

Single-chain Antibodies to the EWS NH₂ Terminus Structurally Discriminate between Intact and Chimeric EWS in Ewing's Sarcoma and Interfere with the Transcriptional Activity of EWS *In vivo*

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Abstract

The chimeric protein EWS-FLI1, arising from chromosomal translocation in Ewing's sarcoma family tumors (ESFT), acts as an aberrant tumorigenic transcription factor. The transforming activity of EWS-FLI1 minimally requires an ETS DNA binding domain and the EWS NH₂ terminus. Proteins interacting with the EWS portion differ between germ-line and chimeric EWS despite their sharing identical sequences in this domain. We explored the use of the phage display technology to isolate anti-EWS-FLI1 specific single-chain antibody fragments (scFvs). Using recombinant EWS-FLI1 as bait, 16 independent specific antibody clones were isolated from combinatorial phage display libraries, of which six were characterized in detail. Despite differing in their complementarity-determining region sequences, all six scFvs bound to the same epitope spanning residues 51 to 75 within the shared minimal transforming EWS domain. Whereas all six scFvs bound efficiently to cellular EWS, reactivity with ESFT-expressed EWS-FLI1 was weak and restricted to denatured protein. One scFv, scFv-I85, when expressed as an intrabody, efficiently suppressed EWS-dependent coactivation of hepatocyte nuclear factor 4- and OCT4-mediated transcription *in vivo* but no effect on known EWS-FLI1 target genes was observed. These data suggest that a prominent EWS epitope exposed on recombinant EWS-FLI1 structurally differs between germ-line and chimeric EWS in mammalian cells and that this region is functionally involved in the transcriptional activity of EWS. Thus, we have generated a tool that will prove useful to specifically differentiate between normal and rearranged EWS in functional studies. (Cancer Res 2006; 66(20): 9862-9)

Introduction

TET gene family members *TLS/FUS*, *EWS*, and *TAFII68* are targets of tumor-specific chromosomal translocations involving several distinct transcription factor genes in sarcomas and leukemias (1). The normal function of TET proteins is not known, but association with components of the transcriptional (2) and RNA processing machineries (3, 4) suggests a role in bridging these components of gene expression. EWS associates with the

transcriptional coactivator cAMP-responsive element binding protein (CREB)-binding protein and hypophosphorylated RNA polymerase II, suggesting that EWS may function as a coactivator of CREB-binding protein-dependent transcription factors, including hepatocyte nuclear factor 4 (HNF4; ref. 5) and OCT4 (6). More recently, EWS has been found in association with DROSHA, suggesting also a role in micro-RNA maturation (7). In cancer-associated TET fusion proteins, the TET COOH-terminal RNA-binding domain is replaced by the DNA-binding domain of the fusion partners generating potent chimeric transcription factors, to which the TET NH₂-terminal domain (NTD) contributes a dispersed transcriptional activation domain. In Ewing's sarcoma family tumors (ESFT), *EWS* is rearranged with members of the *ETS* transcription factor gene family, most frequently *FLI1* (8). The minimal transforming domain of EWS-FLI1, the first 82 NH₂-terminal residues (9), interacts with hsRBP7, but only in the context of rearranged or COOH-terminally deleted EWS but not full-length EWS (2, 10), compatible with differential accessibility and function of the NTD in germ-line and rearranged EWS proteins. In fact, EWS, but not EWS-FLI1, selectively enhances HNF4-dependent transcription (5). In addition, EWS and EWS-FLI1 differentially modulate the effects of BRN-3a on neuronal differentiation and apoptosis (11). These results suggest that the loss of the EWS RNA-binding domain, or its replacement by a transcription factor moiety, results in a structural and, consequently, functional change of the protein, and thus the EWS-FLI1 NTD might be considered a tumor-specific epitope potentially representing a promising target for novel therapeutic agents. We therefore attempted to generate EWS-FLI1 binding agents using phage-display human single-chain antibody fragment (scFv) libraries to isolate peptides with binding specificities for germ-line and/or rearranged EWS-FLI1.

Materials and Methods

Cell lines and transfections. The neuroblastoma cell line SJ-NB7 was kindly supplied by Dr. T. Look (St. Jude Children's Research Hospital, Memphis, TN). The EWS-negative ESFT cell line STA-ET-7.2 has previously been described (12). The hepatoma cell line Hep3B, the human embryonal kidney cell line HEK293, and the ESFT cell line SK-N-MC were obtained from the American Type Culture Collection (Rockville, MD). For analysis of interaction of scFv antibody fragments with EWS or EWS-FLI1 *in vivo*, SJ-NB7 and SK-N-MC cells were transiently cotransfected with plasmid pCMV-SK-EWS or pCMV-SK-EWS-FLI1 encoding full-length EWS or EWS-FLI1 (10) and pIRES2-EGFP vector (Clontech, Palo Alto, CA) encoding scFvs or just the empty vector as a control. As a transfection reagent in all experiments, LipofectAMINE Plus (Invitrogen, Groningen, the Netherlands) was used according to the recommendations of the manufacturer.

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Bacterial expression and purification of human EWS-FLI1 recombinant protein. The glutathione *S*-transferase (GST)-EWS-FLI1 construct used in this study has previously been described (13). According to standard procedures, bacteria carrying the EWS-FLI1 expression vector were grown and induced by isopropyl- β -D-thiogalactopyranoside (Fisher Scientific, Fair Lawn, NJ). Resuspended bacteria were sonicated in radio-immunoprecipitation assay buffer [40 mmol/L HEPES (pH 7.4), 1% NP40, 0.1% SDS, 0.5% Na-deoxycholate (w/v), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, and 2 μ g/mL leupeptin]. The GST-EWS-FLI1 fusion protein in the soluble fraction was then collected by glutathione-Sepharose 4B according to the instructions of the manufacturer (Pharmacia Biotech, Inc., Uppsala, Sweden). Cleavage of the GST from the fusion protein was effected by factor Xa (Roche Diagnostics, Mannheim, Germany) digestion. Reduced glutathione was also added to an aliquot of the soluble fraction to release GST-EWS-FLI1 protein from the Sepharose 4B beads. The purified EWS-FLI1 or GST-EWS-FLI1 proteins were then dialyzed against 1,000 mL of PBS (pH 7.4) at 4°C with three buffer changes.

As an alternative to GST-EWS-FLI1, a transcriptionally active recombinant EWS-FLI1 was used. This protein has been produced in *Escherichia coli*, purified by affinity column chromatography, followed by immobilization-assisted refolding as described in detail (14).

ScFv screening and characterization of isolated clones. *E. coli* strain TG-1 [K12, Δ (*lac-pro*), *supE*, *thi*, *hsd* Δ 5/*traD*36, *proA*⁺*B*⁺, *lacF*⁺, *lacZ* Δ M15] was used for the phage rescue. The nonsuppressor *E. coli* strain HB2151 [K12, *ara*, Δ (*lac-pro*), *thi*/*F*⁺*proA*⁺*B*⁺, *lacF*⁺ Δ M15] was used for the preparation of scFvs. Tomlinson's I and J human scFv libraries were kindly provided by Susan Ellis and Ian Tomlinson (Medical Research Council Centre, Cambridge, United Kingdom). Both libraries are based on a single human framework for V_H (V3-23/DP-47 and JH4b) and V_L (O12/O2/DPK9 and κ 1) with side chain diversity (DVT for TI and NNK for TJ encoded) incorporated at positions in the antigen binding site that makes contact to antigen in known cocrystal structures and highly diverse in the mature repertoire (18 different amino acid positions in total). GST-EWS-FLI1 fusion protein was used as target for bio-panning using a published protocol (15). Libraries were preincubated with GST protein and supernatants subsequently applied to GST-EWS-FLI1-coated tubes to enrich for binders to recombinant EWS-FLI1. After a single round of selection, periplasmic extracts from individual clones were analyzed by indirect ELISA and specificity for EWS-FLI1 was confirmed by competition ELISA (16). Binding of soluble scFv fragments was detected using horseradish peroxidase-conjugated rProtein L (Actigen, Oslo, Norway). Reactivity of phage and derived scFvs with recombinant and native EWS-FLI1, GST, and native EWS was determined by ELISA and immunoblotting. Western blotting and immunoprecipitations were done as previously described (13) using scFv or polyclonal rabbit anti-EWS antibodies 139-2F or SE 677, or mouse hybridoma supernatant 7.3 [provided by C.T. Denny (University of California at Los Angeles, Los Angeles, CA) and O. Delattre (Institut Curie, Paris, France), respectively]. To improve on the binding affinity, affinity maturation of scFv clone I85#5 was done according to Pini et al. (17).

Plasmid constructs and epitope mapping. For mapping of the epitope recognized by the scFv antibodies, recombinant EWS-NTD fragments were expressed in *E. coli* using the pGEX-5X-1 expression vector (Amersham Biosciences, Uppsala, Sweden) and detected with isolated scFvs by immunoblot analyses. Briefly, DNA coding for EWS-NTD fragments C Δ 1 (amino acids 1-82), C Δ 2 (amino acids 1-75), C Δ 3 (amino acids 1-67), C Δ 4 (amino acids 1-61), and C Δ 5 (amino acids 1-54) were derived from the full-length human EWS-NTD cDNA (13) by PCR amplifications and cloned into the pGEX-5X-1 vector.

For intrabody generation, the sequence of the scFv clone that gave the highest ELISA value, scFv-I85, was amplified by PCR using primers containing appropriate restriction sites as well as sequences for the nuclear localization signal and the c-myc tag (5'-CCGGAATTCGCTG-GATTGTTACTC-3' and 5'-CGCGGATCCTTATTAATTCAGATCCTCT-TCCGAGATGAGTTTTTGTCTGGCACCT TTCTCTCTCTTTGG-TGGTGGCGCCCGCGTTGATTTCACCTTGGT-3'). The PCR product was cloned into the mammalian expression vector pIRES2-EGFP (Clontech) following the instructions of the manufacturer.

ELISAs. Nunc 96-well immunoplates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with the appropriate antigen (100 μ L/well) at a concentration of 2 μ g/mL. The remaining protein-binding capacity of each well was blocked at room temperature for 3 hours with 200 μ L of blocking buffer [2% bovine serum albumin (BSA) in PBS]. Purified periplasmic scFv fragment or *E. coli* supernatant containing antibody fragments was diluted with an equal volume of blocking buffer and added to each well (100 μ L/well). Antibody fragments were allowed to bind to the antigen for 1 to 2 hours at room temperature. To determine the amount of soluble scFv antibody that had bound, the microtiter plate was incubated with 100 μ L of 9E10 anti-c-myc monoclonal antibody per well (Sigma, St. Louis, MO), 1 μ g/mL in PBS/1% BSA, followed by alkaline phosphatase-conjugated anti mouse immunoglobulin G (1:5,000 dilution) at room temperature for 1 hour. The plates were washed and then *p*-nitrophenyl phosphate (Sigma) was used as a substrate for the alkaline phosphatase. Absorbance was read at 405 nm. In some assays, bound scFv was detected using horseradish peroxidase-conjugated rProtein L (Actigen) at 1:5,000 dilution and the extent of binding was visualized by addition of 3,3',5,5'-tetramethylbenzidine (Sigma) substrate to the wells. The reaction was stopped by adding 1 mol/L H₂SO₄ after 10 minutes and the absorbance read at 450 nm. All measurements were done in triplicates.

For competition ELISA, bacterial supernatant, competitor, and blocking buffer were mixed in a 1:1:2 ratio and antibody was allowed to bind to the competitor for 3 hours at room temperature before the solution was added to the antigen-coated ELISA plate (100 μ L/well). The remaining steps are as described for indirect ELISA. For capture ELISA, microplate wells were coated with 100 μ L of 2 μ g/mL anti-EWS antibodies and then blocked with 2% BSA/PBS. Nondenatured fusion protein antigens were then added and allowed to bind for 1 hour at room temperature. Purified scFvs (2 μ g/mL) were then applied at 100 μ L/well and remaining steps were as described for indirect and competition ELISAs. The antigens used in assays were purified GST-EWS-FLI1, factor Xa-cleaved GST-EWS-FLI1, GST-SP1, GST-E2F1, refolded bacterial EWS-FLI1 (14), and Flag-tagged EWS-FLI1 generated in baculovirus-infected insect SF9 cells.

Luciferase reporter gene assays. The EWS-negative ESFT cell line STA-ET-7.2, SJ-NB7, Hep3B, and HEK293 cells were used for reporter gene assays. For HNF4 reporter gene assay, HNF4 expression (pCDNA3-HA-HNF4 α) and responsive reporter (pHNF4x8-tk-Luc) constructs were obtained from Dr. Akiyoshi Fukamizu (University of Tsukuba, Tsukuba, Japan). OCT4 expression (pCDNA3/Oct-4) and reporter (pOct-4-10xTATA-Luc) plasmids were kindly provided by Dr. Jungho Kim (Sogang University, Seoul, Korea). Transfections into cell lines were done by lipofection basically according to the protocol of Yoshida (17, 18). Briefly, for reporter gene assay, cells were grown in 24-well plates and cotransfected with various amounts of effector plasmid(s) (pCDNA3-HA-HNF4 α , pCMV-EWS, pIRES-scFv-I85, and pCDNA3/Oct-4). A plasmid encoding β -galactosidase was included in each transfection to control for transfection efficiency. Measurements for luciferase activity were done in a luminometer using the Bright-Glo Luciferase Assay System (Promega, Madison, WI) 48 hours posttransfection. The values were normalized to β -galactosidase activity as an internal control. All experiments were done in triplicate at least thrice for each construct and results represent average relative luciferase activities.

Gene expression studies by reverse transcription-PCR and real-time quantitative PCR. For testing the effects of scFv-I85 on HNF4-dependent gene expression, Hep3B cells were transfected with either pIRES2-EGFP-scFv-I85 or empty pIRES2-EGFP vector, and enhanced green fluorescent protein (EGFP)-positive cells were sorted 48 hours later with a FACS-Aria (BD Biosciences, San Jose, CA). Total RNA was isolated from sorted cell populations with the RNeasy Mini kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was done with 2 μ g of total RNA using random hexamer oligodeoxyribonucleotides (Pharmacia, Uppsala, Sweden) with standard procedures. Primers used for amplification (30 cycles) of published HNF4-dependent genes and PCR product sizes are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) amplification was used as the internal PCR control.

For real-time quantitative PCR, STA-ET-7.2 and SK-N-MC cells were transfected with pIRES2-EGFP-scFv-I85 construct, and sorting, RNA

Table 1. Oligonucleotides and PCR product sizes for determination of HNF4- and EWS-FLI1-dependent gene expression

Gene	Sense primer	Antisense primer	Probe*	Amplicon size (bp)
<i>SERPINA1</i>	TGGGGTGACTCAGTAAATGGTAGA	CAGACAGTCTCTGGGAGAGTACCA		107
<i>SERPINA3</i>	GCAGGAATAAATAAGCCTCAGCAT	TGCCTAAAACCTCTCTGGAAAAAC		156
<i>APOC3</i>	CACCGTTAAGGACAAGTTCTCTGA	AGCAGCTTCTTGCCAGCTTATT		259
<i>TF</i>	CTTGAGAGGGCTGAGAACATAGT	ATATCACATTTGCGAGTTCCATC		436
<i>CP</i>	GTACCACAAGAATTGGAGGCTCTT	TTAGTGGGATCCACAGCAGAATAA		405
<i>GAPDH</i>	CATGTGGCCATGAGGTCACCAC	TGAAGGTCGGAGTCAACGGATTGGT		900
<i>TGFBR2</i>	TCGTCTGTGGACGCGTAT	TGTCAGTGACTATCATGTCGTTATTAACC	AGCAGCATCCCACCGCACGTTT	80
<i>ID2</i>	GACTGCGATCCCACTATTGTCA	GTCCTGGAGCGCTGGTTCT	CCTGCATCACCAGAGACCCGGG	74
<i>MK-STYX</i>	CACTGCCCTTCGAGTGAAGAA	GTGTTATCATACACCACGCACTACTT	TGAATATCTTCTCCCGAGTCTGTGGAC	96
<i>c-MYC</i>	CCCCTGGTGTCCATGAG	GCCTGCCTCTTTCCACAGA	CACCAGCAGCGACTCTGAGAGGAAC	107
<i>B2M</i>	TGAGTATGCCTGCCGTGTGA	TGATGCTGCTTACATGTCTCCGAT	CCATGTGACTTTGTCACAGCCCAAGA TAGTT	85

*FAM-TAMRA-labeled TaqMan PCR probes.

isolation, and cDNA synthesis were done as described above. Primers and probes used for quantification of EWS-FLI1-dependent genes transforming growth factor- β receptor type II (*TGFBR2*), *ID2*, *MK-STYX*, *c-MYC*, and of the internal β 2-microglobulin (*B2M*) control used to normalize for variances in input cDNA are presented in Table 1. Cycling variables on the ABI 7700 or 7900 sequence detection system are as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

Results

ScFv-I85 binds preferentially to GST-EWS-FLI1 and not to other GST fusion proteins. The two phage libraries used to screen for EWS-FLI1 binding scFvs contained at least 10^8 different clones each. Recombinant GST-EWS-FLI1 was used as a bait to enrich for EWS-FLI1 binders after preadsorption of the libraries to GST protein, to exclude GST-specific phage. Soluble scFvs were expressed from 2,688 random clones and screened by ELISA for their specific binding to the EWS-FLI1 portion of GST-EWS-FLI1. Binding activities were detected using both phage supernatants from infected bacterial cultures (data not shown) and soluble scFv (Fig. 1A). One hundred twenty-two clones were identified that specifically bound to GST-EWS-FLI1 but not to GST. The nucleotide sequences of 16 confirmed positive clones with the highest ELISA values were determined. Deduced amino acid sequences of the V_H and V_L genes of all but two recombinant EWS-FLI1-specific scFvs differed slightly in their complementarity-determining regions, indicating that a diversity of binders were present as exemplified for four scFvs in Fig. 1B. Clone scFv-I85 had the highest specific binding to recombinant EWS-FLI1 as well as the lowest nonspecific binding affinity to GST. Therefore, scFv-I85 was used in subsequent experiments. In competition ELISA (Fig. 1C), this clone exhibited specific dose-dependent binding to recombinant GST-EWS-FLI1 before and after GST cleavage with factor Xa. Binding was strongly reduced by prebinding of scFv-I85 to GST-EWS-FLI1, even more to factor Xa-digested EWS-FLI1. Consistent with binding of scFv-I85 to the EWS NH₂ terminus, also GST-EWS competed efficiently with GST-EWS-FLI1 for scFv-I85 binding, whereas no competition was obtained by prebinding to GST. Affinity maturation of scFv-I85 was done by *in vitro* mutagenesis of V_L complementarity-determining region 3 to

obtain clone scFv-I85#7 with a markedly increased reactivity towards GST-EWS-FLI1 (Fig. 1D). Both scFv-I85 and its affinity-matured derivative showed specific binding to GST-EWS-FLI1 and GST fusions of EWS mutants, but not for other GST fusion proteins or GST only (Fig. 1D). These results confirm the specificity of scFv-I85 for EWS-FLI1 and EWS expressed in bacteria.

ScFv-I85 preferentially recognizes endogenous EWS over eukaryotically expressed EWS-FLI1. Immunoblot experiments were done to test for reactivity of scFvs with proteins in whole-cell lysates derived from human tumor cell lines MOLT4, expressing EWS and germ-line FLI1; STA-ET-1 and STA-ET-2.2, positive for EWS and EWS-FLI1 exon 7/6 and exon 9/4 fusions; STA-ET-7.2, expressing only EWS-FLI1 (exon 7/5 fusion); and with EWS-FLI1 expressed in SF9 insect cells. Although scFv-I85 and scFv-I85#7 (not shown) efficiently bound to denatured recombinant EWS-FLI1 from bacteria, no reactivity was obtained with EWS-FLI1 from ESFT cell lines or baculovirus-expressed EWS-FLI1 (Fig. 2A). There was also no reactivity with germ-line FLI1 in MOLT4 cells. Only if immunopurified EWS-FLI1 from a minimum of 1×10^7 cells was applied to the Western blot, a faint signal could be obtained with scFv-I85 (not shown). In contrast, a band of 83 kDa was consistently detected in total cell extracts from 2×10^5 cells from all human cell lines, except STA-ET-7.2 lacking germ-line EWS, and in mouse bone marrow cells. Thus, the 83-kDa band derives from germ-line EWS, consistent with reactivity of scFv-I85 with an evolutionary conserved domain shared between EWS and EWS-FLI1, as already indicated by the results of the competition ELISA (Fig. 1C). Affinity-matured scFv-I85#7 showed stronger reactivity with human EWS than the parental clone without increasing reactivity with endogenous EWS-FLI1 (not shown). Of note, similar results were obtained with the other 15 sequenced scFvs (not shown). To test the capability of scFv-I85 to recognize *ex vivo* native EWS and EWS-FLI1 proteins, it was used in immunoprecipitation experiments from whole-cell lysates of SJ-NB7 and STA-ET-7.2 cells, respectively (Fig. 2B). ScFv-I85 efficiently pulled down germ-line EWS from SJ-NB7 cells, but not EWS-FLI1 from STA-ET-7.2 cells.

Further, we cloned scFv-I85 with a c-myc tag and a nuclear localization sequence into the mammalian expression vector pIRES2-EGFP and transfected it into SJ-NB7 and STA-ET-7.2 cells

(Fig. 2C). Immunoprecipitation of scFv-I85 from transfected cells with an anti-c-myc tag antibody resulted in coprecipitation of EWS from SJ-NB7 cells but not EWS-FLI1 from STA-ET-7.2 cells. This result indicated that scFv-I85, when expressed as intrabody, was capable of binding to EWS *in situ*, but not to EWS-FLI1.

The scFvs share an epitope localized in the EWS-NTD. To delineate the epitope differentially recognized on EWS and EWS-FLI1 proteins by the scFvs, immunoblot analyses of EWS-NTD fragments fused to GST were done (Fig. 3A). COOH-terminal deletions down to amino acid 68 (constructs CΔ1-CΔ3) were tolerated whereas deletion of further seven or more amino acids in CΔ4 and CΔ5 resulted in loss of reactivity with scFv-I85 (Fig. 3B). Further delineation by combined NH₂- and COOH-terminal truncations identified residues 51 to 75 (construct Epi-25,

Fig. 3C) as minimally required for peptide recognition by scFv-I85 with critical residues located between amino acids 58 and 68 (Fig. 3B). Importantly, all six randomly selected scFvs from the 16 sequenced clones showed the same reactivity pattern as scFv-I85, although with slightly different affinities (data not shown).

Reactivity of scFvs with EWS-FLI1 depends on protein folding. The discrepancy in reactivity of scFvs with GST-EWS-FLI1 and endogenous or insect cell-expressed EWS-FLI1 may be either explained by the presence of a stable protein fold maintained under denaturing protein electrophoresis conditions or by eukaryotic protein modification of the scFv-I85 epitope in EWS-FLI1 but not in EWS. To appreciate the contribution of protein folding to antibody accessibility of EWS-FLI1, we compared scFv-I85 reactivity of equal amounts of recombinant GST-EWS-FLI1 fusion protein purified from

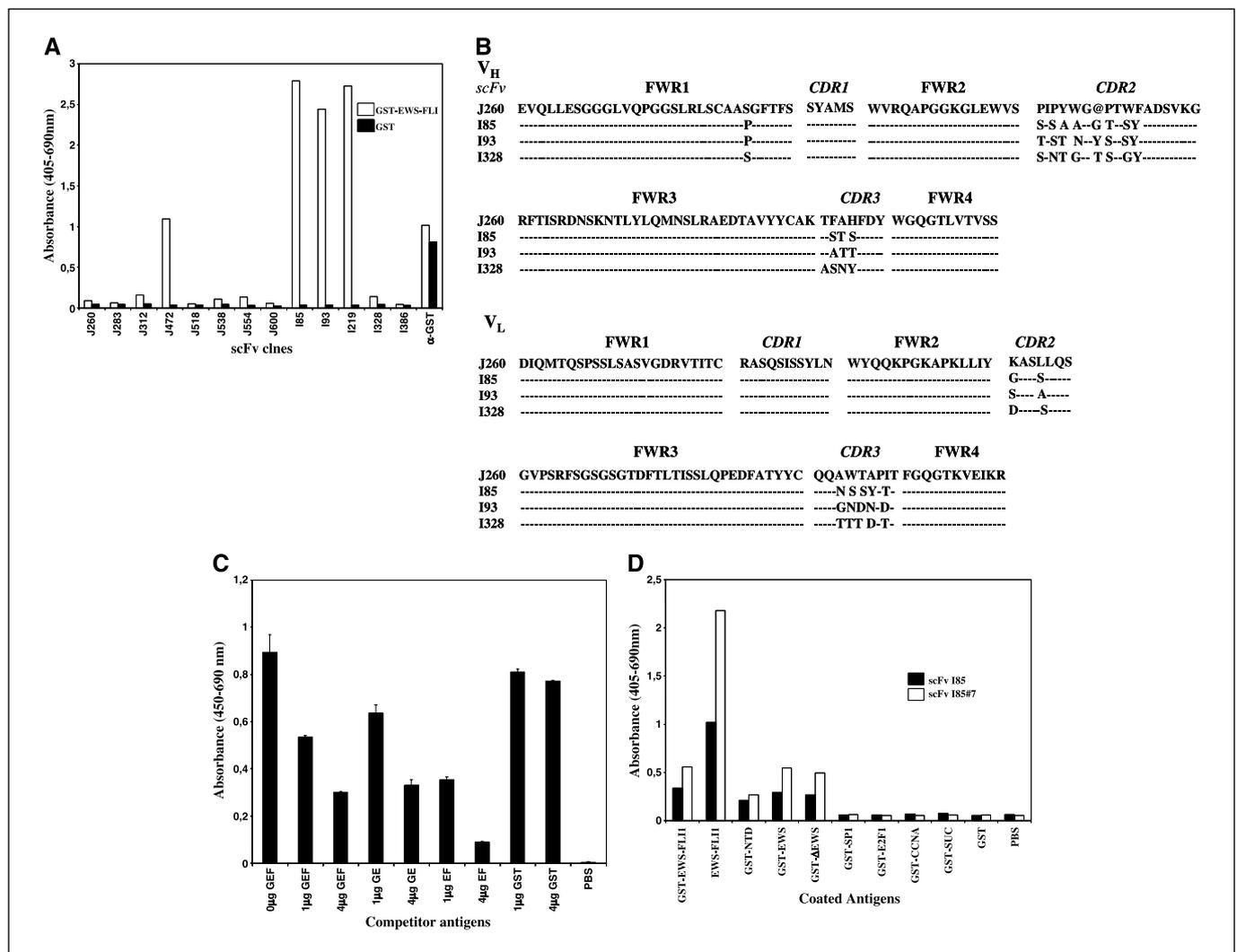


Figure 1. Identification of scFv clones specifically binding to GST-EWS-FLI1. *A*, representative ELISA showing binding specificity of bacterially expressed soluble scFvs to recombinant GST-EWS-FLI1 (white columns) as compared with GST only (black columns). *B*, deduced amino acid sequences of the V_H and V_L domains of representative EWS-FLI1-specific scFv fragments. Dashes, identical residues; heavy chain variable regions (V_H) and light chain variable regions (V_L) are indicated. Framework (FWR) and complementarity-determining regions (CDR) are indicated. *C*, competition ELISA confirming specificity by a dose-dependent binding of scFv-I85 for the recombinant GST-EWS-FLI1 and recombinant EWS-FLI1 on GST cleavage by factor Xa, and also for GST-EWS. ScFv-I85 was preincubated with increasing amounts of GST-EWS-FLI1 (GEF), GST-EWS (GE), GST only (GST), and factor Xa-digested GST-EWS-FLI1 (EF) before adding it to the GST-EWS-FLI1-coated ELISA plate. The decrease in signal with increasing free antigen indicated that the scFv had bound to antigen in solution. The constant signal with increasing free GST showed that scFv was not binding to free GST protein in solution. *D*, a representative ELISA showing specific binding affinity of scFv-I85 and its affinity-matured product, scFv-I85#7, to GST-EWS-FLI1. This effect was seen with other EWS mutants fused to GST (NTD, EWS NH₂-terminal domain, amino acids 1-264; ΔEWS, amino acids 1-82 and 246-264) but not observed with other GST fusion proteins as shown here for GST-SP1, GST-E2F1, GST-CCNA, GST-SUC, and GST only, as well as PBS empty control.

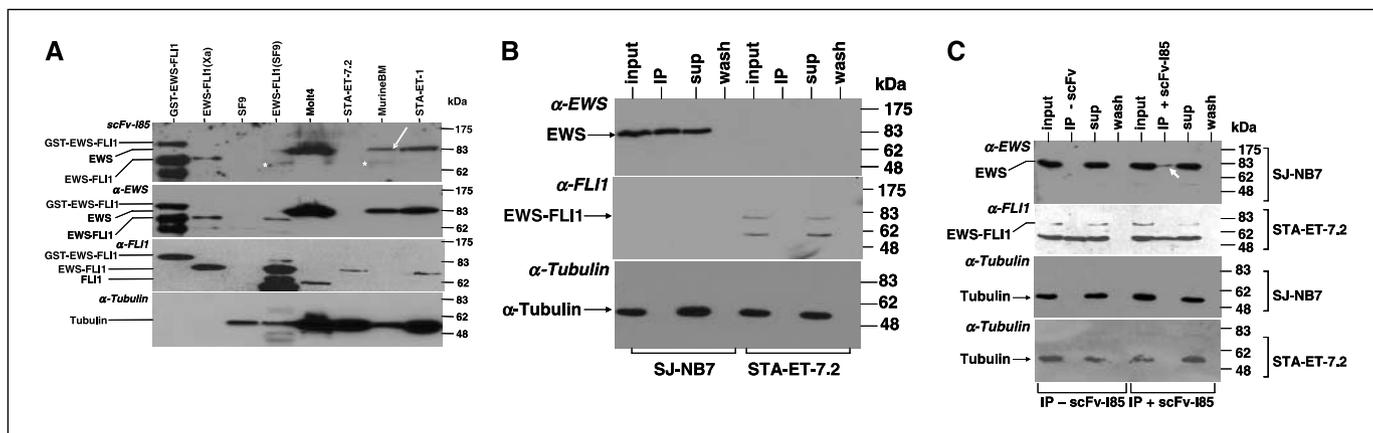


Figure 2. ScFv-I85 binds to denatured EWS but not to EWS-FLI1. *A*, immunoblot showing reactivity of scFv-I85 with GST-EWS-FLI1 with and without factor Xa cleavage, and with a band of 83 kDa in MOLT4 and the ESFT cell line STA-ET-1, as well as in murine bone marrow cells (*arrow*), which, due to its absence in EWS-negative STA-ET-7.2 extracts, was assigned to germ-line EWS. Endogenous EWS-FLI1 was not detectable in any of the three ESFT cell lines nor was baculovirus-expressed EWS-FLI1 in SF9 insect cells. *, unspecific bands. *B*, precipitation of endogenous EWS from EWS-positive SJ-NB7 neuroblastoma cells but not of EWS-FLI1 from STA-ET-7.2 cells using scFv-I85. ScFv-I85 was added to whole-cell extracts (*input*) and subsequently incubated with c-myc antibody-coupled Dynabeads for precipitation (*IP*). Supernatants (*sup*) and wash solution (*wash*) were tested for unbound EWS and EWS-FLI1 proteins. *C*, immunoprecipitation of EWS but not EWS-FLI1 with scFv-I85 intrabody. SJ-NB7 and STA-ET-7.2 cells were transfected with the scFv-I85 construct (+scFv-I85) or empty vector control (-scFv) and scFv-containing complexes were precipitated from whole-cell extracts with anti-c-myc antibody. Germ-line EWS protein (*arrow*) was specifically detected in immunoprecipitates from scFv-I85-transfected SJ-NB7 cells. On the Western blots, germ-line EWS and EWS-FLI1 were detected by antibodies to EWS (α EWS 139-2F) and FLI1 (monoclonal antibody 7.3), respectively.

E. coli under standard conditions after cleavage of the GST domain by factor Xa treatment, affinity-purified recombinant EWS-FLI1 that has been subjected to immobilization-assisted refolding and shown to be transcriptionally active *in vitro* (14), baculovirus-expressed EWS-FLI1, and EWS-FLI1 that has been immunopurified with a FLI1-specific antiserum from the ESFT cell line STA-ET-7.2. In ELISA experiments, in the absence of any detergent, only factor Xa-digested GST-EWS-FLI1, prepared under standard conditions, was recognized by the scFv whereas the refolded recombinant EWS-FLI1 behaved like the eukaryotically expressed EWS-FLI1 (Fig. 4A). However, in immunoprecipitation experiments, in the presence of 0.01% NP40 (not shown) and on immunoblots of Laemmli gels (20% SDS), scFv-I85 reacted also with the refolded protein but not with EWS-FLI1 from ESFT or insect cells (Fig. 4B). These results suggest that refolded recombinant EWS-FLI1 adopts an unstable secondary structure that does not allow access of scFvs in the absence of any

detergents, whereas in EWS-FLI1 expressed in eukaryotic cells, this conformation is stable even under stringent denaturing conditions on the Western blot. Stabilization may be the consequence of posttranslational modification. In the absence of any knowledge about the nature of the thus far unrecognized posttranslational modifications of EWS-FLI1, we did phosphatase and glycosidase treatment of immunoprecipitated EWS-FLI1 from ESFT cells and tested for recognition by the scFv on Western blots. None of these treatments restored reactivity with the scFv excluding these types of modification as being responsible for the stabilization of the scFv-resistant fold of EWS-FLI1 in ESFT cells (not shown).

Expression of scFv-I85 intrabody suppresses EWS-mediated HNF4 and OCT4 transactivation. To investigate the functional consequences of scFv-I85 binding to EWS *in vivo*, we studied EWS-driven coactivation of HNF4-dependent thymidine kinase promoter activity by luciferase assay in the presence and absence of scFv-I85

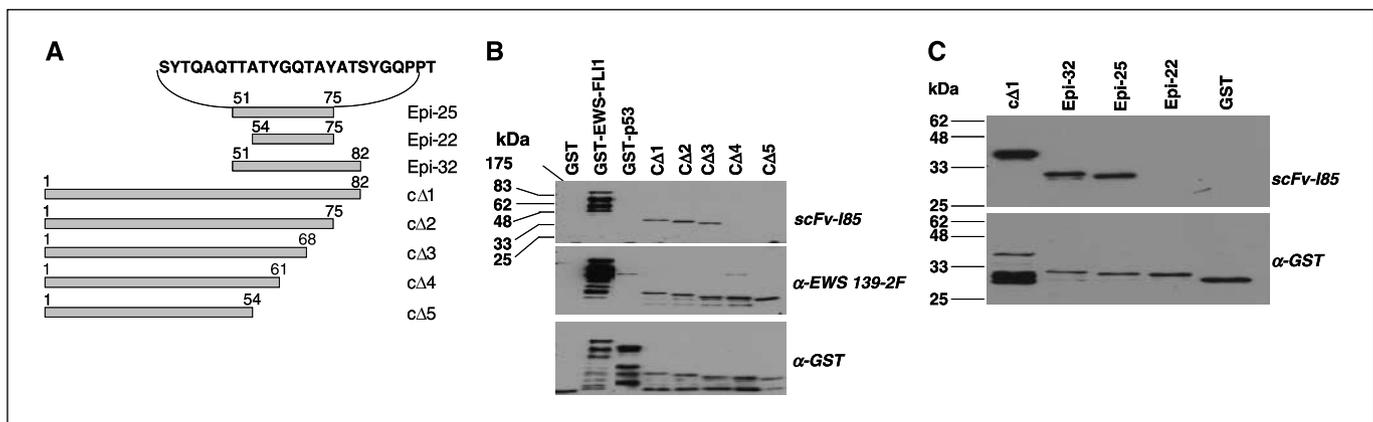


Figure 3. Epitope mapping of scFv-I85 and other scFvs. *A*, schematic representation of deletion constructs generated from EWS-NTD and the amino acid sequence of the minimal recognition epitope. *B*, epitope mapping of scFv-I85 using *E. coli* lysates expressing the EWS deletion constructs (fused to GST) shown in (*A*). *C*, reactivity of scFv-I85 to Epi-25 (amino acids 51-75), Epi-22 (amino acids 54-75), and Epi-32 (amino acids 51-82) constructs (all fused to GST) to determine the minimal recognition epitope. Shown here are Western blots probed with scFv-I85 and α EWS 139-2F. Anti-GST monoclonal antibody was used to control for expression of the fusion constructs. Molecular weight standards are indicated on the left in kilodaltons.

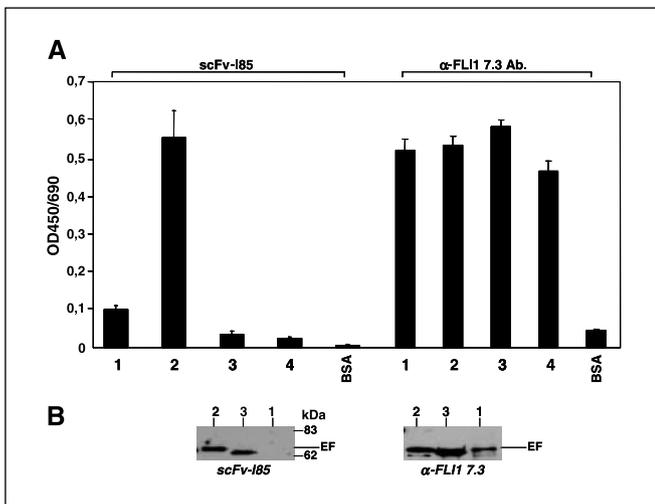


Figure 4. ScFv-I85 reacts with refolded recombinant EWS-FLI1 under denaturing conditions. In ELISA (A), in the absence of any detergent, only factor Xa-cleaved GST-EWS-FLI1 (2) was recognized by scFv-I85 whereas the refolded recombinant EWS-FLI1 (3), insect recombinant (baculovirus-expressed) EWS-FLI1 (1), and immunoprecipitated eukaryotically expressed EWS-FLI1 (4) did not show any reactivity with the scFv-I85 antibody. B, immunoblot showing reactivity of scFv-I85 with immunoprecipitated bacterial EWS-FLI1 proteins (2 and 3) but not insect recombinant EWS-FLI1 (1) under denaturing conditions.

intrabody expression. In both HEK293 (Fig. 5A) and STA-ET-7.2 cells (Fig. 5B), thymidine kinase promoter activity was largely dependent on ectopic HNF4 expression, which was enhanced 2- to 3-fold by cotransfection with an EWS expression plasmid. Coexpression of scFv-I85 intrabody, however, drastically reduced luciferase activity to the level achieved by HNF4 expression alone. Whereas scFv-I85, in the absence of ectopically expressed EWS, did not have any effect on HNF4-driven thymidine kinase reporter activity in STA-ET-7.2 cells lacking endogenous EWS, it further reduced reporter gene activity under these conditions in HEK293 cells, presumably due to interference with the endogenous EWS present in these cells. A similar scFv-I85 intrabody-mediated suppression was observed for OCT4-dependent reporter gene activity in Hep3B cells (Fig. 5C). These results are consistent with a functional knock-down of EWS

coactivator function by intracellular expression of scFv-I85. It should be noted that, in contrast to Hep3B hepatoma cells, EWS did not display any costimulatory effect on OCT4-driven reporter gene activity in SJ-NB7 neuroblastoma and in SK-N-MC and STA-ET-7.2 ESFT cells. Consequently, no influence of scFv-I85 on OCT4 transcriptional activity was observed in these cell lines (data not shown).

To confirm the *in vivo* suppressive activity of scFv-I85 on the transcriptional activity of endogenous EWS, we monitored its effects on the expression of HNF4-regulated genes α 1-antitrypsin (*SERPINA1*), α 1-antichymotrypsin (*SERPINA3*), apolipoprotein-C3 (*APOC3*), transferrin (*TF*), and ceruloplasmin (*CP*; ref. 19) in the hepatoma cell line Hep3B by semiquantitative reverse transcription-PCR (RT-PCR; Fig. 6A). Except for ceruloplasmin, the expression of all tested genes was found to be markedly reduced in scFv-I85 versus control transfected cells. For transferrin, the RT-PCR results were also confirmed by immunoblotting (data not shown). This effect underscores the *in vivo* suppressive activity of the intrabody towards the EWS protein.

Consistent with the lack of reactivity with endogenous EWS-FLI1, scFv-I85 intrabody did not change the expression levels of EWS-FLI1-activated target genes *ID2* (20), *MK-STYX* (21), and *c-MYC* (22), or reactivate expression of the directly EWS-FLI1-suppressed gene *TGFBR2* (23) in STA-ET-7.2 and SK-N-MC ESFT cell lines (Fig. 6B). The lack of interference with EWS-FLI1-mediated transcriptional repression of the *TGFBR2* promoter in luciferase reporter assays (Fig. 6C).

Discussion

The limited availability of native EWS-FLI1 from ESFT cells due to solubility problems and the recent demonstration of transcriptional activity of recombinant EWS-FLI1 (14) prompted us to use bacterially expressed EWS-FLI1 for our antibody screen. The finding that all six randomly selected but independent EWS-FLI1-specific scFv clones recognized the same EWS-derived NH₂-terminal epitope on recombinant EWS-FLI1 suggests that it represents a dominantly exposed and highly accessible domain on the surface of bacterially expressed EWS-FLI1. However, in mammals, this epitope does not seem to be highly immunogenic because none of the three

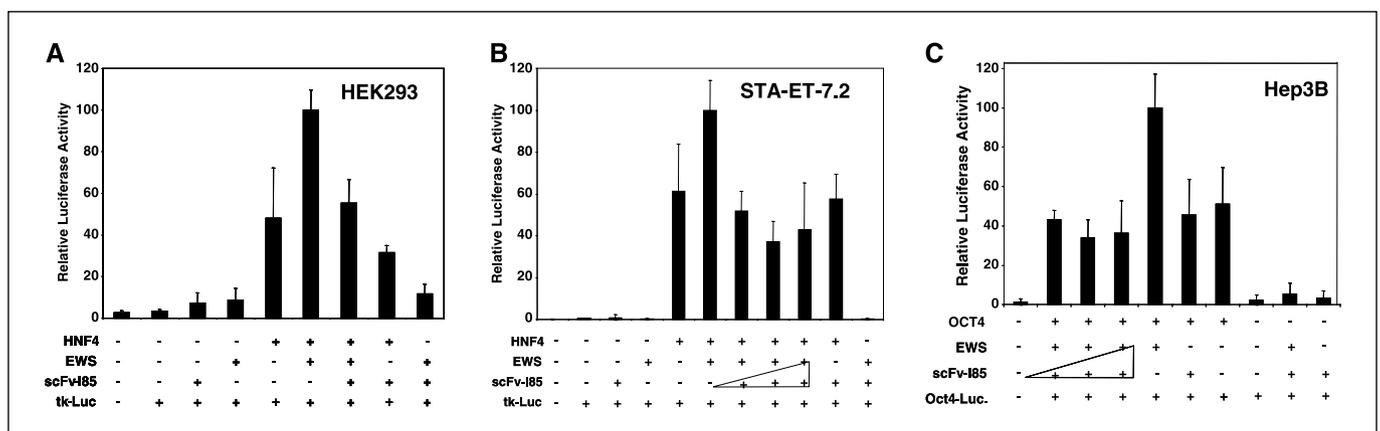


Figure 5. ScFv-I85 suppresses coactivation of HNF4 and OCT4 dependent transcription. HEK293 (A) and STA-ET-7.2 (B) cells were transfected with pHNF4x8-tk-Luc reporter plasmid and HNF4 α and EWS expression plasmids as indicated, followed by luciferase assay. C, Hep3B cells were transfected with pOct4-4-10xTATA-Luc reporter plasmid and OCT4 and EWS plasmids, followed by luciferase assay. Individual values in all cases were normalized to the activity of a cotransfected β -galactosidase reporter plasmid. Columns, mean of at least three independent experiments done in triplicate; bars, SE. In assays using STA-ET-7.2 and Hep3B cells, 50, 100, and 150 ng of scFv-I85 in increasing order were used as indicated.

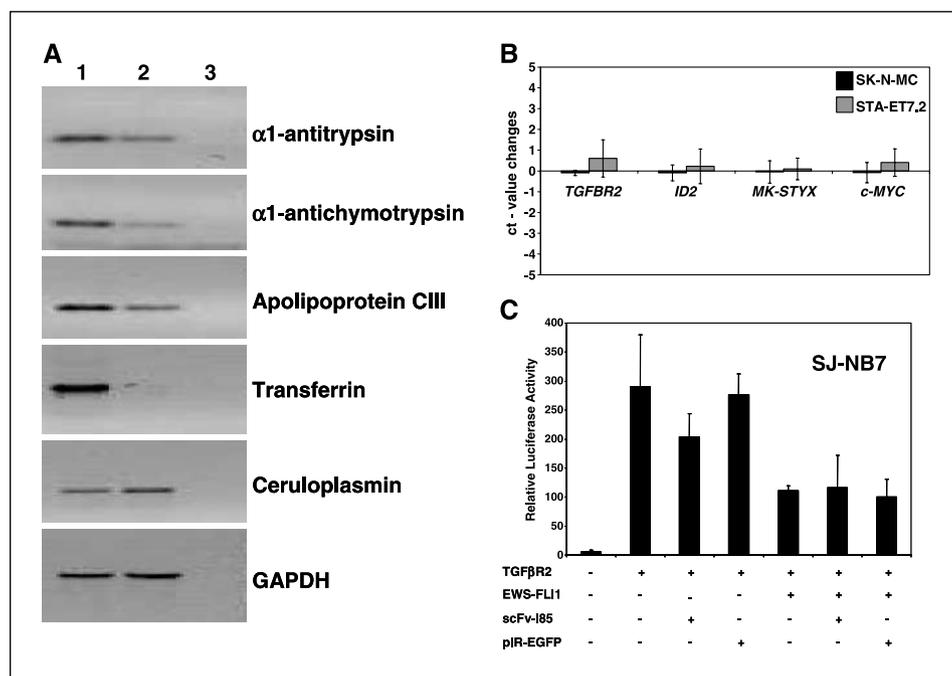


Figure 6. ScFv-I85 intrabody expression impairs EWS-mediated transcriptional coactivation but has no influence on EWS-FLI1-mediated transcription. **A**, representative photographs from ethidium bromide-stained gel of RT-PCR-amplified HNF4-dependent genes from Hep3B cDNA. 1, PCRs of cDNA from pIRES2-EGFP-transfected and GFP-positive sorted Hep3B cells; 2, PCRs of cDNA from sorted scFv-I85-transfected Hep3B cells; 3, negative control PCRs from cDNAs synthesized with H₂O as template. **B**, real-time quantitative PCR showing no influence of scFv-I85 intrabody expression on transcription of known EWS-FLI1 target genes as indicated. SK-N-MC and STA-ET-7.2 ESFT cells were transfected with the scFv-I85 intrabody. Expression of indicated EWS-FLI1 target genes was then analyzed by real-time quantitative PCR. Columns, mean from three independent determinations, each done in duplicate and normalized to β 2-microglobulin expression; bars, SD. **C**, SJ-NB7 cells were transfected with TGF β R2 luciferase reporter plasmid together with pCMV-SK-EWS-FLI1 expression vector, as well as the scFv-I85 intrabody or empty vector control (*pIR-EGFP*). Individual values in all cases were normalized to the activity of a cotransfected β -galactosidase reporter plasmid. Columns, mean of at least three independent experiments done in triplicate; bars, SE.

EWS-specific antisera received from three different sources specifically recognized this epitope (data not shown). This result is consistent with the evolutionary conservation of the epitope between rodents and man and confirms the versatility of phage display libraries to generate antibodies to peptides with low *in vivo* immunogenicity. Reactivity with native mammalian EWS, but hardly with EWS-FLI1 in ESFT, might be explained by two possibilities. EWS-FLI1 RNA is present in ESFT cells at much lower levels than EWS RNA on the Northern blot (12). However, relative EWS and EWS-FLI1 protein abundancies in ESFT cells have not been established thus far. Using immunopurified EWS-FLI1 from ESFT cells and comparing its quantity to GST-EWS-FLI1 on silver-stained gels, we were able to define a minimum of 10-ng endogenous EWS-FLI1 extracted from 1×10^7 cells to obtain scFv-I85 reactivity. However, we failed to determine the corresponding amount of EWS using this method due to our inability to enrich for sufficiently clean germ-line EWS from cells using three different EWS antibodies. Thus, it cannot be excluded that differential reactivity of scFvs on Western blots might be, at least in part, due to strong differences in germ-line and chimeric EWS protein expression in ESFT cells. For native proteins, however, immunoprecipitation, ELISA, reporter gene assay, and target gene expression results are compatible with differential reactivity of scFv-I85 with native germ-line and chimeric EWS. In fact, testing transcriptionally active refolded recombinant EWS-FLI1 under different experimental conditions revealed lack of scFv-I85 reactivity in the absence of any detergent, whereas it was readily detectable under denaturing conditions (Fig. 4). This result suggests that accessibility of the scFv-I85 epitope is dependent on the protein

fold. Because, in contrast to this recombinant protein, cellular EWS-FLI1 remained undetectable by scFv-I85 under all conditions, the higher order structure of the EWS domain within the context of the rearranged protein seems to be stabilized in eukaryotic cells, presumably by posttranslational modification. This lack of reactivity with scFv-I85 seems to be an intrinsic property of EWS fusion proteins in eukaryotic cells, and not restricted to ESFT cells, because it was also observed in SF9 insect cells and in tumor cell lines carrying EWS-ERG, EWS-FEV, and EWS-ATF1 gene fusions (not shown). The minimal peptide recognized by scFv-I85 in recombinant EWS-FLI1 spans amino acids 51 to 75 with critical residues between amino acids 58 and 68. This peptide (TATYQG-TAYAT) contains the seventh of 31 repeats of a degenerate motif with the consensus SYGQQS (8), retaining a tyrosine residue conserved between all 31 repeats and previously shown to be critical for the transcriptional activity of EWS chimeric transcription factors (24). However, previous studies have failed to identify phosphorylation of this residue in EWS fusion proteins (24). Instead, our own preliminary data from mass spectroscopic analysis are consistent with glycosylation of the scFv-I85 epitope in EWS-FLI1 from ESFT cells.⁴ The epitope is not shared by other TET family members, confirming its specificity for EWS.

The critical role of the first 82 EWS NH₂-terminal amino acids in both transactivation (25) and transformation (9) by oncogenic EWS fusion proteins is well established. The epitope differentially

⁴ R. Bachmaier, K. Mechtler, and H. Kovar, unpublished data.

recognized in germ-line and rearranged EWS by our scFvs is part of this domain, suggesting that modifications in this region may be of functional importance to the transforming activity of oncogenic EWS fusion proteins. A structural difference between EWS and EWS-FLI1 NTDs has been suggested to account for differential protein interaction with RNA polymerase II components (10). However, the exact epitope for this interaction has not been mapped. An important prerequisite for the transactivating function of the EWS-NTD is loss of the EWS RNA-binding domain, as inclusion of this domain into EWS chimeric transcription factors *cis*-represses their transcriptional activity (26). This result suggests a role for the EWS RNA-binding domain in determining the accessibility of the NTD for protein interactions, possibly by interfering with posttranslational modifications in this region. It remains to be determined if inclusion of this region into EWS-FLI1 restores reactivity with scFv-I85.

The normal roles of TET family members are not well defined, but functions within the transcriptional and RNA processing apparatus have been suggested. Here, we show that scFv-I85 intrabody expression interferes with the transcriptional coactivator function of germ-line EWS in HNF4- and OCT4-dependent transcription, indicating an essential role of amino acids 51 to 75 in this process. Previously, binding of EWS to CREB-binding protein has been shown to be important for this activity (5). However, the EWS-

CREB-binding protein interaction has been delineated to EWS amino acids 83 to 227. It remains to be determined if binding of scFv-I85 upstream of the CREB-binding protein binding site sterically hinders this interaction or imposes a structural change on this protein interaction domain, or if it unravels an additional functional domain required for coactivation of HNF4 and OCT4. In summary, our study gives a first example for the usefulness of scFvs to structurally distinguish between germ-line EWS and its oncogenic derivatives and to block EWS function *in situ*. They should prove very useful in the exploration of EWS-regulated genes and other functions of EWS, including its role in RNA processing.

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Single-chain Antibodies to the EWS NH₂ Terminus Structurally Discriminate between Intact and Chimeric EWS in Ewing's Sarcoma and Interfere with the Transcriptional Activity of EWS *In vivo*

Dave N.T. Aryee, Michael Kreppel, Radostina Bachmaier, et al.

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