWS/NOR1 in mammalian cells, we next showed that overexpression of EWS/NOR1 caused increased usage of the distal 5′-splice site of pre-mRNA splicing and that EWS/NOR1 interacted with the human splicing protein U1C; neither EWS nor NOR1 had the same activity or interaction as EWS/NOR1. Altogether, our findings reveal that EWS/NOR1 gains a novel activity affecting pre-mRNA splicing.

The EWS gene is involved in various human malignancies by way of chromosomal translocation (1–3). In human extraskeletal myxoid chondrosarcoma with the t(9;22) chromosomal translocation, the translocation creates a fusion gene between EWS and the orphan nuclear receptor NOR1 (4), resulting in a chimeric fusion gene product (EWS/NOR1) containing the N terminus of EWS and full-length NOR1 (5). The function of the EWS protein is not well known; however, the protein possesses a conserved RNA recognition motif (1) and can bind RNA in vitro (6), suggesting that it may be involved in RNA metabolism. NOR1, also called MINOR (7), TEC (8), and CHN (5), was originally identified from rat fetal forebrain undergoing apoptosis in our laboratory (4) and re-identified through its fusion to the EWS gene in myxoid chondrosarcoma (8). NOR1 belongs to the steroid receptor superfamily and transactivates target genes through the NGFI-B response element (AAAGGTCA) (9).

The EWS fusion proteins are believed to lead to malignant transformation by functioning as transcription factors (10, 11) since the EWS fusion proteins share a common structural pattern in which the N-terminal domain of EWS, lacking the RNA-binding domain, is linked to the DNA-binding domain derived from one of a number of transcription factors. Thus, the RNA-binding domain of EWS is replaced by the DNA-binding domain. In addition, the EWS fusion proteins such as EWS/NOR1 (12) and EWS/FLI1 (13) have been shown to possess strong transcriptional activity. The systematic presence of the DNA-binding domain strongly suggests that these fusion proteins exert their oncogenic potential, at least in part, by deregulating the expression of specific target genes.

However, some observations suggest that the transactivation activity of the EWS fusion proteins may not be sufficient to account for their transforming activity. 1) Modified EWS/FLI1 devoid of DNA-binding activity still retains transforming activity (14). 2) The region of EWS that contributes to transactivation by EWS/FLI1 does not coincide with the domain that confers the most efficient transforming activity (15). 3) EWS/NOR1 binds to the same DNA response element as NOR1 (12); however, the target genes for NOR1 do not appear to be directly associated with oncogenesis, and overexpression of NOR1 in transgenic mice leads to massive apoptosis in thymocytes, not tumorigenesis (16). These observations give rise to the question of whether the fusion genes exhibit an oncogenic potential through a transcription-independent manner.

To answer this question, we explored an alternative function of the EWS/NOR1 fusion protein. Using a newly developed functional complementation screening, we surveyed a potential function of the EWS/NOR1 protein in yeast and then verified the function in mammals. Here, we show that the EWS/NOR1 fusion protein gains a novel activity that affects pre-mRNA splicing.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Media—**The Saccharomyces cerevisiae strain W303 (MATa, MATu, and ura3 ade2 leu2 trp1 his3) was used in this study. Yeast culture media including complex, synthetic complete, minimal sporulation, and dropout media were prepared as described previously (17). The yeast cells were transformed by the lithium acetate method. For induction by the inducible GAL1 promoter, the cells were grown in medium containing 2% galactose and 1% raffinose as the carbon source. Genetic manipulations were performed as described by Rose et al. (17). Tetrad analysis was performed using the Singer MSM manual.

**Expression Plasmids and cDNAs—**The human NOR1 cDNA was cloned previously (18), and EWS and U1C cDNAs were isolated from the human placenta cDNA library. The EWS/NOR1 fusion cDNA was generated by PCR-based techniques using the EWS and NOR1 cDNAs. The resulting fusion cDNA was identical to that found in the extraskeletal chondrosarcoma with the t(9;22) chromosomal translocation (5, 8). The pYES2 vector (Invitrogen) was modified by introduction of the ADE2 gene at the unique NheI site. The resulting vector was named pYES2ADE2 (2 µ or, ura3, ADE2). To express EWS, NOR1, or EWS/NOR1 in yeast, each cDNA was inserted downstream of the GAL1 promoter in the pYES2ADE2 plasmid. FLAG-tagged expression vector pCMV-Tag2 or Myc-tagged expression vector pCMV-Tag3 (Stratagene) was used to express tagged proteins in the cultured mammalian cell line. The pEGFP-N1 vector (CLONTECH) was used to detect the EWS/
NOR1 protein as a GFP fusion protein in mammalian cells. **Multistage Selection of Yeast Mutants**—Wild-type yeast haploids containing the pYES2ADE2-EWS/NOR1 plasmid were mutated randomly using a chemical mutagen, ethyl methanesulfonate. After growing on synthetic complete galactose plates (complete amino acid supplements) at 30 °C and growing on synthetic complete—rhamnose plates as replicas-plated onto synthetic complete glucose plates and incubated at 30 °C for 3 days. The white colonies (ADE2 phenotype) on galactose plates as well as the replica-plated colonies that grew poorly on the glucose plates were selected. Isolated colonies were further tested for the plasmid requirement, viz. lethality by 5-fluoroorotic acid (a drug preventing growth of cells expressing the URA3 marker) and growth on synthetic complete medium. Isolated clones were crossed with a wild-type haploid, and then the diploids showing a recessive phenotype (loss of galactose-dependent growth, Ade−, and Ura−) were selected. In the following stage, using meiotic mapping (tetrad analysis), mutants whose dependence was caused by a single mutation were selected. Next, the EWS/NOR1 expression vector was replaced with the empty vector in diploids, and then the plasmid dependence was examined by tetrad analysis. Finally, to reduce extra mutations, mutants were crossed with a wild-type haploid and then sporulated, and tetrads were dissected. This procedure was repeated at least three times.

**Gene Disruption**—The SNU23 gene was disrupted by a one-step replacement method. The ADE2 gene together with SNU23 flanking sequences (500 bp) was genetically engineered into yeast cells and transformed into a wild-type diploid strain, W303. The disruption in Ade− transformants was confirmed by PCR and Southern blot analysis. When sporulated and dissected, this diploid snu23 strain produced only two viable spores/tetrad.

**Western Analyses of Yeast Proteins**—The yeast mutants containing the EWS, NOR1, or EWS/NOR1 expression plasmid grown on galactose medium at permissive temperature (23 °C) and then incubated at 27 °C for 6 h. Yeast protein extracts were prepared by the standard method using glass beads. For Western analyses, protein extracts from yeast cells were electrophoresed on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore Corp.). Since the transactivating domain of EWS/NOR1 was not affected, the Western analyses were performed with the anti-FLAG M2 monoclonal or anti-Myc antibody (Roche Molecular Biochemicals). The proteins were detected by horseradish peroxidase-conjugated secondary anti-goat IgG. Protein bands were visualized using the ECL Plus Western blot analysis system (Amersham Biosciences, Inc.).

**Cell Cycle and Transformation**—HEK293, COS-7, and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and penicillin/streptomycin. Transient transfections were performed with the calcium phosphate transfection kit (Edge BioSystems) or Polyfect transfection reagents (QIAGEN Inc.) according to the protocol supplied by the manufacturer.

**Intracellular Localization**—For visualizing of GFP-tagged EWS/NOR1 protein in COS-7 cells, cells were plated onto chamber slides and transfected with the indicated GFP fusion vector. The following day, cells were washed with phosphate-buffered saline and fixed for 10 min in methanol at −20 °C. For subcellular fractionation, COS-7 cells that had been grown for 24 h after the transfection were washed with ice-cold phosphate-buffered saline and then harvested in hypotonic buffer (20 mM HEPES-KOH, pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 0.5 mM dithiothreitol, and one Complete™ protease inhibitor mixture (Roche Molecular Biochemicals)/25 μl of buffer). The nuclei were pelleted by centrifugation at 300 × g for 10 min, and the supernatant was recovered as a cytoplasmic fraction. The nuclei were resuspended in nuclear extract buffer (20 mM HEPES-KOH, pH 7.9, 0.5 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and Complete™ protease inhibitor mixture) and incubated for 30 min at 4 °C with agitation. After centrifugation at 15,000 × g for 15 min, the supernatant was used as a nuclear fraction. FLAG-tagged EWS/NOR1 protein was detected by the anti-FLAG M2 antibody (Sigma).

**In Vivo Splicing Assay**—For in vivo splicing analysis of EIA pre-mRNA, COS-7 cells were transfected with 7 μg of pCMV-Tag2-E1A and pCMV-Flag2 construct using Polyfect. After transfection, the cells were washed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete™ protease inhibitor mixture) for Western blotting with the anti-FLAG M2 antibody. Cells from the other dish were lysed for total RNA isolation with an RNeasy column kit (QIAGEN Inc.) and then treated with DNaise. Total RNAs were reverse-transcribed with an oligo(dT) primer, and the PCR amplification mixture was carried out with the Taq PCR kit (Roche Molecular Biochemicals)/25 μl of reaction mixture.

**RESULTS**

**Isolation of Yeast Mutants Requiring EWS/NOR1 for Cell Growth**—As the first step in evaluating the function of the EWS/NOR1 fusion protein in humans, we tried to identify functional counterparts of EWS/NOR1 in budding yeast (S. cerevisiae). Yeast functional complementation studies have been widely used to identify human cDNA that complements a specific yeast mutation. We also exploited functional complementation techniques, but inversely; we tried to isolate yeast mutants that are functionally complemented by the human EWS/NOR1 fusion gene. First, we introduced the EWS/NOR1 expression plasmid (pYES2ADE2-EWS/NOR1) (Fig. 1a) into wild-type haploids and thereafter mutated the yeast genome randomly by treatment with a chemical mutagen. To identify EWS/NOR1-dependent mutants, we developed a multistage selection process for screening colonies to assess the EWS/NOR1 dependence for cell growth (Fig. 1b). Since the expression plasmid contains GAL1 promoter-controlled EWS/NOR1 and the markers of URA3 and ADE2, EWS/NOR1 dependence renders cells dependent on galactose for growth, 5-fluoroorotic acid-sensitive, and white in color, respectively. From −40,000 clones (200 colonies/plate × 200 plates) of the independent yeast mutants, we obtained four mutants showing plasmid- and galactose-dependent cell growth as recessive phenotypes.

We further selected the mutants whose dependence was caused by a single mutation. As shown in Fig. 1c, one mutant showed a clear 2+/-2− segregation of the EWS/NOR1-dependent phenotype (white color, galactose-dependent growth, Ura−, and Ade−) upon tetrad analysis when crossed with the wild-type strain. This indicates that the requirement for EWS/NOR1 resulted from a single mutation or closely linked mutations. Next, we replaced the EWS/NOR1 expression plasmid with the empty plasmid in the mutant diploid and tested the dependence by tetrad analysis. Two spores/tetrad from the mutant diploid containing the empty vector did not grow on synthetic complete galactose plates (data not shown), indicating that the EWS/NOR1 expression plasmid-dependent growth is not due to the dependence for plasmid sequences or galac-
tose. Finally, we obtained only one mutant that showed recessive and temperature-sensitive phenotypes of EWS/NOR1 dependence with a 2(+/H11001):2(+/H11002) segregation upon tetrad analysis.

**Determination of the Mutation in the Yeast Mutant** — To determine the mutated gene in the isolated mutant showing EWS/NOR1-dependent cell growth, we introduced a yeast genomic library (YEP13 vector containing wild-type yeast genomic fragments) into the mutant. From 400,000 transformants, we obtained three transformants that grew on glucose plates. Since the EWS/NOR1 expression was suppressed on glucose plates, these transformants were estimated to contain the wild-type gene corresponding to the mutated gene. Recovered plasmids from the three clones had an overlapping region, which corresponded to chromosome IV (bp 283531–293684). To determine the minimal region for the recovery, we created deletion constructs and tested their rescue activity. As shown in Fig. 2, the minimal region was located between bp 283531 and 286087 (construct C); and upon further deletion (bp 283531–285055; construct D), the activity was lost. Within the region between constructs C and D, one open reading frame (SNU23) existed. To test the rescue activity further, the SNU23 gene was isolated by PCR, transferred to the single copy plasmid YCp, and then tested for its activity. As expected, SNU23 in YCp (construct E) showed positive activity, indicating that the mutation is complemented by the SNU23 gene.

To determine the mutation itself, we performed a PCR-based direct sequencing analysis of the SNU23 gene of the mutant yeast clone. Within chromosome IV (bp 284357–285484), which contains the promoter and coding and terminator regions of the SNU23 gene, only one nucleotide mutation was found in the coding region. G at nucleotide 245 of the open reading frame was replaced with A, resulting in one amino acid replacement of Cys (TGC) with Tyr (TAC) at codon 82 (hereafter called C82Y) (Fig. 3a). The replaced amino acid was located on the first Cys residue constituting the core of a zinc finger motif of Snu23p (Fig. 3b). Snu23p is classified as a small nuclear ribonucleoprotein (snRNP) required for pre-mRNA splicing and has an RNA-binding activity and a C2–H2-type zinc finger motif in the middle (19). Although Snu23p showed little amino acid homology to the EWS/NOR1 fusion protein (Fig. 3c), it showed functional similarity to EWS and structural similarity to NOR1 (Fig. 3b); NOR1 has a C2–C2-type zinc finger motif in the middle, and EWS is also classified as an RNA-binding protein. Final confirmation of the mutation was carried out in diploids (MATa/a) that consisted of the mutant haploid (MATa, snu23(C82Y)) and a snu23-null haploid (MATa, snu23Δ::ADE2). These diploids also showed EWS/NOR1 dependence for

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**Fig. 1. Isolation of yeast mutants showing EWS/NOR1-dependent cell growth.** a, shown is the structure of the EWS/NOR1 expression plasmid (pYES2ADE2-EWS/NOR1). b, shown is the strategy for isolating yeast mutants that show EWS/NOR1-dependent cell growth. Wild-type haploids containing pYES2ADE2-EWS/NOR1 were mutated by ethyl methanesulfonate. Because of GAL1 promoter-controlled EWS/NOR1 and the markers of ADE2 and URA3, the dependent clones show phenotypes of galactose-dependent growth (*1), white coloring (*2), and 5-fluoroorotic acid (5FOA) sensitivity (*3), respectively. Further selections were performed as described under “Experimental Procedures.” c, the isolated mutant showing EWS/NOR1 dependence was crossed with a wild-type strain. This diploid cell was sporulated, and tetrads were dissected. The results from 10 dissections (lanes 1–10) are indicated. All spores (A–D) from each tetrad were patched on a synthetic complete galactose plate (Ade+/H11005 15 mg/liter). The segregants showed a 2 (white):2 (red) phenotype, indicating that EWS/NOR1 dependence is caused by a single mutation or closely linked mutations.
cell growth (data not shown), indicating that no mutation other than snu23(C82Y) is involved in the dependence. Altogether, these results indicate that the human EWS/NOR1 fusion protein can complement the defect of yeast snRNP function associated with the snu23(C82Y) mutation.

**EWS/NOR1 Complements the Defect Associated with snu23(C82Y), but Not snu23-null**—Although EWS/NOR1 was capable of complementing the defect associated with snu23(C82Y), we noted that the growth of the mutant was not as robust as that of the wild-type strain. Therefore, we next tested the ability of EWS/NOR1 to rescue the snu23-null yeast strain (snu23/H9004::ADE2). The snu23-null diploids (snu23Δ::ADE2/SNU23) showed inviability of two spores/tetrad, indicating that this is a recessive lethal mutation (data not shown). As shown in Fig. 4, two spores/tetrad from the snu23(C82Y) mutant (lanes 7–12) grew slowly under the EWS/NOR1-expressed condition, but this was not the case for the snu23-null strain (snu23Δ::ADE2) (lanes 13–19). As a control, four spores/tetrad from the wild-type strain grew normally (lanes 1–6). These results indicate that EWS/NOR1 partly substitutes for the function of Snu23p, which probably depends on a zinc finger motif.

Neither EWS Nor NOR1 Complements the Defect Associated with snu23(C82Y)—We next examined the functional complementation activity of the human EWS, NOR1, or EWS/NOR1 protein in the isolated mutant (Fig. 5a). Since the snu23(C82Y) mutation showed a temperature-sensitive phenotype, plasmid shuffling was performed at permissive temperature (23 °C). Under the conditions of gene expression (galactose medium), the mutant containing the EWS/NOR1 expression plasmid grew at nonpermissive temperature (33 °C), but full-length EWS or NOR1 did not rescue the lethality of the mutation. Under the suppressed condition (glucose medium), no mutants containing the EWS, NOR1, or EWS/NOR1 expression plasmid survived. Wild-type yeast cells carrying each expression plas-
mid grew on both galactose and glucose media at 33 °C (data not shown). The expression of each protein was confirmed by Western blotting using anti-His 6 or anti-EWS antibody (Fig. 5b). These results indicate that neither EWS nor NOR1 has the functional complementation activity detected in the EWS/NOR1 fusion protein.

**EWS/NOR1 Localizes to the Nucleus with a Granular Distribution and to Intranuclear Foci**

A yeast functional complementation assay was performed to examine the localization of EWS/NOR1. Tetrads of strain SNU23/SNU23, snu23(C82Y)/SNU23, or snu23Δ::ADE2/SNU23 containing the EWS/NOR1 expression plasmid were analyzed for growth at 30 °C on galactose medium (induced EWS/NOR1 expression). All spores/tetrad from the wild-type strain (SNU23/SNU23) (lanes 1–6) germinated and formed colonies. Two spores/tetrad from the snu23(C82Y)/SNU23 strain (lanes 7–12), which showed EWS/NOR1 dependence, grew slowly compared with wild-type spores. The snu23Δ::ADE2/SNU23 strain generated only two viable spores (lanes 13–19), indicating that EWS/NOR1 does not suppress the defect associated with snu23-null.

**Fig. 4.** EWS/NOR1 suppresses the growth defect associated with snu23(C82Y), but not snu23-null. The functional complementation activity of EWS/NOR1 in the snu23(C82Y)/SNU23 or snu23Δ strain was examined. Tetrads of strain SNU23/SNU23, snu23(C82Y)/SNU23, or snu23Δ::ADE2/SNU23 containing the EWS/NOR1 expression plasmid were analyzed for growth at 30 °C on galactose medium (induced EWS/NOR1 expression). All spores/tetrad from the wild-type strain (SNU23/SNU23) (lanes 1–6) germinated and formed colonies. Two spores/tetrad from the snu23(C82Y)/SNU23 strain (lanes 7–12), which showed EWS/NOR1 dependence, grew slowly compared with wild-type spores. The snu23Δ::ADE2/SNU23 strain generated only two viable spores (lanes 13–19), indicating that EWS/NOR1 does not suppress the defect associated with snu23-null.

**Fig. 5.** Neither EWS nor NOR1 suppresses the growth defect associated with snu23(C82Y). a, the functional complementation activity of EWS, NOR1, and EWS/NOR1 in the snu23(C82Y) mutant was examined. As a negative control, the same strain with the empty vector was used. Plasmid shuffling was performed at permissive temperature (23 °C), and then transformants were patched on galactose medium to induce gene expression (left and center panels) or on glucose medium to suppress expression (right panel) at the indicated temperatures for 3 days. b, the expression of EWS, NOR1, or EWS/NOR1 in the mutant cells was confirmed by Western blot analysis with anti-His8 or anti-EWS monoclonal antibody. Anti-His8 antibody recognizes the consecutive His motif of NOR1 and EWS/NOR1. The yeast transformants were grown on galactose medium at permissive temperature and then incubated at 27 °C for 6 h. The arrowheads mark the positions of EWS, NOR1, and EWS/NOR1.
A number of RNA- and DNA-binding proteins (20), including snRNPs and non-snRNP splicing factors (21), have been shown to localize to the nucleus with a granular distribution or to intranuclear foci. In contrast, EWS is detectable in both the nucleus and the cytoplasm by Western blotting (13) and shows a homogeneous nuclear distribution by immunohistochemistry (22). Therefore, we first examined the intracellular distribution of the EWS/NOR1 protein in mammalian cells using a GFP fusion protein. When expressed in COS-7 cells, GFP-EWS/NOR1 localized within the nucleus and was excluded from nucleoli (Fig. 6a). In the majority of cells, GFP-EWS/NOR1 had a granular nucleoplasmic distribution. Moreover, in a fraction of cells (5–30%), GFP-EWS/NOR1 localized in discrete intranuclear foci. Similar foci and granular distributions were seen when GFP-EWS/NOR1 was expressed in HEK293 and in the human breast cancer cell line MCF-7 (data not shown), indicating that the observed distribution of GFP-EWS/NOR1 is not unique to COS-7 cells. To further confirm the intracellular distribution of EWS/NOR1, COS-7 cells were transfected with the FLAG-tagged EWS/NOR1 expression plasmid and then sequentially extracted as cytoplasmic and nuclear fractions. Using Western blotting with anti-FLAG antibody, EWS/NOR1 was detected in both the nucleus and the cytoplasm (Fig. 6b). These observations indicate that the intracellular distribution of the EWS/NOR1 protein shows characteristics similar to that of the DNA- or RNA-binding proteins, including snRNPs. EWS/NOR1 Affects Pre-mRNA Splicing—To determine whether EWS/NOR1 directly affects pre-mRNA splicing, we next examined the effects of EWS/NOR1 on pre-mRNA splicing of the adenoviral E1A gene in COS-7 cells. Alternative splicing of E1A pre-mRNA generates three major isoforms (13S, 12S, and 9S) and two minor isoforms (11S and 10S) (23). In this established assay, the proteins of interest are coexpressed with an E1A minigene (pCS3-MT-E1A) (24); and thereafter, alternatively spliced isoforms of E1A are detected by reverse transcription-PCR using the primers shown in Fig. 7a.
In COS-7 cells transfected with pCS3-MT-E1A and empty vector, we detected 13 S and 12 S isoforms as major RNA species (Fig. 7b, lane 3), indicating that isoforms generated by proximal splice site selection are detected mainly in COS-7 cells transfected with the E1A minigene. In contrast, overexpression of EWS/NOR1 caused increased usage of the distal 5′-splice site, resulting in greatly increased levels of the 9 S isoform and decreased levels of the relative proportion of the 13 S and 12 S isoforms (lane 6). Both EWS and NOR1 had some effect on the E1A splicing profile; however, this effect was much smaller than that of EWS/NOR1 (lanes 4 and 5). The expression of EWS, NOR1, or EWS/NOR1 was confirmed by Western blotting with the anti-FLAG M2 antibody (lower panel), and all PCRs were repeated with different cycle numbers and varying amounts of input cDNA to verify the results. The identity of the amplified products obtained by reverse transcription-PCR analysis was confirmed by restriction enzyme analysis and Southern blotting with a 32P-labeled E1A probe (data not shown). These results indicate that EWS/NOR1 affects 5′-splice site selection of the E1A minigene and that neither EWS nor NOR1 has this activity.

EWS/NOR1 Interacts with the U1 snRNP-specific Protein U1C—In vivo splicing assay indicates that EWS/NOR1 may affect 5′-splice site selection of pre-mRNA splicing. The key protein for 5′-splice site recognition has been shown to be the U1 snRNP particle (25, 26), which is composed of an RNA backbone, common Sm proteins, and three U1-specific proteins: U1A, U1C, and U1-70K (27). Since the N terminus of EWS has been shown to interact with the splicing protein U1C in vitro (28), we next examined whether EWS/NOR1 interacts with U1C in mammalian cells.

To examine the intercellular association between EWS/NOR1 and U1C, we used the mammalian two-hybrid system, which allows detection of protein-protein interactions by activation of reporter gene expression in mammalian cells. We fused full-length EWS, NOR1, or EWS/NOR1 to the transcriptional activation domain of NF-κB and fused U1C to the DNA-binding domain of GAL4. We transfected these plasmids along with a GAL4-responsive luciferase reporter gene. When EWS or NOR1 was expressed with U1C, a small increase was seen in luciferase activity (Fig. 8a). When EWS/NOR1 was expressed with U1C, an 8-fold increase was seen in activity compared with EWS with U1C, suggesting that EWS/NOR1 binds U1C with high affinity.

To confirm their intracellular association, plasmids expressing Myc-U1C or Myc-luciferase and FLAG-EWS/NOR1 or FLAG-luciferase were cotransfected into HEK293 cells, and lysates from the cotransfected cells were used for immunoprecipitation. An anti-Myc monoclonal antibody co-immunoprecipitated Myc-U1C along with FLAG-EWS/NOR1 (Fig. 8b, lane 7), but did not co-immunoprecipitate Myc-luciferase along with FLAG-EWS/NOR1 or Myc-U1C along with FLAG-luciferase (lanes 5 and 6). A normal mouse IgG did not co-immunoprecipitate Myc-U1C and FLAG-EWS/NOR1 (lane 8). These results indicate that EWS/NOR1 associates with the U1 snRNP-specific protein U1C in mammalian cells and support the idea that EWS/NOR1 affects the pre-mRNA splicing machinery.

DISCUSSION

We have shown that EWS/NOR1 could partly substitute for the function of snRNP in yeast and that EWS/NOR1 affected pre-mRNA splicing in mammals; neither EWS nor NOR1 shared these activities. Although EWS/NOR1 has been believed to lead to malignant transformation via inappropriate activation of NOR1 target genes, our findings indicate that an alternative mechanism may be involved in the oncogenesis by the EWS/NOR1 fusion protein.

A newly developed functional complementation screening in yeast has allowed us to uncover the potential function of EWS/NOR1 complementing the loss of function of snRNP associated with the snu23(C82Y) mutation. Although the EWS/NOR1 protein shows little amino acid homology to Snu23p, the complementation activity may be explained by similarities in protein structure and motif; both NOR1 and Snu23p contain a zinc finger motif in the middle (4, 19), and both EWS and Snu23p are classified as RNA-binding proteins (1, 19). The EWS/NOR1...
fusion protein lacks the RNA-binding domain of EWS, but replaces this region with the full-length NOR1 protein containing a zinc finger motif. Although a zinc finger motif is thought to be a DNA-binding motif, it has also been shown to be involved in RNA binding. For example, nine zinc fingers of transcription factor IIIA bind specifically to the 5 S RNA (29), and also the Wilms’ tumor gene product WT1 binds RNA via the zinc fingers with high affinity and sequence specificity (30). Therefore, it may be possible that the EWS/NOR1 protein retains the RNA-binding activity through its zinc finger motif and may have a function distinct from that of EWS due to the alteration of the RNA-binding specificity or affinity. The observation that EWS/NOR1 and Snu23p show similarity in structural organization and the finding that EWS/NOR1 can complement the defect associated with snu23(C82Y) suggest that EWS/NOR1 may have a function similar to that of Snu23p. In addition, the observation that EWS/NOR1 can rescue a snu23(C82Y) mutant, but not a snu23-null mutant, suggests that EWS/NOR1 can complement only a part of the function of Snu23p, which probably depends on a zinc finger motif.

Snu23p was originally identified from the yeast 25 S U4/U6/U5 tri-snRNP particles, a central unit of the nuclear pre-mRNA splicing machinery, and is also detected in activated spliceosomes, in which U1, U2, U5, and U6 snRNPs particles and non-snRNPs splicing factors assemble (19). The observations that Snu23p is co-immunoprecipitated with unspliced pre-mRNA in vitro (19), that the genetic depletion of Snu23p leads to the accumulation of unspliced pre-mRNA (19), and that the snu23-null strain shows a lethal phenotype indicate that Snu23p is an essential factor for pre-mRNA splicing as one of the snRNPs. Although a counterpart of Snu23 was not identified in the human genetic sequence data bases, our finding that EWS/NOR1 can complement the loss of function of Snu23p(C82Y) gives rise to the hypothesis that EWS/NOR1 directly influences pre-mRNA splicing in humans.

This hypothesis is strongly supported by our findings in mammalian cells that EWS/NOR1 has the ability to affect alternative splicing of the E1A gene and that EWS/NOR1 interacts with the splicing factor U1C with high affinity. This hypothesis is also consistent with the observation that EWS/NOR1 localizes to the nucleus with a granular distribution and to intranuclear foci; these distributions are frequently observed in RNA- or DNA-binding proteins, including snRNPs (21). Since splicing factors are associated with transcriptional initiation factors and RNA polymerase II (31), it is also possible that EWS/NOR1 may participate in the coupled processes of transcription and splicing. The observation that overexpression of EWS/NOR1 causes increased usage of the distal 5'-splice site of the pre-mRNA suggests that EWS/NOR1 may modulate pre-mRNA splicing through the splicing factors that contribute to 5'-splice site selection. This idea is in line with the finding that EWS/NOR1 directly interacts with the splicing protein U1C, one of the components of U1 snRNP particles that has been shown to be responsible for 5'-splice site recognition (25). Furthermore, the observed effect on splice site selection by EWS/NOR1 is similar to that by the human splicing factor heterogeneous nuclear ribonucleoprotein (hnRNP) A1; high concentrations of hnRNP A1 lead to use of distal 5'-sites (32), suggesting that EWS/NOR1 may have an activity similar to that of hnRNP A1 in mammalian cells. Thus, our findings in both yeast and mammals strongly support the idea that EWS/NOR1 directly affects pre-mRNA splicing.

The in vivo splicing assay and the mammalian two-hybrid system demonstrate that neither EWS nor NOR1 has the ability to affect pre-mRNA splicing or to interact with U1C with high affinity, in contrast to EWS/NOR1. These observations are consistent with the finding in yeast that neither EWS nor NOR1 can complement the defect of the snRNP function associated with snu23(C82Y). EWS has been shown to interact weakly with the splicing protein U1C (28) and the essential splicing factor SF1 (also termed ZFM1) (33); both splicing factors are involved in early stages of spliceosome formation. Furthermore, EWS is co-immunoprecipitated with hnRNP A1 and hnRNP C1/C2 (34), which also participate in splicing. These interactions suggest that EWS may play a role in RNA metabolism. However, our observations, along with previous reports (35, 36), have shown that expression of EWS does not affect pre-mRNA splicing in mammalian cells. Additionally, there has been no direct evidence that NOR1, a member of the steroid receptor superfamily, affects pre-mRNA splicing. Together, our results indicate that the EWS/NOR1 fusion protein gains a molecular function distinct from that of EWS or NOR1. Given that transcription and pre-mRNA processing are closely coupled processes in vivo (37), it is possible that this function of EWS/NOR1 may result from a combination of the binding affinity for the splicing factors derived from EWS and the affinity for the transcriptional machinery derived from NOR1.

Although the EWS fusion proteins have been believed to lead to malignant transformation by functioning as transcriptional activators, our finding that EWS/NOR1 influences pre-mRNA splicing suggests an alternative oncogenic mechanism that is independent of alterations in transcriptional control. The contribution of aberrant splicing to tumorigenesis remains unclear; however, emerging data suggest that instability of RNA splicing may be a new pathway that contributes to tumorigenesis. For example, changes in expression patterns of splicing factors as well as alterations in alternative splicing increase with tumor progression in a mouse model of tumorigenesis (38). Alternatively spliced forms of crucial cell cycle regulators such as p53 and cyclin D1 have been found in human cancer cell lines and tumors (39, 40). Abnormal RNA splicing in Ewing’s sarcoma cells has been reported in a variety of molecules such as the fragile histidine triad FHIT tumor suppressor (41) and the p53-inducible P2XM ion channel (42). In addition, recent observations demonstrate that EWS/FLI1 interferes with alteration of the EIA splicing profile induced by hnRNP A1, whereas EWS/FLI1 alone does not affect the splicing pattern of EIA (43). Although this activity of EWS/FLI1 was opposed by that of EWS/NOR1, the findings are consistent, in part, with our findings in that the EWS fusion proteins are involved in alterations in pre-mRNA splicing. Taken together, the modification of RNA metabolism by the EWS fusion proteins gives rise to the intriguing possibility that the instability of pre-mRNA splicing may contribute to the process of oncogenesis.

Our sequential strategy to determine the potential function of EWS/NOR1 allowed us to reveal that it gains a novel biological activity affecting pre-mRNA splicing, which is probably similar to that of Snu23p in yeast. Although counterparts of EWS and NOR1 do not exist in the yeast genome, the observed functional complementation of the snu23 mutation with EWS/NOR1 allows us to speculate on the function of EWS/NOR1 in humans. Our strategy may provide a new approach to the study of genes in search of a function.

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REFERENCES

1. Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovan, H., Joubert, I., de Jong, P., Bouleau, G., Aurias, A., and Thomas, G. (1992) Nature 359, 162–165
2. Jeon, I. S., Davis, J. N., Braun, B. S., Sublett, J. E., Rousell, M. F., Denny, C. T., and Shapiro, D. N. (1995) Oncogene 10, 1229–1234
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