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RUNX3 Facilitates Growth of Ewing Sarcoma Cells

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Abstract

Ewing sarcoma is an aggressive pediatric small round cell tumor that predominantly occurs in bone. Approximately 85% of Ewing sarcomas harbor the EWS/FLI fusion protein, which arises from a chromosomal translocation, t(11:22)(q24;q12). EWS/FLI interacts with numerous lineage-essential transcription factors to maintain mesenchymal progenitors in an undifferentiated state. We previously showed that EWS/FLI binds the osteogenic transcription factor RUNX2 and prevents osteoblast differentiation. In this study, we investigated the role of another Runt-domain protein, RUNX3, in Ewing sarcoma. RUNX3 participates in mesenchymal-derived bone formation and is a context dependent tumor suppressor and oncogene. RUNX3 was detected in all Ewing sarcoma cells examined, whereas RUNX2 was detected in only 73% of specimens. Like RUNX2, RUNX3 binds to EWS/FLI via its Runt domain. EWS/FLI prevented RUNX3 from activating the transcription of a RUNX-responsive reporter, p6OSE2. Stable suppression of RUNX3 expression in the Ewing sarcoma cell line A673 delayed colony growth in anchorage independent soft agar assays and reversed expression of EWS/FLI-responsive genes. These results demonstrate an important role for RUNX3 in Ewing sarcoma.

Keywords

EWS/FLI; RUNX; AML2; Cbfa3

Introduction

Ewing sarcoma is an aggressive small round cell tumor of bone and soft tissues. These tumors predominantly afflict children, adolescents, and young adults with an incidence rate of about 3/1,000,000 people per year (Esiashvili et al., 2008). Treatment typically involves a combination of surgery, radiotherapy and chemotherapy. Ewing sarcoma has a survival rate of ~70%; however, in the 25% of cases where metastasis is present at diagnosis, the survival rate is much lower (~40% with pulmonary metastasis, and <20% with other metastases) (Potratz et al., 2012).

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The majority (85%) of Ewing sarcomas harbor the recurrent chromosomal translocation, t(11;22)(q24;q12) (Sorensen et al., 1994). This gene rearrangement creates a tumor-specific fusion protein, EWS/FLI, by joining the N-terminal transcriptional activation domain of the TET family protein, EWSR1, to the ETS DNA binding domain of transcription factor, FLI1. The resulting fusion protein retains the DNA binding specificity of FLI1, and the potent transactivation domain of EWSR1. The remaining Ewing sarcomas express related fusions between FLI1 and different ETS family proteins (including ERG, ETV1, ETV4, FEV) (Tan and Manley, 2009), or the alternate FUS-ERG fusion (Ng et al., 2007). EWS/FLI functions as a transcription factor regulating a number of target genes. It is not sufficient to transform cells aside from NIH3T3 cells (May et al., 1993); however, it is required to maintain the transformation of Ewing sarcoma cells and their capacity to grow in soft agar (Smith et al., 2006).

EWS/FLI is an attractive therapeutic target because it is expressed exclusively in Ewing sarcoma. Identifying EWS/FLI interaction partners will increase our understanding of how EWS/FLI contributes to tumorigenesis and provide new therapeutic options. Previous work showed that EWS/FLI binds to the Runt domain of RUNX2 (Li et al., 2010b). EWS/FLI represses RUNX2-induced transcriptional activation and inhibits differentiation of mesenchymal progenitor cells into osteoblasts. The suppression of osteoblast lineage differentiation allows EWS/FLI expressing tumors to maintain an undifferentiated state.

RUNX2 and related proteins, RUNX1 and RUNX3, contribute to the development of many tissues and tumors. RUNX1 (AML1, Cbfa1) is essential for hematopoiesis and is involved in numerous chromosomal translocations in acute myeloid leukemias (Goyama et al., 2013; Mitani et al., 1994; Miyoshi et al., 1991; Westendorf et al., 1998). RUNX2 is required for bone development and is highly expressed in numerous solid tumors, including metastatic breast and prostate cancers (Akech et al., 2010; Barnes et al., 2003; Brubaker et al., 2003; Leong et al., 2010). RUNX3 contributes to the development of hematopoietic cells, as well as neuronal and musculoskeletal tissues. Context dependent oncogenic and tumor suppressor functions have been ascribed to RUNX3. RUNX3 was initially found to be a tumor suppressor based on reports that a *RUNX3* knockout mouse developed gastric epithelial hyperplasia (Li et al., 2002). Subsequently RUNX3 was shown to be a tumor suppressor in colorectal (Soong et al., 2009), lung (Lee et al., 2011b), and breast (Chimge and Frenkel, 2013) cancers, as well as in giant cell tumors of the bone (Han and Liang, 2012). Conversely, RUNX3 displayed oncogenic properties in other cancers including ovarian cancer (Lee et al., 2011a), basal cell carcinoma (Salto-Tellez et al., 2006), and head and neck squamous cell carcinoma (Tsunematsu et al., 2009). The role of RUNX3 in bone cancers has not been established.

In this study we investigated the role of RUNX proteins in Ewing sarcoma. EWS/FLI blocks RUNX1 and RUNX2-dependent transcription (Li et al., 2010a); however, here we show that RUNX1 is not expressed in primary Ewing sarcomas. Rather RUNX3 is detected in all Ewing sarcomas with RUNX2 also being present in a fraction of these cancers. Because the Runt domains of RUNX2 and RUNX3 are highly homologous, we hypothesized that EWS/FLI would bind to RUNX3. RUNX3 interacted with EWS/FLI, and RUNX3

suppression delayed anchorage independent growth of Ewing sarcoma cells in vivo. These data demonstrate an oncogenic role for RUNX3 in Ewing sarcoma.

Materials and Methods

Cell Culture—C2C12, COS, and A673 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM). A4573, MHH, TC-71, SK-ES, and RD-ES cells were cultured in RPMI-1640. SK-NMC cells were maintained in Minimum Essential Medium (MEM). All media were supplemented with 10% FBS, 100 Units/mL penicillin, and 100µg/mL streptomycin. Drs. Stephen Lessnick and David Loeb kindly provided Ewing sarcoma cell lines.

Plasmids—Flag-tagged RUNX3 constructs were previously described (Pande et al., 2009). FLI1-Flag was kindly provided by Tamara Nowling (Nowling et al., 2008). HA-EWS/FLI was generated by subcloning EWS/FLI into a pCMV5 expression vector containing an HA tag. PCR primers were designed to amplify EWS/FLI, with HindIII and XbaI restriction enzymes sites added to the ends of the sequences for cloning. The primer sequences are: Forward 5'-CCCAAGCTTGGCTCCACGGATTACAGTAC-3', and Reverse 5'-GCTCTAGACTACTGCTGCCCCGTAGCTGCTGC-3'.

Immunocytochemistry—C2C12 cells were plated on glass coverslips, and transiently transfected with the indicated expression constructs (pcDNA3, pcDNA3-RUNX3-Flag, pCMV5-HA-EWS/FLI) using Lipofectamine (Life Technologies). After 48 hours, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% TritonX-100, blocked in immunofluorescence buffer (3% BSA, 20mM MgCl₂, and 0.3% Tween-20 in PBS) and incubated with the anti-Flag (1:800, Sigma-Aldrich, M2 antibody) and anti-Flil (1:100, Santa Cruz, C-19) primary antibodies, followed by incubation with Alexa Fluor488-conjugated, goat anti-mouse and Alexa555-conjugated, goat anti-rabbit secondary antibodies (Life Technologies). The coverslips were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Images were collected on a Zeiss LSM510 confocal microscope.

Immunoprecipitation—Cos cells were transiently transfected with the indicated expression constructs using Lipofectamine (Life Technologies) and lysed in 1% TritonX-100 in PBS for five minutes on ice. All lysates were sonicated and cleared by centrifugation. Four percent of the volume was removed to measure protein expression levels before immunoprecipitation. Lysates were pre-cleared for 30 minutes with Protein G Dynabeads (Life Technologies) and immunoprecipitated with the indicated antibody. Bead-conjugated Flag antibodies (Sigma, M8823) were added to lysates for 1 hour. HA (Covance, MMS-101R) and FLI1 (Santa Cruz, SC-356) antibodies were incubated with the lysates overnight and protein G Dynabeads (Life Technologies) were added for 1 hour. Immunoprecipitants were washed three times with lysis buffer, and protein was eluted from beads in loading buffer (375mM Tris HCL, 9% SDS, 50% glycerol, 0.03% bromophenol blue, and β-mercaptoethanol) at 95°C.

Immunoblotting—Cells were lysed in modified RIPA buffer (50mM Tris-HCL, pH7.4, 150mM NaCl, 1%NP-40, 0.25% sodium deoxycholate, 1mM EDTA). Total protein was quantified using the detergent-compatible protein assay (Bio-Rad). Proteins lysates (25µg) and immunoprecipitated products were resolved by 10% SDS-PAGE, electro-transferred to Immobilon-P membranes (Millipore), blocked for 30 min in TBST containing 5% non-fat dried milk, and incubated in the indicated primary antibody overnight. Membranes were washed three times for ten minutes in TBST, incubated with HRP conjugated secondary antibodies (Santa Cruz Biotechnology), washed twice with TBST and once with TBS, and developed with ECL Prime (Amersham) and a FluorChem M imager (Cell Bioscience). The primary antibodies used are RUNX2 (AML3- as described in (Meyers et al., 1993)), RUNX3 (Abcam, R3-5G4, 1:1000), Tubulin (DSHB, E7,1:10,000), HA (Covance, MMS-101R, 1:1,000), Flag (Sigma, F1804, 1:1000), and FLI1 (Santa Cruz, SC-356, 1:1,000).

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs were performed with seven Ewing sarcoma cell lines as previously described (Matsumoto-Taniura et al., 1996). Briefly, cells were lysed in microextraction buffer (20mM HEPES pH 7.7, 450mM NaCl, 0.2mM EDTA, 0.5mM DTT, 25% glycerol), sonicated, and centrifuged to collect protein supernatants. Double-stranded DNA probes containing the RUNX binding sequence (5'-AATTCGAGTATTGTGGTTAATACG-3') (Meyers et al., 1993) were labeled with [α -³²P] dATP using Klenow polymerase (New England Biolabs). Lysates were incubated with radiolabeled probe, with or without 100-fold excess of unlabeled double-stranded DNA probes (cold competitor) as indicated. For supershift assays, 2µg of rabbit IgG, anti-RUNX1 (Meyers et al., 1993), anti-RUNX2 (AML3 (Meyers et al., 1993)), or anti-RUNX3 (Millipore, PC286)) was added to reactions as indicated. Samples were resolved on a 4% TBE gel, which was dried and imaged using a Typhoon FLA 7000 (GE Healthcare).

RNA Isolation and RT-qPCR—Four primary Ewing sarcoma tumors were flash frozen in liquid nitrogen, and crushed with mortar and pestle. RNA was extracted from these primary tumors and from cultured cells using TRIzol (Life Technologies) according to manufacturer's instructions. The RNA was reverse transcribed into cDNA using the SuperScript III First Strand Synthesis System (Invitrogen). The resulting cDNAs were placed into semi-quantitative real-time PCR (Bio-Rad iQ SYBR Green Supermix). Transcript levels were normalized to GAPDH levels. The sequences of primers used are: GAPDH (5'-ATGTTTCGTCATGGGTGTGAA-3' and 5'-TGTGGTCATGAGTCCTTCCA-3'), EWS/FLI (5'-GCACCTCCATCCTACCCTCCT-3' and 5'-AGGGTTGGCTAGGCGACTGCT-3'), RUNX3 (5'-AGGCAATGACGAGAACTACTCC-3' and 5'-CGAAGGTCGTTGAACCTGG-3'), CDKN1A/p21 (5'-TGTCCGTCAGAACCCATGC-3' and 5'-AAAGTCGAAGTTCCATCGCTC-3') NKX2-2 (5'-TGCCTCTCCTTCTGAACCTTGG-3' and 5'-GCGAAATCTGCCACCAGTTG-3'), EPHB3 (5'-AGCAACCTGGTCTGCAAAGT-3' and 5'-TCCATAGCTCATGACCTCCC-3').

Luciferase Assays—C2C12 cells were transiently transfected in triplicate with pOSE2-luc (a RUNX-responsive reporter), pRL-null, and the indicated expression plasmids

(pcDNA3, FLI1, EWS/FLI, RUNX3) using Lipofectamine (Life Technologies). After 48 hours, cells were lysed in passive lysis buffer (Promega), and the firefly and renilla luciferase activities were measured by dual luciferase assay (Promega) according to manufacturer's protocol. Firefly luciferase activities were normalized to renilla luciferase values.

Establishment of RUNX3 knockdown cells—RUNX3 Sigma Mission shRNAs (“sh813”: NM_004350.2-813s21c1 “sh1400”: NM_004350.2-1400s21c1, “sh3300”: NM_004350.2-3330s21c1) and a control non-target shRNA (shNT) were purchased from Sigma and transduced into A673 Ewing sarcoma cells with lentiviruses. Lentiviruses were made by transiently transfecting HEK 293T cells with the indicated shRNA construct, pMD-G (VSV-G), and EXQV with Lipofectamine 2000. Virus-containing supernatants were collected 72 hours later, filtered, and placed on A673 Ewing sarcoma cells in the presence of 6µg/mL polybrene. Cells were incubated with 0.4µg/mL of puromycin for 6 days to select for populations transduced with the virus. RUNX3 expression was determined by immunoblotting as described above.

High Throughput RNA Sequencing & Bioinformatic analysis—Total RNA was isolated from A673 Ewing sarcoma cells containing control shNT and RUNX3 sh813 shRNAs. Gene expression was determined with high throughput RNA sequencing using the TrueSeq method (‘poly A RNA Seq’). RNA libraries were prepared according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina). Briefly, poly-A mRNA was purified from total RNA using oligo dT magnetic beads. Purified mRNA was fragmented at 95°C for 8 minutes, eluted from the beads and primed for first strand cDNA synthesis. RNA fragments were copied into first strand cDNA using SuperScript III reverse transcriptase and random primers (Invitrogen). Next, second strand cDNA synthesis was performed using DNA polymerase I and RNase H. The double-stranded cDNA was purified using a single AMPure XP bead (Agencourt) clean-up step. The cDNA ends were repaired and phosphorylated using Klenow, T4 polymerase, and T4 polynucleotide kinase followed by a single AMPure XP bead clean-up. Blunt-ended cDNAs were modified to include a single 3' adenylate (A) residue using Klenow exo- (3' to 5' exo minus). Paired-end DNA adaptors (Illumina) with a single “T” base overhang at the 3' end were immediately ligated to the ‘A tailed’ cDNA population. Unique indexes, included in the standard TruSeq Kits (12-Set A and 12-Set B) were incorporated at the adaptor ligation step for multiplex sample loading on the flow cells. The resulting constructs were purified by two consecutive AMPure XP bead clean-up steps. The adapter-modified DNA fragments were enriched by 12 cycles of PCR using primers included in the Illumina Sample Prep Kit. The concentration and size distribution of the libraries was determined on an Agilent Bioanalyzer DNA 1000 chip. A final quantification, using Qubit fluorometry (Invitrogen), confirmed sample concentrations. Libraries were loaded onto paired end flow cells at concentrations of 8-10 pM to generate cluster densities of 700,000/mm² following the standard protocol for the Illumina cBot and cBot paired end cluster kit version 3. Flow cells were sequenced as 51 × 2 paired end reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and HCS v2.0.12 data collection software. Base-calling was performed using Illumina's RTA version 1.17.21.3. The RNA-Seq data were analyzed using MAPRSeq v.1.2.1, the

Bionformatics Core standard tool, which includes alignment with TopHat 2.0.6 (Kim et al., 2013) and gene counts with the HTSeq software. Normalization and differential expression analysis were performed using edgeR 2.6.2 (Robinson et al., 2010). Analysis was limited to genes with reads per kilobase per million (RPKM) ≥ 1 to exclude genes that are not expressed at an appreciable level. Data were deposited in the gene expression omnibus (GEO).

Soft Agar Colony Formation Assay—A base layer of 2mL 0.35% agarose (SeaPlaque GTG agarose, Lonza) in IMEM medium was plated in 35mm dishes. A673 cells (5×10^4) stably expressing either the NT shRNA or one of three RUNX3 shRNAs (sh813, sh1400, sh3300) were seeded in 0.35% agarose in IMEM medium containing 0.4 μ g/mL of puromycin and placed on top of the base layer. Cells were fed with IMEM medium containing 10% FBS, 100 Units/mL penicillin, 100 μ g/mL streptomycin, and 0.4 μ g/mL puromycin every 4 days for 12 days. Cells were plated in triplicate. Images were taken from three different fields from each plate. Colony size was quantified from digital images with image analysis software (Bioquant Osteo, Nashville TN), using a lower threshold of 300 square microns as the detectable limit for a colony.

Results

Ewing Sarcoma Cells Express RUNX3

The Ewing sarcoma fusion protein, EWS/FLI, binds the Runt domain of RUNX2, represses RUNX1 and RUNX2-driven transcription, and blocks osteoblast differentiation (Li et al., 2010a; Li et al., 2010b). Here we assessed expression levels of RUNX proteins (RUNX1, RUNX2, and RUNX3) in a panel of seven Ewing sarcoma cell lines (SK-ES, RD-ES, MHH, A673, TC-71, SK-N-MC, and A4573) by EMSA, which is a sensitive assay that measures functional protein levels through DNA interactions. RUNX-DNA complexes were detected in all of the Ewing sarcoma cells. Interestingly, the RUNX3 antibody produced supershifts in all seven cell lines, indicating that RUNX3 is expressed and binds DNA in these Ewing sarcoma cells (Figure 1A). Four cell lines (A673, TC-71, SK-ES, and A4573) also expressed RUNX2, but none expressed detectable levels of RUNX1. Western blotting confirmed RUNX3 and RUNX2 expression in the six cell lines tested (Figure 1B). RUNX3 mRNA was also detected in primary Ewing sarcoma tumors (Figure 1C). All four of these Ewing sarcoma tumors (ES1-4) expressed more RUNX3 mRNA than that observed in the Ewing sarcoma A673 cell line, which has high levels of RUNX3 protein. RUNX1 and RUNX2 mRNA are also expressed in primary tumors, but at lower levels than RUNX3.

RUNX3 Binds to EWS/FLI

To determine if RUNX3 binds to EWS/FLI, COS cells were transiently transfected with RUNX3-Flag and EWS/FLI-HA expression vectors. HA-EWS/FLI was easily detected in the Flag (RUNX3) immunoprecipitation complexes (Figure 2A). Reciprocally, RUNX3 was present in HA-EWS/FLI protein complexes (Figure 2B). A series of RUNX3 C-terminal truncation mutants revealed that the N-terminus and the RUNT domain are sufficient for interactions with EWS/FLI (Figure 2B), similar to what was observed with the RUNX2/EWS/FLI interaction (Li et al., 2010b). The interaction between RUNX3 and

EWS/FLI was confirmed by immunofluorescent co-localization of RUNX3-Flag and EWS/FLI-HA in C2C12 mesenchymal progenitor cells. Both proteins were predominantly present in the nucleus, and a large proportion of the RUNX3 co-localized with EWS/FLI (Figure 3). Together these data demonstrate that RUNX3 associates with EWS/FLI.

EWS/FLI Inhibits RUNX3 Transcriptional Activity

The functional significance of the interaction between EWS/FLI and RUNX3 was tested in a luciferase assay using the RUNX reporter, p6OSE2, which contains six RUNX binding elements driving luciferase expression. As expected, RUNX3 activated the reporter. EWS/FLI modestly repressed the basal activity of the RUNX reporter and blocked the RUNX3 dependent activation (Figure 4). In contrast, wildtype FLI1 activated the RUNX reporter in the presence or absence of exogenous RUNX3. These data indicate wildtype FLI1 cooperates with RUNX proteins present in these cells, but that EWS/FLI represses RUNX3 transcriptional activity.

RUNX3 Suppression Reduces Ewing Sarcoma Cell Growth in Soft Agar

The physical and functional interaction between RUNX3 and EWS/FLI suggested a role for RUNX3 in Ewing sarcoma tumorigenesis. To determine the requirement of RUNX3 for proliferation of Ewing sarcomas, we stably suppressed its expression in the A673 Ewing sarcoma cell line by RNA interference. Four cell lines were generated expressing a non-target shRNA (shNT), or each of three RUNX3 shRNAs: sh813, sh1400, sh3300. Compared to the shNT control, sh813 was the most efficient at reducing RUNX3 protein levels, but sh1400 and sh3300 also reduced RUNX3 expression (Figure 5A). Control or RUNX3-suppressed A673 cells were placed into soft agar and an anchorage-independent growth assay. After 12 days, control cells (shNT) produced large visible colonies. In contrast, cells containing any of the RUNX3 shRNAs were less efficient at forming colonies, with colony sizes being 60% smaller (Figure 5B and 5C). These data demonstrate RUNX3 promotes the tumorigenic potential of Ewing sarcoma cells. Gene expression analysis of shNT and sh813 cells indicated that 995 genes were expressed at 2-fold higher levels in the sh813 cells compared to shNT samples and 292 were expressed at 2-fold lower levels in the RUNX3 suppressed cells. The ten most differentially regulated genes in each list are shown in Table 1. The expression levels of three of the EWS/FLI target genes (NKX2-2, EPHB3, CDKN1 (p21)) were validated by qPCR using mRNA from the all three RUNX3 suppressed cells (sh813, sh1400 and sh3300) (Figures 5D-F). Together these data demonstrate that RUNX3 suppression disrupts expression of many EWS/FLI regulated genes and Ewing sarcoma growth.

Discussion

Ewing sarcomas are aggressive, poorly differentiated tumors. The majority harbor the recurrent chromosomal translocation t(11;22) and express the resulting fusion protein, EWS/FLI. EWS/FLI is a disordered protein, making it a challenge to design structure-based small molecule inhibitors (Erkizan et al., 2009; Erkizan et al., 2010). Identifying EWS/FLI interaction partners will enhance the development of targeted therapies and increase understanding of Ewing sarcoma etiology. EWS/FLI interacts with numerous protein

partners that contribute to altered cellular fate and tumor formation (Erkizan et al., 2010). We previously reported that EWS/FLI binds to the osteogenic transcription factor RUNX2 and inhibits differentiation of osteoblast progenitors (Li et al., 2010b). Here we demonstrate that only some Ewing sarcomas express RUNX2, but all express RUNX3. Like RUNX2, RUNX3 associates with EWS/FLI. Suppression of RUNX3 reduced growth of an Ewing sarcoma cell line in soft agar assays, and disrupted expression of EWS/FLI-regulated genes. These data indicate an oncogenic role for RUNX3 in Ewing sarcoma.

Physical interactions between RUNT and ETS proteins are common and well established. For example, RUNX1 binds ETS1 (Gu et al., 2000), FLI1 (Huang et al., 2009), NERF-2 and ELF-1 (Cho et al., 2004). ChIP-seq data show that RUNX and ETS1 recognition sequences are adjacent to each other in many promoters (Hollenhorst et al., 2009). We have shown that EWS/FLI associates with all three RUNX factors and represses RUNX-driven transcriptional activation of the p6OSE2-luc reporter, which contains six RUNX recognition sequences, but no ETS recognition sites. These data indicate that RUNX3 may recruit EWS-FLI to RUNX binding sites and expand the range of influence of EWS-FLI on the genome. While EWS/FLI suppresses activation by RUNX3, FLI1 activates RUNX-driven expression of the reporter. The FLI portion of the fusion protein appears to be sufficient for interactions with RUNX factors (data not shown). The cooperative effect of FLI1 with RUNX3 is consistent with the effects of other ETS factors on RUNX proteins (Kim et al., 1999). EWSR1 residues in the fusion protein likely alter co-factor interactions in Ewing sarcomas.

Our RNA-seq data indicate that RUNX3 may also affect EWS/FLI-dependent gene transcription. Notably, RUNX3 suppression disrupted the expression of numerous EWS/FLI-responsive genes and reversed their expression patterns (Table 2). For example, four genes (PPP1R1A, NKX2-2, EPHB3, and NR0B1) that EWS/FLI induces were repressed in RUNX3 suppressed cells, and twelve genes that EWS/FLI represses were expressed at higher levels RUNX3 shRNA cells. The exception to this reverse regulation was BCL11B, which was elevated with RUNX3 suppression and is activated by EWS-FLI (Wiles et al., 2013). Analysis of some EWS/FLI target genes whose expression levels are altered by RUNX3 suppression identified both RUNX3 and EWS/FLI binding sites in promoter sequences. The RUNX3 binding motif (TGT/CGGT) was present one or more times in the CDKN1 (p21), EPHB3, IER3, NKX2-2, PGF, and PHLDA1 promoters. The core EWS/FLI consensus sequence (AGGAA) was also present in the proximal promoters of the same genes (Guillon et al., 2009). The exception once again was the promoter of BCL11B, the only gene whose transcription was not reversed by RUNX3 suppression. It contains the core EWS/FLI binding element, but no RUNX3 binding sites. Additional gene expression and ChIP-seq experiments on Ewing sarcoma cells are needed to fully understand how RUNX3 affects EWS/FLI function and gene expression signatures of Ewing sarcomas, but these data suggest that the activities of these transcription factors are linked and contribute to gene expression signatures of Ewing tumors.

Our results suggest an oncogenic role for RUNX3 in Ewing sarcoma tumors. RUNX3 suppression by RNA interference inhibited growth of an Ewing sarcoma cell line. Enhanced expression of the cell cycle inhibitor and tumor suppressor CNDK1 (p21) in RUNX3-suppressed cells may contribute to growth suppression. Reduced expression of the

homeobox transcription factor, NKX2-2, may also contribute to the reduction in RUNX3 sh813 colony size because it is required for the Ewing sarcoma cell proliferation in soft agar (Owen et al., 2008; Smith et al., 2006). Although more investigation of these molecular mechanisms is needed, these data establish an important role for RUNX3 in Ewing sarcoma. Targeting RUNX3 or its target genes may be an effective strategy in reducing the oncogenic potential of EWS/FLI and reducing the growth of Ewing sarcoma tumors.

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Figure 1. Ewing sarcomas express RUNX3

A. Lysates from seven Ewing sarcoma cell lines were incubated with radiolabeled DNA probes containing RUNX binding sites in the presence or absence of an unlabeled probe (cold competitor) or antibodies to RUNX1, RUNX2, or RUNX3. *represents non-specific bands. B. Western blots of Ewing Sarcoma cell lines and the U2OS osteosarcoma cell line show expression levels of RUNX2 and RUNX3. C. Messenger RNA levels of RUNX1, RUNX2, and RUNX3 were measured by qPCR in four Ewing sarcoma primary tumors (ES1-4), and an Ewing sarcoma cell line (A673).

Figure 2. RUNX3 binds EWS/FLI

A. Cos cells were transfected with RUNX3-Flag and/or EWS/FLI-HA expression constructs and subjected to immunoprecipitation with FLAG antibodies and western blotting with FLAG or HA antibodies. B. Cos cells were transfected with an EWS/FLI-HA vector in the presence or absence of plasmid producing full-length (FL) RUNX3-Flag or C-terminal RUNX3 deletion. Extracts were immunoprecipitated with HA antibodies and western blotting was performed with FLAG or HA antibodies.

Figure 3. RUNX3 co-localizes with EWS/FLI in cell nuclei

C2C12 cells overexpressing RUNX3-Flag and EWS/FLI-HA or a control vector, were stained with Flag and FLI1 antibodies. DAPI counterstains were used to identify chromatin in cell nuclei.

Figure 4. EWS/FLI blocks RUNX3 transcriptional activity

C2C12 cells were co-transfected with p6OSE2-luc, pRL-luc, and the indicated expression constructs. Firefly luciferase values were normalized to renilla luciferase values. * $p < 0.05$ relative to vector only control in unpaired t-tests.

Figure 5. RUNX3 suppression reduces Ewing sarcoma cell growth in soft agar and disrupts EWS/FLI transcriptional activity

A. Western blot showing RUNX3 expression levels in A673 cells stably expressing one of three RUNX3 shRNAs (sh813, sh1400, sh3300) or a non-target shRNA (shNT). B. Representative images of soft agar colony sizes in A673 cell cultures expressing the indicated shRNAs. C. Average colony sizes of A673 cells expressing the indicated shRNAs after 12 days in soft agar. D-F. NKX2-2, EPHB3 and CDKN1 (p21) mRNA expression in A673 cells expressing the indicated shRNAs was measured by qPCR.

Table 1

Top differentially expressed genes in RUNX3-suppressed A673 Ewing sarcoma cells.

10 Most Induced Genes		10 Most Repressed Genes	
Gene	Fold Change (sh813/NT)	Gene	Fold Change (NT/sh813)
C2CD4A	48.09	SCARNA10	10.60
FAIM2	39.34	CAMSAP3	9.14
IL1B	25.33	GPR123	7.23
MMP1	23.84	SNORD59A	6.74
NT5E	18.65	MIR1470	6.74
DKK3	17.27	GNG7	6.46
F2RL2	12.81	CHST1	6.18
SNORD12	11.42	MATK	6.18
EBI3	10.90	PADI2	6.06
DKK1	10.53	CHP2	5.74

Table 2

Regulation of EWS/FLI target genes in RUNX3-suppressed A673 Ewing sarcoma cells.

Gene	Fold Change (sh813/NT)	Transcriptional Regulation by EWS/FLI	Reference(s)
RUNX3	0.28	Induced by EWS/FLI	(Smith et al., 2006)
PPP1R1A	0.39	Induced by EWS/FLI	(Smith et al., 2006)
NKX2-2	0.41	Induced by EWS/FLI	(Smith et al., 2006)
EPHB3	0.47	Induced by EWS/FLI	(Smith et al., 2006)
NR0B1	0.51	Induced by EWS/FLI	(Smith et al., 2006)
CDKN1A	1.69	Repressed by EWS/FLI	(Li et al., 2010c; Nakatani et al., 2003)
BCL11B	2.15	Induced by EWS/FLI	(Wiles et al., 2013)
PHLDA1	2.99	Repressed by EWS/FLI	(Smith et al., 2006)
IER3	3.21	Repressed by EWS/FLI	(Smith et al., 2006)
EHD3	3.99	Repressed by EWS/FLI	(Smith et al., 2006)
TGFBR2	4.50	Repressed by EWS/FLI	(Smith et al., 2006)
PTX3	5.10	Repressed by EWS/FLI	(Smith et al., 2006)
CRYAB	5.70	Repressed by EWS/FLI	(Smith et al., 2006)
ADM	5.93	Repressed by EWS/FLI	(Smith et al., 2006)
CD44	7.24	Repressed by EWS/FLI	(Smith et al., 2006)
PGF	7.85	Repressed by EWS/FLI	(Smith et al., 2006)
DKK1	10.53	Repressed by EWS/FLI	(Smith et al., 2006)
NTSE	18.65	Repressed by EWS/FLI	(Smith et al., 2006)