Desmoplastic small round cell tumor (DSRCT) is defined by a chimeric transcription factor, resulting from fusion of the N-terminal domain of the Ewing's sarcoma gene EWS to the three C-terminal zinc fingers of the Wilms' tumor suppressor WT1. Although DNA-binding sites have been defined for the uninterrupted WT1 zinc finger domains, the most prevalent isoforms of both WT1 and EWS–WT1 have an insertion of three amino acids [lysine, threonine, and serine (KTS)], which abrogates binding to known consensus sequences and transactivation of known target genes. Here, we used cDNA subtractive hybridization to identify an endogenous gene, LRRC15, which is specifically up-regulated after inducible expression of EWS–WT1(+KTS) in cancer cell lines, and is expressed within primary DSRCT cells. The chimeric protein binds in vitro and in vivo to a specific element upstream of LRRC15, leading to dramatic transcriptional activation. Mutagenesis studies define the optimal binding site of the (+KTS) isoform of EWS–WT1 as 5'-GGAGG(A/G)-3'. LRRC15 encodes a leucine-rich transmembrane protein, present at the leading edge of migrating cells, the expression of which in normal tissues is restricted to the invasive cytotrophoblast layer of the placenta; small interfering (siRNA)-mediated suppression of LRRC15 expression in breast cancer cells leads to abrogation of invasiveness in vitro. Together, these observations define the consequence of (KTS) insertion within WT1-derived zinc fingers, and identify a novel EWS–WT1 transcriptional target implicated in tumor invasiveness.

[Keywords: DSRCT; EWS–WT1; alternative splicing; promoter; zinc finger; invasion]

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Figure 1. Induction of LRRC15 by EWS–WT1(+KTS). (A) Schematic representation of EWS, WT1, and the EWS–WT1 translocation, fusing the N-terminal domain [NTD] of EWS [exons 1–7] to the last three zinc fingers of WT1 [exons 8–10]. The KTS alternative splice inserts three amino acids [lysine, threonine, and serine] between zinc fingers 3 and 4, and is retained in the translocation product. (B) cDNA subtraction results after induction of EWS–WT1(+KTS). Of 62 clones initially found to be differentially expressed upon primary hybridization screening, 32 were confirmed to be induced by Northern blot analysis. These represented only two genes: LRRC15 and EWS–WT1 itself. (C) Northern blot analysis of U2OS cells demonstrating induction of endogenous LRRC15 mRNA, 12 h after inducible expression of EWS–WT1(+KTS), but not EWS–WT1(−KTS), WT1(+KTS), or WT1(−KTS). Blot was hybridized with probes for LRRC15, WT1 [detecting both WT1 and EWS–WT1], and GAPDH [loading control]. (D) RNA in situ hybridization analysis of LRRC15 in two primary DSRCT samples [magnification, 80×]. Adjacent sections were stained with hematoxylin and eosin [H&E], showing nests of tumor cells surrounded by reactive stroma. LRRC15 expression is restricted to tumor cells. No staining was observed with a control [LRRC15 sense] probe.
snRNPs within subnuclear speckles and its coimmunoprecipitation with the splicing factor U2AF65 [Larsson et al. 1995; Davies et al. 1998]. Although the precise function of WT1(−KTS) remains to be defined, the physiological importance of this isoform is demonstrated by the developmental defects in individuals with Frasier syndrome, who have a splice junction mutation, and by the distinct abnormalities of mice engineered to lack either WT1(−KTS) or WT1(+KTS) isoforms [Barbaux et al. 1997; Hammes et al. 1998; Klamt et al. 1998; Hammes et al. 2001].

The EWS–WT1 translocation includes zinc fingers 2–4 of WT1, preserving the alternative insertion of KTS. As for WT1, EWS–WT1(+KTS) is the most abundant isoform, expressed at a ratio of ∼2:1 with respect to the [−KTS] variant (Gerald et al. 1995). Studies of EWS–WT1(−KTS) have demonstrated binding to the consensus sequence 5′-GC(G/C)G/G-GGG-3′, and induction of target genes, including PDGFA, IGFl1R, IL2/15Rβ, and BAIAP3 [Kamieli et al. 1996; Lee et al. 1997; Finkeltov et al. 2002; Palmer et al. 2002; Wong et al. 2002]. These transcripts are not induced by WT1(−KTS) itself, suggesting that the absence of WT1 zinc ﬁnger 1 in the chimera may lead to distinct DNA-binding speciﬁcity in vivo. Comparable speciﬁcity is not observed in vitro, with WT1(−KTS) and EWS–WT1(−KTS) binding to GC-rich DNA sequences and transactivating similar promoter reporters in transient transfection assays, a discordance presumably related to the importance of chromatin context. The identiﬁcation of endogenous transcriptional target genes for these transcription factors is therefore critical to deﬁning their physiological properties.

In contrast to the [−KTS] isoform, EWS–WT1(+KTS) has not been shown to bind to a speciﬁc DNA sequence or regulate gene expression. To dissect its functional properties, we undertook a cDNA subtractive hybridization, as an unbiased screen for endogenous transcripts with expression that is altered after inducible expression of this isoform. LRRc15, a gene encoding a leucine-rich, transmembrane protein was found to be dramatically induced by EWS–WT1(+KTS). We used this potential EWS–WT1(+KTS) target gene to demonstrate binding of EWS–WT1(+KTS) to a speciﬁc DNA sequence, both in vitro and in vivo, and its ability to mediate potent transcriptional activation. A role for LRRc15 in cellular invasion is suggested by its striking expression within the cytotrophoblast cells of the placenta, which invade the maternal decidua during implantation, and by the reduced invasion of breast cancer cells after small interfering siRNA-mediated suppression of LRRc15 expression. EWS–WT1(+KTS) therefore encodes a transcriptional activator with a distinct DNA recognition sequence, the induction of which with LRRc15 contributes to the malignant properties of DSRCT.

Results

Induction of LRRC15 by EWS–WT1(+KTS)

To identify endogenous transcripts induced by EWS–WT1(+KTS), we generated U2OS osteosarcoma cells in which expression of this isoform is driven by a tetracycline-repressible promoter [UED5 cells]. Poly(A)+ RNA was isolated from subconfluent cells grown in the presence of tetracycline or 12 h after drug withdrawal, and subjected to cDNA subtractive hybridization [PCR-select, Clontech]. Differentially expressed products of 100 bp to 2 kb in size were cloned, sequenced, and used to probe Northern blots to conﬁrm altered expression after tetracycline withdrawal. Of 32 clones validated in this screen, the EWS–WT1(+KTS) transcript itself accounted for three clones, the remaining 29 clones identiﬁed a single novel transcript (see below, Fig. 1B) that we called LRRC15 [leucine-rich repeat containing 15, Hugo Gene Nomenclature Committee-approved gene symbol, http://www.gene.ucl.ac.uk/nomenclature]. Strong induction of LRRC15 is observed in two independent EWS–WT1(+KTS)-inducible cell lines (UED5 and UED26 cells), but not in cells with inducible expression of the EWS–WT1(−KTS) isoform (UF-1 cells), or with inducible constructs encoding the parental WT1(+KTS) [UD28 cells] or WT1(−KTS) [UB27 cells] isoforms (Fig. 1C). The time course of LRRC15 expression upon tetracycline withdrawal closely follows that of EWS–WT1(+KTS) itself, with earliest expression detectable at 6 h [data not shown]. As an initial test for physiological signiﬁcance, we screened primary DSRCT specimens for expression of the LRRC15 transcript. In two snap-frozen primary tumor specimens with high-quality RNA, LRRC15 expression is readily evident within the characteristic nests of tumor cells, but not in the surrounding reactive stroma [Fig. 1D]. LRRC15 expression was detectable by using either RNA in situ hybridization or reverse transcriptase PCR (RT–PCR) analysis in four of eight primary DSRCT specimens in which the EWS–WT1 chimera itself was successfully ampliﬁed. The reason for absence of LRRC15 expression in the remaining cases is unknown and may reﬂect the need for additional factors regulating LRRC15 expression in some DSRCTs. Thus, LRRC15 was initially identiﬁed by virtue of its speciﬁc and dramatic induction by EWS–WT1(+KTS) in heterologous cell lines, and found to be expressed in a subset of primary DSRCT tumors. LRRC15 expression is below detection in all normal tissues except for placenta [see below].

To clone the full-length LRRC15 transcript, we generated a cDNA library from UED5 cells and performed 5′ and 3′ RACE, combined with database analysis, yielding a 6-kb transcript with an open reading frame (ORF) of 1741 bp and encoding a predicted protein of 581 amino acids. The gene is encoded by three exons, with the entire coding region contained within exon three. While this work was ongoing, the rat ortholog, Lib, was reported as a gene induced in rat C6 astrocyte cells upon addition of β-amyloid [Satoh et al. 2002]. LRRC15 is a member of the leucine-rich repeat [LRR] superfamily, encoding transmembrane proteins thought to function in a broad array of cell–cell interactions [Kobe and Deisenhofer 1994, 1995]. Of note, two close homologs are colocated in tandem with LRRC15 at 3q29: GP5 [GenBank accession no. Z23091] and CPN2 [GenBank accession no.
Transcriptional activation of LRRC15 by EWS–WT1(+KTS)

To determine whether LRRC15 constitutes a direct transcriptional target of EWS–WT1(+KTS), we first tested its potential regulatory sequences for responsiveness by using promoter reporter constructs. However, luciferase constructs containing 2.5 kb of genomic sequence upstream of the transcriptional start site failed to show induction after cotransfection with EWS–WT1(+KTS), as did the entire sequence of introns 1 and 2. To search for more distant regulatory sequences, we isolated a BAC centered around LRRC15 and spanning 120 kb of genomic sequence (573k19). After EcoRI, BglII, or HindIII restriction, fragments of the BAC were cloned into the promoter-less luciferase reporter plasmid pGL3 to generate a library, which was then screened in pools for responsiveness to EWS–WT1(+KTS) in U2OS cells. An initial pool of eight clones [H6] demonstrated a mean of approximately sevenfold activation after cotransfection with CMV-driven EWS–WT1(+KTS) [background less than threefold; Fig. 3B]. Individual clone HC6 demonstrated 13-fold activation by EWS–WT1(+KTS), with further subcloning to generate HC62 [35-fold activation], and finally HC63, a minimal 142-bp region within HC62 with 50-fold transcriptional activation. Mapping of this sequence [AC108676: nucleotides 75,838–75,979] within the genomic contig showed it to be 70 kb upstream of the LRRC15 transcriptional start site [AC125362: nucleotide 33,964].

The (+KTS) isoforms of both WT1 and EWS–WT1 have not been shown to bind to a specific DNA sequence. To determine whether transcriptional activation of the HC63 element results from direct DNA binding by EWS–WT1(+KTS), we first tested in vitro binding by using electrophoretic mobility shift assays [EMSA] and bacterially synthesized DNA-binding domains of the chimera or parental WT1 [Fig. 3C]. Binding to HC63 is restricted to the (+KTS) isoform of EWS–WT1, with a minimal gel shift observed after incubation with the (−KTS) variant. The parental WT1(+KTS) DNA-binding domain (including zinc finger 1) does not bind to HC63. Binding by the (+KTS) isoform of EWS–WT1 is specifically competed with a molar excess of unlabeled probe, and supershifting of protein–DNA complexes was achieved by using antibodies against the WT1 domain of the chimera, but not with nonspecific antibodies [Fig. 3C]. Of note, two gel shifted bands are observed after incubation of EWS–WT1(+KTS) with HC63, consistent with the presence of two binding sites [see below].

To confirm that EWS–WT1(+KTS) binds to HC63 in vivo, we used chromatin immunoprecipitation [ChIP] to enrich for DNA fragments bound by the chimera. UED5 cells were grown in the absence of tetracycline and treated with formaldehyde, and cross-linked protein–DNA complexes were immunoprecipitated by using antibodies against either the WT1 zinc finger domain [C19] or the C-terminal HA-epitope tag [αHA]. Selective enrichment of the HC63 sequence is observed using both antibodies, relative to control [β-actin] sequences [Fig. 3D]. No enrichment is observed in the absence of antibody or in mock immunoprecipitated samples [rabbit preimmune serum], nor is there enrichment of HC63 template from U2OS cells with induced expression of EWS–WT1(−KTS). We also examined an unrelated site [AC125362: 47844–48021] within the BAC but outside HC6, demonstrating no enrichment with this template [data not shown]. Thus, EWS–WT1(+KTS) encodes a DNA-binding protein that binds in vitro and in vivo to a specific DNA sequence, mediating transcriptional activation. HC63 is located at some distance from the LRRC15 transcriptional start, suggesting that regulation of gene expression may involve chromatin looping, as shown recently for regulation of the β-globin gene by its H52 enhancer [Carter et al. 2002].

Definition of EWS–WT1(+KTS) binding consensus sequence

To define the precise binding sequence for EWS–WT1(+KTS), EMSA was performed by using overlapping, radiolabeled double-stranded probes spanning HC63 [Fig. 4A]. Two binding sites were identified: the first between nucleotides 41 and 70 [HC63-1], and the second between nucleotides 81 and 110 [HC63-2]. Extensive in vitro mutagenesis of HC63-1, combined with EMSA, identified six essential residues: E(KTS)RE1 [HC63 nucleotides 55–60; Fig. 4B]. Substitution of G6, G7, G8, and G9 to adenine or substitution of A6 to thymine results in complete or significant loss of binding. Analysis of HC63-2 identified a very closely related sequence required for DNA binding; E(KTS)RE2 [HC63 nucleotides 91–96; Fig. 4C]. The two sequences differ only at position 6 [Fig. 4E]. Substitution of A6 to thymine in E(KTS)RE1 results in loss of binding [Fig. 4B], whereas substitution of G6 to adenine in E(KTS)RE2 has a minimal effect [Fig. 4C]. Hence, position 6 is required for DNA binding, with A6 interchangeable with G6. On either side of the 6-bp sequence, substitution of G[−1] or T [+1] has a minimal effect on DNA binding. The effect of mutations in the binding site was confirmed by using EMSA competition assays. In-
corporation of G_{2}A into an unlabeled oligonucleotide prevents competition for binding to radiolabeled HC63-2, whereas G_{6}A competes effectively (Fig. 4D). Taken together, these experiments suggest a novel recognition consensus sequence for EWS–WT1(+KTS): 5’-GGAG(A/G)-3’ [Fig. 4E].
To test whether the E[KTS]RE1 and E[KTS]RE2 sequences are indeed responsible for the transactivation of HC63 by EWS–WT1(+KTS), we disrupted these sequences, either alone or in combination, within the full-length HC63-reporter [Fig. 4F]. Triple substitutions of G1, G2, and G3 to adenine were made within both E[KTS]RE1 and E[KTS]RE2. Wild-type or mutant reporters were cotransfected along with CMV-driven EWS–WT1(+KTS) into U2OS cells. Disruption of E[KTS]RE1 alone greatly reduces EWS–WT1(+KTS)-mediated transactivation of HC63, whereas disruption of both binding sites essentially abrogates EWS–WT1(+KTS)-dependent transactivation of HC63 [Fig. 4G]. Thus, two sequences, E[KTS]RE1 and E[KTS]RE2, are responsible for activation of this responsive element by EWS–WT1(+KTS).

The discovery of a specific DNA-binding sequence for EWS–WT1(+KTS) has implications for the functional properties of the parental WT1(+KTS) protein, which includes an additional N-terminal zinc finger. To gain insight into the binding properties conferred by addition or omission of WT1-derived zinc fingers, we tested combinations of these for binding to HC63-1 [Fig. 5]. Among the zinc fingers present in the chimera [2-3-[KTS]-4], zinc finger 2 is essential for binding, because the 3-[KTS]-4 combination is unable to bind HC63-1. Surprisingly, fingers 2 and 3 consistently demonstrate increased in vitro binding, compared with 2-3-[KTS]-4. The reduction in in vitro binding associated with the presence of [KTS]-4 may be compensated by additional specificity in vivo [see below]. By analogy with the crystal structure of the related EGR1 protein [Pavletich and Pabo 1991], the KTS insertion places the fourth zinc finger out of register with respect to the major groove of DNA. When the fourth zinc finger is in phase, as in 2-3-4, binding to HC63-1 is greatly reduced. Similarly, presence of zinc finger 1, in both 1-2-3-[KTS]-4 and 1-2-3, prevents recognition of the E[KTS]RE sites. Taken together, these observations suggest that zinc fingers 2 and 3 are required for optimal binding of the E[KTS]RE consensus. Zinc finger 4 may modulate binding when placed out of phase by the KTS insertion, but the presence of either zinc fingers 1 or 4 within the major groove abolishes binding by the adjacent fingers 2 and 3.

**Contribution of LRRC15 to cellular invasion**

Identification of LRRC15 as the only transcript induced after EWS–WT1(+KTS) expression in our cDNA subtraction assay raised the possibility that its functional properties may contribute to transformation by the chimera. Like other EWS-dependent translocation products, tumorigenicity assays have not been well defined for EWS–WT1, with only the (+KTS) isoform mediating a modest transforming effect in NIH3T3 cells [Kim et al. 1998]. Cell type-specific oncogenic effects have been postulated, but the cell of origin for either Ewing’s sarcoma or DSRCT remains to be defined. To gain insight into the functional properties of LRRC15, we therefore first searched for normal tissues demonstrating physiological expression of this gene. Consistent with previous studies of the rat ortholog Lib [Satoh et al. 2002], Northern blot analysis demonstrated LRRC15 expression only in placenta [data not shown]. To define the specific cell type expressing LRRC15, we undertook RNA in situ hybridization experiments by using sections of mouse placenta. Remarkably, Lrc15 expression is restricted to the cytotrophoblast cell layer [Fig. 6A]. These cells constitute the invasive layer of the placenta, which invades the maternal decidua during implantation of the embryo [Morris et al. 1998].

The unique physiological expression pattern of LRRC15 suggested a potential role in cellular invasion. Expression of LRRC15 in established human cancer-derived cell lines is uncommon, but we identified high levels of expression in Hs467T breast carcinoma cells by Northern blotting [data not shown]. These cells are highly invasive, penetrating matrigel-coated pores to an extent comparable to that of the HT1080 fibrosarcoma cell line commonly used to calibrate such assays [Albini et al. 1987; Kobayashi et al. 1992]. Treatment of Hs467T cells with siRNA specific to LRRC15 successfully reduced levels of the endogenous transcript [Fig. 6B]. Invasion of LRRC15-knockdown cells through matrigel was reduced by 65%, whereas cells treated with a nonspecific siRNA showed no change [Fig. 6B]. As a control, migration of Hs467T cells through uncoated plastic pores, an index of cellular motility rather than invasion, was not altered by reduction of LRRC15 expression. These observations suggest that LRRC15 induction by EWS–WT1(+KTS) contributes to the invasive phenotype of DSRCT.

**Discussion**

We have used cells with inducible expression of EWS–WT1(+KTS) and subtractive cDNA hybridization to identify the first transcriptional target for a (+KTS) isoform of the Wilms’ tumor-derived zinc fingers. The dem-
onstration of direct in vivo binding of EWS–WT1(+KTS) to a specific DNA recognition site has implications for understanding the functional properties of the (+KTS) variants. The properties of LRRC15 itself provide additional insight into the mechanisms by which the EWS–WT1 translocation drives malignant transformation in DSRCT.

C2H2 zinc finger transcription factor family represents the most common class of DNA-binding motifs found in eukaryotes (Lander et al. 2001; Tupler et al. 2001).
2001; Venter et al. 2001; Waterston et al. 2002). Many zinc fingers are thought to recognize a 3–4-bp DNA sequence, although some may also be involved in RNA or protein interactions. Structural studies of the Zif268/EGR1 binding domain, which is closely related to zinc fingers 2, 3, and 4 of WT1, indicate that it is wrapped around DNA, with each zinc finger residing within the major groove [Pavletich and Pabo 1991]. However, in-
stead of a fixed nucleotide recognition code for each zinc finger in isolation, recent studies have shown that neighboring zinc fingers can act synergistically to bind DNA. Thus, a change in the binding capability of one zinc finger can potentially affect the sequence specificity of the protein as a whole [Isalan et al. 1997, 1998; Elrod-Erickson et al. 1998]. The effect of the [KTS] insertion between WT1 zinc fingers 3 and 4 is therefore likely to be complex. By altering the spacing between zinc fingers 3 and 4, it may shift zinc finger 4 away from the major groove, and its effects on the flanking zinc fingers 3 and 4 may be associated with altered DNA binding by the entire zinc finger domain.

Our results indicate that in vivo recognition of the novel 5’-GGAGG[A/G]-3’ consensus by EWS–WT1(+KTS) is mediated primarily by zinc fingers 2 and 3. We note, however, that the 5’-GGAGG[A/G]-3’ consensus occurs ~80 times in the 70-kb genomic sequence between HC63 and the LRR1C15 transcriptional start site; yet, transcriptional activation appears restricted to the E[KTS]RE sites within the HC63 genomic fragment. Specific in vivo binding is therefore likely to be modulated by additional DNA-binding factors, flanking sequences, as well as a favorable chromatin context defining accessible sites. In vitro zinc finger studies have suggested that high-affinity and high-specificity DNA binding requires at least three zinc fingers (Wang and Pabo 1999; Joung et al. 2000). However, flanking zinc fingers 2 and 3 with additional WT1-derived zinc fingers within the correct phase for binding to the major groove of DNA abrogates their binding to E[KTS]RE. For instance, addition of zinc finger 1, as in WT1(+KTS), or zinc finger 4, as in EWS–WT1(−KTS), reduces binding affinity. Stabilization of DNA binding by zinc fingers 2 and 3 might therefore be mediated by an alternative mechanism, such as zinc finger–protein interactions. It is likely that the insertion of KTS shifts zinc finger 4 out of register, preventing its contact with DNA but making it accessible for a protein–protein stabilizing interaction. According to this model, the loss of zinc finger 1 resulting from the EWS–WT1 translocation, together with the displacement of zinc finger 4 by the KTS insertion, would allow for high-affinity binding to a unique DNA recognition element and transcriptional activation of novel genes.

Given these considerations, it is not surprising that the DNA recognition sequence derived for each finger within the setting of the parental WT1 protein is not predictive of the sequence recognized by EWS–WT1(+KTS). Our approach in first identifying an endogenous target gene, and then searching for a responsive

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**Figure 5.** Characterization of zinc finger binding to E[KTS]RE. (A) EMSA analysis of combinations of WT1-derived zinc finger proteins. Binding is shown by using HC63-1, which contains the EWS–WT1(+KTS) binding sequence E[KTS]RE, and using consensus sequences previously identified for EWS–WT1(−KTS) (called E-WRE1) or for WT1(−KTS) (called WTE). A loading control for expression of zinc finger proteins is shown by using Western blotting with anti-GST antibody [αGST]. (B) Relative binding affinity of combinations of WT1-derived zinc finger proteins for the E[KTS]RE sequence. Results from multiple EMSA experiments using HC63-1 probe are represented schematically.
regulatory sequence, was therefore unbiased and succeeded in defining the novel E(KTS)RE consensus sequence. A similar strategy may be important in determining whether the parental tumor suppressor, WT1(KTS) is capable of direct DNA binding and regulation of gene expression. WT1(KTS) and EWS-WT1(KTS) differ in their subnuclear localization. Presence of WT1(KTS) within nuclear bodies containing snRNPs and its coimmunoprecipitation with the splicing factor U2AF65 have implied a role in some aspect of premRNA processing [Larsson et al. 1995; Davies et al. 1998]. Nonetheless, our results raise the possibility that WT1(KTS) may also bind a specific DNA sequence defined by zinc fingers 1–3. Previous studies have searched

Figure 6. Role of LRRC15 in cellular invasion. (A) RNA in situ hybridization analysis of Lrrc15 in mouse placenta [magnification, 80×]. Adjacent section is stained with H&E showing cytotrophoblast layer [cy], stroma [s], and maternal decidua [md]. Lrrc15 expression is restricted to cytotrophoblast cells. No staining is observed with a control [Lrrc15 sense] probe. (B, top) Effect of LRRC15 expression on matrigel invasion by Hs467T breast cancer cells. Quantitative real-time RT–PCR [TaqMan] analysis of LRRC15 transcript from Hs467T cells, 72 h after treatment with specific siRNA duplexes, nonspecific duplexes, or untreated [mock]. The expression of GAPDH was used to normalize for variances in input cDNA. (Bottom) Results of transwell migration assays are shown for matrigel-coated plates [correlated with cellular invasion] or for uncoated plastic [correlated with cellular migration]. Hs467T cells treated with LRRC15 siRNA or nonspecific siRNA, or mock-treated controls are compared with the highly invasive HT1080 cells and the noninvasive NIH 3T3 cells. Standard deviations are derived from three independent experiments.
for WT1(+KTS) DNA-binding sites, but no specific site has been confirmed to date [Bickmore et al. 1992].

The identification of a transcriptional target for EWS-WT1(+KTS) also provides new insight into the transforming properties of this tumor-specific translocation product. LRRC15 encodes an N-terminal transmembrane domain, 15 LRRs, and a short C-terminal cytoplasmic tail. LRR-containing cell surface proteins have diverse functions, with many linked to a role in cell adhesion or signaling, including the family member LG11, which is inactivated during malignant progression in brain tumors [Chernova et al. 1998]. The LRR superfamily member that is closest in amino acid sequence to LRRC15 is GP5. These two genes share the same genomic structure and are adjacent to each other, consistent with a tandem gene duplication event. GP5 encodes a subunit of the GPlb-V-IX receptor that binds von Willebrand factor and is important in platelet aggregation [Roth et al. 1996; Gurney et al. 2002], and it may act to negatively regulate the response of the activated receptor [Ramakrishnan et al. 1999]. We have shown that LRRC15 also encodes a transmembrane glycoprotein, expressed at the leading edge of migrating cells. Although functional analyses suggest that LRRC15 is likely to be involved in cellular invasion, further work will be required to identify potential ligands or associated cofactors.

The normal expression pattern of LRRC15 appears to be restricted to invasive cells in the placental cytotrophoblast layer. As such, the induction of LRRC15 by EWS–WT1(+KTS) may represent the misappropriation of a physiological invasion mechanism by an oncogenic chimera. It is of interest that the rat ortholog, Lib, was identified in the highly invasive C6 glioblastoma cell line, where its expression is induced by addition of cytokines or β-amyloid [Sato et al. 2002]. In vitro, we have demonstrated that LRRC15 contributes to the invasion of breast cancer cells through matrigel, a reconstituted basement membrane matrix derived from mouse sarcoma cells. Matrigel invasion serves as a useful in vitro assay for cell invasion. LRRC15 further promotes invasion and motility of breast cancer cells expressing WT1(+KTS), but no specific sites have been identified which contained LRRC15 sequence. A plasmid library was generated by subcloning into pCR2.1 [Invitrogen] and predicted target cDNAs were confirmed using Northern blotting. Frozen mouse tissue specimens were obtained from the Dana-Farber/Harvard Cancer Center Pathology Core Facility and frozen tumor specimens of DSRCT from either the same source or the Memorial Sloan-Kettering tumor bank.

LRRC15 cloning and characterization

To clone full-length cDNAs corresponding to subtraction products, poly(A)+-selected RNA from U2OS cells expressing EWS–WT1(+KTS) was used to generate a cDNA library in ASAP-1 [Novagen]. Approximately 5 × 10⁶ pfu was screened by using a subtracted fragment as a probe by standard methods. The additional nucleotide sequence obtained was used to interrogate the NCBI database in which an exact match to a genomic contig (accession no. AC025389) is shown. Bioinformatics programs [http://genes.mit.edu and http://compbio.ornl.gov/Grail] were used to find an ORF of 1741 bp within 6-kb transcript in which the subtracted cDNA mapped to the 3' UTR. To verify that the predicted transcript was full length, poly(A)+-selected RNA from U2OS cells expressing EWS–WT1(+KTS) was also used to perform 5'-rapid amplification of cDNA ends [RACE] reactions (Clontech). Three primers were used: 4R (5'-CAGGGGAATTCCTGCCTGAATGAC-3'), 12R (5'-CTGTCCGTGTTGAGGATCTCGAGGCTCATGG-3'), and 5R (5'-CGGCCATGACGAGATCACTAAGACGCG-3'). 5 independent clones were sequenced for each resulting product.

The full-length LRRC15 ORF was cloned, along with a 3' HA tag into the CMV-driven pCDNA3 plasmid [Invitrogen]. Stable cell lines were prepared by cotransfecting LRRC15 expression constructs with the selection plasmid pBABE puro, followed by selection using 1 µg/mL puromycin. Immunofluorescence was performed by using antibodies to the HA-epitope (16B12) to detect HA-tagged LRRC15, FITC-conjugated antiamyloid antibody [Jackson ImmunoResearch], and TRITC-conjugated phalloidin (Sigma). Photographs were taken by using a Zeiss Axioscope 510 imaging system. Combinations of oligosaccharide digesting enzymes were added to cellular extracts of HT1080 cells expressing LRRC15, according to the manufacturer's instructions [Prozyme], and LRRC15 protein was visualized by SDS-PAGE and Western blotting.

LRRC15 promoter constructs and luciferase reporter assays

To clone sequences upstream of the LRRC15 ORF, the human BAC DNA pools released IV [Research Genetics] was screened by PCR with primers LRRC15F (5'-GAATTTCCTCCCTCTCATGGGAATTC-3') and LRRC15R (5'-GGTGAGAACAATCTCCTCTTAAAGG-3'), and a clone (573k19) was identified which contained LRRC15 sequence. A plasmid library was generated from this bacterial artificial chromosome (BAC) DNA digested with either EcoRI, BglII, or HindIII by subcloning into the promoter-less luciferase vector pGL3 [Promega]. Pools were

Materials and methods

Generation of cell lines and cDNA subtraction

Full-length EWS–WT1(+KTS) cDNA was generated by using RT–PCR from a primary DSRCT, confirmed by nucleotide sequencing, and fused to a C-terminal hemagglutinin (HA) epitope into the pCDNA3 vector [Invitrogen]. U2OS cells with tetracycline-repressible EWS–WT1(+KTS) expression were generated and maintained in 1 µg/mL tetracycline as described previously [Englert et al. 1995; Lee et al. 1997]. UEDS, a clone with tightly regulated, 100- to 200-fold inducible expression of EWS–WT1(+KTS) was used for analysis. Immunofluorescence microscopy was performed as described [Lee et al. 1997]. Tetracycline was withdrawn for 12 h; total RNA was isolated with RNA STAT60 [Tel-Test]; and poly(A)+-selected RNA was obtained by using oligo(dT) resin [Invitrogen]. cDNA subtraction was performed by using the PCR-select kit [Clontech]. A cDNA-subtracted plasmid library was generated by subcloning into pCRII [Invitrogen] and predicted target cDNAs were confirmed using Northern blotting. Frozen mouse tissue specimens were obtained from the Dana-Farber/Harvard Cancer Center Pathology Core Facility and frozen tumor specimens of DSRCT from either the same source or the Memorial Sloan-Kettering tumor bank.
screened for luciferase activity, and candidates were rescreened as individual clones. Substitutions were introduced by PCR to change the guanines at positions 1, 2, and 4 of E(KTS)RE1 to adenines, and the guanines at positions 1, 2, and 4 of E(KTS)RE2 to adenines. For luciferase reporter assays, 1 µg of CMV-driven expression constructs or empty vector was cotransfected with 0.2 µg of the promoter reporter into U2OS cells by using the calcium DNA precipitation method. Equal amounts of CMV-driven constructs were transfected in each experiment, and cotransfection of a Renilla luciferase reporter (20 ng) was used to allow standardization for transfection efficiency. All experiments were performed in triplicate.

EMSAs and ChIP

Given the insolvency of full-length WT1 protein, the zinc finger domains of EWS–WT1(+KTS) and EWS–WT1(−KTS) were cloned into the pGEX3X GST-expression vector (Pharmacia) and expressed in Escherichia coli BL21(DE3). Deletion mutant zinc finger proteins were also generated containing zinc fingers 2 and 3, zinc fingers 3 and 4 with the KTS alternative splice, and zinc fingers 1, 2, and 3. For mutational analysis of the EWS–WT1(+KTS)-binding site, oligonucleotides containing single base pair substitutions within the E(KTS)RE were annealed, subcloned, and verified by nucleotide sequencing. End-labeled probes (20,000 cpm) were incubated with 200 ng of GST-WT1 protein in binding buffer as described [Palmer et al. 2002]. After incubation for 30 min at 4°C, binding reactions were electrophoresed on a 5% polyacrylamide gel in 0.5X TBE buffer for 2 h for competition experiments, 100-fold molar excess of unlabeled annealed oligonucleotides were added to 200 ng of GST-WT1 in binding buffer. Competitor DNA was incubated for 10 min at room temperature prior to addition of labeled probe, followed by incubation for an additional 20 min at 4°C. ChIP was performed as described [Palmer et al. 2002]. The following primer sets were used: HC63 nucleotides 95549–95691 and primer sets were used: HC63 nucleotides 95549–95691 of AC025389; following primer sets were used: HC63 nucleotides 95549–95691 of AC025389; lowing primer sets were used: HC63 nucleotides 95549–95691 of AC025389; following primer sets were used: HC63 nucleotides 95549–95691 of AC025389; following primer sets were used: HC63 nucleotides 95549–95691 of AC025389.

Accession numbers

LRRC15 mRNA sequence is available in the Third Party Annotation section of the DDBJ/EMBL/GenBank databases under the accession number TPA: BK001325.

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