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Mutation in the DNA-binding domain of the *EWS-Oct-4* oncogene results in dominant negative activity that interferes with EWS-Oct-4-mediated transactivation

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The EWS-Oct-4 protein is a chimeric molecule in which the amino terminal domain (NTD) of the EWS becomes fused to the carboxy terminal domain (CTD) of the Oct-4 transcription factor. It was identified in human bone and soft-tissue tumors associated with t(6;22)(p21;q12). Using *in vitro* and *in vivo* systems, we found that the EWS-Oct-4 protein self-associates. The major domains required for self-association mapped to the EWS NTD (amino acids 70–163) and the POU DNA-binding domain. EWS-Oct-4 protein also associated with EWS-Oct-4 (V351P), which contains a mutation in the POU DNA-binding domain. Using electrophoretic mobility shift assays, we found that the EWS-Oct-4 (V351P) mutant interfered with wild-type EWS-Oct-4 DNA-binding activity. In addition, we found that EWS-Oct-4-mediated transcriptional activation was inhibited by EWS-Oct-4 (V351P) protein *in vivo*. Thus, this mutation in the POU DNA-binding domain results in a dominant negative protein. These findings suggest that the biological functions of the EWS-Oct-4 oncogene can be modulated by the dominant negative mutant EWS-Oct-4 (V351P).

Key words: EWS-Oct-4; self-association; dominant negative; chromosomal translocation; transactivation; transformation; bone and soft-tissue tumors

Oncogene activation can arise from a variety of structural changes to the genome including mutations, gene amplifications, and translocations. Chromosome translocations are an abnormality caused by rearrangements between nonhomologous chromosomes. Tumors of the bone and soft tissue represent a heterogeneous group of mesenchymal lesions and some of them have been shown to exhibit a tumor-specific chromosomal translocation harboring t(6;22)(p21;q12), which contains the *EWS* [Ewing sarcoma; also known as *EWSR1* (Ewing sarcoma breakpoint region 1)] gene at 22q12 and the *Oct-4* gene at 6p21.[1] Striking features of these tumors include the diffuse proliferation pattern of undifferentiated tumor cells, and positive immunoreactivity for vimentin, S-100 and neuron-specific enolase. Although these bone and soft tissue tumors are characterized genetically by the reciprocal translocation of chromosomes 6 and 22, only the *EWS-Oct-4* fusion transcript has been identified in tumors; the reciprocal *Oct-4-EWS* fusion has not been detected.[1] The *EWS-Oct-4* gene encodes a nuclear protein that binds DNA with the same sequence specificity as its partner Oct-4 protein.[2] Comparisons between the transactivation properties of EWS-Oct-4 and Oct-4, indicate that the former exhibits higher transactivation activity for a known target reporter gene containing an Oct-4 binding site. In addition, ectopic expression of EWS-Oct-4 results in increased tumorigenic growth potential in nude mice.[2]

EWS is a member of the TET gene family[3] and as a result of chromosome translocation, it becomes fused to genes postulated to function as transcription factors.[4] In each case, translocation produces chimeric molecules containing the N-terminal domain (NTD) of EWS fused to the DNA-binding domain of its partner. Despite involvement of the *EWS* gene in human cancers, its biological function *in vivo* remains unknown. The *EWS* gene encodes a 656-amino acid protein that contains 3 arginine- and glycine-rich tracts, as well as an 85-amino acid RNA-recognition motif at its C-terminus. The EWS NTD (amino acids 1–285) of EWS is composed almost exclusively (~90%) of tyrosine, glycine, alanine, serine, threonine and proline residues, organized in a repeated and degenerate polypeptide motif with the consensus NSYGQQS. This domain exhibits weak homology to the C-terminal region of eukaryotic RNA polymerase II.[5] The EWS protein was believed to be involved in transcriptional regulation, since it is associated with (*i*) both TFIID (transcription factor IID) and RNA polymerase II,[3] (*ii*) HNF4 (hepatocyte nuclear factor 4),[6] (*iii*) Brn-3a,[7,8] (*iv*) Oct-4,[9] and (*v*) the transcriptional co-activator CBP (CREB-binding protein).[6,10] Recently, targeted disruption of the *EWS* gene has demonstrated that *EWS* is required for meiosis and B cell development.[11] In addition, the *EWS* gene product was also shown to be necessary for proper mitotic spindle integrity.[12]

Oct-4, also referred to as *Oct-3*, is a member of the POU family of transcription factors and it is expressed in pluripotent embryonic stem cells and germ cells.[13–18] Members of the POU transcription factor family share the conserved POU DNA-binding domain, originally identified in the transcription factors Pit-1, Oct-1, Oct-2 and Unc-86.[19] During differentiation, Oct-4 functions as a master switch by regulating gene expression in pluripotent cells or cells that can develop pluripotent potential.[20,21] Furthermore, it was recently reported that Oct-4 is a key player in the genesis of human testicular germ cell tumors (TGCTs).[22,23] Human TGCTs are the most common malignancy in adolescent and young adult Caucasian males and are the cause of 1 in 7 deaths in this group.[24,25] The *Oct-4* transcript is consistently detected in a specific set of human TGCTs of adolescents and young adults: the seminomas and embryonal carcinomas.[26] In addition, the precursor lesions of human TGCT, known as CIS, also express *Oct-4*.[26] Expression of *Oct-4* has also been reported in human primary breast carcinomas, human breast cancer cell lines, and other types of carcinoma cell lines, suggesting that it may be implicated in tumorigenesis by upregulating downstream target genes.[22,27–29] Consistent with these findings, expression of *Oct-4* in a heterologous cell system transformed non-tumorigenic cells and endowed tumorigenicity in nude mice. Activation of *Oct-4* in adult mice using a doxycycline-dependent expression system resulted in dysplastic growth of epithelial tissues that are dependent on continuous *Oct-4* expression.[30]

During our characterization of the *EWS-Oct-4* oncogene we discovered that this product can self-associate and that the responsible regions map to the EWS NTD (amino acids 70–163) and Oct-4 POU DNA-binding domains. Mutation in the POU DNA-binding

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domain of EWS-Oct-4 yielded protein unable to bind the EWS-Oct-4 consensus site but which could still associate with wild-type EWS-Oct-4. Interestingly, the EWS-Oct-4 (V351P) mutant protein interfered with wild-type EWS-Oct-4 DNA-binding activity. Transcriptional activation by EWS-Oct-4 was also inhibited by coexpression of EWS-Oct-4 (V351P) *in vivo*, suggesting that it acts as a dominant negative. These results indicate that the biological activity of EWS-Oct-4 can be modulated by the DNA-binding domain mutant EWS-Oct-4 (V351P).

Material and methods

Material and general methods

Restriction endonucleases, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs. PfuTurbo polymerase was purchased from Stratagene and [γ-^{32}P] ATP (3000 Ci/mmol) was obtained from PerkinElmer. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations and SDS-polyacrylamide gel electrophoresis of proteins were carried out by standard methods.[31] Subclones generated from PCR products were sequenced by the chain termination method using double-stranded DNA templates to ensure the absence of mutations.

Plasmid constructs

The construction of GST-EWS-Oct-4 has been described previously.[2] To construct pTRC-HisA/$(His)_6$-Flag-EWS-Oct-4, Flag-tagged EWS-Oct-4 was PCR-amplified from pKSII/Flag-EWS-Oct-4[2] using the primers 5′-BamHIFlagE-O (5′-GATC*GGATCC*ATGGATTACAAGGATGAC-3′, the *Bam*HI site is underlined) and 3′-hOct-4-437 (5′-CTAG*GAATTC*TCAGTTTGAATGCATGGG-3′, the *Eco*RI site is underlined), followed by ligation into the vector TOPO2.1 (Invitrogen, Carlsbad, CA). pTOPO2.1/Flag-EWS-Oct-4 was digested with *Bam*HI and *Eco*RI, and the digested fragment was subcloned into the same sites of pTRC-HisA (Invitrogen) to generate pTRC-HisA/$(His)_6$-Flag-EWS-Oct-4.

The plasmids pEF-BOS/GST[32] and pcDNA3/Flag-EWS-Oct-4[2] have been described previously. To generate pEF-BOS/GST-EWS-Oct-4, pGEX(4T-1)/EWS-Oct-4[2] was digested with *Bam*HI and *Not*1 and the digested fragment was cloned into the same sites of pEF-BOS/GST.

To generate an expression vector for the fusion protein GST-EWS-Oct-4 (1–211), sequence encoding EWS-Oct-4 (1–211) was PCR-amplified from pcDNA3/EWS-Oct-4 using the primers 5′-EWS-1 (5′-GATC*GAATTC*ATGGCGTCCACGGATTAC-3′, the *Eco*RI site is underlined) and 3′-E-O NTD (5′-GATC*CTCGAG*CGGGTTTTGCTCCAGCTT-3′, the *Xho*I site is underlined). The PCR product was digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX(4T-1). To construct an expression vector for the fusion protein GST-EWS-Oct-4 (CTD), sequence encoding EWS-Oct-4 (CTD) was PCR-amplified from pcDNA3/EWS-Oct-4 using the primers 5′-E-O CTD (5′-GATC*GGATCC*AGCGACTATGCACAACGA-3′, the *Bam*HI site is underlined) and 3′-hOct-4-437 (5′-CTAG*GAATTC*TCAGTTTGAATGCATGGG-3′, the *Eco*RI site is underlined). The PCR product was digested with *Bam*HI and *Eco*RI, and then cloned into the same sites of pGEX(4T-1). The plasmids encoding GST-EWS-Oct-4 (1–163),[33] GST-EWS-Oct-4 (1–35),[33] GST-EWS-Oct-4 (70–163)[34] and GST-EWS-Oct-4 (POU)[35] have been described previously.

The plasmids pcDNA3/Flag-EWS-Oct-4 (V351P) and pEGFP (N1)-EWS-Oct-4 have been described previously.[2] To generate pEGFP (N1)-EWS-Oct-4 (V351P), the EWS-Oct-4 (V351P) fragment was PCR-amplified using the primers 5′ BamHIFlagE-O (5′-GATC*GGATCC*ATGGATTACAAGGATGAC-3′, the *Bam*HI site is underlined) and 3′ hOct4 CTD GFP (5′-GATC*GGATCC*GCTCCGTTTGAATGCATGGG-3′, the *Bam*HI site is underlined) from pcDNA3/Flag-EWS-Oct-4 (V351P) and ligated into the TOPO2.1 vector. To generate pEGFP (N1)-EWS-Oct-4 (V351P), pTOPO2.1/Flag-EWS-Oct-4 (V351P) was digested with *Bam*HI and then the digested fragment was subcloned into the same sites of pEGFP (N1) (Clontech, Palo Alto, CA).

Expression and purification of GST fusion proteins

Glutathione *S*-transferase (GST) fusion proteins GST-EWS-Oct-4 and GST-hOct-4 were expressed in *Escherichia coli*, as described previously.[35] After binding to glutathione-Sepharose and washing, the proteins were eluted with reduced glutathione (Sigma, St. Louis, MO). Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA). The purity and sizes of eluted proteins were evaluated by Coomassie staining of sodium dodecyl sulphate (SDS)-polyacrylamide gels.

GST pull-down assays and co-affinity precipitation

In vitro GST pull-down assays were performed as described previously.[9] For *in vivo* co-affinity precipitation experiments, 293T cells were cotransfected with 0.1 μg of pcDNA3/Flag-EWS-Oct-4 together with either 2 μg of pEF-BOS/GST or pEF-Bos/GST-EWS-Oct-4 using VivaMagic Reagent (Vivagen Co. Ltd., Korea). Co-affinity precipitation assays were also performed as described,[9,32] and bound proteins were detected by immunoblotting with anti-Flag (M2) antibodies.[32]

In vitro *transcription and translation*

In vitro transcription and translation of full-length Flag tagged EWS-Oct-4 and modified Flag-tagged EWS-Oct-4 (V351P) were performed in rabbit reticulocyte lysates using the TNT T7-coupled reticulocyte system, as specified by the manufacturer (Promega, Madison, WI).

Electrophoretic mobility shift assays

Probes (5′-GGCACTTC*ACTAGCAT*AACAATGAGGGCTC-3′ and 5′-GAGCCCTCATTGTT*ATGCTAGT*GAAGTGCC-3′; underlines indicate the Oct-4 recognition site) for electrophoretic mobility shift assays (EMSAs) were prepared from synthetic oligonucleotides for which the sequences have been described previously.[36] The probe was prepared by end-labelling annealed complementary oligonucleotides with [γ-^{32}P] ATP using T4 polynucleotide kinase. DNA binding reactions were performed with *in vitro* translated EWS-Oct-4 and EWS-Oct-4 (V351P) for 30 min at 4°C in binding buffer containing 10 mM Tris·HCl (pH 8.0), 40 mM KCl, 6% glycerol, 1mM DTT, 0.05% NP-40, and 10 ng/μl of poly(dIñdC)ñ(dIñdC). Following binding, the reaction mixtures were run on 4% polyacrylamide gels (acrylamide/bisacrylamide ratio. 37:1) in 0.5× TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA) buffer at 150 V for 2 to 3 hr at 4°C. The gels were dried and exposed to Kodak X-Omat film at −70°C using an intensifying screen.

Subcellular localization

Subcellular localization analyses of EWS-Oct-4 and EWS-Oct-4 (V351P) proteins were performed as described previously.[35] Briefly, 293T cells were plated on glass coverslips and transfected with the respective DNA plasmids using VivaMagic Reagent (Vivagen Co. Ltd.). After incubation for 48 hr, the cells were washed in phosphate-buffered saline (PBS) and fixed for 10 min at −20°C in a mixture of acetone and methanol (1:1, v/v). To detect EWS-Oct-4 or EWS-Oct-4 (V351P), fluorescence was detected with a fluorescence microscope (Olympus, IX71) equipped with a DP71 digital camera (Olympus, Japan).

Reporter gene assays

Cells were transiently transfected with plasmids using VivaMagic Reagent. Luciferase assays were performed with the Dual-luciferase Assay System (Promega). *Renilla* luciferase activities were used to normalize transfection efficiencies.

Establishment of a ZHBTc4/Flag-EWS-Oct-4 cell line expressing Flag-tagged EWS-Oct-4, and RT-PCR

To generate pCAG-IP/Flag-EWS-Oct-4, pKSII/Flag-EWS-Oct-4[2] was digested with *Sal*I, repaired with the klenow fragment, and redigested with *Not*I. pCAG-IP was also digested with *Xho*I, repaired with the klenow fragment, and then redigested with *Not*I. The excised Flag-EWS-Oct-4 insert was directly ligated with the *Not*I and blunted *Xho*I sites of the pCAG-IP vector. The pCAG-IP/Flag-EWS-Oct-4 was digested with *Sfi*I and *Bal*I to generate pCAG-IPΔPuro/Flag-EWS-Oct-4. ZHBTc4/Flag-EWS-Oct-4 cells (a stable cell line expressing Flag-tagged EWS-Oct-4 protein) was generated as described previously.[2] To investigate the dominant negative effect of EWS-Oct-4 (V351P) *in vivo*, ZHBTc4/Flag-EWS-Oct-4 cells were transiently transfected with pCAG-IP/EGFP or pCAG-IP/EWS-Oct-4 (V351P)-EGFP plasmids by electroporation using the MicroPorator (Digital Biotech, Ltd., Korea). Total RNAs were prepared from ZHBTc4/Flag-EWS-Oct-4 cells expressing EWS-Oct-4 (V351P) or vector alone using an RNeasy mini kit (Qiagen, Valencia, CA) with on-column DNase treatment, and messenger RNA was purified with an Oligodex-dT mRNA mini kit (Qiagen). cDNA synthesis was performed using a Superscript First-strand Synthesis System for RT-PCR (Invitrogen). RT-PCR reactions for *fgf-4*, *Hprt* and β-*actin* genes were performed with gene-specific primer sets as described previously.[2,35]

Results

EWS-Oct-4 is a self-associated protein

The chimeric EWS-Oct-4 oncoprotein comprises 437 amino acids, which form 4 major functional domains (Fig. 1*a*). A transactivation domain [amino acids 1-193; labeled as EWS (NTD)] is located at the N-terminus and the truncated Oct-4 NTD (amino acids 200-210; labeled as N) is found within the central region of the protein. The C-terminal portion of EWS-Oct-4 contains a POU DNA-binding domain (amino acids 211–366; labeled as POU) and the C-terminal regulatory domain (amino acids 367–437; labeled as C). As shown in Fig. 1*a*, several EWS- or Oct-4-interacting proteins have been reported independently,[9,32–34,37–40] yet it is not clear whether these proteins interact directly with EWS-Oct-4 to modulate its function. Previously, we demonstrated the POU DNA-binding domain of Oct-4 is involved in binding to EWS.[9] To determine whether EWS-Oct-4 can self-associate, we performed *in vitro* GST pull-down assays. Bacterially expressed GST or GST-EWS-Oct-4 fusion proteins immobilized on glutathione-Sepharose beads were mixed with Flag-EWS-Oct-4. Flag-EWS-Oct-4 was expressed as a 6 histidine-tagged protein in *E. coli* and purified by Ni^{+2}-NTA (Ni^{+2}-nitrilotriacetate)-agarose resin. After extensive washing, the bound proteins were eluted and then analyzed by 10% SDS-PAGE, followed by transfer to a membrane. Immunoblotting with anti-Flag antibody (M2; Sigma-Aldrich) revealed that $(His)_6$-tagged Flag-EWS-Oct-4 associated with GST-EWS-Oct-4, but not with GST alone (Fig. 1*b*).

To determine whether EWS-Oct-4 protein self-associated *in vivo*, we transfected 293T cells with an expression vector driving synthesis of EWS-Oct-4 and then performed co-affinity precipitations (Fig. 2*a*). We used the 293T cell line because EWS-Oct-4-positive bone and soft-tissue tumor cell lines or their equivalents, were unavailable. For these assays, we co-transfected 293T cells with mammalian expression vectors for GST-fused EWS-Oct-4 (pEF-BOS/GST-EWS-Oct-4) or the GST domain alone (pEF-BOS/GST) and pcDNA3/Flag-EWS-Oct-4. The cells were harvested 48 hr after transfection and then the cell lysates were incubated with glutathione beads. Immunoblotting of the eluates with anti-Flag antibody revealed that Flag-EWS-Oct-4 co-precipitated with GST-EWS-Oct-4, but not with GST alone (Fig. 2*b*, top panel). The 3 proteins were identified in the transfected cells by probing for Flag tag (Flag-EWS-Oct-4; Fig. 2*b*, middle panel) or GST (GST-EWS-Oct-4 and GST; Fig. 2*b*, bottom panel). These results demonstrated that EWS-Oct-4 can self-associate *in vivo* as well as *in vitro*.

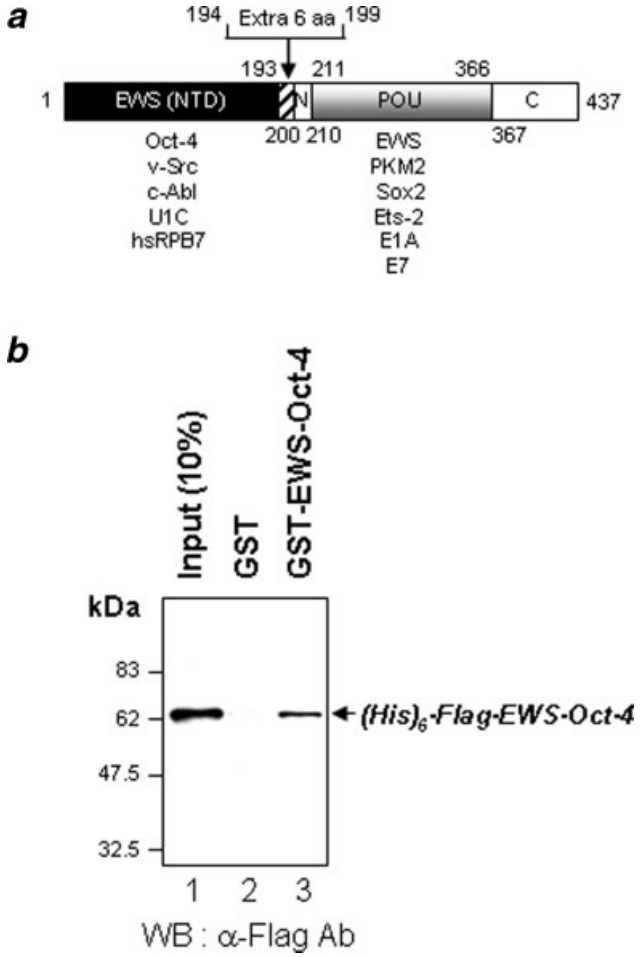

Figure 1 – Self-association of EWS-Oct-4 *in vitro*. (*a*) Schematic representation of EWS-Oct-4 organization and putative interactions. Functionally important domains of the EWS-Oct-4 chimera are depicted and putative interacting proteins are shown at the bottom of the figure. (*b*) Interaction between EWS-Oct-4 and GST-EWS-Oct-4 *in vitro*. Purified $(His)_6$-tagged Flag-EWS-Oct-4 protein was incubated with either 2 μg of GST (lane 2) or GST-EWS-Oct-4 (lane 3) bound to glutathione Sepharose beads. Approximately 10% of the $(His)_6$-Flag-EWS-Oct-4 protein used in the experiment was loaded in lane 1 as a reference. The EWS-Oct-4 proteins were separated by 10% SDS-PAGE and transferred onto membranes. Bound $(His)_6$-Flag-EWS-Oct-4 protein was detected with anti-Flag antibody. The positions of the molecular weight markers are indicated on the left (NEB broad range markers) and the position of $(His)_6$-Flag-EWS-Oct-4 is indicated to the right. Three independent experiments were performed, all of which gave similar results. Lane 1, 10% input; lane 2, GST alone; lane 3, GST-EWS-Oct-4. WB, Western blotting; Ab, antibody.

EWS-Oct-4 contains at least 2 independent self-association motifs

To begin identification of the domain(s) within EWS-Oct-4 that mediates self-association, we performed *in vitro* GST pull-down assays with a series of EWS-Oct-4 deletion mutants (Fig. 3*a*). Carboxyl- and amino-terminal deletions of EWS-Oct-4 were generated and fused in-frame to the GST domain. These GST-fusion proteins were expressed individually in *E. coli*, purified, and then coupled to glutathione-Sepharose beads. After incubation with recombinant $(His)_6$- and Flag-tagged EWS-Oct-4 protein, followed by extensive washing, we found specific association between EWS-Oct-4 protein and 4 of the GST fusions, *i.e.*, fusions containing the EWS-Oct-4 (1-211), EWS-Oct-4 (1-163), EWS-Oct-4 (70-163) and EWS-Oct-4 (POU) domains (Fig. 3*b*). In contrast, no interactions were observed between EWS-Oct-4 and GST

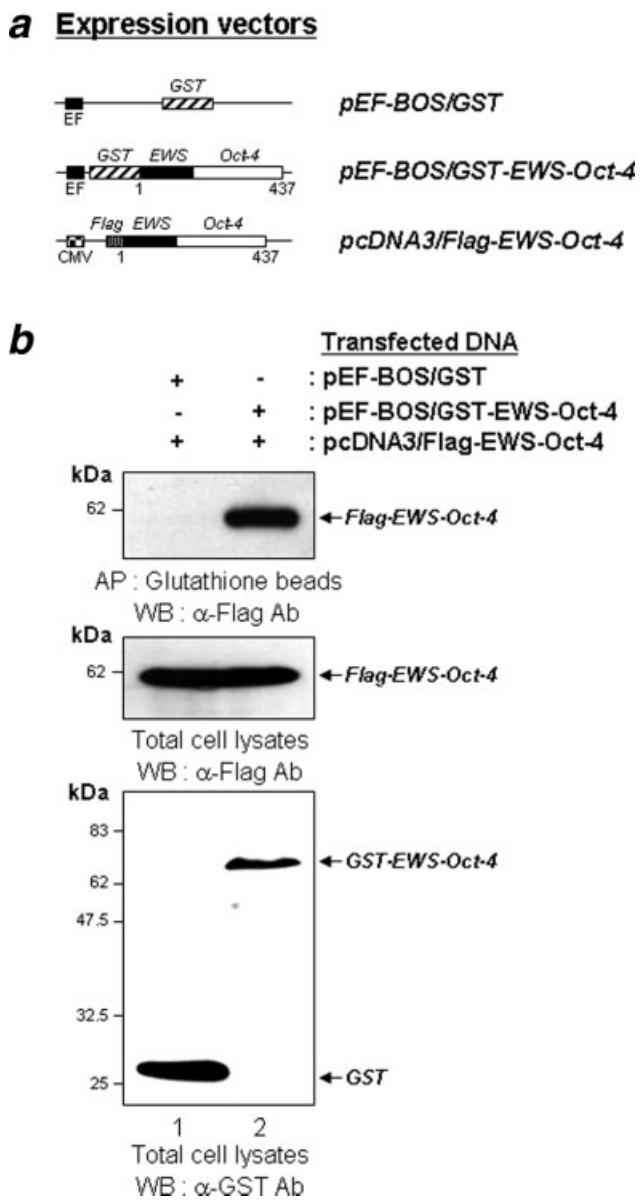

Figure 2 – Self-association of EWS-Oct-4 *in vivo*. (*a*) Schematic representation of the expression vectors used. Expression vector pEF-BOS/GST-EWS-Oct-4 encodes a GST-EWS-Oct-4 fusion protein. The pEF-BOS/GST expression vector was used as a control. The expression vector driving the production of Flag-tagged EWS-Oct-4 is also shown. The positions of the first and last amino acids of EWS-Oct-4 are indicated below each construct. (*b*) Co-affinity purification of Flag-tagged EWS-Oct-4 with GST-EWS-Oct-4 from transfected cells. Forty-eight hours after transfection of 293T cells with 0.2 μg of pcDNA3/Flag-EWS-Oct-4 and either 2 μg of pEF-BOS/GST or pEF-BOS/GST-EWS-Oct-4, cell extracts were prepared as described in Material and Methods and affinity-precipitated with glutathione-Sepharose beads. After separation by 12% SDS-polyacrylamide gel electrophoresis, the proteins were Western blotted with an anti-Flag or an anti-GST (B-14; Santa Cruz Biotechnology) antibody. The identities of the transfected DNAs are indicated above the panel. The positions of the molecular weight markers are indicated to the left and the positions of Flag-tagged EWS-Oct-4, GST and GST-fusion EWS-Oct-4 are indicated by the arrows on the right. Three independent experiments gave similar results. AP, affinity precipitation; WB, Western blotting; Ab, antibody.

alone (Fig. 3*b* lane 2), GST-EWS-Oct-4 (1-35) (lane 5) or GST-EWS-Oct-4 (CTD) (lane 8). These results suggest that EWS-Oct-4 contains at least 2 sites (amino acids 70–163 and the POU domain) that can self-associate independently with EWS-Oct-4.

Based on these results, it is likely that the interaction surface of the EWS N-terminus is the POU DNA binding domain, and *vice versa*. To investigate this, EWS-Oct-4 (70-163) was expressed as a 6 histidine-containing fusion protein in *E. coli* and purified by Ni^{+2}-NTA-agarose resin. A GST-pull down assay was then performed using recombinant $(His)_6$-EWS-Oct-4 (70-163) with GST, GST-EWS-Oct-4 (70-163), or GST-EWS-Oct-4 (POU) fusion proteins. As shown in Figure 3*c*, $(His)_6$-EWS-Oct-4 (70-163) protein interacts with GST-EWS-Oct-4 (POU), but not with GST-EWS-Oct-4 (70-163) or GST alone, indicating that theEWS-Oct-4 (70-163) interacts with EWS-Oct-4 (POU), not with EWS-Oct-4 (70-163).

To determine whether the POU domain of EWS-Oct-4 is able to associate with EWS-Oct-4 (POU) as well as EWS-Oct-4 (70-163), GST pull-down assays were performed with $(His)_6$-EWS-Oct-4 (POU) protein. As shown in Figure 3*d*, the GST fusion protein containing the POU domain was able to retain $(His)_6$-EWS-Oct-4 (POU) protein. These results suggest that the self-association of EWS-Oct-4 can be mediated independently through the EWS-Oct-4 (POU) and the EWS-Oct-4 (70-163) or the EWS-Oct-4 (POU) and the EWS-Oct-4 (POU) domains. The GST-fusion proteins utilized in this assay were fractionated on 15% SDS-PAGE and visualized by Coomassie Blue staining, which confirmed that similar amounts of protein had been used in each pull-down assay (Fig. 3*e*).

EWS-Oct-4 (V351P) mutant protein can form a heteromeric complex with wild-type EWS-Oct-4 in vivo

Site-directed mutagenesis was used to generate an amino acid change hypothesized to affect the DNA-binding function of EWS-Oct-4. Specifically, valine 351 lies within the POU DNA-binding domain that is predicted to be involved in interactions with DNA[41]; this amino acid was mutated to proline (Fig. 4*a*). To investigate whether the EWS-Oct-4 mutant protein [EWS-Oct-4 (V351P)] can associate with wild-type EWS-Oct-4, we transiently transfected 293T cells with expression vectors that express both proteins and then performed an affinity-precipitation. 293T cells were co-transfected with pCMV-Tag2A/Flag-EWS-Oct-4 (V351P) and pEF-BOS/GST-EWS-Oct-4 or pEF-BOS/GST. Cells were lysed 48 hr after transfection and the GST- EWS-Oct-4 fusion protein was affinity-precipitated with glutathione-Sepharose beads. Immunoblotting was performed using anti-Flag antibody to detect the presence of Flag-EWS-Oct-4 (V351P), which was found to co-precipitate specifically with GST-EWS-Oct-4 (Fig. 4*b*, top panel, lane 2), but not with GST alone (Fig. 4*b*, top panel, lane 1). The 3 proteins were identified in the transfected cells by probing for Flag tag [Flag-EWS-Oct-4 (V351P); middle panel] or GST (GST and GST-EWS-Oct-4; lower panel). These results demonstrate that the EWS-Oct-4 (V351P) mutant protein formed a heterodimer with wild-type EWS-Oct-4 *in vivo*.

To determine whether the EWS-Oct-4 (V351P) mutant protein self-associates *in vivo*, we performed co-affinity precipitations on the extracts of 293T cells transfected with a vector for the expression of EWS-Oct-4 (V351P) (Fig. 4*c*). The cells were harvested 48 hr after transfection and cell lysates were prepared. The cell lysates were then incubated with glutathione beads. Immunoblotting of the eluates from the beads with anti-Flag antibody revealed that Flag-EWS-Oct-4 (V351P) co-precipitated with GST-EWS-Oct-4 (V351P), but not with GST alone (Fig. 4*c*, top panel). The 3 proteins were identified in the transfected cells by probing for the Flag tag [Flag-EWS-Oct-4 (V351P); Fig. 4*c*, middle panel], GST [GST-EWS-Oct-4 (V351P) and GST alone; Fig. 4*c*, bottom panel]. These results demonstrated that the EWS-Oct-4 (V351P) mutant protein can also self-associate *in vivo*.

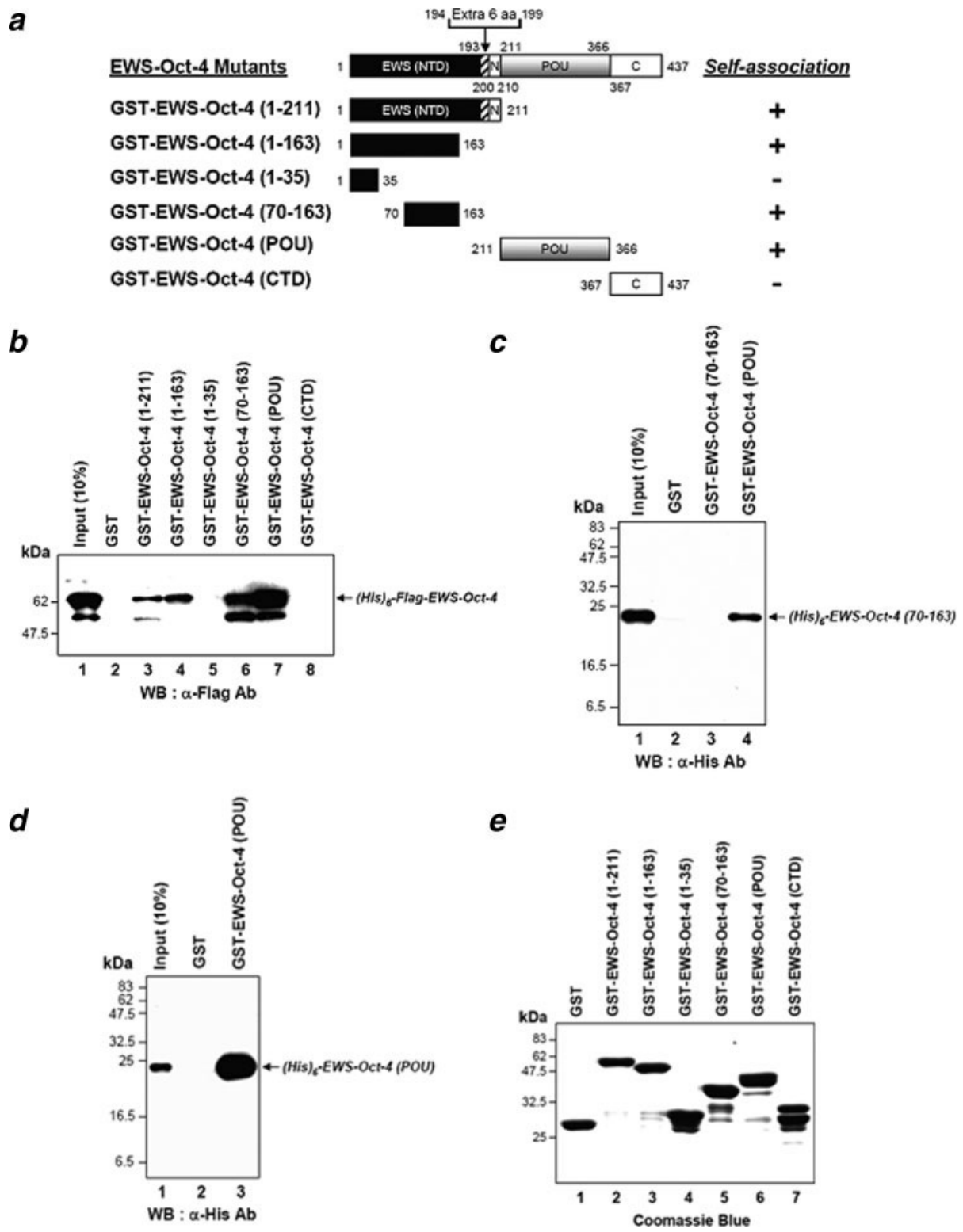

FIGURE 3.

EWS-Oct-4 (V351P) mutant protein interferes with wild-type EWS-Oct-4 DNA-binding activity

In order to test whether EWS-Oct-4 (V351P) can bind to an authentic EWS-Oct-4 DNA recognition sequence, we studied its DNA-binding properties using EMSAs. An oligonucleotide containing the consensus EWS-Oct-4 DNA-binding sequence was synthesized and used as a target in the binding reaction. Cell-free rabbit reticulocyte lysates were used to translated synthetic RNAs produced by *in vitro* transcription. The resultant *in vitro*-translated Flag-tagged EWS-Oct-4 and EWS-Oct-4 (V351P) proteins were

quantified by SDS-PAGE and Western blot using an anti-Flag antibody (Fig. 5*a*). Quantitation of *in vitro* translated proteins was performed using the ChemiDoc™ XRS System (Bio-Rad). We confirmed that similar amounts of *in vitro* translated EWS-Oct-4 and EWS-Oct-4 (V351P) were added to each assay.

The *in vitro* translated proteins were assayed for DNA binding activity by EMSA analysis. EMSAs were performed using a single concentration of probe, and increasing amounts of *in vitro* translated protein (Fig. 5*b*). We found that wild-type EWS-Oct-4 protein successfully bound DNA (Fig. 5*b*, lanes 4–6), whereas the V351 to P351 mutation within the POU DNA-binding domain reduced DNA-binding activity (Fig. 5*b*, lanes 7–9). Control reticulocyte lysates programmed with vector [pcDNA3 (Invitrogen)] alone exhibited very low levels of DNA-binding (Fig. 5*b*, lanes 1-3). Thus, the DNA-binding activity of EWS-Oct-4 requires the presence of V351 within the POU DNA-binding domain.

Since this mutant protein can dimerize with wild-type EWS-Oct-4 (Fig. 4*b*), we investigated whether it might interfere with DNA binding by EWS-Oct-4. We performed EMSAs using *in vitro*-translated EWS-Oct-4 and increasing amounts of EWS-Oct-4 (V351P) protein. Pre-incubation of EWS-Oct-4 with a 2.5-, 5-, 10-, or 20-fold molar excess of EWS-Oct-4 (V351P) resulted in increased interference with EWS-Oct-4 DNA binding activity (Fig. 5*c*). These data show that self-associated proteins containing V351P mutation function in a dominant negative manner and inhibit EWS-Oct-4 DNA-binding activity.

EWS-Oct-4-mediated transactivation is inhibited by EWS-Oct-4 (V351P) protein

It has been reported that EWS-Oct-4 protein is present in the nucleus.[2] To determine whether mutant EWS-Oct-4 (V351P) protein exhibits altered intracellular localization, 293T cells were transfected with pEGFP (N1) (Clontech), pEGFP (N1)-EWS-Oct-4 or pEGFP (N1)-EWS-Oct-4 (V351P), the latter 2 being fusion constructs that express a C-terminal EGFP. Similar to EWS-Oct-4 protein, EGFP-tagged EWS-Oct-4 (V351P) clearly localized to the nucleus (Fig. 6*a*, panels c, f and i). Consistent with a previous report,[2] EGFP-tagged EWS-Oct-4 was found to be a nuclear-specific protein (Fig. 6*a*, panels b, e and h), whereas EGFP alone was found in both the nucleus and cytoplasm of these cells (Fig. 6*a*, panels a, d and g).

The data in Figures 4 and 5 indicate that the EWS-Oct-4 (V351P) mutant is capable of forming a heterodimer with EWS-Oct-4 and that it can interfere with its DNA-binding activity. To determine whether this mutant functioned as a dominant negative by inhibiting EWS-Oct-4-mediated transactivation in cells, we established a cotransfection assay using the Oct-4-responsive reporter construct pOct-4(10x)TATA luc, which contains 10 copies of the Oct-4 binding site and a TATA box cloned upstream of the luciferase gene.[9] The effect of mutant EWS-Oct-4 (V351P) expression on expression from this reporter plasmid was examined by transfecting 293T cells with pcDNA3/EWS-Oct-4 in the presence or absence of pcDNA3/EWS-Oct-4 (V351P). As shown in Figure 6*b*, EWS-Oct-4 activated gene expression from the pOct-4(10x)TATA luc reporter by ~320-fold (lane 2). This activation is mediated via EWS-Oct-4 binding sites in the pOct-4(10x)TATA luc reporter and activation was not observed with the POU DNA-binding domain mutant EWS-Oct-4 (V351P).[2] However, when the EWS-Oct-4 (V351P) protein was co-expressed with EWS-Oct-4, activation of reporter gene expression was reduced to ≤15% (Fig. 6*b*, lanes 3–7).

To investigate further whether ectopic expression of EWS-Oct-4 (V351P) modulates expression of endogeneous EWS-Oct-4 downstream target genes *in vivo*, we transiently transfected ZHBTc4/Flag-EWS-Oct-4 cells (Oct-4-null ZHBTc4 ES cells expressing Flag-tagged EWS-Oct-4 protein) with the pCAG-IP/EGFP or pCAG-IP/EWS-Oct-4 (V351P)-EGFP construct. Consistent with a previous report,[2] expression of *fgf-4* was detected in ZHBTc4/Flag-EWS-Oct-4 cells (Fig. 6*c*, lane 1). However, when the EWS-Oct-4 (V351P)-EGFP protein was co-expressed with EWS-Oct-4, *fgf-4* expression was clearly down-regulated (Fig. 6*c*, lane 3). The expression of the *fgf-4* gene was unaffected in ZHBTc4/Flag-EWS-Oct-4 cells transfected with the EGFP vector alone (Fig. 6*c*, lane 2). These results strongly indicate that EWS-Oct-4 (V351P) down-regulates EWS-Oct-4-mediated transactivation and functions as a dominant-negative regulator for wild-type EWS-Oct-4 protein *in vitro* and *in vivo*.

Discussion

To increase our understanding of *EWS-Oct-4* oncogene function, we have characterized the EWS-Oct-4 fusion protein produced by a chromosomal translocation found in human bone and soft-tissue tumors. In this report, we demonstrated EWS-Oct-4 associates with itself *in vitro* and *in vivo*, and EWS-Oct-4-mediated transcriptional activation can be antagonized by the POU DNA-binding domain mutant EWS-Oct-4 (V351P). Together this information suggests that the biological activity of the EWS-Oct-4 chimeric protein can be regulated by its dominant negative protein.

Functional inactivation of the EWS-Oct-4 oncoprotein by the POU DNA-binding domain mutant EWS-Oct-4 (V351P) could be explained by one of following 4 models. First, it is possible that the mutant protein causes mislocalization of wild-type protein.[42,43] Since transactivation activity of EWS-Oct-4 requires EWS-Oct-4

Figure 3 – Mapping the EWS-Oct-4 self-association domains. (*a*) Schematic representation of the GST-EWS-Oct-4 fusion proteins and their ability to self-associate with each other. Numbers refer to amino acid residues, and binding ability is indicated by + or −. (*b*) Involvement of 2 independent domains of EWS-Oct-4 in the self-association. Recombinant $(His)_6$-tagged EWS-Oct-4 protein was incubated with 2 μg of GST (lane 2), GST-EWS-Oct-4 (1–211) (lane 3), GST-EWS-Oct-4 (1–163) (lane 4), GST-EWS-Oct-4 (1–35) (lane 5), GST-EWS-Oct-4 (70–163) (lane 6), GST-EWS-Oct-4 (POU) (lane 7), or GST-EWS-Oct-4 (CTD) (lane 8) proteins bound to glutathione-Sepharose beads. Aliquots of the input (10%, lane 1) and the pellets (lanes 2–8) obtained from GST pull-down assay, were analyzed by 10% SDS-PAGE, and the bound proteins were detected by Western blot using anti-Flag antibody. The positions of molecular mass markers and $(His)_6$-Flag-EWS-Oct-4 are indicated. Three independent experiments were performed, all of which gave similar results. WB, Western blotting. (*c*) Binding of EWS-Oct-4 (70–163) domain to the EWS-Oct-4 (POU) domain. Recombinant $(His)_6$-EWS-Oct-4 (70–163) protein was incubated with 2 μg of GST (lane 2), GST-EWS-Oct-4 (70–163) (lane 3), or GST-EWS-Oct-4 (POU) (lane 4) proteins bound to glutathione-Sepharose beads. Aliquots of the input (10%, lane 1) and the pellets (lanes 2–4) obtained from the GST pull-down assay were analyzed by 15% SDS-PAGE, and the bound proteins were detected by Western blot using anti-His antibody (H-15, Santa Cruz Biotechnology). The positions of the molecular weight markers and $(His)_6$-EWS-Oct-4 (70–163) are indicated. Three independent experiments were performed, all of which gave similar results. WB, Western blotting. (*d*) Strong binding affinity between EWS-Oct-4 (POU) domains. Recombinant $(His)_6$-EWS-Oct-4 (POU) protein was incubated with 2 μg of GST (lane 2), or GST-EWS-Oct-4 (POU) (lane 3) proteins bound to glutathione-Sepharose beads. Aliquots of the input (10%, lane 1) and the pellets (lanes 2 and 3) obtained from the GST pull-down assay were analyzed by 15% SDS-PAGE, and the bound proteins were detected by Western blot using anti-His antibody (H-15, Santa Cruz Biotechnology). The positions of the molecular weight markers and $(His)_6$-EWS-Oct-4 (POU) are indicated. Three independent experiments were performed, all of which gave similar results. WB, Western blotting. (*e*) Coomassie Blue staining of the GST-EWS-Oct-4 deletions. The relative abundances of GST-fusion proteins utilized were determined by separation on 15% SDS-PAGE and visualization with Coomassie Blue staining. Lane 1, GST alone; lane 2, GST-EWS-Oct-4 (1–211); lane 3, GST-EWS-Oct-4 (1–163); lane 4, GST-EWS-Oct-4 (1–35); lane 5, GST-EWS-Oct-4 (70–163); lane 6, GST-EWS-Oct-4 (POU); lane 7, GST-EWS-Oct-4 (CTD).

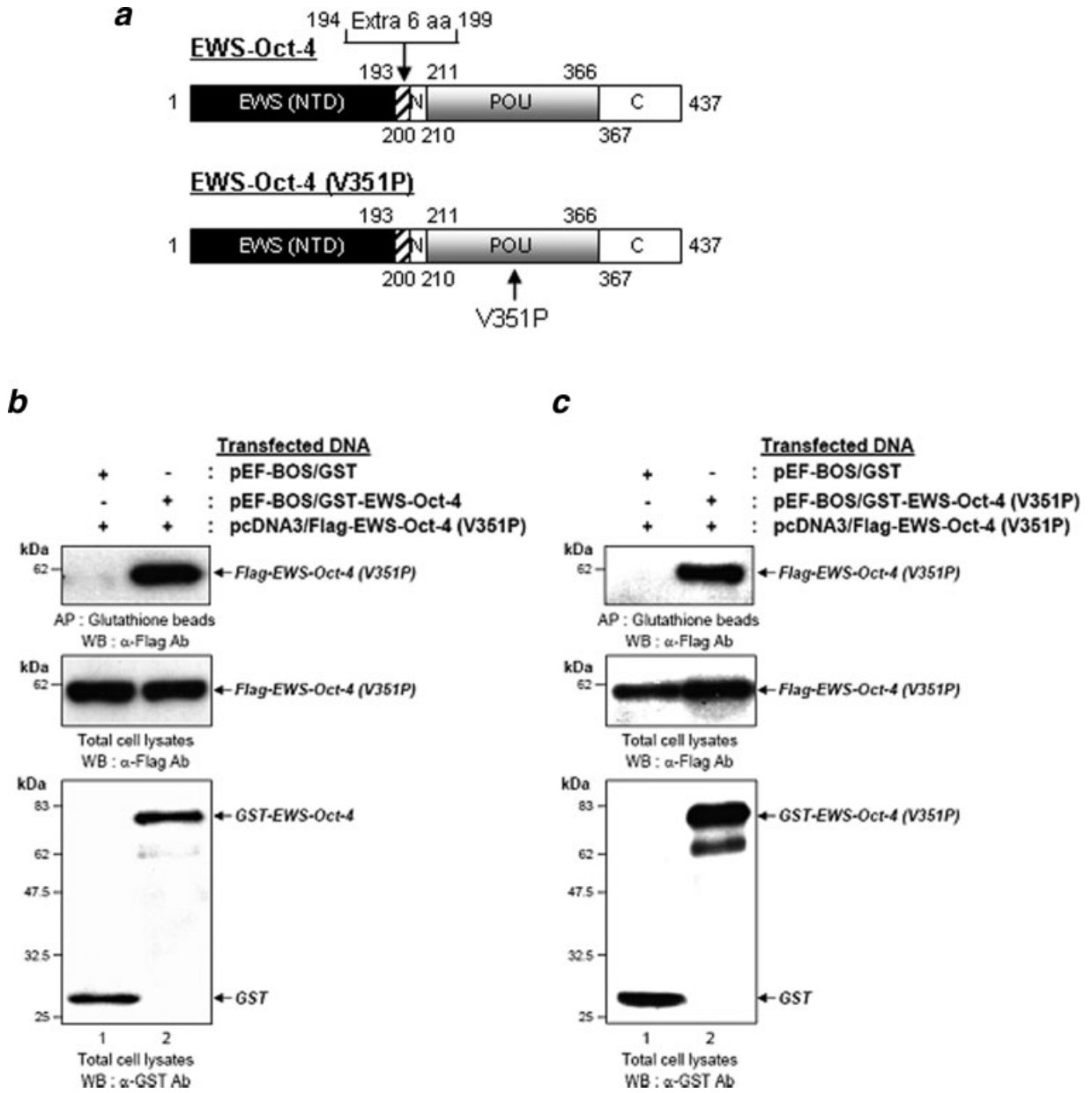

FIGURE 4 – Association between EWS-Oct-4 (V351P) mutant protein and wild-type EWS-Oct-4 *in vivo*. (*a*) Schematic representation of expression vectors used for production of wild-type or mutant EWS-Oct-4 proteins. Numbers refer to amino acid residues, and the arrow and V351P label represent the Val-351 to Pro-351 missense mutation in the POU DNA-binding domain of EWS-Oct-4. (*b*) Physical association between EWS-Oct-4 and EWS-Oct-4 (V351P) *in vivo*. Forty-eight hours after transfection of 293T cells with 0.2 μg of pCMV-Tag2A/EWS-Oct-4 (V351P) and either 2 μg of pEF-BOS/GST or pEF-BOS/GST-EWS-Oct-4, cell extracts were prepared as described in Material and Methods, followed by affinity precipitation with glutathione-Sepharose beads. After separation with SDS-polyacrylamide gel electrophoresis, the proteins were Western blotted with an anti-Flag or an anti-GST antibody. The identities of the transfected DNAs are indicated above the panel. The positions of the molecular weight markers are indicated to the left and the positions of Flag-tagged EWS-Oct-4 (V351P), GST, and GST-fusion EWS-Oct-4 are indicated by the arrows on the right. Three independent experiments gave similar results. AP, affinity precipitation; WB, Western blotting; Ab, antibody. (*c*) Physical association of EWS-Oct-4 (V351P) proteins *in vivo*. Forty-eight hours after transfection of 293T cells with 0.2 μg of pCMV-Tag2A/EWS-Oct-4 (V351P) and either 2 μg of pEF-BOS/GST or pEF-BOS/GST-EWS-Oct-4 (V351P), cell extracts were prepared as described in Material and Methods, followed by affinity precipitation with glutathione-Sepharose beads. After separation by SDS-polyacrylamide gel electrophoresis, the proteins were subjected to Western blot analysis with an anti-Flag or an anti-GST antibody. The identities of the transfected DNAs are indicated above the panel. The positions of the molecular weight markers are indicated to the left and the positions of Flag-tagged EWS-Oct-4 (V351P), GST, and GST-fusion EWS-Oct-4 (V351P) are indicated by the arrows on the right. Three independent experiments gave similar results. AP, affinity precipitation; WB, Western blotting; Ab, antibody.

localization to the nucleus, we examined whether EWS-Oct-4 (V351P) altered the subcellular localization of EWS-Oct-4. However, since EWS-Oct-4 (V351P) also localized to nucleus (Fig. 6*a*), it appears unlikely that the mutant protein would alter nuclear localization of wild-type EWS-Oct-4.

Second, it is possible that the mutant protein competes with wild-type target protein for DNA-binding sites in promoter region, or that it binds at or near the transcription start sites blocking interaction between EWS-Oct-4 and general transcription factors.[44] However, it is unlikely that these proteins compete for binding to

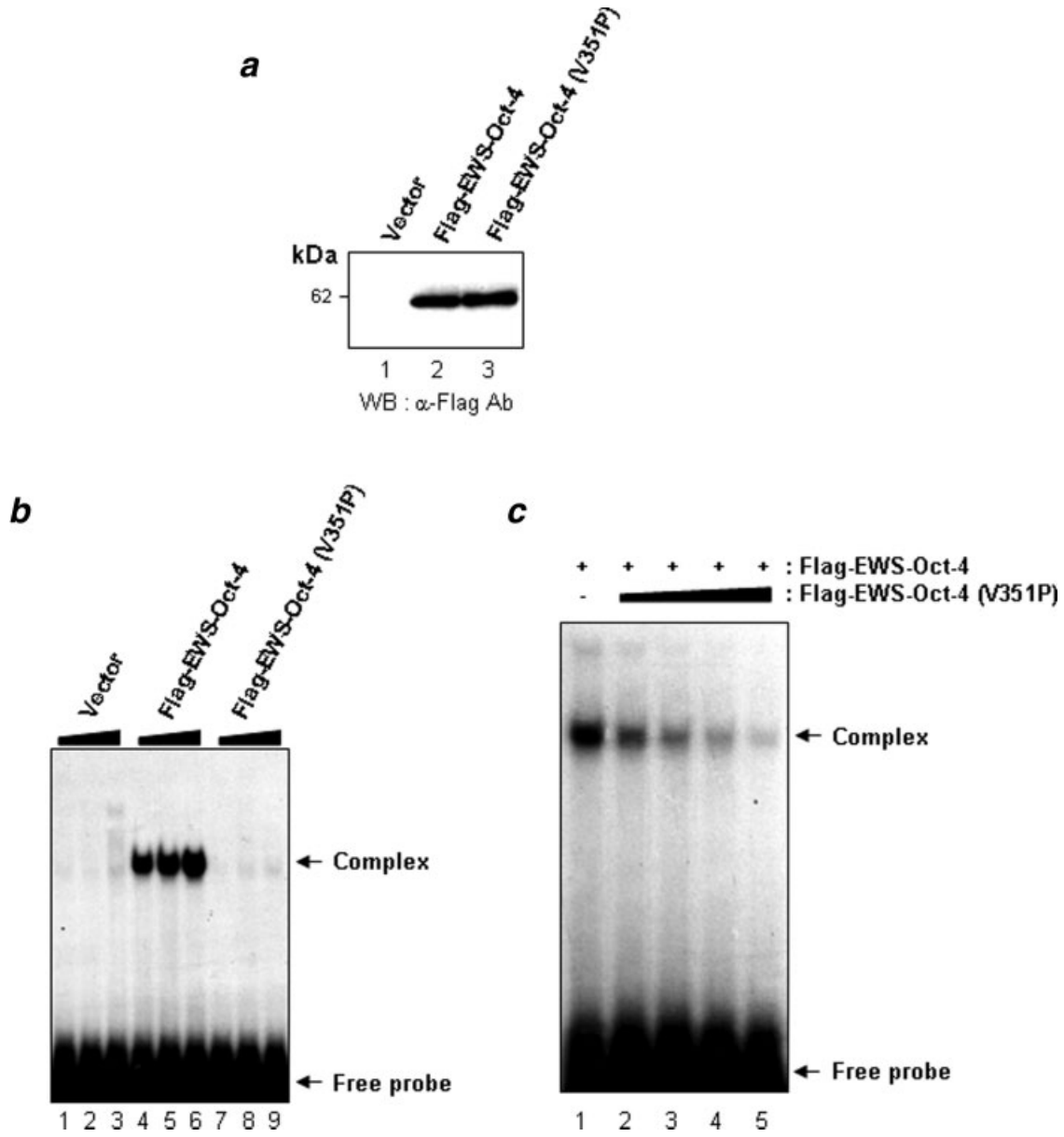

FIGURE 5 – Interference with wild-type EWS-Oct-4 DNA-binding activity by mutant EWS-Oct-4 (V351P) protein. (*a*) Western blot analyses of wild-type and mutant EWS-Oct-4 proteins translated *in vitro*. Rabbit reticulocyte lysates were programmed with vectors encoding Flag-EWS-Oct-4 or Flag-EWS-Oct-4 (V351P) mRNA (indicated above the panel). Four microliters of translation products were analyzed by 10% SDS-PAGE, followed by Western blotting and detection with anti-Flag antibody. Migration of pre-stained molecular weight markers is indicated to the left (kDa). WB, Western blotting. (*b*) Effect of the Val351 to P351 substitution on DNA-binding activity. Following *in vitro* translation, protein concentrations were normalized such that equimolar amounts of EWS-Oct-4 proteins were present in the same volumes used in EMSA. The oligonucleotide used in this experiment contained one EWS-Oct-4 binding site. Radiolabeled probe was incubated with either reticulocyte lysate programmed with vector (lane 1, 0.2 μl; lane 2, 1 μl; lane 3, 5 μl), reticulocyte lysate programmed with Flag-EWS-Oct-4 mRNA (lane 4, 0.2 μl; lane 5, 1 μl; lane 6, 5 μl) or reticulocyte lysate programmed with Flag-EWS-Oct-4 (V351P) mRNA (lane 7, 0.2 μl; lane 8, 1 μl; lane 9, 5 μl). Protein-DNA complexes were resolved on nondenaturing 4% polyacrylamide (acrylamide:bisacrylamide ratio, 37:1) gels run at 4°C in 0.5X TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA). The positions of the free probe and protein-DNA complex are indicated. Three independent experiments gave similar results. (*c*) Inhibition of EWS-Oct-4 DNA-binding activity by the EWS-Oct-4 (V351P) mutant. EMSA analyses of EWS-Oct-4 were performed as in Fig. 5B. Lane 1, EWS-Oct-4 (0.2 μl of reticulocyte lysate programmed with Flag-EWS-Oct-4 mRNA); lane 2, EWS-Oct-4 (0.2 μl) preincubated with a 2.5-fold excess of EWS-Oct-4 (V351P); lane 3, EWS-Oct-4 (0.2 μl) preincubated with a 5-fold excess of EWS-Oct-4 (V351P); lane 4, EWS-Oct-4 (0.2 μl) preincubated with a 10-fold excess of EWS-Oct-4 (V351P); lane 5, EWS-Oct-4 (0.2 μl) preincubated with a 20-fold excess of EWS-Oct-4 (V351P). For all reactions the total amount of reticulocyte lysate was adjusted to 4.2 μl with control lysate programmed with vector. Three independent experiments were performed, all of which produced similar results.

the EWS-Oct-4 recognition site or bind near transcription start sites, since the EWS-Oct-4 (V351P) mutant protein was unable to bind DNA (Fig. 5*b*) and could not activate reporter gene expression.[2]

Third, it is possible that the mutant protein quenches the activity of transcriptional activators or co-factors responsible for EWS-Oct-4 function.[43] However, quenching of EWS-Oct-4 co-activator activity does not appear to be a limiting factor in our experiments,

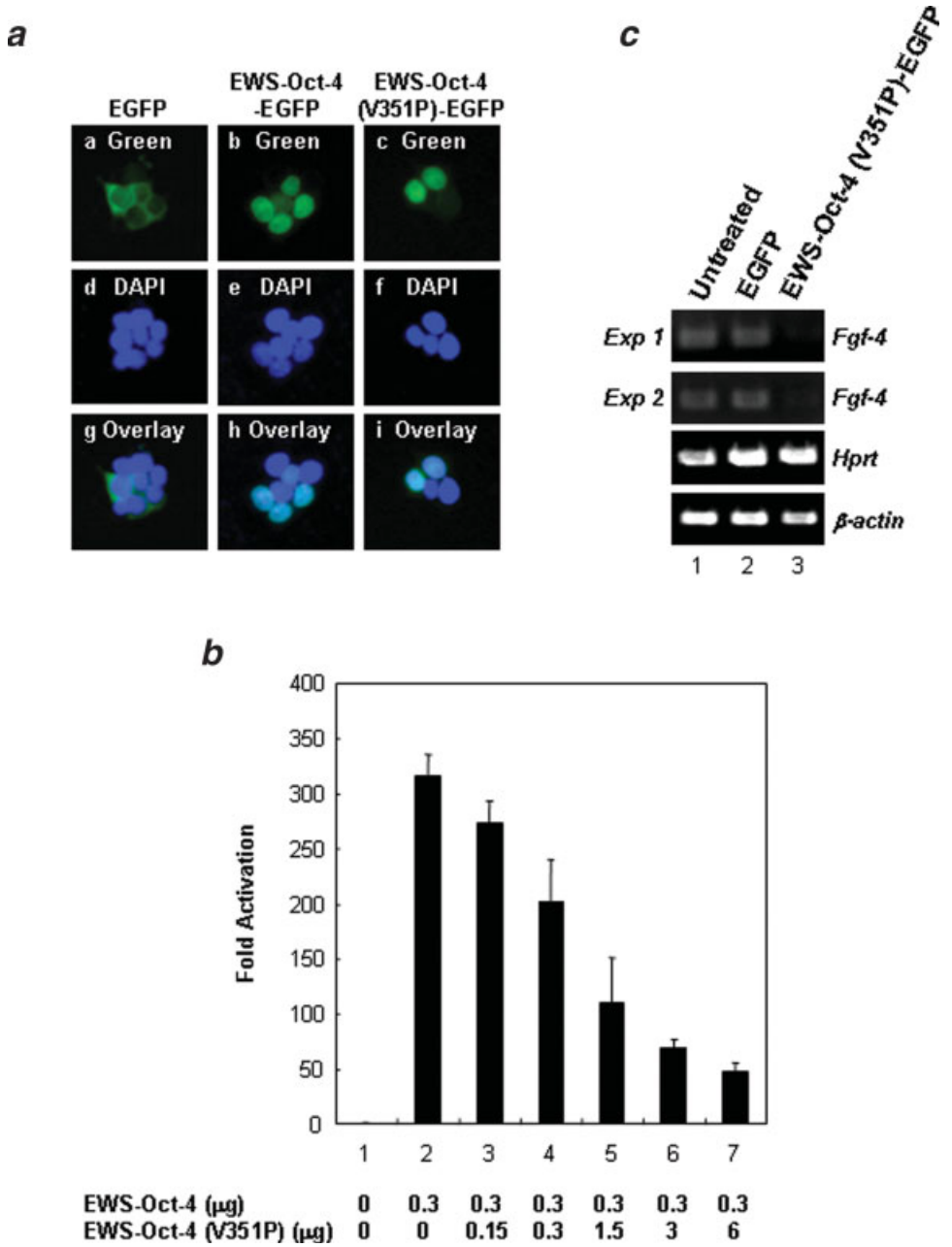

FIGURE 6 – Inhibition of EWS-Oct-4-mediated transcription by EWS-Oct-4 (V351P). (*a*) Subcellular localization of EWS-Oct-4 (V351P) mutant protein. 293T cells grown on coverslips were transfected with mammalian expression vectors encoding EGFP (*a*), EGFP-tagged EWS-Oct-4 (*b*) or EWS-Oct-4 (V351P) (*c*). Subcellular distributions of transfected proteins were analyzed by fluorescence microscopy (top panels a, b and c). Cell nuclei were stained with DAPI (middle panels d, e and f) and merged images are also shown (bottom panels g, h and i). Three independent experiments generated similar results. (*b*) EWS-Oct-4 (V351P) protein interferes with transcriptional activation by EWS-Oct-4. 293T cells were co-transfected with 0.3 μg of EWS-Oct-4 and various amounts (0–6 μg) of EWS-Oct-4 (V351P). For all reactions, the total amount of transfected DNA was maintained by adjustment with empty vector. After 48 hr the cells were harvested and luciferase assays performed. The averages of representative duplicated experiments are presented and error bars are shown. Three independent experiments were performed, all of which gave similar results. (*c*) Down-regulation of EWS-Oct-4 downstream target gene expression by EWS-Oct-4 (V351P) *in vivo*. RT-PCR analysis of *fgf-4* mRNA was performed in ZHBTc4/Flag-EWS-Oct-4 cells transfected with the EGFP vector or EWS-Oct-4 (V351P)-EGFP fusion proteins. *Hprt* and β-*actin* were used for normalization. Following amplification, an aliquot of each product was analyzed by staining the gel with ethidium bromide. The transfected cell lines from which the input RNAs used in the RTs were derived are shown above the panel.

since increasing amounts of EWS-Oct-4 result in dose-dependent transcriptional activation.[2]

Finally, a fourth possible model suggests the formation of nonfunctional oligomers between wild-type and mutant proteins.[45] Mutation in the POU DNA-binding domain resulted in reduced DNA-binding activity (Fig. 5*b*), but did not affect formation of the EWS-Oct-4 complex (Fig. 4). Interestingly, mutant EWS-Oct-4 (V351P) protein interfered with DNA binding by wild-type EWS-Oct-4 *in vitro* (Fig. 5*c*). These results suggest that EWS-Oct-4 (V351P) mutant oligomerizes with wild-type protein to form nonproductive complexes. The net result is antagonism of wild-type EWS-Oct-4 activity and a dominant-negative mode of action.

The connection between embryonic genes and human cancers has been a topic of great interest since the processes underlying proliferation, differentiation, and tumorigenesis have long been thought to be inter-related.[46] Adult neoplasms often re-express genes normally expressed only during embryonic development.[27] In addition, a recent study has found that a gene crucial for embryonic development can quickly become a potent cancer promoter in adult mice following a genetic misalignment, indicating that embryonic genes can become cancer inducers.[47] Although Oct-4 is normally expressed in embryonic stem cells and germ cells, and it is required for maintaining their pluripotency, it can also promote tumorigenesis when expressed inappropriately.[22,23,27,29] In addition, Oct-4 is expressed in human tumors, including testicular germ cell tumor and breast carcinoma, and it plays a part in human cancer development.[22] The tumorigenic potential of the chimeric EWS-Oct-4 gene product is consistent with the idea that this protein plays a crucial role in the formation of bone and soft tissue tumors. Furthermore, the rapid growth of cell lines expressing EWS-Oct-4 in nude mice indicates that it is a potent oncogene.[2] Even though EWS-Oct-4 is not an original embryonic gene, this chimeric gene encodes a nuclear protein that binds DNA with the same sequence specificity as the parental Oct-4 protein.[2] It also has a higher transactivation activity than Oct-4 against a known target reporter gene containing Oct-4 binding sites.[2] Thus, it would be interesting to test whether EWS-Oct-4 stimulates activation of an ESC (embryonic stem cell)-like transcriptional program in human differentiated adult cells, inducing pathogenic self-renewal characteristic of cancer stem cells.

In conclusion, the present study demonstrates that mutant EWS-Oct-4 exerts a dominant negative effect by preventing wild-type EWS-Oct-4 from binding to the DNA of its target genes. Regulation of EWS-Oct-4 function remains less understood. We propose a model whereby EWS-Oct-4 (V351P) mutant protein inhibits wild-type EWS-Oct-4 DNA binding and regulates its activity by direct association. It is currently unclear how wild-type EWS-Oct-4-mediated DNA binding is inhibited by EWS-Oct-4 (V351P), however, EWS-Oct-4 (V351P) might either mask the DNA-binding surface or, alternatively, it may inhibit binding by altering conformation of the DNA binding motif or allosterically affecting the DNA-binding surface. EWS-Oct-4 can self-associate and the responsible regions map to the EWS NTD (amino acids 70–163) and Oct-4 POU DNA-binding domains. Thus, it would be interesting to determine whether oligomerization of EWS-Oct-4 is necessary for binding DNA. However, it is clear that a complex network of intermolecular protein-protein interaction serve to modify the DNA-binding potential of EWS-Oct-4. Targeting EWS-Oct-4 domains essential for its activity may lead to the development of novel therapeutic strategies for this aggressive disease.

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