Ewing Family Tumors (EFTs) are characterized by a translocation between the RNA binding protein EWS and one of five ETS transcription factors, most commonly FLI1. The fusion protein produced by the translocation has been thought to act as an aberrant transcription factor leading to changes in gene expression and cellular transformation. In this study we investigated the specific processes EWS/FLI1 utilizes to alter gene expression. Using both heterologous NIH 3T3 and human EFT cell lines, we have demonstrated by quantitative pre-mRNA analysis that EWS/FLI1 repressed the expression of previously validated direct target genes at the level of transcript synthesis. Chromatin immunoprecipitation (ChIP) experiments showed that EWS/FLI1 decreases the amount of Pol II at the promoter of down regulated genes in both murine and human model systems. However in down regulated target genes, there was a significant disparity between the modulation of cognate mRNA and pre-mRNAs, suggesting that these genes could also be regulated at a post-transcriptional level. Confirming this, we found that EWS/FLI1 decreased the transcript half-life of IGFBP3, a down regulated direct target gene in human tumor derived Ewing’s sarcoma cell lines. Additionally, we have shown through re-expression experiments that full EWS/FLI1 mediated transcriptional repression requires intact EWS and ETS domains. Together these data demonstrate that EWS/FLI1 can dictate steady state target gene expression by modulating both transcript synthesis and degradation.

The Ewing Family Tumors (EFTs) are a family of malignant diseases that have traditionally been labeled Ewing’s Sarcoma, primitive neuroectodermal tumors (PNET), and Askin tumors (1). The genetic hallmark of EFTs is a reciprocal translocation between the N-terminus of the EWS gene and the C-terminus of one of five ETS transcription factor family members: FLI1, ERG, ETV-1, ETV4 or FEV (2-3). Forced expression of EWS/FLI1 results in transformation and tumorigenesis in NIH 3T3 cells (4). When either the EWS or FLI1 domains are deleted, transformation is lost (5). Also, when EWS/FLI1 expression is reduced via RNAi, EFT cell lines typically undergo growth arrest (6-7). Taken together, these data have bolstered the consensus view that EWS/ETS fusions are necessary both for EFT formation and for continued growth.

Functions of normal EWS and ETS proteins have molded current views of how EWS/ETS fusions promote cellular transformation. ETS proteins belong to a well characterized family of winged helix-loop-helix transcription factors that are related through a highly conserved 85 amino acid ETS domain. They typically work by forming heteromeric complexes with other transcription factors and can act as either transcription activators or repressors. The ETS domain mediates site-specific DNA binding, recognizing the GGA(A/T) consensus sequence, as well as facilitating protein-protein interactions (8-9). The ETS proteins are involved in a wide variety of functions that include cellular proliferation, differentiation, cell cycle control, development and tumorigenesis (8,10-11) (12-13). As a result of chromosomal translocation, the normal N-terminus of FLI1 is replaced by N-terminal sequences from EWS. However in all EFT fusions, the ETS domain is invariably present.

EWS is a member of the ubiquitously expressed TET family of proteins, consisting of FUS (TLS), EWS, and TAF15 (hTAFII68). The N-terminus of these proteins, which is retained in
EFT fusion proteins, is a highly disordered peptide consisting of a series of degenerate repeats with a consensus sequence of NSYGGQS (14). This domain can act as a potent transcriptional activator in heterologous model systems (15). The C-terminus of this family which is not present in EFT fusions, consists of an RNA-binding domain and several RGG-box domains. While the functions of TET proteins have yet to be completely elucidated, evidence of interactions with TFIIID, RNA Polymerase II (Pol II) (16-17) and elements of the RNA splicing machinery (18-19) indicate these proteins may play a role in transcription and/or mRNA splicing.

Given this information, the prevailing theory is that EWS/FLI1 causes transformation by acting as an aberrant transcription factor. Much of this theory is based on the replacement of the FLI1 activation domain with the more potent EWS activation domain while the FLI1 DNA-binding domain remains intact and functional. EWS/FLI1 has been shown to bind directly to the promoters of several of its target genes and also is able to modulate gene expression in reporter assays (20-21). Additionally, EWS/FLI1 retains the ability of native EWS to interact with Pol II (22). Despite this evidence, the explicit molecular mechanisms through which EWS/ETS fusions modulate gene expression remain ill defined.

Previous microarray and chromatin immunoprecipitation (ChIP) experiments have identified several direct targets of EWS/FLI1 in both human and murine cell lines. Uridine phosphorylase (Upp) was identified as a direct target gene necessary for tumorigenesis that is up regulated by EWS/FLI1 in NIH 3T3 cells (23). While most research has focused on genes up regulated there are actually more genes repressed by EWS/FLI1 in microarray analysis. In NIH 3T3 cells, thrombospondin 2 (Tsp2) is a direct target gene of EWS/FLI1 and several of its binding sites are present in the Tsp2 promoter (24). Forced expression of thrombospondins antagonized EFT tumor growth in a murine model system confirming the potential biologic impact of down regulated target genes. In EFT cell lines, insulin-like growth factor binding protein 3 (IGFBP3), an important regulator of cell proliferation and apoptosis, has been shown to be directly down regulated by EWS/FLI1 (25). Using these previously identified direct target genes in well characterized cellular model systems, we now find that EWS/ETS fusions can modulate target gene expression through both transcriptional and post-transcriptional mechanisms.

**Experimental Procedures**

**Plasmid Constructs and Viruses** - The retroviral triple FLAG-tagged EWS/FLI1 construct and all lentiviral EWS/FLI1 shRNA constructs were previously described (4,24). NIH 3T3 cells were transduced with retrovirus and then selected for 6-8 days in G418. A4573 and A673 cells were transduced with lentivirus and grown with no antibiotic selection. A4573 cells were harvested two days post-transduction. A673 cells were harvested between two days and two weeks post-transduction.

**RNA Analysis and Quantitative Real Time PCR** - RNA was harvested using the RNeasy MiniPrep kit (Qiagen, Hilden, Germany). To determine the half-life of RNA transcripts, cells were treated with 10μM of α-Amanitin and incubated for 0-16 hours before harvesting RNA. Approximately 2μg of total RNA was used to make first strand cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Primer sequences available in Supplementary Materials.

**Chromatin Immunoprecipitation (ChIP)** - Briefly, cells were crosslinked in 1% formaldehyde for 15 minutes before adding 0.14M glycyne to stop the reaction. Cells were washed serially with PBS, Mg-NI, Mg-NI-NP40 and Ca-NI before micrococcal nuclease digestion for 1 hour. Chromatin was spun down and resuspended in lysis buffer before sonication. For each IP, 2μg of antibody was added to 100ul of chromatin and 900ul of dilution buffer and then rotated overnight at 4°C. 50ul of Protein-A agarose beads were added to each IP reaction and then rotated for two hours at 4°C. The beads were washed 8 times for 5 minutes each prior to 2 10 minute elutions at 65°C. Inputs and elutions were incubated with RNase for 10 minutes and then Proteinase K for 1 hour at 56°C. The inputs and samples were incubated at 100°C for 15 minutes to reverse crosslinks and then column
purified for qRT-PCR. Detailed protocol and primer sequences are available in Supplementary Materials.

**Immunoblot and ChIP Antibodies** - Immunoblot antibodies used were monoclonal anti-FLAG M2 (Sigma, St Louis, MO, USA), anti-FLI1 (BD-Biosciences, Sparks, MD, USA) and anti-Actin C-11 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). ChIP antibodies used were N-20 (Santa Cruz) and Pol II (SA Biosciences).

**RESULTS**

**EWS/FLI1 partially regulates murine gene expression at the level of transcription.** Molecular genetic studies of EWS/FLI1 have relied on two types of model systems: (i) exogenous expression of EWS/FLI1 in heterologous cells; (ii) targeted inhibition of endogenous EWS/FLI1 in EFT cells. Since each approach has advantages and drawbacks, we utilized them both with the aim of identifying common mechanisms through which EWS/FLI1 regulates target genes. The murine NIH 3T3 cell line was chosen as a heterologous host because it is one of the few somatic cell lines that can tolerate stable expression of EWS/FLI1 without undergoing growth arrest or death. In addition, when transduced with EWS/ETS fusions, NIH 3T3 cells phenotypically change and take on an EFT-like morphology (26).

EWS/FLI1 regulates the expression of many genes (23-25,27-30). However these studies typically assessed the steady state levels of specific transcripts, which for each gene, is the net effect of mRNA synthesis balanced against mRNA destruction. Since intronic sequences are rapidly spliced out during transcription, the levels of pre-mRNA can be used as an assessment of newly made transcripts (31-32). By comparing pre-mRNA and mature mRNA it is possible to assess whether gene regulation can be attributed to changes in RNA synthesis or RNA stability. For each gene if pre-mRNA and mRNA levels parallel each other, these data would indicate that regulation occurs primarily through changes in RNA synthesis. Alternatively if levels of pre-mRNA are unchanged while mRNA is differentially regulated, the data would suggest that there is no change in the rate of RNA synthesis and that gene regulation is occurring at the level of RNA stability.

We have previously shown that in NIH 3T3 cells, EWS/FLI1 directly up regulates Upp and down regulates Tsp2 (23-24). Quantitative pre-mRNA analyses were performed to determine whether these expression changes were occurring at the level of transcription. NIH 3T3 cells were transduced with either a retrovirus containing FLAG-tagged EWS/FLI1 or an empty vector control (Tk neo) (Figure 1A). To compare the amount of Upp mRNA in NIH 3T3 cells expressing EWS/FLI1 and control cells, quantitative reverse transcriptase PCR (qRT-PCR) was employed using primers designed from adjacent exons, spanning intron 3. Cells expressing EWS/FLI1 showed a 12-fold up regulation of Upp mRNA compared to control cells. In similar fashion, primers spanning the up- and downstream intron/exon junctions of exon 5 were used to determine the amount of Upp pre-mRNA. These assays showed a nearly 8-fold up regulation of Upp pre-mRNA in NIH 3T3 cells expressing EWS/FLI1 compared to control cells (Figure 1B and C).

Microarray experiments have identified a larger set of down regulated genes than those up regulated by EWS/FLI1. While EWS/FLI1 may be modulating many of these genes indirectly, some of these genes have shown to be direct targets and to be involved in the tumorigenic effects of EWS/FLI1 (24-25). In order to determine whether down regulated direct targets are modulated by EWS/FLI1 in a similar manner to up regulated targets, levels of pre- and mature mRNAs of Tsp2 were determined in our NIH 3T3 model system.

Mature Tsp2 mRNA was found to be down regulated 8-fold by qRT-PCR using primers spanning the exon 6/7 boundary in NIH 3T3 cells expressing EWS/FLI1 compared to control cells. Using primers spanning the intron 6/exon 7 boundary, Tsp2 pre-mRNA was down regulated by EWS/FLI1 but only by 1.7-fold when compared to control cells (Figure 1B and D). While both Tsp2 mRNA and pre-mRNA were substantially down regulated by EWS/FLI1, Tsp2 pre-mRNA only demonstrated a down regulation of approximately 20% of the levels seen in mRNA.
In sum, these results indicate that EWS/FLI1 modulates the transcription of both up- and down regulated EWS/FLI1 target genes in an NIH 3T3 model system. However the finding that for Tsp2, the changes in pre-mRNA levels were less than its cognate mRNA, suggests that EWS/FLI1 may also modulate targets through mechanisms other than transcript synthesis.

**EWS/FLI1 alters transcription and RNA stability in EFT cells.** While NIH 3T3 cells have served as a tractable model system, there are several drawbacks inherent to their use. Though like EFTs, NIH 3T3 cells are of mesenchymal lineage, they are decidedly not the cell of origin of EFTs. Although microarray analyses have identified genes that are regulated by ectopic expression of EWS/FLI1 in NIH 3T3 cells, this gene expression profile does not match the profile seen in native EFT cell lines (33).

An alternative to using an EWS/FLI1 transduced heterologous system, is to start with EFT derived cell lines and knock down endogenous EWS/FLI1 by RNAi. This approach has the advantage of assessing EWS/FLI1 function in a native cell background. However, decreased expression of EWS/FLI1 typically results in progressive growth arrest of EFT cell lines within seven days. The use of engineered lentiviruses allowed for efficient introduction of shRNA constructs into EFT cell lines so assessment of target gene expression could be performed in unselected polyclonal transductants before growth arrest became apparent.

A lentiviral construct was created that contains a CMV driven GFP marker and a previously validated shRNA targeting the 3’ portion of EWS/FLI1 under the transcriptional control of the U6 polymerase III promoter (U6 818) (34). Viral stocks were used to knock down expression of EWS/FLI1 in two distinct EFT cell lines, A4573 and A673. A4573 cells like most EFT cell lines, undergo growth arrest after silencing of EWS/FLI1 expression. A673 cells continue to grow in anchorage dependent cell culture after knock down of EWS/FLI1.

A4573 and A673 cells were treated with either lentivirus containing the shRNA (U6 818) or an empty vector control (U6). The transduction efficiency was determined by GFP expression and was typically in the range of 80 to 90%. The ability of transduced shRNA constructs to attenuate EWS/FLI1 expression was determined by anti-FLI1 immunoblot and qRT-PCR (Figure 2A and data not shown). Typical EWS/FLI1 knock down efficiencies ranged from 60% to 85%. A4573 cells were harvested 48 hours after transduction since this was the optimal point of EWS/FLI1 knock down before the cells began to show signs of cell growth arrest. Transduced A673 cells grow and maintain significant EWS/FLI1 knock down for at least two weeks post-transduction, and could be harvested at any point during this time period.

Assessment of pre-mRNA and mRNA levels of IGFBP3, a gene previously shown to be down regulated by EWS/FLI1 in EFT cell lines, was performed by qRT-PCR (25). These assays used primers designed to span the exon 3/exon 4 junction of IGFBP3 to measure mRNA or exon 3/intron 3 junction to measure pre-mRNA (Figure 2B). In A4573s, the knock down of EWS/FLI1 released the suppression of IGFBP3, causing a 31-fold increase in expression of the mRNA. When primers were used to measure pre-mRNA, the increase of IGFBP3 only reached about 10-fold (Figure 2C). In A673 cells, the up regulation of IGFBP3 after EWS/FLI1 knock down was even more dramatic. IGFBP3 mRNA was up regulated 320-fold while its corresponding pre-mRNA rose 8-fold (Figure 2D).

These data were similar to results from our NIH 3T3 model system in demonstrating that for down regulated EWS/FLI1 targets, a decrease in RNA synthesis does indeed occur. However the fall in pre-mRNAs was significantly less than that seen for cognate mature mRNAs in all cell systems. These data suggest that EWS/FLI1 could be suppressing some - and perhaps many - of its target genes by modulating both transcript synthesis and degradation.

**EWS/FLI1 alters Polymerase II recruitment at Tsp2 in NIH 3T3 cells.** To assess how EWS/FLI1 might be modulating the transcript synthesis of target genes, RNA polymerase II (Pol II) was assayed by chromatin immunoprecipitation (ChIP). The recruitment of Pol II to the promoter is an important step of gene regulation that transcription factors commonly influence (reviewed in (35-36)). By reducing the amount of Pol II available at a
promoter, a transcription factor can reduce the expression of a gene.

ChIP assays were performed to determine Pol II occupancy at the promoters of Upp and Tsp2 in NIH 3T3 cells with and without EWS/FLI1 (Figure 3). Cells were fixed in situ with formaldehyde and harvested for chromatin. After shearing by sonication, cross-linked chromatin was immunoprecipitated with antisera recognizing the carboxy-terminal domain (CTD) of Pol II. Formaldehyde cross links were then reversed and the amount of enrichment over unpurified chromatin was determined by quantitative PCR (qPCR). GAPDH was used as a positive control since it is both highly expressed and its expression is unaffected by EWS/FLI1. The GAPDH promoter showed Pol II occupancy ranging from approximately 0.2 to 1%, reflecting experiment to experiment variability. However there was never a significant consistent difference between NIH 3T3 cells expressing EWS/FLI1 and control cells. In similar fashion, a region of mouse chromosome 6 (Untra6) that lacked any known genes was used as a negative control and consistently showed low background levels of Pol II occupancy ranging from 0.001% to 0.02%. In comparing relative Pol II occupancy of GAPDH and Untra6 across all experiments, there was a 50- to 80-fold difference, which is an indicator of the effective dynamic range of our assay.

Pol II occupancy was then assayed at the Upp and Tsp2 promoters to assess the effects of EWS/FLI1 on transcription initiation for an up regulated and down regulated target gene respectively. While Pol II occupancy at the Upp promoter was low in both EWS/FLI1 expressing cells as well as control cells, they still remained above the Untra6 negative controls. In a representative ChIP, the occupancy was measured as 0.012% and 0.014% in EWS/FLI1 expression cells and control cells, respectively (Figure 3A). Averaging across six ChIP experiments, no significant difference in Pol II occupancy at the Upp promoter could be seen between EWS/FLI1 and control cells (Figure 3B).

By contrast, ChIP analysis of the Tsp2 promoter consistently detected less Pol II occupancy in EWS/FLI1 expressing NIH 3T3 cells compared to controls. In a representative ChIP, control cells showed 0.28% occupancy of Pol II at the Tsp2 promoter while EWS/FLI1 expressing cells only showed 0.06% Pol II occupancy (Figure 3C). Over a series of three experiments, the expression of EWS/FLI1 resulted in an average 3.5-fold decrease in the amount of Pol II at the Tsp2 promoter (Figure 3D).

EWS/FLI1 modulates Pol II occupancy at the IGFBP3 promoter in EFT cell lines. We have demonstrated that EWS/FLI1 suppresses the expression of IGFBP3 pre-mRNA in EFT cell lines though not to the extent that it does IGFBP3 mature mRNA. Nevertheless this result suggests that EWS/FLI1 may be down regulating IGFBP3 at least partly at the level of transcript synthesis. To pursue this further, Pol II occupancy at the IGFBP3 promoter was determined by ChIP using human EFT cell lines in which EWS/FLI1 expression was modulated by RNAi. Similar to the NIH 3T3 model, the GAPDH promoter was used as a positive control and a region of the human chromosome 12 lacking known genes was used as a negative control (IGX). ChIP experiments starting with lentiviral transduction of EWS/FLI1 shRNA constructs and ending with site-specific qPCR readout, were performed at least in triplicate.

The A673 cell line showed the least increase in IGFBP3 pre-mRNA in response to EWS/FLI1 knock down and showed the lowest levels of Pol II occupancy (Figure 4A and B). A representative ChIP found the GAPDH promoter Pol II occupancy to be 0.40% and 0.45% for EWS/FLI1 knock down and control cells, respectively, while the negative was 0.041% and 0.035%. This represents an approximate 10-fold enrichment between positive and negative controls. The occupancy of Pol II at the IGFBP3 promoter was 0.084% and 0.04% in cells with and without EWS/FLI1 knock down. Over the course of three ChIPs the fold difference of the IGFBP3 promoter Pol II occupancy between EWS/FLI1 knock down and control cells averaged 2.2-fold (Figure 4B).

Similar results were obtained using the A4573 EFT cell line. The dynamic range between GAPDH and IGX negative location controls was again approximately 10-fold (Figure 4C and D). In a representative ChIP the percent
occupancy of the GAPDH promoter was 0.20% and 0.23% for EWS/FLI1 knock down and control cells, respectively and 0.034% and 0.025% for corresponding negative controls. The occupancy of Pol II at the IGFBP3 promoter was measured at 0.107% in EWS/FLI1 knock down cells and 0.059% in control cells. Averaging over three A4573 ChIPs showed a Pol II occupancy change of 1.8-fold in response to EWS/FLI1 knock down.

In addition to recruiting Pol II to promoters, EWS/FLI1 can modulate Pol II occupancy within the coding regions of an up regulated target gene (37). To determine whether this could also be occurring with down regulated target genes, Pol II occupancy was assessed within the coding region of IGFBP3 in A4573 cells.

In comparison to ChIPs assaying promoter Pol II occupancy, these experiments showed considerably less signal (Figure 4E and F). For a typical run, Pol II within GAPDH coding region showed 0.084% and 0.064% occupancy and the negative control only measured 0.009% and 0.005%. Nevertheless, there was a consistent enrichment of about 10-fold in Pol II within the actively transcribed gene over background, which matched the levels of enrichment seen with the promoter studies. In a typical experiment using primers to the first intron of IGFBP3 approximately 1kb downstream of the transcription start site, we found Pol II occupancy of 0.001% and 0.0012% in EWS/FLI1 knock down and negative control A4573 cells, respectively. Averaging over three experiments, modulation of EWS/FLI1 expression resulted in only a 1.4-fold difference in Pol II occupancy within the IGFBP3 gene. This result suggests that, at this location in the gene, any pause site or elongation rate was having only a small affect on the levels of transcription.

EWS/FLI1 alters IGFBP3 RNA half-life in the A673 EFT cell line. In both NIH 3T3 and EFT model systems, the degree to which EWS/FLI1 modulated down regulated target gene pre-mRNA levels was consistently less than that seen for the corresponding mature transcripts. Furthermore, the change in Pol II occupancy at target gene promoters in response to EWS/FLI1, appeared modest compared to the change in mRNA levels. These observations suggested that EWS/FLI1 might be changing the expression of some target genes at least in part, through post-transcriptional mechanisms.

To investigate this possibility further, the effect of EWS/FLI1 on target gene transcript degradation rates was assessed using α-Amanitin a specific Pol II inhibitor. The effect of EWS/FLI1 on IGFBP3 mRNA half-life in the A673 EFT cell line was studied primarily because these cells can continue to grow in cell culture even after knocking down EWS/FLI1 expression. As with our earlier expression studies, A673 cells were first transduced with either EWS/FLI1 or empty vector shRNA constructs. After assaying transduction and knock down efficiencies, unselected polyclonal transductants were treated with α-Amanitin. Cells were then harvested at time points ranging from 0 to 16 hours post treatment and IGFBP3 mRNA levels were determined by qRT-PCR. Log transformed mRNA levels were plotted against time. The slope of this curve was used to calculate IGFBP3 transcript half-life. In order to account for any cell death over the duration of the time course, levels of the 18S rRNA, a Pol I transcript unaffected by α-Amanitin, was used to normalize the results. From start to finish, this experiment was repeated six times.

In A673 cells, the knockdown of EWS/FLI1 resulted in a consistent increase in the half-life of IGFBP3 mRNA (Figure 5). While the measured half-lives ranged from 5 to 14 hours in A673 cells transduced with empty vector, cells in which EWS/FLI1 knocked down varied between 15 to 47 hours. For each of the six experiments, the ratio of the IGFBP3 half-life with and without EWS/FLI1 knock down, was calculated (Figure 5A). The geometric mean of the ratio over all six experiments indicated that EWS/FLI1 decreased IGFBP3 mRNA half-life 2.9-fold (95% confidence limits 1.8-4.9).

Intact EWS and ETS domains are required for full repression of IGFBP3 by EWS/FLI1. Using an RNAi knock down strategy, it is possible study EWS/FLI1 target gene regulation in a native EFT cell context. To extend this model further, knock down re-expression studies were performed to identify critical domains in the EWS/FLI1 fusion necessary for repression of IGFBP3. In addition
to wild type EWS/FLI1, two mutant fusions were used representing loss of function of EWS or FLI1 domains respectively. In EWS(DAF)/FLI1, critical tyrosine residues contained in the degenerate hexapeptide repeats of the EWS transcriptional activation domain are changed to alanine, resulting in a mutant that is defective in both transcriptional activation and cellular transformation (38). The EWS/FLI1(TPM) mutant contains alteration of three amino acids within the FLI1 ETS domain: R337N, R340N, Y341V. As a result this mutant has lost the capacity to directly bind to DNA and to transform cells (39).

A673 cells were first transduced with lentiviruses containing a GFP expression construct and an shRNA (H1 EF4), that specifically targeted the 3’ untranslated region found on endogenous EWS/FLI1 transcripts but not on those from exogenous constructs (40). Flow sorted (>95% GFP positive) polyclonal populations were expanded and transduced with amphotropic retroviruses containing intact or mutant EWS/FLI1. Cells transduced with either empty lentiviral (H1) or retroviral (Tk neo) vectors served as negative controls. After antibiotic selection, polyclonal populations were harvested for protein and total RNA. The expression of EWS/FLI1 and IGFBP3 transcripts was determined using qRT-PCR. These experiments were repeated in duplicate starting from shRNA knock down through IGFBP3 quantitation.

Transduction of the HI EF4 shRNA construct resulted in an average 220-fold increase in IGFBP3 mRNA which is similar to that found with the U6 818 shRNA (Figure 6A). Re-expression of wild-type EWS/FLI1 resulted in a 15-fold decrease in IGFBP3 mRNA levels compared to the Tk neo empty vector control further validating that EWS/FLI1 transcriptionally represses this target gene (Figure 6B). When the EWS(DAF)/FLI1 mutant was expressed, a 3-fold decrease in IGFBP3 mRNA levels was observed. In contrast, expression of the EWS/FLI1(TPM) mutant resulted in a 2-fold increase in IGFBP3 mRNA levels. Immunoblots of transduced cells showed that mutant constructs were expressed at levels greater than wild type EWS/FLI1 indicating that the loss of function seen with the mutant fusions was not simply due to protein instability (Figure 6C). Together these data demonstrate that both EWS and ETS domains are necessary for full down regulation of IGFBP3 mRNA.

**DISCUSSION**

For the last 15 years, the hypothesis that EWS/FLI1 transforms cells by acting as an aberrant transcription factor has stood. However trying to mechanistically dissect how these fusion proteins function at a molecular genetic level has been notoriously difficult. With this in mind, we began our investigation from a broad perspective by querying whether EWS/FLI1 regulated target genes at a transcriptional or post-transcriptional level. To do this we compared the levels of mRNA and pre-mRNA of EWS/FLI1 direct target genes in both murine and human model systems. Our results in both systems fit the view that EWS/FLI1 can modulate target gene expression both at the level of transcript synthesis and at the level of mRNA degradation.

The strategy of using pre-mRNA levels as a proxy for new transcript synthesis has been previously validated by other investigators. However this approach has limitations. First since pre-mRNA levels are assayed by qRT-PCR using site-specific primers spanning intron-exon borders, it assumes that EWS/FLI1 does not significantly alter splicing rates at each specific location. Second, it is well known that for each gene, there is intron to intron variability in splicing rates. This makes it difficult to derive quantitative meaning from comparisons of pre-mRNA and mRNA levels. For example, EWS/FLI1 down regulates IGFBP3 mRNA levels 40-fold more than pre-mRNA levels in A673 cells. However this difference can only be interpreted qualitatively that both transcriptional and post-transcriptional regulation is occurring. The relative proportion of each mechanism cannot be gleaned from this assay.

To gain further insight into transcript synthesis dynamics of EWS/FLI1 target genes, Pol II ChIP analyses were performed. The relative low level of enrichment of Pol II at the promoters of Upp and Tsp2 in NIH 3T3 cells and IGFBP3 in EFT cell lines when compared to GAPDH, suggests that with or without EWS/FLI1, these genes are being transcribed at
modest rates. In spite of showing an almost 8-fold increase in Upp pre-mRNA in response to EWS/FLI1 in NIH 3T3 cells, there was no significant increase in Pol II occupancy. This suggests that in this cellular context, EWS/FLI1 may be exerting its effect on Upp at stages distal to recruitment of Pol II such as promoter escape, elongation rate, or movement through pause sites.

In contrast to Upp, we found that, for down regulated target genes, EWS/FLI1 did alter promoter Pol II occupancy in both NIH 3T3 and EFT model systems. Though they were reproducible, the differences in Pol II occupancy were small, approximately 3.5-fold for Tsp2 in NIH 3T3 cells and about 2-fold for IGFBP3 in EFT cell lines. EWS/FLI1 may also change the Pol II occupancy within the IGFBP3 coding region in A4573 cells but the changes were near the limit of detectability. Due to the lower level of IGFBP3 Pol II ChIP enrichment in A673 cells, we were unable to determine the level of Pol II occupancy in the IGFBP3 coding region. Together with our pre-mRNA analyses, these data suggest that EWS/FLI1 down regulates some target genes through a decrease in transcript synthesis.

Though transcript synthesis may be playing a role in dictating the steady-state levels of EWS/FLI1 down regulated targets, our pre-mRNA studies suggested that additional regulatory mechanisms might be at work as well. We confirmed this by finding that in A673 cells, EWS/FLI1 decreased the half-life of IGFBP3 transcripts by almost 3-fold. Though there was considerable experiment to experiment variation, the decrement in half-life was clearly statistically significant. The relatively high degree of variation is probably due to the serial manipulations needed in our model system, EFT cells first being lentivirally transduced with shRNA constructs and then treated with α-Amanitin. By sequentially coupling these two procedures, the net effect on overall variability of the assay is cumulative.

When we tried to perform similar analyses with the A4573 EFT cell line, we were unable to obtain consistent results. This may be due to the fact that A4573 cells like most EFT cell lines, undergo growth arrest in response to EWS/FLI1 knock down. Factoring in this additional parameter could have increased the overall variability of the assay to the point that it was no longer interpretable. Alternatively, half-life modulation of EWS/FLI1 down regulated target genes may take time to develop fully. Qualitatively comparing the change in IGFBP3 pre-mRNA and mRNA levels in response to EWS/FLI1 knock down in the two EFT cell lines, suggests that this might be true. Though the change in pre-mRNA in both cell lines was about 10-fold, IGFBP3 mRNA changed approximately 30-fold in A4573 cells that by necessity were harvested two days after EWS/FLI1 knock down, where an over 300-fold change was found in A673 cells that could be harvested two weeks after knock down. In effect, the ability of A673 cells to survive in tissue culture with greatly reduced levels of endogenous EWS/FLI1, allowed us to uncover an additional mechanism of target gene modulation.

On a mechanistic level, the loss of repression of IGFBP3 in A673 cells expressing a shRNA construct targeting endogenous EWS/FLI1, can be reestablished by transducing EWS/FLI1 which drives down IGFBP3 mRNA levels. However neither transduction of EWS nor ETS EWS/FLI1 mutants were able to fully repress IGFBP3 to the extent seen with the wild type fusion. This suggests that both these domains play functional roles in this process perhaps by promoting protein-protein and DNA binding interactions through EWS and ETS domains respectively. The fact that IGFBP3 levels consistently increased in cells transduced with the EWS/FLI1(TPM) construct, also suggests that this mutant might be acting in a dominant negative fashion by sequestering factors involved with EWS/FLI1 target gene repression.

There are multiple ways through which cells can regulate transcript degradation rates ranging from modulating components of the mRNA metabolism machinery, to altering expression of specific microRNAs. For example, EWS/FLI1 has been shown to down regulate miRNA145 in human pediatric mesenchymal stem cells (41). Though explicitly defining the components through which EWS/FLI1 modulates target gene half life is beyond the scope of this manuscript, such investigations will depend on having well characterized EFT model systems that can exist in the absence of
EWS/FLI1 for more than one or two days. This will be a challenge.

Our work suggest that EWS/FLI1 may be able to induce large gene expression changes by causing smaller alterations in multiple stages of gene regulation. The regulation of casein by prolactin is a case in point of such synergy (42). Prolactin was able to up regulate casein mRNA 6-12-fold but only increased casein transcription by 2-4-fold. On further investigation, the authors also found that prolactin increased casein mRNA half-life and concluded that both mechanisms were necessary for proper regulation.

EWS/FLI1 may work in a similar multifaceted manner, at least for some down regulated target genes. From a pragmatic point of view, this is not a welcoming prospect. Studying multiple regulatory mechanisms each with a limited dynamic response to EWS/FLI1, will certainly be more challenging than investigating a single mode of action with a large dynamic range. If indeed EWS/FLI1 is working via multiple molecular mechanisms, effective therapeutic targeting of this fusion could prove to be difficult. The key to meeting these challenges will be to devise better EFT model systems in which EWS/FLI1 target gene modulation can be studied at multiple mechanistic levels.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. EWS/FLI1 can modulate target gene pre-mRNA and mRNA expression in NIH 3T3 cells. 
(a) Immunoblot analysis of NIH 3T3 cells transduced with triple FLAG-tagged EWS/FLI1 or empty vector control (Tk neo). EWS/FLI1 could be detected up to two months post-transduction. (b) Schematic of primer design to distinguish between mRNA and pre-mRNA for Upp and Tsp2. (c) qRT-PCR analysis of mRNA and pre-mRNA mean fold difference in transcript level of Upp in NIH 3T3 cells transduced with EWS/FLI compared to empty vector. (d) qRT-PCR analysis of mRNA and pre-mRNA levels of Tsp2 in NIH 3T3 cells transduced with EWS/FLI or empty vector control. To best show difference in down regulated genes, the Tsp2 data was log2 transformed. Upp and Tsp2 qRT-PCR levels were normalized to GAPDH. Error bars depicted calculated standard deviations.

Figure 2. EWS/FLI1 knock down de-represses IGFBP3 to a greater extent at mRNA level than at pre-mRNA level in EFT cell lines. (a) Immunoblot of EFTs transduced with shRNA against EWS/FLI1 (U6 818) demonstrating reduced EWS/FLI1 expression versus empty vector (U6). A4573 lysates were harvested 48 hours post-transduction. A673 lysates were harvest 48 hours and 7 days post-transduction. A4573 cells contain a type III EWS/FLI1 fusion which is slightly larger than the type I fusion found in A673 cells. (b) Schematic of primers designed to distinguish IGFBP3 mRNA and pre-mRNA. (c, d) mean difference in IGFBP3 mRNA and pre-mRNA levels by qRT-PCR analysis, in A457 or A673 cells transduced with EWS/FLI1 shRNA or empty vector. IGFBP3 qRT-PCR values were normalized to GAPDH.

Figure 3. Pol II occupancy in response to EWS/FLI1 differs in up and down regulated target genes in NIH 3T3 cells. (a) qPCR analysis of representative Pol II ChIP at the Upp promoter in NIH 3T3 cells. Pol II percent occupancy was determined for the promoter region of positive control (GAPDH), negative control (Untra6), and Upp. (b) Mean Pol II percent occupancy fold change at Upp promoter in response to EWS/FLI1 averaged over six independent ChIP experiments. (c) qPCR analysis of representative Pol II ChIP at the Tsp2 promoter in NIH 3T3 cells. Pol II percent occupancy was determined for the promoter region of positive control (GAPDH), negative control (Untra6), and Tsp2. (d) Mean Tsp2 promoter Pol II percent occupancy fold change in response to EWS/FLI1 averaged over three independent ChIP experiments.

Figure 4. EWS/FLI1 decreases Pol II occupancy in the promoter and coding regions of IGFBP3 in EFT cell lines. (a) qPCR analysis of a representative Pol II ChIP at the IGFBP3 promoter in A673 cells treated with either EWS/FLI1 targeted shRNA (U6 818) or empty vector (U6). Pol II occupancy was determined for the promoter region of positive control (GAPDH), negative control (IGX), and the promoter region of IGFBP3. (b) Mean IGFBP3 promoter Pol II occupancy fold change averaged over three independent A673 ChIP experiments. (c) qPCR analysis of a representative Pol II ChIP at the IGFBP3 promoter in A4573 cells. (d) Mean IGFBP3 promoter Pol II occupancy fold change over three independent A4573 ChIP experiments. (e) qPCR analysis of a representative Pol II ChIP at the coding region of IGFBP3 in A4573 cells. (f) Mean Pol II occupancy fold change over three independent A4573 IGFBP3 coding region ChIP experiments.

Figure 5. EWS/FLI1 alters the RNA half-life of IGFBP3 in A673 cells. (a) Six independent experiments were performed to determine the RNA half-life of IGFBP3 in A673 cells. The fold difference was calculated from the difference between the slopes of the RNA decay rate in A673 cells transduced with shRNA targeting EWS/FLI1 compared to empty vector control cells. (b) The geometric mean of fold change across all six experiments graphed with the 95% confidence interval.
Figure 6. Re-expression of wild-type EWS/FLI1 and EWS mutant suppresses IGFBP3 expression while introduction of ETS domain mutant increases mRNA levels. (a) Mean difference in EWS/FLI1 and IGFBP3 levels by qRT-PCR analysis in A673 cells transduced with lentiviral shRNA against EWS/FLI1 (H1 EF4) or empty vector control (H1). Total RNA was harvested 14 days post-transduction. EWS/FLI1 and IGFBP3 qRT-PCR values were normalized to GAPDH. Error bars represent range of experimental values. (b) Mean difference in IGFBP3 mRNA levels by qRT-PCR analysis in A673 cells transduced with lentiviral shRNA against EWS/FLI1 (H1 EF4), then transduced with retroviral empty vector (Tk neo), EWS mutant (DAF), FLAG-tagged ETS mutant (TPM), or triple FLAG-tagged wild-type EWS/FLI1. Levels are relative to A673 H1 EF4 cells transduced with retroviral empty vector. IGFBP3 qRT-PCR values were normalized to GAPDH. (c) FLI1 and FLAG immunoblot analysis of EWS/FLI1 levels in A673 cells transduced as described in (b). Lysates were harvested 14 days post-lentiviral transduction. FLI1 immunoblot reveals exogenous EWS/FLI1 constructs display higher levels of expression than the endogenous protein. Higher wild type EWS/FLI1 signal in FLAG immunoblot compared to TPM mutant is due to the presence of a triple FLAG-tag. The DAF mutant is not FLAG-tagged therefore no signal is observed.
Figure 3

A 3T3

% Pol II Occupancy

GAPDH Untra6 Upp

EWS/FLI1 Tk neo

B

Fold Difference

Upp

C 3T3

% Pol II Occupancy

GAPDH Untra6 Tsp2

EWS/FLI1 Tk neo

D

Fold Difference

Tsp2
Figure 4

A

% Pol II Occupancy

Promoter: GAPDH  IGX  IGFBP3

B

Fold Difference

IGFBP3

C

% Pol II Occupancy

Promoter: GAPDH  IGX  IGFBP3

D

Fold Difference

IGFBP3

E

% Pol II Occupancy

Coding Region: GAPDH  IGX  IGFBP3

F

Fold Difference

IGFBP3
Figure 6

(A) Fold Difference
- H1
- H1 EF4

(EWS/FLI1, IGFBP3)

(B) Fold Difference
- EWS/FLI1
- IGFBP3

(C) Western Blots
- Tk neo
- DAF
- TPM
- EWS/FLI1

α-FLI1
α-actin
α-FLAG
α-actin
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