A zebrafish transgenic model of Ewing’s sarcoma reveals conserved mediators of EWS-FLI1 tumorigenesis

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SUMMARY

Ewing’s sarcoma, a malignant bone tumor of children and young adults, is a member of the small-round-blue-cell tumor family. Ewing’s sarcoma family tumors (ESFTs), which include peripheral primitive neuroectodermal tumors (PNETs), are characterized by chromosomal translocations that generate fusions between the EWS gene and ETS-family transcription factors, most commonly FLI1. The EWS-FLI1 fusion oncoprotein represents an attractive therapeutic target for treatment of Ewing’s sarcoma. The cell of origin of ESFT and the molecular mechanisms by which EWS-FLI1 mediates tumorigenesis remain unknown, and few animal models of Ewing’s sarcoma exist. Here, we report the use of zebrafish as a vertebrate model of EWS-FLI1 function and tumorigenesis. Mosaic expression of the human EWS-FLI1 fusion protein in zebrafish caused the development of tumors with histology strongly resembling that of human Ewing’s sarcoma. The incidence of tumors increased in a p53 mutant background, suggesting that the p53 pathway suppresses EWS-FLI1-driven tumorigenesis. Gene expression profiling of the zebrafish tumors defined a set of genes that might be regulated by EWS-FLI1, including the zebrafish ortholog of a crucial EWS-FLI1 target gene in humans. Stable zebrafish transgenic lines expressing EWS-FLI1 under the control of the heat-shock promoter exhibit altered embryonic development and defective convergence and extension, suggesting that EWS-FLI1 interacts with conserved developmental pathways. These results indicate that functional targets of EWS-FLI1 that mediate tumorigenesis are conserved from zebrafish to human and provide a novel context in which to study the function of this fusion oncogene.

INTRODUCTION

Chromosomal translocations that create fusion oncogenes have long been recognized as a cause of human cancers such as leukemias and sarcomas (Mitelman et al., 2007), and recently have been described in prostate (Tomlins et al., 2005; Brenner and Chinnaian, 2009) and lung cancer (Soda et al., 2007). Drugs that target oncogenic fusions have also led to dramatic improvements in the treatment of certain cancers (Druker, 2009). In Ewing’s sarcoma, a malignant bone tumor that most commonly occurs in adolescents and young adults, the discovery of a characteristic chromosomal translocation t(11;22)(q24;q12) (Delattre et al., 1992) marked a turning point in the understanding of the biology of the disease. In the majority of Ewing’s tumors, the N-terminal portion of EWS becomes fused to the C-terminal portion of FLI-1, which includes an ETS family DNA binding domain (Delattre et al., 1992). In some (~15%) Ewing’s tumors, alternative chromosomal translocations create fusions between EWS and other ETS family transcription factors. These alternative fusions suggest that EWS mediates a critical change in ETS family transcription factors that allows them to function aberrantly and promote tumor development, possibly through incorrect regulation of target gene expression.

Ewing’s sarcoma is classified as a small-round-blue-cell tumor (SRBCT), a group that also includes medulloblastoma, undifferentiated neuroblastoma, alveolar rhabdomyosarcoma and some types of leukemia. The histology of tumors in this family is that of poorly differentiated cells, scant cytoplasm and round nuclei that stain darkly with hematoxylin. Ewing’s sarcomas are frequently associated with bone; however, they can also be found in other tissues, obscuring the identification of a definite cell of origin.

The discovery of EWS-ETS translocations in Ewing’s sarcoma family tumors (ESFTs) creates an opportunity for the development of targeted therapy of Ewing’s sarcoma, either through direct inhibition of EWS-FLI1, or through the discovery of critical downstream effectors that might themselves be candidates for targeted therapy (Uren and Toretsky, 2005; Erkizan et al., 2009). EWS-FLI1 is believed to function primarily as a transcription factor. Although microarray studies have identified a large number of potential EWS-FLI1 targets, they have also revealed a few overlapping genes that are consistently regulated by EWS-FLI1 across these varying cellular backgrounds (Ohali et al., 2004; Staeg et al., 2004; Baird et al., 2005; Smith et al., 2006; Hancock and Lessnick, 2008). These studies have also indicated that neural genes are frequently induced by EWS-FLI1 (Hulieskov et al., 2005; Siligan et al., 2005). In fact, a neuronally expressed homeobox transcription factor, NKX2.2, is required for EWS-FLI1-induced transformation and tumorigenesis in a mouse xenograft model (Smith et al., 2006).

Cell culture experiments have demonstrated that heterologous expression of EWS-FLI1 is toxic to many cell types by inducing growth arrest or apoptosis (Deneen and Denny, 2001; Lessnick et al., 2002). These observations, along with the relative lack of differentiation in Ewing’s tumor cells, have led to the hypothesis that only a subset of undifferentiated precursor cell is capable of
responding to EWS-FLI1 to generate Ewing’s sarcoma. Mouse mesenchymal progenitor cells are one type of cell that tolerates EWS-FLI1 expression and generate Ewing’s-like tumors when transplanted into mice (Riggi et al., 2005). Although this result is a promising step toward understanding the possible origins of Ewing’s sarcoma, mesenchymal progenitor cells remain relatively uncharacterized at a molecular level, therefore the cellular context required for EWS-FLI1 to exert its function remains unknown. Thus, the molecular characteristics that are required for EWS-FLI1 responsiveness, as well as the downstream mechanisms by which EWS-FLI1 gives rise to tumors, remain to be elucidated.

Several mouse models of EWS-FLI1 transgenic expression have been reported. Expression of EWS-FLI1 in hematopoietic tissues in an Mx1-Cre conditional mouse model induced myeloid or erythroid leukemia in transgenic mice (Torchia et al., 2007). Recently, EWS-FLI1 was conditionally expressed in mice under the control of a primitive mesenchymal cell promoter, resulting in limb defects and accelerated development of undifferentiated sarcoma when introduced into the p53 mutant background (Lin et al., 2008). Further studies in which EWS-FLI1 is expressed in a different cell type or at a specific developmental stage are required to more fully recapitulate Ewing’s sarcoma in the mouse.

Models of several types of cancers have recently been developed in the zebrafish Danio rerio, including melanoma, leukemia and rhabdomyosarcoma (Amatruda and Patton, 2008). Here, we report that expression of human EWS-FLI1 in zebrafish causes tumors strongly resembling human Ewing’s sarcoma. We identify an EWS-FLI1 gene expression signature in the zebrafish tumors, which reveals striking conservation of key EWS-FLI1 targets between fish and human Ewing’s tumors. Additionally, we show that EWS-FLI1 exerts powerful effects on embryonic development, indicating that EWS-FLI1 functions through conserved developmental signaling pathways.

RESULTS
Expression of a human EWS-FLI1 transgene causes small-round-blue-cell tumors in zebrafish

The molecular basis of Ewing’s sarcoma is unknown despite the strong association with the EWS-FLI1 oncogene. To develop an animal model in which to study the function of EWS-FLI1, we created transgenic zebrafish carrying the human EWS-FLI1 fusion protein, using the Tol2 transposon system (Kawakami et al., 2004). Three different transgenes were generated, two of which use the zebrafish heat-shock (hsp70) promoter, and another using the β-actin promoter, to drive expression of a FLAG-tagged human EWS-FLI1 (Fig. 1A).

From over 300 wild-type embryos injected with the three different EWS-FLI1 transgenes and raised to adulthood, we identified two fish with tumors. One of the fish was identified by the appearance of outgrowths covering the head at 15 months of age. Sectioning and staining revealed the presence of diffuse tumor cells covering the surface of the head, as well as within the head, gills, and body cavity (Table 1; Fig. 1C,D). This histology resembles that of existing zebrafish models of leukemia (Langenau et al., 2005; Chen et al., 2007). The other tumor identified in a wild-type fish was a solid tumor that arose in proximity to the eye at 12 months of age (Table 1; Fig. 1E,F). Both of these histologies are consistent with the SRBCT morphological category. These tumor types rarely occur spontaneously in zebrafish (Amsterdam et al., 2009). The identification of zebrafish tumors that histologically resemble Ewing’s family tumors was encouraging, but the low incidence of tumors precluded the analysis of a large number of tumors.

Because we observed a low incidence of tumors, we suspected that cellular tumor suppressive mechanisms might be inhibiting tumor development in the wild-type zebrafish. We identified the p53 pathway as a likely candidate for such tumor suppression. Mutations in tp53 occur in a subset of primary Ewing’s tumors (Hamelin et al., 1994; Huang et al., 2005) and research suggests that EWS-FLI1 can induce a p53-dependent growth arrest (Lessnick et al., 2002). Additionally, mutation of tp53 has been shown to be a sensitized background for the development of other tumors in the zebrafish, including rhabdomyosarcoma and melanoma models (Patton et al., 2005; Langenau et al., 2007), as well as in a mouse model of EWS-FLI1-driven tumor development (Lin et al., 2008). Zebrafish with a mutation in tp53 are predisposed to develop malignant peripheral nerve sheath tumors (MPNSTs) (Bergmann et al., 2005), composed of spindle cells with abundant cytoplasm. Based on the histology of the two tumors we identified in wild-type EWS-FLI1 transgenic fish, we anticipated that the histology of a tumor driven by EWS-FLI1 would be distinct from this tumor type. Therefore, we injected the EWS-FLI1 transposons into p53-deficient zebrafish embryos and raised these fish to adulthood.

From 6 to 19 months of age, we identified 48 tumors from approximately 150 tp53 mutant zebrafish injected with one of the three EWS-FLI1 transposons (Table 1). Of these, 33 were classified based on their histology as malignant peripheral nerve sheath tumors (MPNSTs). Diffuse, leukemia-like tumors were identified in four fish (Fig. 1G). The remaining ten tumors exhibited SRBCT histology, but were not diffuse and instead present as solid tumor masses (Fig. 1H). The identification of both solid and diffuse tumors suggests that EWS-FLI1 can induce the formation of at least two tumor types in the zebrafish. Both MPNSTs and SRBCTs were identified at similar ages of onset (average age at sacrifice 13.1 months each). The small round blue cell morphology was not identified in any tumors occurring in uninjected tp53 mutants. Therefore, this histological phenotype is associated with the presence of EWS-FLI1 and is consistent with the pathology of human Ewing’s sarcoma.

To address the similarity of the diffuse tumors to leukemias, we performed quantitative RT-PCR for two genes known to be highly expressed in other zebrafish models of leukemia, rag2 and lck (Langenau et al., 2005). Both genes were more highly expressed in RNA from samples classified as leukemia-like tumors (supplementary material Fig. S2). The tumors classified as zebrafish solid SRBCTs did show slightly increased expression of rag2 and lck compared with levels in the wild type but were substantially lower than expression in the leukemia-like tumors. Thus, the expression of two conserved markers for leukemia is consistent with the histological characterization of the leukemia-like tumors identified in zebrafish.

SRBCTs are transplantable

To show that the zebrafish Ewing’s sarcomas have malignant potential, we performed transplantation experiments. Following dissection and manual dissociation of two SRBCTs derived from tp53 mutants, approximately 1 million cells were injected intraperitoneally
into irradiated wild-type adult zebrafish. Recently, a number of transplantation experiments have been performed in zebrafish tumor models with transplanted tumors typically developing within 2 to 8 weeks post-transplant [reviewed by Taylor and Zon (Taylor and Zon, 2009)]. Both of our primary transplants formed new tumors within 3-5 weeks, consistent with these previously reported results. These tumors were then subjected to further rounds of transplantation (supplementary material Table S1). Even when the number of injected tumor cells was reduced more than 10 times (see number of cells transplanted in 1° vs 4° or 8° transplant in supplementary material Table S1), the tumors continued to regenerate. One of the two secondary transplants continued to generate new tumors in each host and was serially transplanted nine times. These successful transplantation experiments indicate that the tumor cells continue to proliferate. Although one tumor (D85) appeared to have diminished capacity to regenerate with subsequent transplants, the second tumor (D86) maintained an average tumor development time of 17 days over eight rounds of transplantation. Both primary tumors were initially categorized as solid SRBCTs based on their histology. The subsequent tumors continued to exhibit similar histology, although some tumor variation was observed (Fig. 2). After several rounds of transplantation, tumors appeared to have increased

Fig. 1. EWS-FLI1 induces SRBCTs in zebrafish. (A) Schematic of Tol2 transposons including the hsp70 or the β-actin promoter, FLAG-tag (black rectangle), human EWS-FLI1 coding sequence, IRES-GFP sequence (gray rectangle), flanked by Tol2 recombination sites (triangles). (B-H) H&E staining of sagittal sections of adult zebrafish. (B) Control adult zebrafish. Wild-type zebrafish injected with EWS-FLI1 developed an invasive leukemia like-tumor (C magnified in D) or a solid small round blue cell tumor of the eye (arrow in E) (E magnified in F). tp53 mutants injected with EWS-FLI1 transposons also develop leukemia (G) or solid tumors (H) with similar histology. Scale bars: 200 µm (B,C,E), 50 µm (D,F-H).
cytoplasm in some regions (see 6° and 8° in Fig. 2B). Both primary
tumors were derived from tp53 mutant fish, but formed new tumors
in the recipient wild-type hosts. From these transplantation
experiments, we conclude that, although p53 might serve as a barrier
to transformation by EWS-FLI1, this effect is probably autonomous
to the tumor cell and not dependent on the p53 status of the
surrounding normal tissue.

**Zebrafish SRBCTs express the EWS-FLI1 transgene**

To determine whether the tumors express the EWS-FLI1 mRNA, we
tested for the presence of EWS-FLI1 using RT-PCR. EWS-FLI1
mRNA was present at detectable levels in four of six of the SRBCT
primary or transplanted tumors analyzed (supplementary material
Fig. S1 and data not shown). By contrast, only one of ten MPNST
tumors exhibited EWS-FLI1 expression (supplementary material
Fig. S1 and data not shown). We conclude that the SRBCTs were
caused by the presence of the transgene, but that the MPNSTs were
attributable to the mutation in tp53. Those tumors for which we
were unable to detect EWS-FLI1 mRNA might have undetectable
levels of expression, normal tissue co-isolated with the tumor tissue
that obscures detection or might have expressed EWS-FLI1 during
tumor initiation but lost expression in later stages of tumor growth.
Together, the pathology of these tumors and expression of EWS-
FLI1 mRNA in the majority of tumor samples indicate that these
tumors are a model of Ewing’s sarcoma in the zebrafish.

**Gene expression profiling of zebrafish SRBCTs**

The histology of the zebrafish tumors strongly resembles that of
Ewing’s sarcoma and other human SRBCTs. Based on the model
that EWS-FLI1 acts as a transcription factor, we hypothesized
that this similarity in cellular morphology was caused by changes
in gene expression driven by EWS-FLI1. We isolated tissue from
three solid SRBCTs and hybridized the RNA to Affymetrix
zebrafish gene expression arrays. The most highly expressed genes
in these zebrafish tumors were ribosomal proteins and
components of the electron transport chain, consistent with a
general requirement for protein translation and energy production
in proliferating cells.

Using a mutation in the conserved tumor suppressor p53 as a
sensitized genetic background that is likely to affect the expression

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**Table 1. Tumor spectrum of EWS-FLI1 transgenic zebrafish**

<table>
<thead>
<tr>
<th>Transgene</th>
<th>MPNST</th>
<th>Solid tumor</th>
<th>Leukemia-like</th>
<th>Other</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53+/+</td>
<td>hsp70:EWS-FLI1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>hsp70:EWS-FLI1:IRES-GFP</td>
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<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>β-actin:EWS-FLI1:IRES-GFP</td>
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<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>hsp70:EWS-FLI1:IRES-GFP</td>
<td>14</td>
<td>2</td>
<td>2</td>
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<td>β-actin:EWS-FLI1:IRES-GFP</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Approximate number of injected embryos that were raised to adulthood and monitored for tumor development.
of many genes that contribute to tumor initiation or development. Although the precise relationship between EWS-FLI1 and p53 remains unknown, we wanted to eliminate those genes with expression affected by the loss of p53. Therefore, to determine those genes that might be regulated more specifically by EWS-FLI1 and not generally overexpressed in tumors, we compared the gene expression profile with three of the MPNSTs that occurred in the tp53 mutants.

To examine total variation within these samples, we determined the correlation coefficients among the biological replicates (supplementary material Table S2). The MPNST samples were well correlated (mean R=0.95). The SRBCT samples had a lower average correlation (mean R=0.90), suggesting more variation among samples. Increased variation might be expected from mosaic integration of the transgene and differences in the tissue of origin, because two tumors presented in the abdominal cavity and one tumor presented in the eye.

Comparing these two tumor types revealed 421 probes that were differentially expressed between the SRBCTs and the malignant peripheral nerve sheath tumors (Fig. 3A,B) (supplementary material Table S3). These probes were annotated to 376 zebrafish genes, which we will refer to as the zebrafish SRBCT up- and downregulated genes. The majority (243 genes) were downregulated in SRBCTs, suggesting repression by EWS-FLI1. Although EWS-FLI1 is predicted to be a transcriptional activator, our finding of a larger proportion of genes with reduced expression in EWS-FLI1-driven tumors is in agreement with gene expression studies in human and mouse systems. These downregulated genes probably reflect indirect effects on gene expression. In fact, the SRBTC upregulated gene set is significantly enriched for genes with predicted roles in transcription; at least 17 genes involved in the regulation of transcription (2.44-fold enrichment, P<0.0026) are contained within this set. Therefore, many of the gene expression changes, particularly those genes that are repressed, might be indirect targets of EWS-FLI1 through these transcription factors.

Although a specific cell of origin of Ewing’s sarcoma has not yet been identified, multiple studies have found that Ewing’s sarcoma cells often express genes associated with neural development (Staeg et al., 2004; Hu-Lieskovan et al., 2005; Siligan et al., 2005). Indeed, the zebrafish SRBCT upregulated gene list is enriched for genes involved in nervous system development and neurogenesis (3.87-fold, P=0.0024), including nkd2.2a, olig2, sox3 and asc1a/b (supplementary material Table S3). We performed quantitative RT-PCR to validate higher expression of nkd2.2 and olig2 in the zebrafish SRBCTs (Fig. 3C). The downregulated gene list does not exhibit enrichment in this category, indicating that a neural-associated gene expression profile is correlated with the presence of EWS-FLI1.

To compare these zebrafish tumors to human studies of EWS-FLI1-regulated gene expression, the differentially expressed zebrafish genes were mapped to their human homologs. The zebrafish probes were mapped to 97 human homologs (upregulated in SRBCTs) and 162 human homologs (downregulated). To compare these genes with the combined results from several human studies, we used a gene set generated by a meta-analysis of multiple microarray experiments that identified a core EWS-FLI1 gene expression signature of 503 EWS-FLI1 upregulated genes and 293 EWS-FLI1 downregulated genes (Hancock and Lessnick, 2008). Comparing the genes from the human upregulated gene signature with the zebrafish SRBCT-enriched genes revealed an overlap of six genes: NKX2-2, MYC, MAPT, SALL2, PADI2 and POLI3F1. The number of common genes reflects a slight, although not statistically significant, overlap (1.7-fold) between the two gene lists. However, the identity of the overlapping genes suggests that they are biologically relevant. NKX2-2 is one of very few genes that have been shown to be a critically important downstream target of EWS-FLI1 in tumorigenesis (Smith et al., 2006), and mediates transcriptional repression downstream of EWS-FLI1 (Owen et al., 2008). The oncogene MYC has also been previously identified to be upregulated by EWS-FLI1 (Dauphinot et al., 2001; Zwerner et al., 2008). The microtubule associated protein tau (MAPT) and NKX2-2 were in a group of only ten genes identified in an analysis comparing Ewing’s family tumors and cell lines with peripheral blood (Cheung et al., 2007).

Comparing gene lists directly might fail to identify those genes with expression that does not meet a certain criteria in some experiments, but do exhibit the same pattern of change in gene expression. Published analyses of expression profiling of EWS-FLI1 models have produced gene sets with very limited overlap. An alternative way to compare expression profiling experiments is to use gene set enrichment analysis (GSEA), which provides a more
comprehensive look at the way a candidate gene set behaves in another dataset (Mootha et al., 2003; Subramanian et al., 2005). We used publicly available microarray data from several studies of EWS-FLI1-regulated gene expression in human cell lines or tumors. Three datasets were generated using gene expression profiling following the knockdown of EWS-FLI1 in the Ewing’s sarcoma cell lines (Smith et al., 2006; Tirode et al., 2007; Kauer et al., 2009). In two of the three datasets, one of our gene sets (either up- or downregulated) was significantly enriched (Table 2). An additional dataset generated by inducible expression of EWS-FLI1 in a bone marrow stromal cell line (Miyagawa et al., 2008) was enriched for the zebrafish upregulated gene set. Thus, at least one zebrafish gene set was enriched in 3 of 4 datasets testing EWS-FLI1-induced gene expression profiles in cell culture systems.

The datasets used in the above analyses were generated in cell culture systems. To determine whether our data also showed similarity to human Ewing’s tumors, we used publicly available gene expression dataset from a panel of 96 human sarcomas which included five Ewing’s tumors and four malignant peripheral nerve sheath tumors (Henderson et al., 2005). The zebrafish SRBCT upregulated genes were enriched in the Ewing’s sarcoma tumor samples (Table 2). Two published datasets compared Ewing’s tumors to bone marrow stromal cells, a possible cell of origin for Ewing’s sarcoma (Tirode et al., 2007; Miyagawa et al., 2008). In both of these datasets, the Ewing’s sarcoma samples were enriched for the zebrafish upregulated gene set; the Miyagawa dataset was also enriched for the downregulated gene set (Table 2). Two additional datasets compared Ewing’s sarcoma with either neuroblastoma or rhabdomyosarcoma (Baer et al., 2004; Staege et al., 2004) and did not show enrichment of the zebrafish gene sets (data not shown). Taken together with the similar histology of the zebrafish SRBCTs and human Ewing’s sarcomas, these data highlight the strong cross-species conservation of cellular and transcriptional responses to EWS-FLI1.

**EWS-FLI1 impairs zebrafish embryonic development**

The Ewing’s-like tumors that we identified in the adult zebrafish were probably derived from a subset of cells because of the mosaic nature of transposon integration. Although all fish were screened by PCR of embryos for germline transmission of the EWS-FLI1 transgene, no founders were initially identified. Because EWS-FLI1 has a toxic effect on many types of human cells, we hypothesized that it might also be toxic to germ cells or early embryonic cells. Indeed, expression of human or zebrafish versions of EWS-FLI1 by mRNA injection in zebrafish embryos caused abnormal development, mitotic defects and cell death (Embree et al., 2009). Such effects would prevent the identification of stable transgenic lines, as well as preclude further analysis of the effects of EWS-FLI1 on embryonic development. Alternatively, our PCR screening method might not have been robust enough to detect the transgene in pools of embryos, particularly if the rate of transmission was low. To address this possibility, we used a transgenesis marker, cmlc2:EGFP that expresses GFP in the heart beginning at about 24 hours post fertilization (h.p.f.). We generated an additional transposon in which the hsp70 promoter drives expression of EWS-FLI1 and the cmlc2 promoter drives GFP expression from a separate cassette. Following injection of this transposon, we identified a founder that transmitted progeny with GFP expression in the heart. This transgenic line is denoted Tg(hsp70:EWS-FLI1:ires:EGFP; cmlc2:EGFP)sw3 and will be referred to as hsp70:EWS-FLI1 for simplicity.

To determine the effect of transgenic EWS-FLI1 on embryonic gene expression, we heat shocked the transgenic embryos at 50% epiboly, collected total RNA 4 hours later, and performed microarray analysis (supplementary material Tables S4, S5). There was a modest overlap in genes upregulated in zebrafish SRBCTs and transgenic embryos, or downregulated in the tumors and the transgenic embryos (supplementary material Table S6). This modest overlap is probably due to the fact that the highly dynamic context of a developing embryo, in which many different tissue-specific enhancers and pathways might be active, differs from that of a tumor derived from one particular lineage. In a rapidly developing embryo, indirect effects of EWS-FLI1 expression might in fact predominate, making the comparison with human Ewing’s tumors more problematic.

We speculated that the embryonic developmental phenotype of EWS-FLI1 transgenic embryos might therefore provide a more robust readout of the effects of EWS-FLI1 on conserved signaling

<table>
<thead>
<tr>
<th>Expression dataset</th>
<th>Reference</th>
<th>Zebrafish SRBCT upregulated</th>
<th>Zebrafish SRBCT downregulated</th>
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<tbody>
<tr>
<td>Knockdown of EWS-FLI1 in Ewing’s cell lines</td>
<td>Smith et al., 2006</td>
<td>70</td>
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<tr>
<td></td>
<td>Kauer et al., 2009</td>
<td>70</td>
<td>0.98</td>
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<tr>
<td></td>
<td>Miyagawa et al., 2008</td>
<td>83</td>
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<td>1.36</td>
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<td>Ewing’s tumors vs bone marrow stromal cells</td>
<td>Tirode et al., 2007</td>
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<td>Ewing’s cell lines vs bone marrow stromal immortalized cell line</td>
<td>Miyagawa et al., 2008</td>
<td>83</td>
<td>1.79</td>
</tr>
</tbody>
</table>

NES, normalized enrichment score; FDR, false discovery rate; NS, not statistically significant; Size, number of genes with zebrafish orthologs in the human expression dataset.
The heat-shock promoter used to express EWS-FLI1 can exhibit basal transcripational activity. We tested whether EWS-FLI1 was expressed in these embryos in the presence and absence of heat shock. RT-PCR of RNA from GFP-positive F1 embryos reveals that EWS-FLI1 is expressed in these embryos even in the absence of heat shock, and also that expression increases following heat shock (Fig. 4E). We evaluated embryonic development following heat shock at the 50% epiboly stage to determine whether increased EWS-FLI1 expression would further perturb embryonic development. Consistent with the increase in EWS-FLI1 gene expression after heat shock, these embryos exhibited more severe developmental defects. Similarly to previous results (Embree et al., 2009), embryonic expression of EWS-FLI1 caused mitotic defects (11/25 mitoses in transgenic embryos exhibited abnormal or monopolar spindles, compared with 1/25 in control embryos; \( P=0.002 \) by Fisher’s exact test) (supplementary material Fig. S3). At 2 days post fertilization, the embryos have a shorter anterior-posterior axis, as well as reduced head development (Fig. 5A,B). At an earlier stage (18 somites), a reduced anterior-posterior axis is already evident, with extremely reduced tail extension. At this stage, in situ hybridization for myoD reveals that the somites are wider (\( P<0.02 \)) (Fig. 5C-E). The average angle between the head and the tail is also significantly greater (17.5° vs 60.8°; \( P<0.001 \)) in the transgenic embryos compared with the control embryos (Fig. 5C,D,F). Reduced body length and increased somite width are phenotypes that are frequently associated with convergence and extension defects. Convergence and extension movements are controlled largely by the non-canonical Wnt signaling pathway (Roszko et al., 2009). Therefore, EWS-FLI1 might be modulating non-canonical Wnt signaling to perturb embryonic development in this zebrafish transgenic line.

**DISCUSSION**

The identification of human EWS-FLI1-driven tumors in zebrafish shows that critical downstream transcriptional targets and protein interactions that allow EWS-FLI1 to generate tumors are conserved between zebrafish and humans. The developmental phenotypes caused by EWS-FLI1 in zebrafish embryos provides further support for this conservation and provides a second, independent context in which to dissect the downstream targets of EWS-FLI1. Creating regulated or inducible models of EWS-FLI1 expression will improve this system by allowing us to induce EWS-FLI1 in a temporal or tissue-specific fashion to increase the tumor incidence. A model in which we could reliably produce SRBCTs in the zebrafish would provide an ideal platform for chemical or genetic screening for EWS-FLI1 inhibitors.

The identification of both solid and infiltrating tumors suggests that EWS-FLI1 can induce the formation of both of these tumor types in the zebrafish. Both of these tumor types have also occurred in transgenic mice expressing EWS-FLI1 using tissue-specific promoters, suggesting that the cellular context in which EWS-FLI1 is expressed dictates the tumor type that subsequently develops (Torchia et al., 2007; Lin et al., 2008). The finding that EWS-FLI1 promotes leukemia in both mouse and zebrafish indicates that this property of EWS-FLI1 is conserved. Although EWS-FLI1-associated leukemia is not observed in humans, a closely related fusion of TLS-ERG is associated with acute myeloid leukemia (Ichikawa et al., 1994). Rare cases of pre-B-cell lymphoblastic leukemia with the EWS-FLI1 translocation have also been reported.
These cases suggest a connection between EWS translocation proteins and cancers of the hematopoietic lineage. Differences in cellular context, cellular niche, expression level, or temporal expression in animal models may account for the lack of EWS-FLI1-associated leukemia in humans.

Serial transplantation of two zebrafish tumors demonstrated the independent growth of the tumor cells. The histological variation observed following several rounds of transplantation could be caused by tumor evolution, stochastic differences in subpopulations of cells transplanted, or differences in signals or environment within the host tissue. Although some rounds of transplantation required a longer time for tumor development, the number of transplant recipients was not large enough to confirm that this difference is statistically significant. Presently, serial transplantation studies performed in zebrafish are becoming a powerful tool to study cancer in this model organism [reviewed by Taylor and Zon (Taylor and Zon, 2009)]. Most zebrafish cancer models to date have been shown to be serially transplantable; however, some differences are becoming evident; a KRAS-induced MPD was unable to be serially transplanted (Le et al., 2007) and a T-ALL line showed increasing malignancy with subsequent transplants (Frazer et al., 2009). Understanding the mechanisms underlying engraftment and proliferation in zebrafish cancer models might shed light on tumor progression and metastasis in human cancer.

In the zebrafish model, we identified tumors in animals injected with a hsp70:EWS-FLI1 transgene, even in the absence of heat shock. In these tumors, the heat shock promoter is probably acting as a minimal promoter that allows EWS-FLI1 expression to be controlled by local enhancers at the genomic site of transgene integration. Under this model, the two different histologies of SRBCTs that we identified might arise because of trapping of different types of tissue-specific enhancers by the hsp70:EWS-FLI1 transgene. Identification of these enhancers, and the tissue type they define, could perhaps help clarify the Ewing’s sarcoma cell of origin.

Mutation of tp53 provided a sensitized background for the formation of SRBCTs in zebrafish. Although mutations in tp53 occur in only a subset of Ewing’s sarcomas (Huang et al., 2005), the p53 pathway might also be inhibited by other mechanisms (Ban et al., 2008; Li et al., 2010). Interestingly, the solid SRBCTs that we identified most frequently arose in proximity to the eye and abdomen, the locations in which MPNSTs also develop in tp53 mutant zebrafish (Berghmans et al., 2005). This cell type might be predisposed to tumor development in the zebrafish, or the MPNSTs and SRBCTs might both be able to arise from the same type of nerve sheath cell. An alternative possibility is that the tumors originate as a result of the loss of p53, but that the presence of EWS-FLI1 drives the tumor cells to a less differentiated, more primitive state, which results in the SRBCT histology. These possibilities cannot be distinguished from this study.

The microarray analysis provides firm support for the validity of this system as a model of human Ewing’s sarcoma. The identification of relevant target genes and the enrichment of the zebrafish gene set in both Ewing’s sarcoma cell lines and primary tumors reveal that EWS-FLI1 produces similar gene expression changes in the zebrafish tumors. By comparing the gene expression profiles to MPNSTs, we eliminated those genes that are generally affected in tumors and also those genes that might be changed because of the absence of p53. Therefore, additional gene expression changes caused by EWS-FLI1 might also exist if they share these properties. Our gene set is therefore a

![Image of zebrafish embryos](image-url)
We identified some genes that were upregulated in zebrafish SBRCTs but downregulated in human EWS-FLI1-dependent expression datasets, or vice versa. Differences in regulatory elements, protein-protein interactions, cellular niche and growth factors, and many other differences might exist between animal models and humans. Of course, animal models will almost never completely recapitulate the human conditions. In this context, the similarity of these tumors suggests that these differences are not crucial to tumorigenesis. Therefore, the gene expression profile we found is likely to include relevant biological targets downstream of EWS-FLI1. These gene sets also show enrichment in multiple, independent datasets generated from experiments in human cell lines. Even among the various microarray analyses performed from human cell lines, the differences between experiments are much greater than the similarities. A meta-analysis used to generate a EWS-FLI1 core-expression signature concluded that the core was more similar to primary Ewing’s sarcoma, than any of the individual experiments (Hancock and Lessnick, 2008). Therefore, each additional model of Ewing’s sarcoma provides new information toward understanding the transcriptional consequences of EWS-FLI1 and, by extension, facilitates the development of improved, targeted therapies of Ewing’s sarcoma.

The stable EWS-FLI1-expressing transgenic line that we established represents a valuable new tool for the study of EWS-FLI1 cellular function. Many signaling pathways that function in zebrafish embryonic development have been dissected. The phenotypes observed in the EWS-FLI1 embryos are consistent with defects in convergence and extension during gastrulation. The non-canonical Wnt-PCP and BMP signaling pathways as well as other genes, including those encoding Stat3, heterotrimeric G proteins and cell adhesion molecules contribute to proper convergence and extension in zebrafish embryos (Roszko et al., 2009). The convergence and extension defect we observed in transgenic EWS-FLI1 embryos suggests that EWS-FLI1 directly interferes with genes that function during this process; a finding made possible by the expression of the transgene in the context of a developing embryo, in which the interplay of multiple signaling pathways determines embryo morphology. Future experiments will be required to fully dissect the relationship between EWS-FLI1 and these morphological defects.

The phenotype we observed in the hsp70:EWS-FLI1 line was not identical to that previously reported following mRNA injection (Embree et al., 2009). Similarly to our analysis, embryos with severe trunk defects, including a shorter axis, were observed, as well as abnormal mitoses. We speculate that mRNA injection results in a higher level of EWS-FLI1 expression as well as an earlier onset of expression that might account for some of these differences.

This animal model of EWS-FLI1 function is amenable to future pathway and genetic analysis and provides a new approach to dissecting the role of EWS-FLI1. Genes that contribute to cancer frequently have crucial roles in embryonic development; therefore, elucidating the mechanism by which EWS-FLI1 perturbs development will also shed light on its contribution to tumorigenesis. Importantly, these zebrafish models represent the first vertebrate model for EWS-FLI1 function that is amenable to genetic and chemical screening. A system to perform suppressor screens for EWS-FLI1-induced phenotypes in a whole animal context has until now, been elusive. Future genetic and chemical screens for EWS-FLI1 suppressors in the zebrafish should reveal novel downstream effectors and therapeutics for the treatment of Ewing’s sarcoma.

METHODS

Zebrafish care, maintenance and mutants

Zebrafish were raised according to standard protocols (Westerfield, 2000). For mutation of tps3, the allele TPS3<sup>[M]</sup> was used (Berghmans et al., 2005). A morpholino targeting the 5’ end of the FLAG-EWS-FLI1 mRNA (5’-TGTCATCGTCGTCTTGTAG-TCCAT-3’) was purchased from GeneTools (Philomath, OR). Heat shock of transgenic embryos was performed by incubating embryos at 39°C for 30 minutes at 50% epiboly.

Plasmid construction

The plasmid containing a triple-FLAG-tagged human EWS-FLI1 of the type 1 fusion, which fuses codon 265 of EWS to codon 219 of FLI1 was provided by Chris Denny, University of California-Los Angeles, Los Angeles, CA. This plasmid was digested with SmaI and Clal and ligated to the plasmid containing the 1.5 kb hsp70 promoter digested with EcoRV and Clal fragment (provided by Jon Kuwada, University of Michigan, Ann Arbor, MI). This plasmid was then digested with SmaI and Clal and ligated into pT2KX1G following digestion with Xhol and Clal to create pTZ109 (hsp70:EWS-FLI1). pTZ109 was digested with NotI and Xhol to release the ~3 kb hsp70:EWS-FLI1 fragment, which was then ligated into the 3.8 kb Tol2 transposon backbone of pT2KH digested with Xhol and NotI (provided by Koichi Kawakami, National Institute of Genetics, Shizuoka, Japan) to create pTZ307 (hsp70:EWS-FLI1). To create pME-EWS-FLI1, triple-FLAG-EWS-FLI1 was PCR amplified using primers with Gateway recombination sites attB1-EF1 (5’-GGGGACACGTGTACAAAAAAGCAGGCTATG-GACTACAAGAGCAAGGATG-3’) and attB2-EF1 (5’-GGGG-GACCTTTTGTAACAGAAGGCTAATGAGTCGTCAGTTCAGGATGGGGTTGT-3’) and recombined with pDONR221 using BP Clonase II (Invitrogen, Carlsbad, CA). To generate constructs for transposition, entry vectors p5E-bactin2 or p5E-hsp70l respectively, pME-EWS-FLI1, and p3E-RES-EGFPpA (Kwan et al., 2007) were recombined with destination vector pDEST1ol2A or pDEST1ol2CG2, which includes cmrl2EFGFP, (provided by Koichi Kawakami) in an LR reaction with LR Clonase II Plus (Invitrogen). Transposase RNA was synthesized from plasmid pCS2FA using the MMessage mMachne kit (Applied Biosystems/Ambion, Foster City, CA). Plasmids were injected at a concentration of 10-100 ng/μl with transposase RNA at 250 ng/μl.

Fixation and staining

Fish were euthanized in Tricaine, dissected, fixed in 4% paraformaldehyde in 1× PBS for 2 days at 4°C, decalcified in 0.25 M EDTA for 4-5 days at room temperature, embedded, sagittally sectioned and stained with hematoxylin and eosin (H and E). For examination of mitotic spindles, Tg(HSP:EWS-FLI1) embryos were heat shocked at the 50% epiboly stage and fixed in 4% paraformaldehyde at 24 h.p.f. Anti-tubulin staining was performed as described (Verduzzo and Amatruda, 2011). In situ hybridization
was performed as described (Thisse and Thisse, 2008). Embryos were photographed and measurements of somite width and head-tail angle were performed in ImageJ.

Tumor transplants

The tumor was removed, minced and washed in 0.9× PBS with FBS. Cells were counted and injected intraperitoneally into adult fish 2 days after irradiation (20 Gy).

RNA isolation, RT-PCR and microarray analysis

RNA from tumors or embryos was isolated using Trizol followed by RNaseasy purification (Qiagen). For leukemia-like tumors, only fixed tissue was available. The tumor-containing regions were scraped from the paraffin-embedded sections and then the RNA was purified as described above. RT-PCR for human EWS-FLI1 was performed using primers hEWS-FLI1-R: CTAGTAGT-AGCTGCTAAAGTGTTG and hEWS-FlI1-Fnt1: ATCTCTACA-GCCAAGCTCAGAGT, which are not present in the zebrafish genome. qPCR primers were resuspended at 500 nM each and validated by performing a tenfold dilution curve qPCR, starting with 2000 ng cDNA, down to 2 ng cDNA, in duplicate. RPL13a primers for reference were previously validated (Neumann et al., 2011). All qPCRs were performed with three technical replicates on and Applied Biosystems 7500 Real Time PCR system using TaqFast SYBR green supermix from Bio-Rad (catalog no. 172-5101). Reactions were 20 µl total consisting of 2 µl primer mixes, 10 µl SYBR supermix, and 2000 ng cDNA. The Forward (F) and Reverse (R) primers used were: sox2, (F) GGAACCTTCTG-TCCGAGACG, (R) GCCAGGGTGATCTTGCTCCTT; nkx2.2a, (F) CTAGTGGCCTCGAACCAC, (R) CGACGTGTCTT-GAGA GTTC, olig2, (F) ATGTGATATTCCC GGATGC, (R) CAAACAAACACTGCACGA; rag2, (F) CAAACAGCTCTC-AGATT TCG, (R) CCAGGTCTAGTAAGGA AAC; lck, (F) GCCCTCCAGTGCAGAATTT, (R) TTGTATATGGCA-ACCACGC; rpl13a, (F) TCTGGAGACTGTAAGGATATGC, (R) AGACGACAACTAC TGGACAG.

For microarray experiments, MNST samples were taken from tumors in three un.injected tp53 mutant adults. Solid small round blue cell tumors were isolated from one primary tumor and transplant recipient tumor from two independent primary tumors. Total RNA (2.5 µg) from tumors was reverse-transcribed using a T7-Oligo(dt) Promoter Primer, followed by RNaseH-mediated second-strand cDNA synthesis. Biotinylated cRNA probe was prepared with MessageAmp II (Ambion), fragmented and hybridized to Affymetrix GeneChip Zebrafish Genome Arrays according to the manufacturer’s protocol. Array data normalized using the RMA method and preprocessed using Chipster (Finnish IT Center for Science CSC). In accordance with the MIAME guidelines, CEL files have been deposited at the GEO repository (Barrett and Edgar, 2006), under accession number GSE31217. An empirical Bayes two groups test (P<0.05, Benjamini-Hochberg corrected) was used to generate the list of 421 differentially expressed probes. Human homologs of zebrafish genes were compiled from three sources: Zebrafish Gene and Microarray Annotation Project (ZGMAP, http://zfblast1.danio.tchlab.org/zgMap/Affymetrix.aspx), AILUN Cross-species mapping (http://ailun.stanford.edu/crossSpecies.php), and Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) (Huang et al., 2009a; Huang et al., 2009b). Gene set enrichment analysis (GSEA, http://www.broadinstitute.org/gsea/) was used to compare the zebrafish EWS-FLI1 up- and downregulated genes to human microarray data (Subramanian et al., 2005). Analysis of overrepresented gene categories was performed using Gene Set Analysis Toolkit V2 (http://bioinfo.vanderbilt.edu/webgestalt).

Publicly available datasets used for gene set enrichment analysis (GSEA)

The datasets used for GSEA are available as follows. Expression profiling of Ewing’s sarcoma and rhabdomyosarcoma (Baer et al., 2004), GEO GSE967; expression profiling of human mesenchymal tumors (Henderson et al., 2005), Array Express: E-MEXP-353; knockdown of EWS-FLI1 in Ewing’s cell lines and expression profiling of Ewing’s tumors and Bone Marrow Stem Cells (Tirole et al., 2007), GEO: GSE7007. Additional BMSC samples (Shahdadfar et al., 2005), E-MEXP-215; siRNA knockdown of EWS-FLI1 in Ewing’s cell lines (Kauer et al., 2009), Array Express: E-GEOD-14543; knockdown of EWS-FLI1 in Ewing’s cell lines (Smith et al., 2006), from http://www.huntsmancancer.org/publicweb/content/lessnick/mscSupplementalSmith2006_files/msc...
Supplemental-Smith-2006.html. Inducible Expression of EWS-FLI1 in immortalized bone marrow stromal cell line and expression profiling of Ewing's cell lines (Miyagawa et al., 2008), GEO: GSE8865 and GSE8596. Expression profiling of Ewing's sarcoma and Neuroblastoma (Staeger et al., 2004), Array Express: E-GEOD-15757.

ACKNOWLEDGEMENTS
We thank Chris Denny, Jon Kuwada, Chi-Bin Chien, Nathan Lawson and Tom Look for reagents.

FUNDING
This work was supported by Kevin's Ewing's sarcoma Fund; Curing Kid's Cancer; the Amon G. Carter Foundation; the American Cancer Society [grant number ACS-IRG 60219604] (J.F.A.); the National Institutes of Health [grant number 1R01CA135731] (J.F.A.); The Cancer Prevention and Research Institute of Texas [grant number RP110395]; Welch Foundation [grant number I-1679] (J.F.A.); and a Post-Doctoral Fellowship from the American Cancer Society [grant number PF0826701D] (S.W.L.).

COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
J.F.A. and S.W.L. conceived and designed the experiments. S.W.L., A.N.B., A.M.K. and G.L.C. performed the experiments. J.F.A., S.W.L. and D.R. analyzed the data. S.W.L. and J.F.A. wrote the paper.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.007401/-/DC1

REFERENCES


Leacock Supplementary Figure S1
Table S1. Serial transplantation of SRBCTs

<table>
<thead>
<tr>
<th></th>
<th>Number of cells transplanted</th>
<th>Time to tumor</th>
</tr>
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<tbody>
<tr>
<td><strong>D85</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1° recipient</td>
<td>$3 \times 10^6$</td>
<td>33 days</td>
</tr>
<tr>
<td>2° recipient</td>
<td>$1.7 \times 10^6$</td>
<td>57 days</td>
</tr>
<tr>
<td><strong>D86</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1° recipient</td>
<td>$3 \times 10^6$</td>
<td>23 days</td>
</tr>
<tr>
<td>2° recipient</td>
<td>$1.75 \times 10^6$</td>
<td>21 days</td>
</tr>
<tr>
<td>3° recipient</td>
<td>$1 \times 10^6$</td>
<td>16 days</td>
</tr>
<tr>
<td>4° recipient</td>
<td>$0.25 \times 10^6$</td>
<td>12 days</td>
</tr>
<tr>
<td>5° recipient</td>
<td>$0.6 \times 10^6$</td>
<td>15 days</td>
</tr>
<tr>
<td>6° recipient</td>
<td>$1 \times 10^6$</td>
<td>33 days</td>
</tr>
<tr>
<td>7° recipient</td>
<td>$1 \times 10^6$</td>
<td>14 days</td>
</tr>
<tr>
<td>8° recipient</td>
<td>$0.3 \times 10^6$</td>
<td>16 days</td>
</tr>
<tr>
<td>9° recipient</td>
<td>$1 \times 10^6$</td>
<td>15 days</td>
</tr>
</tbody>
</table>
Table S2. Correlation coefficients (R) between biological replicates in microarray analysis

<table>
<thead>
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<th>MPNST D259</th>
<th>MPSNT D335</th>
<th>MPNST D336</th>
</tr>
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<tbody>
<tr>
<td>0.95</td>
<td>0.94</td>
<td>0.97</td>
</tr>
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</table>

**Average correlation: 0.95**

<table>
<thead>
<tr>
<th>SRBCT D167</th>
<th>SRBCT D394</th>
<th>SRBCT D161</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.94</td>
<td>0.89</td>
<td>0.86</td>
</tr>
</tbody>
</table>

**Average correlation: 0.90**

Correlations between all genes from individual biological replicate microarrays within each tumor type are shown. The average of the three correlations is also shown.