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Expression profiling of EWS/FLI identifies *NKX2.2* as a critical target gene in Ewing's sarcoma

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Summary

Our understanding of Ewing's sarcoma development mediated by the EWS/FLI fusion protein has been limited by a lack of knowledge regarding the tumor cell of origin. To circumvent this, we analyzed the function of EWS/FLI in Ewing's sarcoma itself. By combining retroviral-mediated RNA interference with reexpression studies, we show that ongoing EWS/FLI expression is required for the tumorigenic phenotype of Ewing's sarcoma. We used this system to define the full complement of EWS/FLI-regulated genes in Ewing's sarcoma. Functional analysis revealed that *NKX2.2* is an EWS/FLI-regulated gene that is necessary for oncogenic transformation in this tumor. Thus, we developed a highly validated transcriptional profile for the EWS/FLI fusion protein and identified a critical target gene in Ewing's sarcoma development.

Introduction

Ewing's sarcoma is a highly aggressive bone-associated tumor of children and young adults. Most cases of Ewing's sarcoma have a recurrent chromosomal translocation, t(11;22)(q24;q12), that encodes the EWS/FLI fusion protein (Delattre et al., 1992). The FLI portion contains an ETS family DNA binding domain, while the EWS portion functions as a strong transcriptional activation domain (Delattre et al., 1992; Lessnick et al., 1995; May et al., 1993a, 1993b). EWS/FLI is thus an aberrant transcription factor that dysregulates target genes involved in tumor development.

One difficulty in understanding EWS/FLI function has been the lack of an appropriate model system in which to study the fusion. Because the cell of origin of Ewing's sarcoma is unknown, most studies have relied on heterologous cell types with uncertain relevance to the human disease. For example, when introduced into NIH3T3 mouse fibroblasts, EWS/FLI causes oncogenic transformation (May et al., 1993a). When introduced into other cell types, such as primary human or mouse fibroblasts, EWS/FLI causes growth arrest or cell death (Deneen and Denny, 2001; Lessnick et al., 2002). Cellular background is therefore important in determining the phenotypic response to EWS/FLI expression. This suggests that there may be different gene expression changes associated with EWS/FLI expression related to the cell type used.

A number of potential EWS/FLI target genes have been identified in heterologous cell systems (e.g., Braun et al., 1995; Deneen et al., 2003; Lessnick et al., 2002; May et al., 1997; Thompson et al., 1996). However, it has been difficult to define the role of these target genes in Ewing's sarcoma development. Much of this difficulty has been due to the relatively subtle oncogenic effects of many of these targets in NIH3T3 cells, such as *MFNG*, *EAT2*, and *UPP* (Deneen et al., 2003; May et al., 1997; Thompson et al., 1996). Indeed, the subtle oncogenic effects of these targets have suggested a model for Ewing's sarcoma development whereby EWS/FLI upregulates multiple weak oncogenes, and it is only through the additive effects of these targets that cancer development occurs. This model predicts that loss of any one of these targets in Ewing's sarcoma cells would have only a minimal effect on oncogenic transformation.

Rather than studying EWS/FLI in a heterologous cell type, we developed a system to analyze the effects of EWS/FLI in Ewing's sarcoma itself. We used this system to determine the genes that

SIGNIFICANCE

Ewing's sarcoma is an important model for pediatric cancer development because it is virtually defined by a single oncogenic event, the EWS/FLI fusion. EWS/FLI is an aberrant transcription factor, but only a few targets have been identified, and these have mostly come from studies in heterologous cell types. We describe a generalizable approach to study the fusion in Ewing's sarcoma itself and use this system to comprehensively define the transcriptional profile of EWS/FLI. We identified a critical target gene that has not been previously implicated in cancer development. The approach we describe has clear advantages over prior model systems and can be used to study other oncogenic transcription factors in their "native" context as well.

are dysregulated by EWS/FLI (both directly and indirectly). Functional studies revealed that one of these, *NKX2.2*, is absolutely required for oncogenic transformation in Ewing's sarcoma cells but is not sufficient to mediate oncogenesis by itself. This supports a model in which EWS/FLI upregulates multiple target genes, and each gene contributes a nonredundant function toward cancer development. This has diagnostic and therapeutic implications for this important pediatric cancer.

Results

EWS/FLI is required for Ewing's sarcoma oncogenic transformation

To analyze the effects of EWS/FLI in its native cellular background, we used retroviral-mediated RNAi to "knock down" endogenous fusion expression. We designed a construct (designated EF-2-RNAi) directed against the 3′ untranslated region (3′ UTR) of EWS/FLI (Figure 1A). Wild-type FLI is not expressed in Ewing's sarcoma cells (Figure 1B), and so EF-2-RNAi targets only EWS/FLI. Retroviruses targeting luciferase or ERG, neither of which are expressed in these cells (data not shown), served as negative controls and were designated luc-RNAi and ERG-RNAi, respectively. Following infection and puromycin selection of A673 Ewing's sarcoma cells, we found that EF-2-RNAi effectively knocked down both EWS/FLI transcript (Figure 1C) and protein levels (Figure 1D).

To investigate the function of EWS/FLI in Ewing's sarcoma, the phenotype of A673 Ewing's sarcoma cells harboring the EF-2-RNAi construct was assessed. These cells maintained a growth rate that was identical to cells infected with control retroviruses (Figure 2A). The level of EWS/FLI expression in the knockdown cells was the same at the beginning and the end of the growth curve time course (data not shown). Thus, the normal growth of A673 cells harboring the EF-2-RNAi construct was not due to silencing or loss of the construct. Over a prolonged period of time (weeks to months), we detect diminished activity of the EF-2-RNAi construct, suggesting either a subtle growth effect that is not detected in the assays performed above, or nonspecific silencing of the RNAi construct occurs (data not shown).

To determine if oncogenic transformation is dependent on EWS/FLI, we infected A673 cells or 293EBNA (transformed human embryonic kidney cells that lack EWS/FLI) with EF-2-RNAi or control retroviruses. Following selection, cells were seeded in soft agar to assess oncogenic transformation. We found that A673 cells were severely restricted in their colony forming efficiency by the EF-2-RNAi (Figure 2B). Loss of transformation was dependent on the presence of EWS/FLI because 293EBNA cells were unaffected by the EF-2-RNAi retrovirus (Figure 2B).

We next performed xenograft tumor formation assays. A673 Ewing's sarcoma cells were engineered to express the luciferase protein and then infected with the EF-2-RNAi retrovirus (or ERG-RNAi as a control). The control cells formed large tumors in immunodeficient mice that could be observed with bioluminescent imaging (Figure 2C). EF-2-RNAi-infected cells formed smaller tumors that were delayed in development as compared to control cells (Figure 2C). The EF-2-RNAi tumors that formed were "escapee" tumors that lost the RNAi effect and regained EWS/FLI expression (Figure 2D). We did not note a correlation between the level of reexpressed EWS/FLI protein and tumor size in the escapee tumors. Taken together, these data demonstrate that ongoing EWS/FLI expression is required for the transformed phenotype of Ewing's sarcoma cells.

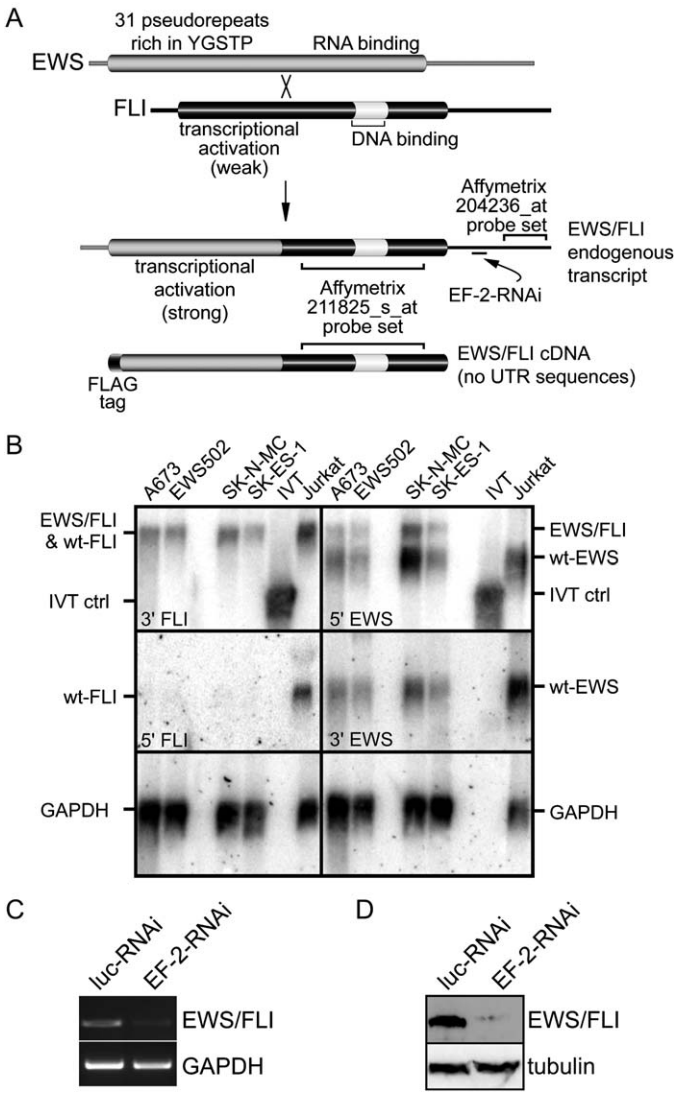

Figure 1. Targeting of EWS/FLI by retroviral RNAi

A: Schematic diagram of wild-type EWS, wild-type FLI, and the EWS/FLI fusion transcripts. The locations of two Affymetrix probe sets that interrogate EWS/FLI expression levels are indicated. The location of the EF-2-RNAi is also indicated. A depiction of the EWS/FLI cDNA is provided.

B: Northern blot analysis of mRNA isolated from the Ewing's sarcoma cell lines A673, EWS502, SK-N-MC, and SK-ES-1 demonstrates that EWS/FLI is detected by both the 3′ FLI and 5′ EWS probes. Wild-type EWS is detected by both the 5′ EWS and 3′ EWS probes in all cell lines tested. Wild-type FLI is only detectable in Jurkat T cells using the 5′ FLI probe, but not in any of the Ewing's sarcoma cell lines. IVT indicates in vitro transcribed EWS/FLI transcript as a control. GAPDH is shown as a loading control.

C: Infection of A673 Ewing's sarcoma cells with the EF-2-RNAi retrovirus results in significantly decreased transcript levels as compared to control luc-RNAi retrovirus, as determined by semiquantitative RT-PCR. GAPDH amplifies equally from each sample.

D: Infection of A673 Ewing's sarcoma cells with EF-2-RNAi retrovirus results in a significant reduction in protein levels as compared to control luc-RNAi retrovirus, as determined by Western blot using an anti-FLI antibody. Tubulin is shown as a loading control.

To determine if the loss of transformation is due to knockdown of EWS/FLI, or to "off-target" or other nonspecific effects, we

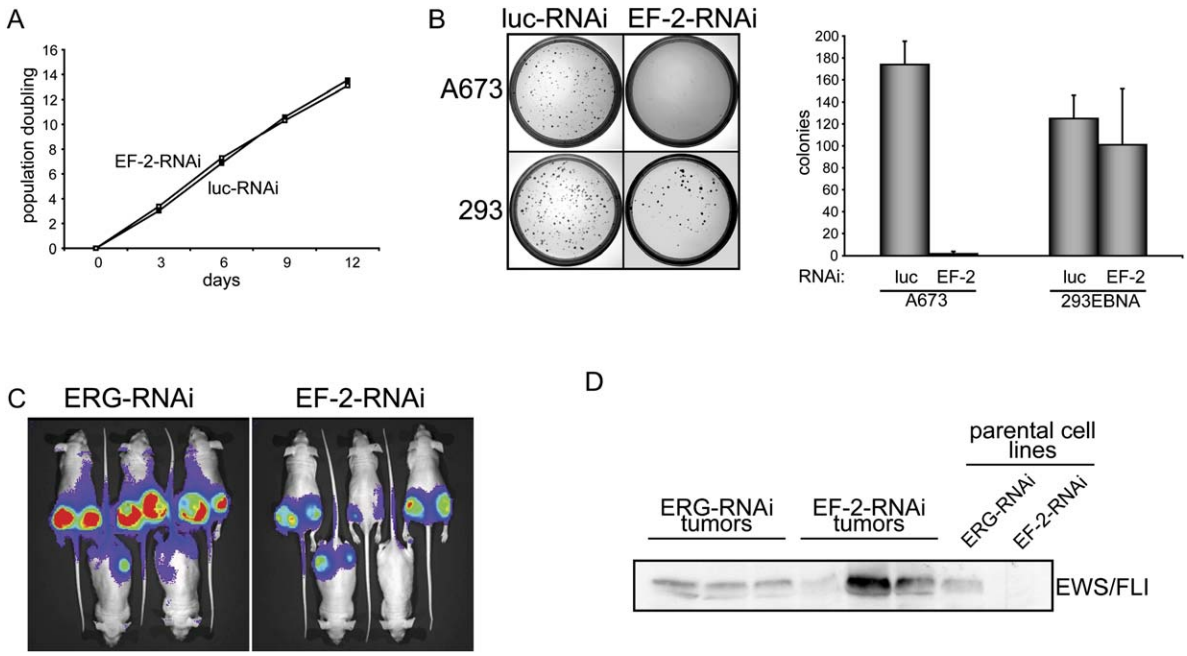

Figure 2. RNAi against EWS/FLI blocks transformation

A: Growth assays (3T5 assays; Lessnick et al., 2002) for A673 Ewing's sarcoma cells infected with either the EF-2-RNAi or the luc-RNAi control are shown.

B: Knockdown of EWS/FLI with the EF-2-RNAi blocks transformation in A673 Ewing's sarcoma cells, but not in control 293 cells, as measured by soft agar assay. Error bars indicate standard deviation.

C and D: Xenograft growth of A673 cells harboring the EF-2-RNAi is reduced as compared to those infected with ERG-RNAi retrovirus, as measured by bioluminescent imaging. However, the tumors that form have reexpressed EWS/FLI protein, as measured by Western blot with an anti-FLI antibody. The parental A673 cell lines containing each RNAi retrovirus are shown as a comparison.

performed "knockdown/rescue" experiments. A673 cells infected with the EF-2-RNAi retrovirus were infected with a second retrovirus containing an EWS/FLI cDNA. Because the cDNA does not contain the 3′ untranslated region of FLI (the region to which the RNAi is directed), the EF-2-RNAi construct does not affect this exogenous transcript (see Figure 1A). We found that wild-type EWS/FLI completely rescued the transformed phenotype, but the R2L2 DNA binding mutant could not (Figure 3A; Bailly et al., 1994).

We extended this analysis to include an inducible EWS/FLI cDNA construct. We generated a clonal A673 cell line (clone 1-1, hereafter designated "Tet-A673") that contained the FLAG-tagged EWS/FLI cDNA under the control of a tetracycline-repressible promoter (Figure 3B). This clone had undetectable background levels of FLAG-tagged EWS/FLI when uninduced, produced detectible tagged EWS/FLI by 12 hr of induction, and demonstrated maximal expression by 24 hr (Figure 3C). The amount of induced EWS/FLI was very similar to endogenous wild-type levels (Figure 3B).

We repeated the knockdown/rescue experiments with Tet-A673 cells containing either the ERG-RNAi control or EF-2-RNAi retroviruses. Growth assays demonstrated that uninduced Tet-A673 cells grew similarly with either retroviral construct, supporting the results obtained in the noninducible cells (Figure 3D). Induction of EWS/FLI in the presence of the EF-2-RNAi construct did not change the growth rate, as expected (Figure 3D). Interestingly, when EWS/FLI expression was induced in the presence of the control ERG-RNAi construct (thus adding excess EWS/FLI protein in addition to endogenous levels of the fusion), cell growth was significantly diminished, demonstrating that supraphysiologic levels of EWS/FLI are a hindrance to cell growth (Figure 3D). The same effect was observed in cells that did not contain the ERG-RNAi retrovirus, thus demonstrating that this was not a synthetic effect related to the retroviral construct (data not shown).

Soft agar assays demonstrated that inducible EWS/FLI rescued the loss of transformation caused by EF-2-RNAi (Figure 3E). The transformation observed in the uninduced cells with the EF-2-RNAi is likely due to incomplete suppression of the inducible construct in setting of 3 weeks of growth in soft agar. We observed evidence of "leaky" expression in soft agar previously (Lessnick et al., 2002). Furthermore, expression of exogenous EWS/FLI in the presence of endogenous EWS/FLI (i.e., supraphysiologic expression levels) prevented growth in soft agar, again supporting the notion that EWS/FLI levels must be tightly regulated for growth and transformation. These data further support the hypothesis that ongoing EWS/FLI expression is required for the transformed phenotype of Ewing's sarcoma.

Transcriptional signature of EWS/FLI

Next, the gene expression profile of EWS/FLI as it relates to oncogenic transformation was determined. We, and others, have previously shown that significant transcriptional consequences are associated with changes in cell growth (e.g., Lessnick et al., 2002; Zhang et al., 2004). Because A673 cells expressing the EWS/FLI knockdown construct maintain normal growth even though they are no longer transformed (Figures 2A and 3B), there should be minimal contribution of growth effects to the transcriptional profile.

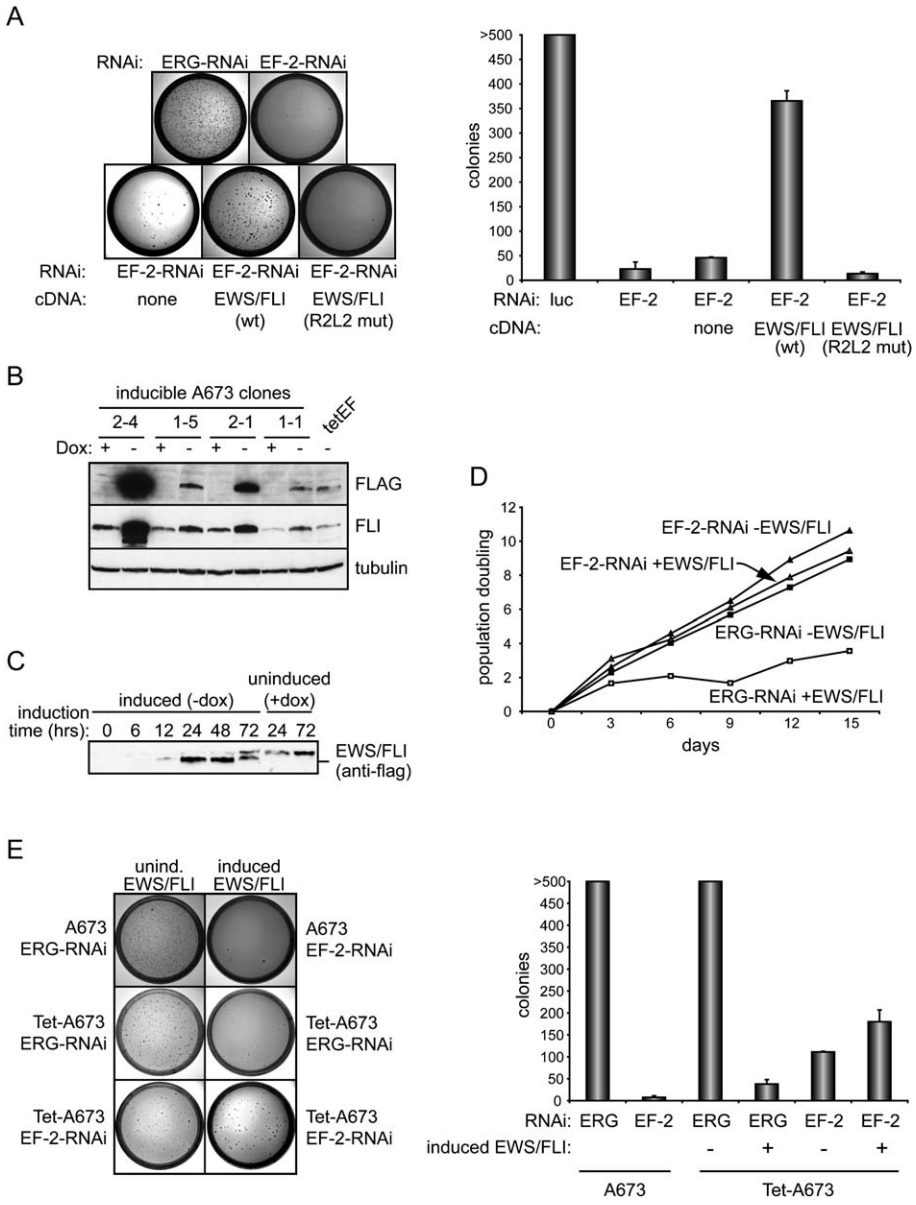

Figure 3. Rescue of EWS/FLI RNAi by an EWS/FLI cDNA

A: A673 cells infected with EF-2-RNAi are inhibited for oncogenic transformation, as compared to cells infected with the control ERG-RNAi retrovirus, as measured by soft agar assays. The transformation defect in A673 cells with EF-2-RNAi is rescued following expression of wild-type (wt) EWS/FLI cDNA, but not with an empty retrovirus (no cDNA) or the EWS/FLI DNA binding mutant R2L2. Error bars indicate standard deviation of duplicate plates.

B: Induction of exogenous EWS/FLI in four different inducible A673 clones in response to doxycycline removal. Western blotting with an anti-FLAG antibody only measures the inducible protein, while blotting with anti-FLI measures both the basal levels of EWS/FLI and the inducible fusion levels. Clone 1-1 (subsequently called "Tet-A673") induces similar levels of EWS/FLI as are induced in tetEF cells, which have been previously shown to express wild-type levels of EWS/FLI protein (Lessnick et al., 2002). Tubulin is shown as a loading control.

C: Western blotting with an anti-FLAG antibody demonstrates that inducible EWS/FLI protein is detectable by 12 hr postinduction and reaches maximal levels by 24 hr in Tet-A673 cells. A nonspecific background band is present at a slightly higher molecular weight than the EWS/FLI band. This band appears to correlate with either cell density or time in culture, as it is present in the later, but not in the earlier, time points. It is not EWS/FLI as it is present in both induced and uninduced samples.

D: 3T5 growth analysis reveals that Tet-A673 cells infected with the EF-2-RNAi retrovirus grow nearly identically in the presence (+EWS/FLI) or absence (−EWS/FLI) of induced EWS/FLI protein. Tet-A673 cells infected with a control ERG-RNAi retrovirus grow well without induced EWS/FLI protein but become severely growth restricted following induction of additional EWS/FLI.

E: Soft agar assays demonstrate that Tet-A673 cells harboring the EF-2-RNAi construct form colonies in soft agar following the induction of EWS/FLI. Tet-A673 cells harboring the ERG-RNAi control become restricted in their colony forming ability following expression of inducible EWS/FLI protein. Error bars indicate standard deviation of duplicate plates.

While we have demonstrated that off-target effects do not mediate the loss of transformation observed in A673 cells infected with the EF-2-RNAi retrovirus, off-target effects may still be present (Jackson et al., 2003; Huppi et al., 2005). To control for this, we identified a second EWS/FLI RNAi construct (designated EF-4-RNAi) that provides significant levels of EWS/FLI knockdown and also disrupts oncogenic transformation (data not shown). We reasoned that different RNAi constructs will have distinct off-target effects, and identification of genes that are altered similarly by both RNAi constructs would control for these (Huppi et al., 2005).

A673 cells infected with either the EF-2-RNAi or EF-4-RNAi viruses were prepared, in duplicate with their associated controls, and were subjected to microarray analysis. Because these cells were grown in tissue culture for several weeks, these experiments assessed the transcriptional changes that were stably altered by the EWS/FLI fusion. We called these cells "stable knockdown" cells.

To identify genes that were up- or downregulated in the presence of the EWS/FLI RNAi constructs, we sorted genes using the signal-to-noise metric followed by permutation testing. We found that EWS/FLI upregulated 320 genes and downregulated 1151 genes at the 95% confidence level (Figure 4A). The fact that EWS/FLI downregulated so many more genes than it upregulated was counterintuitive since prior work (performed in murine fibroblasts) suggested that EWS/FLI functions as a transcriptional activator to mediate oncogenic transformation (Lessnick et al., 1995; May et al., 1993b).

The "stable knockdown" results are likely to include both direct and indirect EWS/FLI target genes. To determine which genes are directly regulated by EWS/FLI, we attempted to perform chromatin immunoprecipitation experiments. Unfortunately, we

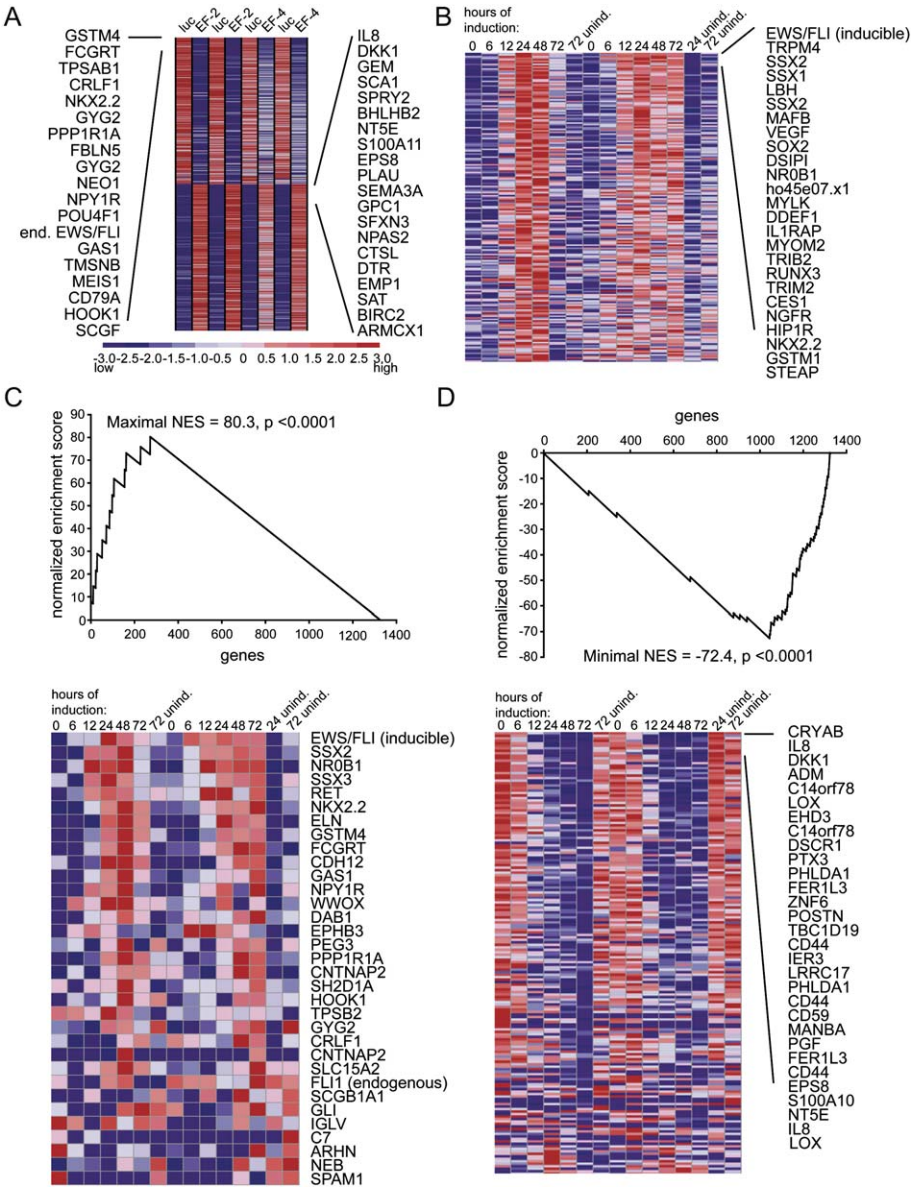

Figure 4. Microarray analysis of A673 cells with EWS/FLI RNAi

A: Oligonucleotide microarray data of A673 cells with either EF-2-RNAi, EF-4-RNAi, or luc-RNAi control. Genes were rank ordered using the signal-to-noise metric. The top 20 genes that either increase with increased EWS/FLI (left side list) or decrease with increased EWS/FLI (right side list) are shown. ''End. EWS/FLI'' indicates the probe set that is measuring endogenous EWS/FLI transcript levels. The color scale is shown (and is used for all subsequent oligonucleotide expression data figures).

B: Microarray data of Tet-A673 cells harboring the EF-2-RNAi retrovirus, and subsequently induced to express the EWS/FLI cDNA for the time periods shown. Columns labeled ''72 unind.'' and ''24 unind.'' indicate samples that were grown for the specified times but were not induced to express EWS/FLI. Genes were rank ordered using the Pearson correlation coefficient on the basis of their similarity to reexpression of EWS/FLI. The top 25 genes are listed.

C: Gene set enrichment analysis (GSEA) using the 33 genes that were upregulated by EWS/FLI in all four replicates from Figure 4A as the gene set (see text for details). The rank-ordered list was the list from Figure 4B. The high maximal normalized enrichment score (NES) with the low p value indicates highly significant similarity between the two data sets. The color plot representation of the genes is shown as well (lower portion of panel).

D: GSEA using the 180 genes that were downregulated by EWS/FLI in all four replicates from Figure 4A as the gene set. The rank-ordered list was the list from Figure 4B. The very low minimal NES with the very low p value again indicates highly significant similarity between the two data sets. The color plot representation of the genes is shown as well (lower portion of panel).

found unacceptably high levels of background immunoprecipitation with commercially available EWS/FLI antibodies and were unable to adequately complete these experiments.

As an alternate approach, we used an ''inducible rescue'' experiment to enrich for genes that are likely to be direct EWS/FLI targets. Endogenous EWS/FLI was knocked down with the EF-2-RNAi retrovirus in Tet-A673 cells. The exogenous EWS/FLI cDNA was then induced, and samples were collected at various times after induction, processed, and hybridized to oligonucleotide microarrays.

Induction of the exogenous EWS/FLI transcript was monitored with the 211825_s_at probe set (see Figure 1A). We identified 1326 genes with expression changes of at least 2.5-fold and rank ordered these based on similarity of expression to the induced exogenous EWS/FLI transcript using the Pearson correlation coefficient as the distance metric (Figure 4B).

To compare the ''stable knockdown'' and ''inducible rescue'' experiments, we performed a modified gene set enrichment analysis (GSEA; Mootha et al., 2003). We first identified the most reproducibly altered genes in the ''stable knockdown'' A673 cells described above. Using a 2.5-fold change cutoff value, we identified 33 genes that were upregulated, and 180 genes that were downregulated by EWS/FLI in each of the four replicates (see Figure 4A). We then compared these to the 1326 rank-ordered genes from the ''inducible rescue'' cells.

If the two data sets are well correlated, we expect the EWS/FLI-regulated genes in the stable knockdown experiment to be enriched at the top of the rank-ordered list from the inducible rescue experiment. This correlation was quantified using a normalized running sum statistic called the normalized enrichment score (NES). The maximal and minimal NES were determined. The best possible NES is 100 (indicating perfect correlation), and the worst possible NES is −100 (indicating perfect inverse correlation). An empiric p value was derived based on the number of times a maximal NES (or minimal NES) was obtained from 10,000 randomly chosen gene sets that was the same, or

greater than, the experimentally determined value. Additional details are presented in the Supplemental Data available with this article online.

Comparison of the 33 EWS/FLI-upregulated genes to the inducible rescue experiment yielded a maximal NES of 80.3 ($p < 0.0001$), indicating excellent correlation (Figure 4C). Comparison of the 180 EWS/FLI downregulated genes to the inducible rescue experiment gave a minimal NES of −72.4 ($p < 0.0001$), indicating a highly significant inverse correlation as one would expect for downregulated genes (Figure 4D). Thus, the results from the stable knockdown and inducible rescue experiments are highly similar. We therefore conclude that the vast majority of EWS/FLI-regulated genes are regulated in the same time frame as EWS/FLI, suggesting that many of these are likely to be direct EWS/FLI target genes.

To validate the microarray data using an alternate technique, and to extend the results to other Ewing's sarcoma cell lines, we performed quantitative RT-PCR (qRT-PCR) analyses on a random set of genes from Figure 4C. EWS/FLI was knocked down in A673, SK-N-MC, and EWS-502 Ewing's sarcoma cells with the EF-2-RNAi retrovirus. Amplification of each gene was compared in these cells to control cells expressing the luc-RNAi control. We found that knockdown of EWS/FLI resulted in similar decreases in each gene tested (Table S1). These results confirm the initial microarray data and suggest that EWS/FLI regulates similar genes in other Ewing's sarcoma cell lines as well.

Inspection of the data revealed that EWS/FLI upregulated multiple genes related to neural differentiation, consistent with the neural crest phenotype of Ewing's sarcoma tumors. These included *NKX2.2*, *NPY1R*, *RET*, *EPHB3*, *DAB1*, *CDH12*, and *CNTNAP2*. These data support the previous assertion that EWS/FLI itself induces the neural phenotype of Ewing's sarcoma, rather than the phenotype occurring as a consequence of the tumor's cell of origin (Teitell et al., 1999).

Identification of *SSX* family members (which are fused to *SYT* in synovial sarcoma, another sarcoma of uncertain origin) suggests the potential for common mechanisms of oncogenic transformation across the fusion-associated solid tumors of adolescents and young adults. The upregulation of *GYG2*, which can nucleate the initial step of glycogen formation through autoglucosylation, suggests a molecular mechanism for the high levels of glycogen observed in Ewing's sarcoma (Mu et al., 1997; Navas-Palacios et al., 1984). We also identified upregulation of the *SH2D1A* gene, which encodes an SH2-domain-only protein that is highly similar to *EAT2*, which has been previously identified as an EWS/FLI target gene (Braun et al., 1995). *EAT2* itself was not represented on the microarray used.

With respect to other previously identified EWS/FLI target genes, *TGFBR2* was repressed by EWS/FLI, as previously reported (Hahm et al., 1999). *MYC*, *ID2*, *MFNG*, *KRT15*, *UBE2C*, *CYP2F1*, and *CDKN1C* were not significantly altered (Arvand et al., 1998; Bailly et al., 1994; Dauphinot et al., 2001; Fukuma et al., 2003; May et al., 1997). Uridine phosphorylase (*UPP1*), *MMP3* (stromelysin 1), and *PDGFC* were downregulated, rather than upregulated as previously reported (Braun et al., 1995; Deneen et al., 2003; Zwerner and May, 2001). It should be noted that many of these were identified in alternate models of EWS/FLI expression (e.g., in NIH3T3 cells) rather than in Ewing's sarcoma itself.

To extend the analysis of previously reported EWS/FLI target genes beyond those listed above, we compared the microarray data we obtained in A673 Ewing's sarcoma cells to publicly available data generated in two alternate systems: human rhabdomyosarcoma cells and primary human fibroblasts expressing inducible EWS/FLI protein (RD-EF and tet-EF, respectively; Hu-Lieskovan et al., 2005b; Lessnick et al., 2002). The list of EWS/FLI-upregulated genes in each data set was compared to the EWS/FLI-upregulated genes in both the stable knockdown and the inducible rescue A673 cells using chi-square analysis. We found small but highly significant overlaps between EWS/FLI-regulated genes in both of these heterologous models and the A673 systems (Table 1; see Supplemental Data for details of the analysis and complete data sets). Thus, the A673 system identified genes that were found in other EWS/FLI model systems, supporting the validity of our model. The A673 model also identified many genes that were not observed in these heterologous systems, thus demonstrating the importance of this model.

Table 1. Comparisons between A673 and other model systems

	RD-EF	Tet-EF
A673 stable knockdown	5.2×10^{-4}	1.5×10^{-3}
A673 inducible rescue	2×10^{-8}	1.4×10^{-4}

The gene expression data from the A673 "stable knockdown" (Figure 4A) and "inducible rescue" (Figure 4B) were compared to the EWS/FLI-upregulated genes identified in human rhabdomyosarcoma cells and primary human fibroblasts expressing inducible EWS/FLI (RD-EF and tet-EF cells, respectively; Hu-Lieskovan et al., 2005b; Lessnick et al., 2002). The chi-square p values for the overlapping gene sets are shown. A complete description of the analysis and a full presentation of the data are provided in the Supplemental Data.

Comparison of EWS/FLI signatures to Ewing's sarcoma

Ewing's sarcoma is highly associated with the EWS/FLI oncoprotein. If the genes we identified are valid targets, they should also be expressed in Ewing's sarcoma, but not in other pediatric tumors. To test this, we compared our data with publicly available microarray data on small round blue cell tumors of childhood (SRBCT; Khan et al., 2001), which included Ewing's sarcoma. The genes in the SRBCT data set were sorted to distinguish between Ewing's sarcoma and the other tumors, using the signal-to-noise metric as a distance measure (Lessnick et al., 2002). The data sets were mapped to their UniGene identifiers to allow for comparisons between different microarray platforms.

The stable knockdown and inducible rescue rank-ordered lists were compared to the SRBCT list using the Spearman correlation coefficient (Table 2). When used in this way, the Spearman coefficient quantifies the correlation of gene rank position between data sets. An empiric p value was derived by repeatedly shuffling the rank order of one of the two data sets in a pair, and determining the number of times that a correlation coefficient was obtained that was higher than the experimentally determined coefficient.

While both of our data sets showed significant similarity to the human tumor data set, the inducible rescue data were more closely correlated to the human tumor data than the stable knockdown data, with a Spearman correlation coefficient of 0.43 versus 0.30, respectively (Table 2). These values were highly statistically significant, with p values of <0.0001 for each. Thus, the inducible rescue data more accurately identified

Table 2. Spearman correlation coefficient comparisons between "stable knockdown," "inducible rescue," and small round blue cell tumor data

SRBCT versus	Spearman correlation coefficient	p value	Number of common genes
A673 stable knockdown	0.30	<0.0001	313
A673 inducible rescue	0.43	<0.0001	209

The complete gene expression data from Figure 4A (stable knockdown) and Figure 4B (inducible rescue) were rank ordered, as described in the text, and compared to data from small round blue cell tumors (Khan et al., 2001). The Spearman correlation coefficient is shown. The p value was an empirically determined value based on 10,000 randomly generated rank orders of the same data. The number of common genes indicates how many genes were present in each pairwise comparison.

Ewing's sarcoma-specific genes than the stable knockdown data.

NKX2.2 is required for oncogenic transformation

Because loss of EWS/FLI results in loss of transformation, EWS/FLI-regulated genes should include those required for Ewing's sarcoma development. Because prior data suggested that transcriptional activation is critical to the function of EWS/FLI as an oncoprotein, we focused our efforts on genes that were upregulated by the fusion (Figure 4C). Using an RNAi approach to analyze the oncogenic role of candidate genes, we identified NKX2.2 as a critical mediator of transformation mediated by EWS/FLI (see below).

NKX2.2 is a homeobox containing-protein that has roles in neuronal development but has never been implicated as having a role in tumorigenesis (Briscoe et al., 1999). As shown in Table S1, NKX2.2 transcript levels are regulated by EWS/FLI in multiple Ewing's sarcoma cell lines. NKX2.2 protein is also decreased following knockdown of EWS/FLI in multiple Ewing's sarcoma cell lines (Figure 5A and data not shown). Thus, *NKX2.2* is an EWS/FLI target in multiple Ewing's sarcoma cell lines, although whether it is directly, or indirectly, regulated by EWS/FLI remains to be determined.

We developed a retroviral NKX2.2 RNAi construct (designated NKX-RNAi), and introduced it into A673 Ewing's sarcoma cells. This construct resulted in a 73% reduction of endogenous NKX2.2 transcript levels (data not shown), which is similar to the 84% reduction of NKX2.2 transcript levels following EWS/FLI knockdown. NKX2.2 protein levels were similarly reduced by NKX-RNAi (Figure 5B).

Knockdown of NKX2.2 had minimal effects on the growth rate of A673 Ewing's sarcoma cells (Figure 5C). There was no significant increase in cell death observed (data not shown). This is similar to what we observed with EWS/FLI knockdown (Figure 2A).

Introduction of NKX-RNAi into multiple Ewing's sarcoma cells resulted in a near complete loss of oncogenic transformation both in soft agar assays (Figure 5D) and in a xenograft model of Ewing's sarcoma (Figure 5E). Transformation was rescued when NKX2.2 was reexpressed using a cDNA that does not contain the endogenous 3′ UTR and so is unaffected by the RNAi (Figure 5F). Taken together, these data show that NKX2.2 is necessary for oncogenic transformation in Ewing's sarcoma.

To determine if NKX2.2 is sufficient for transformation, we knocked down EWS/FLI with the EF-2-RNAi retrovirus and reintroduced NKX2.2 expression from the cDNA-containing retroviral vector. We found that NKX2.2 could not rescue the loss of transformation resulting from EWS/FLI knockdown (Figure 5G). We also found that NKX2.2 did not induce oncogenic transformation in NIH3T3 murine fibroblasts (data not shown). Thus, although NKX2.2 is necessary for oncogenic transformation in Ewing's sarcoma, it is not sufficient.

We reasoned that, if *NKX2.2* were a critical EWS/FLI target gene, then it should be expressed in patient-derived primary Ewing's sarcoma tumor samples as well. We performed reverse-transcriptase polymerase chain reaction (RT-PCR) for NKX2.2 in four Ewing's sarcoma tumor samples. NKX2.2 was expressed in each of the tumor samples, but not in normal human fibroblasts (Figure 6A). We then analyzed the expression pattern of NKX2.2 in a recently published microarray data set containing 181 sarcoma tumor samples, including 20 Ewing's sarcomas (Baird et al., 2005). NKX2.2 was expressed in most (18 of 20) Ewing's sarcoma samples, but was expressed in only 7 of 161 of the other tumors (Figure 6B). In this data set, then, NKX2.2 is an excellent marker of Ewing's sarcoma, with a sensitivity of 90% and a specificity of 96%. These data support the assertion that *NKX2.2* is a critical EWS/FLI target gene required for oncogenic transformation in Ewing's sarcoma and also suggest that NKX2.2 may serve as a diagnostic marker for this disease.

Discussion

The identification of EWS/FLI over a decade ago suggested that more specific and less toxic therapies for Ewing's sarcoma would be feasible (Delattre et al., 1992). Unfortunately, this hope has not been realized. One particular difficulty has been the uncertainty regarding Ewing's sarcoma's cell of origin and the lack of an appropriate cell type in which to study the fusion protein. We circumvented this difficulty by analyzing EWS/FLI in patient-derived Ewing's sarcoma cells using a loss-of-function approach with retroviral-mediated RNAi.

We found that ongoing expression of EWS/FLI is absolutely required for tumorigenesis in Ewing's sarcoma. Loss of EWS/FLI resulted in loss of transformation, while reexpression of EWS/FLI rescued this phenotype. In contrast to prior studies conducted in NIH3T3 cells (Jaishankar et al., 1999; Welford et al., 2001), we found that the DNA binding function of EWS/FLI was required for its oncogenic function. In addition, our results demonstrate that EWS/FLI does not function via a "hit and run" mechanism whereby it initiates the transformation process and then is no longer required. This finding also demonstrates that EWS/FLI expression does not result in "oncogene addiction," whereby the brief inactivation of an oncogene is sufficient to result in sustained loss of the tumorigenic phenotype (Jain et al., 2002). Because EWS/FLI is required for the oncogenic phenotype of Ewing's sarcoma, targeting the fusion may be a useful therapeutic modality for this disease, which is supported by a recent publication (Hu-Lieskovan et al., 2005a).

Interestingly, EWS/FLI expression is not required for the growth of A673 Ewing's sarcoma cells in tissue culture. In these cells, at least, the oncogenic function of EWS/FLI has been dissociated from other potential growth effects. We have noticed that growth is adversely effected by loss of EWS/FLI in some Ewing's sarcoma cell lines (data not shown). The differences in cellular background that determine cellular response to loss

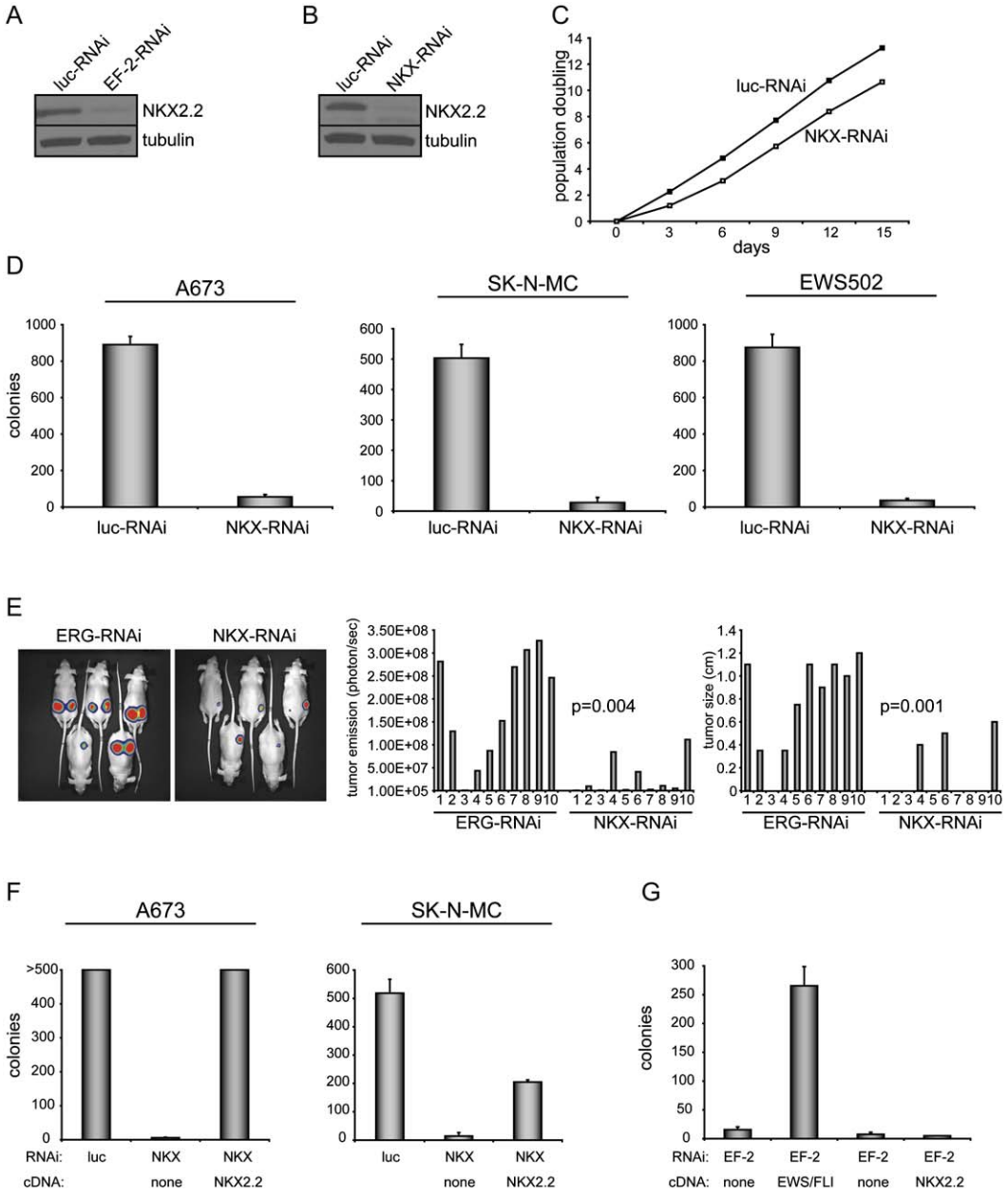

Figure 5. *NKX2.2* is a critical EWS/FLI target gene required for oncogenesis

A: Western blot of A673 cells harboring the EF-2-RNAi retrovirus, or the luc-RNAi control, demonstrates that NKX2.2 protein levels are significantly decreased following knockdown of EWS/FLI. Tubulin is shown as a loading control.

B: Western blot analysis of A673 cells demonstrates that the NKX-RNAi retrovirus efficiently knocks down endogenous NKX2.2 protein. Tubulin is shown as a loading control.

C: Growth analysis using a 3T5 assay (Lessnick et al., 2002) demonstrates that A673 cells harboring the NKX-RNAi retrovirus grow nearly as well as cells containing the luc-RNAi control virus.

D: Soft agar assays using A673, SK-N-MC, and EWS502 Ewing's sarcoma cells demonstrate that knockdown of NKX2.2 with the NKX-RNAi retrovirus results in a near complete loss of transformation as compared to the control luc-RNAi containing cells. A graphical representation of colony formation is shown. Error bars indicate standard deviation of duplicate plates.

E: Murine xenograft experiments using TC71 Ewing's sarcoma cells expressing a luc-neo fusion protein that were subsequently infected with either the ERG-RNAi or NKX-RNAi retroviruses demonstrate that NKX2.2 is required for tumor formation in immunodeficient mice. Bioluminescent imaging data is shown in the left panel. Tumor photon emission is shown in the middle panel, and tumor size is shown in the right panel, where numbers indicate separate tumor injection sites. p values from Student's t tests are also shown.

F: Soft agar assays of A673 and SK-N-MC cells demonstrate that transformation is rescued by reexpression of NKX2.2 using a cDNA that does not contain the 3′ UTR (the region to which NKX-RNAi is directed). Error bars indicate standard deviation of duplicate plates.

G: A673 cells infected with the EF-2-RNAi retrovirus were subsequently infected with either control empty retroviruses (labeled "none"), retrovirus expressing EWS/FLI, or retrovirus expressing NKX2.2. Only expression of EWS/FLI rescued the loss of transformation induced by the EF-2-RNAi retrovirus. Error bars indicate standard deviation.

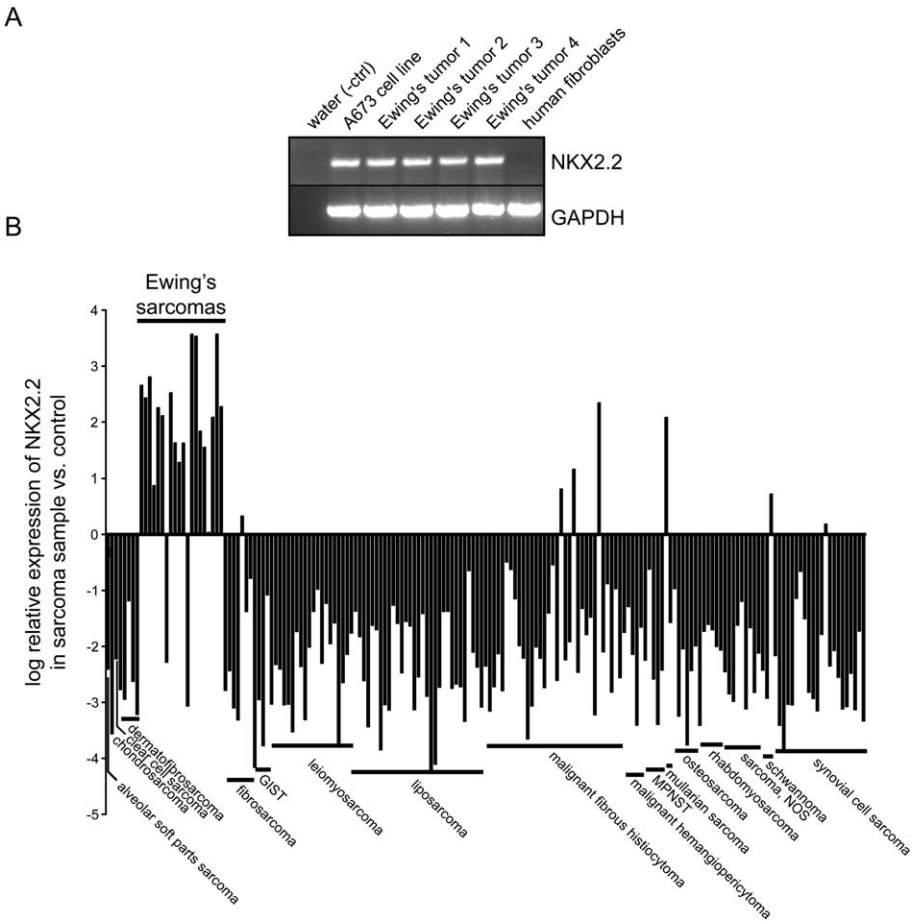

Figure 6. NKX2.2 is expressed in Ewing's sarcoma tumor samples

A: RT-PCR analysis demonstrates that NKX2.2 transcript is expressed in four different patient-derived Ewing's sarcoma tumor samples, but not in human fibroblasts. GAPDH was used as an amplification control.

B: Graphical representation of NKX2.2 expression levels across 181 sarcoma tumor samples, including 20 cases of Ewing's sarcoma. Tumor types are indicated. Primary data from Baird et al. (2005).

of EWS/FLI are unknown. It is fortunate that A673 cells maintain a normal growth rate following EWS/FLI knockdown, because that has provided the unique opportunity to isolate and analyze the transformation effects of EWS/FLI without the confounding effects of growth arrest or cell death.

One surprising finding in the microarray data was that EWS/FLI downregulates three to four times as many genes as it upregulates. This was unanticipated because most prior studies suggested that EWS/FLI functions as a transcriptional activator (e.g., Lessnick et al., 1995; May et al., 1993b). This observation was also noted in a recent publication that used transfected siRNA against EWS/FLI (Prieur et al., 2004). There are at least three models to account for this observation. First, EWS/FLI may contain a transcriptional repressive domain that has not yet been identified. This domain may function to repress some genes, while the transcriptional activation domain may stimulate the expression of others. Second, EWS/FLI may function as a dominant-negative at some promoters by competing with other ETS family members for DNA binding, and thus preventing these ETS factors from stimulating transcription. Third, EWS/FLI may upregulate a transcriptional repressor. In this case, transcriptional repression would be an indirect effect. Future work will be required to distinguish between these models.

EWS/FLI is known to function as an aberrant transcription factor, but the mechanisms by which EWS/FLI-regulated genes mediate Ewing's sarcoma development are largely unknown. Indeed, there are multiple possibilities. For example, EWS/FLI may upregulate a "strong" oncogene that would be both necessary and sufficient for transformation. Strong oncogenes such as *MYC* and/or *CCND1* (encoding cyclin D1) appear to be upregulated by EWS/FLI in some models (Dauphinot et al., 2001; Hu-Lieskovan et al., 2005b; Lessnick et al., 2002), but not in others (Arvand et al., 2001; Prieur et al., 2004; this report). Alternately, EWS/FLI may upregulate multiple "weak" oncogenes, each of which contributes partially to transformation. These models are not mutually exclusive. Loss of any one of these would then have a small quantitative effect on transformation. This appears to be the case in NIH3T3 cells, where EWS/FLI upregulates genes such as *MFNG*, *EAT2*, and *UPP1*, each of which appear to contribute partially to oncogenic transformation (Deneen et al., 2003; May et al., 1997; Thompson et al., 1996). The observation that NKX2.2 is necessary, but not sufficient, for transformation suggests that, in Ewing's sarcoma cells, EWS/FLI regulates multiple nonredundant cooperating genes. Based on this model, we hypothesize that other EWS/FLI-regulated genes will also participate in this process. Another possibility to be explored is that some of the downregulated target genes may function as tumor suppressors to prevent oncogenic transformation.

In addition to its role in oncogenic transformation described in this report, NKX2.2 is also interesting because of its previously defined role in neural development. NKX2.2 is a member of the NK2 family of homeobox genes (Kim and Nirenberg, 1989). NKX2.2 is expressed in the developing forebrain and spinal

cord and is thought to underlie neuronal development, patterning, and fate specification of neurons and oligodendrocytes (McMahon, 2000; Price et al., 1992; Qi et al., 2001). NKX2.2 has not been previously implicated in cancer development. While the cell of origin of Ewing's sarcoma is unknown, one prevailing theory is that it is derived from the neural crest (e.g., Cavenzzana et al., 1987; Collini et al., 2003; Staege et al., 2004). Importantly, however, the neural crest phenotype may be a *consequence* of EWS/FLI expression, rather than being related to the cell of origin of the tumor (Hu-Lieskovan et al., 2005b; Teitell et al., 1999; Thompson et al., 1999). As an EWS/FLI target gene, *NKX2.2* may thus contribute to the neural characteristics of the tumor. Future work will be directed toward understanding the mechanism by which NKX2.2 participates in oncogenesis of Ewing's sarcoma, and whether it plays a role in the neural phenotype as well.

Based on the work presented in this report, *NKX2.2* may be either a direct or an indirect EWS/FLI target gene. *NKX2.2* expression is tightly correlated with EWS/FLI expression in the inducible rescue data set, suggesting that *NKX2.2* is a direct target. We attempted to perform luciferase assays with the *NKX2.2* promoter in human embryonic kidney cells (293EBNA) but were unable to document direct regulation (data not shown). While one interpretation of this result is that *NKX2.2* is not a direct EWS/FLI target gene, alternate explanations include the following: (1) the EWS/FLI-response element was not in our construct; (2) a required cofactor was not present in the heterologous cells; or (3) a repressor was present in the heterologous cells that prevented EWS/FLI from demonstrating transcriptional activation in this setting. Additional work will be required to understand the detailed mechanism of *NKX2.2* gene regulation in Ewing's sarcoma.

The identification of *NKX2.2* as a critical EWS/FLI target gene has important diagnostic and therapeutic implications. First, the demonstration that NKX2.2 is not only expressed in Ewing's sarcoma cell lines, but is also expressed in primary patient-derived tumor samples, suggests that it may serve as a diagnostic marker for this tumor. This is important because the diagnosis of Ewing's sarcoma is primarily based on the histologic appearance of the tumor and an appropriate immunohistochemical staining pattern. The main immunohistochemical marker for Ewing's sarcoma is CD99 (also called MIC2; Ambros et al., 1991). CD99 expression is not specific to Ewing's sarcoma and can also be found on lymphocytes and other hematopoietic cells, endothelial cells, and other tumor types (Choi et al., 2001; Dworzak et al., 1994; Matias-Guiu et al., 1998; Schenkel et al., 2002). While EWS/FLI and other translocations are thought to be highly specific for Ewing's sarcoma, molecular tests for these translocations are not universally applied to biopsy specimens. A new marker would increase the diagnostic specificity for this tumor.

NKX2.2 may also serve as a therapeutic target for Ewing's sarcoma. We have shown that loss of NKX2.2 expression via RNAi results in a loss of oncogenic transformation. Therapeutic approaches directed against NKX2.2 may have clinical value for patients with this disease. With some important exceptions, transcription factors have been difficult to therapeutically target. It seems likely that NKX2.2 will regulate downstream genes that are involved in the oncogenesis of Ewing's sarcoma. Identification and analysis of these gene products may identify more clinically tractable targets. Future work will be directed toward this goal as well.

Experimental procedures

Constructs and retroviruses

The RNAi retroviral vector pSRP contains an H1 promoter for expression of shRNAs, and a puromycin resistant marker. Oligonucleotide sequences that were cloned downstream of the H1 promoter are provided in the Supplemental Data. For some experiments, the puromycin resistance cassette was replaced with a hygromycin resistance cassette. The NKX-RNAi retrovirus used the pMKO.1puro vector (Masutomi et al., 2003). NKX-RNAi oligonucleotide sequences are provided in the Supplemental Data.

A FLAG epitope-tagged EWS/FLI cDNA (type 4 breakpoint; May et al., 1993a), or the R2L2 mutant (Bailly et al., 1994), was cloned into the pMSCV-puro retroviral vector (Clontech). A full-length NKX2.2 cDNA was cloned into the retroviral expression vector pQCXIN (Clontech). The inducible EWS/FLI construct, retroviral production, and retroviral infection were previously described (Lessnick et al., 2002).

Cell culture

Ewing's sarcoma cell lines were grown as described (Lessnick et al., 2002). Following retroviral infection, polyclonal cell populations were prepared by growth in the appropriate selective media. Soft agar assays were performed as described (Lessnick et al., 2002).

Tet-A673 cells were generated by infection with pREV-TETOFF (Clontech) and selection with G418. A single clone was then infected with FLAG-EWS/FLI-pREV-TRE and selected with hygromycin in the presence of 1 μg/ml doxycycline and G418 (Lessnick et al., 2002). Individual clones were tested for induction of FLAG-EWS/FLI as previously described (Lessnick et al., 2002).

Xenograft imaging

A673 or TC71 cells were infected with pMMP-LucNeo and selected with G418 (Rubin et al., 2003). They were then infected with either EF-2-RNAi, NKX-RNAi, or ERG-RNAi retroviruses and selected with puromycin. Following selection, 1×10^6 cells were injected into the flanks of nude mice. Mice were imaged weekly using a Xenogen IVIS 100 imaging system, per the manufacturer's directions. Animal experiments were performed following approval from the University of Utah Institutional Animal Care and Use Committee.

Northern blot analysis

Positive control EWS/FLI RNA was prepared using an in vitro transcription reaction. One microgram of mRNA (Ewing's cells), 1 μg of total RNA (Jurkatt cells), or 0.01 μg of in vitro transcribed EWS/FLI RNA was used. Probes for EWS were prepared using PCR to amplify coding bases 1 to 793 (for 5′ EWS) or coding bases 802 to 1607 (for 3′ EWS). The 5′ FLI probe was prepared using PCR to amplify coding bases 44 to 604. The 3′ FLI probe was a 475 bp PvuII-HindIII fragment derived from the EWS/FLI cDNA.

RT-PCR

Total RNA from the indicated sources was amplified and detected using SYBR green fluorescence for quantitative analysis. For nonquantitative analysis, total RNA was amplified for 35 cycles, and the PCR products were subjected to agarose gel electrophoresis. Primer sequences are available on request. Deidentified patient samples were obtained through an approved University of Utah Institutional Review Board Protocol.

Immunodetection

The following antibodies were used for immunodetection: M2-anti-FLAG (Sigma), anti-FLI-1 (BD PharMingen 554266), anti-α-tubulin (Santa Cruz sc-5286), anti-NKX2.2 (Santa Cruz sc-15015).

Microarray analysis

A complete description of the microarray analysis is provided in the Supplemental Data. The complete set of microarray data is available online at http://www.ncbi.nlm.nih.gov/projects/geo/ (accession number GSE4565).

Supplemental data

The Supplemental Data can be found with this article online at http://www.cancercell.org/cgi/content/full/9/5/405/DC1/.

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Accession numbers

The accession number for the complete set of microarray data (available online at http://www.ncbi.nlm.nih.gov/projects/geo/) is GSE4565.