CONTRIBUTIONS OF EWS/FLI TARGET GENES AND CELLULAR CONTEXT IN EWING SARCOMA PATHOGENESIS

by

Elizabeth Callahan Toomey

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Elizabeth Callahan Toomey**

has been approved by the following supervisory committee members:

Stephen L. Lessnick	, Chair	**May 22, 2013** Date Approved
Donald E. Ayer	, Member	**May 22, 2013** Date Approved
John Kimble Frazer	, Member	**June 8, 2013** Date Approved
Roland Dean Tantin	, Member	**May 22, 2013** Date Approved
Bryan E. Welm	, Member	**May 22, 2013** Date Approved

and by **Bradley Cairns**, Chair of

the Department of **Oncological Sciences**

and by Donna M. White, Interim Dean of The Graduate School.

ABSTRACT

Adult cancers often arise due to the accumulation of mutations over time, while pediatric malignancies occur early in life and may result from disruption of normal developmental processes. Ewing sarcoma is an undifferentiated neoplasm and the second most common bone associated tumor in children and young adults. This is an aggressive disease and effective treatment for those with metastatic disease is lacking. The EWS/FLI translocation encodes an oncogenic transcription factor and the principle driver of the disease. This fusion protein relies on a very specific cellular context in order to dysregulate transcription of several thousand genes including many developmental regulators. The presence of the translocation in a permissive cell type may be sufficient to drive the transformation process. The specific oncogenic contributions made by EWS/FLI target genes and identification of the cell of origin – which remains unknown – will lead to targeted treatment options based on the molecular underpinnings of the disease. Toward this goal, we identified BCL11B as an up-regulated EWS/FLI target necessary for maintenance of transformation. Genome wide expression profiling of BCL11B regulated genes revealed that BCL11B is responsible for a significant portion of the EWS/FLI repressed gene signature. The normal function of BCL11B as a transcriptional repressor in many developmental processes, suggests that inappropriate expression of BCL11B in the incipient Ewing sarcoma cell leads to aberrant expression of genes involved in several cell lineages, thus contributing to the poorly differentiated

phenotype in Ewing sarcoma. Next we shifted our focus away from EWS/FLI target genes to identify genes present in the precursor cell that maintain Ewing sarcoma in an undifferentiated state and may foster a permissive cellular environment for EWS/FLI. ZEB2 expression is not dependent on EWS/FLI levels, but it is highly expressed in Ewing sarcoma tumors. ZEB2 represses epithelial gene expression in Ewing sarcoma cells. Reduction of ZEB2 allows for expression of epithelial genes and corresponding decreases in cell mobility and actin cytoskeleton rearrangements. Furthermore, the emergence of these epithelial phenotypes decreased the metastatic potential of Ewing sarcoma cells in a mouse metastasis model. This heretofore unrecognized epithelial plasticity may provide insight into the histogenesis of this disease.

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CHAPTER 1

INTRODUCTION

Malignancies affecting the pediatric and young adult population have a unique biology when compared to those occurring in older individuals. Sarcomas, tumors that are thought to arise from mesenchymal tissues, are the most common solid tumor in this age group (Bleyer *et al*, 2008). A more complete understanding of the molecular mechanisms that contribute to cancer formation in this young population will inform better treatment strategies for this subset of patients. The work in this thesis will focus on a specific class of sarcoma, Ewing sarcoma. Ewing sarcoma is most commonly diagnosed in the second decade of life and is often bone-associated, but can also occur in soft tissue such as kidney and pancreas (Taylor *et al*, 2011). This highly aggressive neoplasm is characterized by the t(11;22)(q24;q12) chromosomal translocation resulting in the oncogenic transcription factor, EWS/FLI (Delattre *et al*, 1992; Turc-Carel *et al*, 1988). A detailed review of Ewing sarcoma is presented in Chapter 2.

In 2007, when I began my thesis research, the Ewing sarcoma field had made great progress in understanding the EWS/FLI oncogene using mutational analysis to demonstrate the involvement of various domains in transformation. The ETS (E26) DNA binding domain present in the FLI (Friend leukemia virus intergration1) portion of the fusion as well as the transcription regulatory domain contributed by EWS (Ewing sarcoma breakpoint region 1) (Delattre *et al*, 1992; Lessnick *et al*, 1995; May *et al*, 1993a; May *et al*, 1993b; Sankar *et al*, 2012) is absolutely required for transformation. The main research focus at this time was concentrated on identifying EWS/FLI target genes, and the experimental system used to do this was shifting from NIH3T3 cells and human fibroblasts made to ectopically express EWS/FLI to a more relevant system based in patient derived Ewing sarcoma cell lines. In this context, authentic EWS/FLI target

genes could be identified by knocking down EWS/FLI using RNA interference (RNAi) and then re-expressing an EWS/FLI cDNA that was resistant to the RNAi effect. This identified thousands of genes that were dysregulated by EWS/FLI in its native cellular context (Smith *et al*, 2006). While this represented a major advancement, the cell of origin for Ewing sarcoma remained elusive. This limitation prevented study of the development of the disease by modeling tumorigenesis *in vitro* or using animal models.

EWS/FLI expression results in a poorly differentiated sarcoma. This malignant transformation is achieved by dysregulation of thousands of target genes. Among the genes with aberrant expression are several genes involved in developmental signaling pathways such as sonic hedgehog (Beauchamp *et al*, 2009; Zwerner *et al*, 2008), transforming growth factor beta (Hahm *et al*, 1999; Sankar *et al*, 2012), and WNT (Navarro *et al*, 2010). Further contributing to the undifferentiated phenotype and unknown histogenesis, genes from multiple lineages are expressed in Ewing's tumors. Principal components analysis clusters Ewing sarcoma patient tumors and cell lines with other primitive cell types and separate from any differentiated tissue (Tirode *et al*, 2007; von Levetzow *et al*, 2011). Among adult tissues, Ewing sarcoma samples have gene expression signatures most closely related to various brain and endothelial tissues. The observation that EWS/FLI induces the expression of several neural genes has led to debate over whether the neural gene expression reflects the cell of origin or is a consequence of the translocation (Staege *et al*, 2004).

The transcriptional dysregulation caused by the EWS/FLI fusion is presumed to be the main, or perhaps, sole driver in Ewing sarcoma development. Aside from the translocation, Ewing sarcoma tumors have simple karyotypes (Taylor *et al*, 2011). Copy

number analysis shows very few recurrent chromosomal gains or losses (Jahromi *et al*, 2012). A more directed approach assessing known tumor suppressors, *p53* and *p16/p14ARF*, demonstrated that each of these loci is mutated in less than 15% of patient samples, respectively (Huang *et al*, 2005). Mutations in known oncogenes are even less common. One report sequenced 29 known oncogenes in 75 Ewing sarcoma tumors and found that only 4% had any sequence abnormalities (Shukla *et al*, 2012). These studies suggest that there is no common cooperating mutation in this malignancy. This finding supports the assertion that EWS/FLI and the appropriate cellular background may be the only necessary factors in Ewing sarcoma development.

The cellular context in which this fusion occurs has proven critical in the formation of Ewing sarcoma. However currently the cell of origin remains undefined. These tumors are poorly differentiated and thought to arise in a primitive cell type. Such a primitive cell would contain an open chromatin structure (Gaspar-Maia *et al*, 2011) thus allowing for the massive transcriptional dysregulation caused by EWS/FLI. Over the years, EWS/FLI has been ectopically expressed in various cell types with different outcomes. EWS/FLI expression in primary mouse fibroblasts results in apoptosis (Deneen & Denny, 2001) and growth arrest in primary human fibroblasts (Lessnick *et al*, 2002). Neuroblastoma cell lines forced to expresses EWS/FLI adopt a gene expression profile more similar to Ewing sarcoma than the neuroblastoma parent cell line (Rorie *et al*, 2004). Two murine-derived cells, NIH3T3 cells and bone marrow derived mesenchymal stem cells (MSCs), are the only cells that undergo transformation in response to EWS/FLI expression (May *et al*, 1993a; Riggi *et al*, 2005). To date, human MSCs and neural crest stem cells (NCSCs) are the only primary human cell types to

foster EWS/FLI expression. Importantly, however, these cells are not transformed by the stable expression of the translocation (Miyagawa *et al*, 2008; Riggi *et al*, 2008; von Levetzow *et al*, 2011). Despite this fact, MSCs and NCSCs, are the leading candidates for the cell of origin in Ewing sarcoma. Of note, NCSCs and MCSs may not be mutually exclusive cell types. Experimental evidence in both mouse and human cells has demonstrated that NCSCs can give rise to mesenchymal stem cells which can then differentiate along mesenchymal lineages (Lee *et al*, 2007; Takashima *et al*, 2007).

The majority of the Ewing sarcoma field at this time backs MSCs as the cell of origin. Support for this hypothesis comes from the finding that MSCs are able to maintain proliferation in the presence of EWS/FLI and when expressing the fusion, they take on a gene expression profile that resembles Ewing sarcoma cell lines and tumors (Miyagawa *et al*, 2008; Riggi *et al*, 2008). Complementary experimental data show the reciprocal effect. When EWS/FLI levels are reduced in Ewing sarcoma cell lines, the gene expression profile shifts toward that of a MSC (Tirode *et al*, 2007). This mesenchymal gene expression profile also correlates with a more mesenchymal phenotype marked by an increase in migration and adhesion with corresponding cytoskeletal rearrangements (Chaturvedi *et al*, 2012). In addition, Ewing sarcoma cells are able to differentiate along mesenchymal lineages when EWS/FLI is knocked-down (Tirode *et al*, 2007). However, the fact that these cells are not transformed by EWS/FLI cannot be ignored. This coupled with the observation that Ewing sarcoma cells cannot undergo mesenchymal lineage differentiation in the presence of EWS/FLI expression (Tirode *et al*, 2007), but MSCs retain this differentiation capacity when expressing EWS/FLI (Riggi *et al*, 2008) suggests

that MSCs may be further along the differentiation spectrum than the *bona fide* Ewing sarcoma cell of origin (Figure 1.1).

Ewing sarcoma has an undisputable relationship to the mesenchymal lineage while there is a less well appreciated connection to the epithelial lineage. This later relationship has not been investigated experimentally, but pathologists have noted expression of several epithelial proteins in patient tumor samples. These studies have noted the expression of desmosomal proteins, desmoglein and desmoplakin, tight junction proteins, claudin, occuldin, and tight junction protein 1 (ZO-1), and epithelial cytokeratins in a significant subset of samples (Machado *et al*, 2012; Schuetz *et al*, 2005). Despite retaining epithelial and mesenchymal features, Ewing sarcomas are overwhelmingly undifferentiated however these observations suggest some degree of cellular plasticity in these tumors.

Interestingly, it has recently been demonstrated that carcinomas, tumors arising from epithelial tissues, must retain some degree of cellular plasticity to successfully grow and metastasize (Ocana *et al*, 2012; Tsai *et al*, 2012). In this setting, relatively well differentiated epithelial tumor cells must undergo an epithelial-mesenchymal transition (EMT) in order to disrupt intercellular adhesions and gain invasive and migratory ability (Thiery, 2002). These qualities allow for escape from the primary tumor and survival in the circulatory system, but this does not coincide with active proliferation (Muller *et al*, 2005). A mesenchymal-epithelial transition (MET) at the secondary site is proposed to facilitate reactivation of a proliferative state allowing for metastatic colonization.

The contribution of EMT to cancer metastasis has been backed by research for some time now, but the MET in this pathologic setting has remained controversial due to

lack of experimental evidence. The concept of EMT and MET during development has been recognized for almost 50 years since they were first observed in early chick embryogenesis by Elizabeth Hay and colleagues (Sartor *et al*, 2010). EMT and MET are dynamic processes that don't necessarily refer to cell fate specification but reflect phenotypic changes in cell shape and mobility that occur throughout embryogenesis. Neural crest migration is achieved through an EMT in neuroepithelial cells of the dorsal neural tube. These acquired mesenchymal qualities allow for migration throughout the embryo until the neural crest cells undergo MET at their destination to allow for further differentiation into diverse tissues including neurons, melanocytes and craniofacial bones (Acloque *et al*, 2009). The induction of the developmental EMT by certain transcription factors and the downstream effectors is mirrored in cancer metastasis. The involvement of MET in tumor progression was initially only supported by pathology studies that had observed most epithelial tumor metastases retained the epithelial differentiation state of the primary tumor. This held true for metastases derived from primary tumors that had evidence of cells undergoing EMT – which presumably were the cells that gave rise to the metastases with epithelial characteristics (Brabletz *et al*, 2001).

Over a decade passed before experimental evidence provided support for the clinically observed MET. An elegant yet simple study using a mouse model of squamous cell carcinoma demonstrated that as expected, expression of TWIST1, a well characterized EMT inducing transcription factor (EMT-TF), in the tumor resulted in the loss of epithelial morphology and emergence of invasive edges. The expression of the transgene was controlled by doxycycline (dox). When dox was applied locally, at the primary tumor, the cells underwent EMT at the primary site and formed pulmonary

metastases. These secondary lesions no longer expressed TWIST1. In contrast, when dox was given systemically, resulting in constitutive expression of the transgene, significantly fewer mice developed distant disease. The authors demonstrate that the cells constitutively expressing TWIST1 are present in the lung, but suggest that the expression of TWIST1 prevents colonization. While this forced expression is not biologically relevant, primary human tumor samples with matched lymph node metastases were consistent with this mouse model. For example, metastatic lesions expressed less than 50% of the TWIST1 seen in the primary tumor for breast carcinomas (Tsai *et al*, 2012). An independent study recognized a similar phenomenon with a newly identified EMT-TF, paired-related homeobox 1 (PRRX1) (Ocana *et al*, 2012).

Sarcoma metastases do not fit neatly into this EMT-MET paradigm, but the concept of cellular plasticity can likely be applied more broadly. EWS/FLI prevents mesenchymal phenotypes, and as a result, the oncogenic driver in Ewing sarcoma counter-intuitively prevents cellular qualities required for metastasis (Chaturvedi *et al*, 2012). In spite of this, Ewing sarcomas are highly metastatic. Approximately 20-25% of patients present with overt metastasis, and the remainder are considered to have mircometastatic disease (Arndt & Crist, 1999). This conclusion was reached in the era prior to the use systemic chemotherapy in Ewing sarcoma when the majority of patients would relapse with distant disease after surgical resection of the primary tumor (Dahlin *et al*, 1961; Wang & Schulz, 1953). Presence of metastasis correlates with very poor prognosis in Ewing sarcoma. Patients with localized disease have a four-year overall survival rate close to 90%, but this drops to less than 20% for patients with disseminated disease (Kolb *et al*, 2003). The poorly differentiated state of Ewing sarcoma retaining

both epithelial and mesenchymal features potentially primes these tumor cells for successful metastasis.

The first broad goal of my thesis work was to understand the contributions of EWS/FLI target genes in "creating" a Ewing sarcoma cell. More specifically, EWS/FLI dysregulates the expression of thousands of transcripts; some of these genes are relevant to the disease process, but many are not. I had a particular interest in genes that were involved in developmental processes or fate specification in that they would be part of the bigger picture relating to Ewing sarcoma as a disease of normal development gone awry. I investigated B-cell chronic lymphocytic leukemia/lymphoma 11B (BCL11B), a gene that is up-regulated by EWS/FLI, and its contribution to the maintenance of transformation in Ewing sarcoma. BCL11B represented a promising EWS/FLI target gene for further investigation because it is one of the few genes that is regulated by EWS/FLI in Ewing sarcoma cell lines, in addition to the two proposed cells of origin – MSCs (Kauer *et al*, 2009) and NCSCs (von Levetzow *et al*, 2011). BCL11B is required for the normal development of skin (Golonzhka *et al*, 2009a), teeth (Golonzhka *et al*, 2009b), the central nervous system (CNS) (Arlotta *et al*, 2005; Chen *et al*, 2008), and T-cell maturation (Li *et al*, 2010) – placing it among the EWS/FLI targets involved in developmental processes in diverse tissues. This work is presented in Chapter 3.

The second major goal of this work – presented in Chapter 4 - was to investigate genes expressed in the cell of origin that contribute to the oncogenic phenotype, again with a focus on developmentally relevant genes. I identified the EMT-TF zinc finger E-box binding homeobox 2 (ZEB2), as a gene that is highly expressed in Ewing sarcoma patient tumors. ZEB2 expression is not regulated by EWS/FLI, and thus it is expressed in

the cell of origin before the translocation event, potentially contributing to a cellular environment permissive for EWS/FLI expression. ZEB2 represses the epithelial phenotype in Ewing sarcoma making its expression complementary to that of EWS/FLI in the formation of a tumor cell with metastatic competence. Both proteins contribute to the undifferentiated nature of the tumor. EWS/FLI and its targets initiate and sustain tumor proliferation, and ZEB2 facilitates metastasis. The work presented here highlights the oncogenic power of EWS/FLI, with an emphasis on developmental dysregulation, and the importance of the appropriate cellular context in Ewing sarcomagenesis and its high propensity for metastasis.

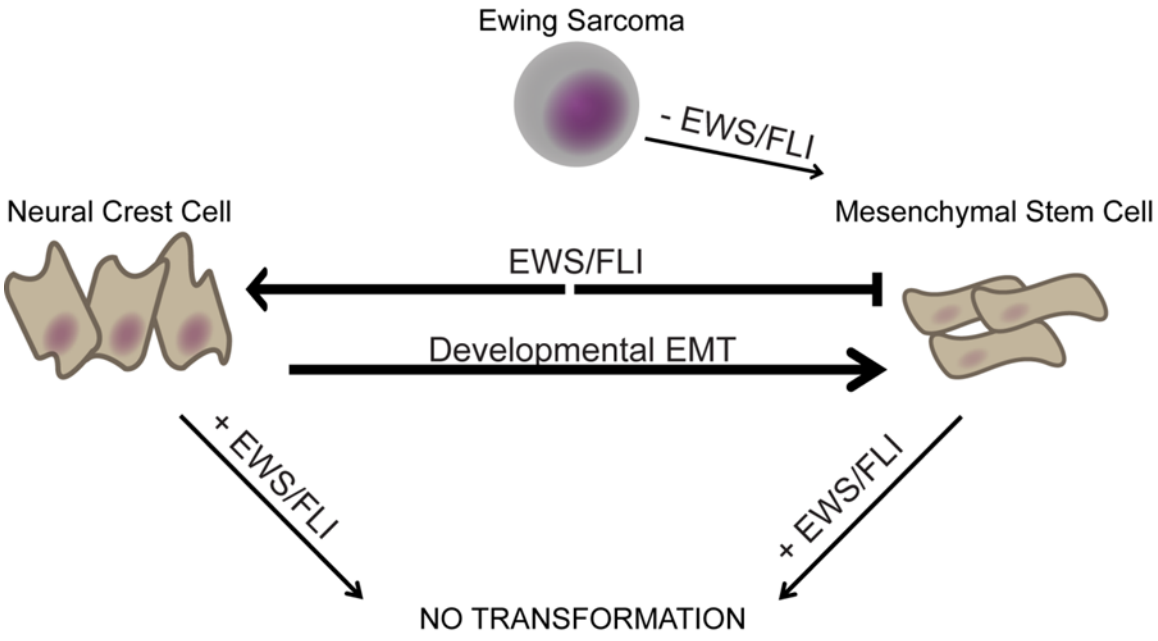

Figure 1.1: Summary model of Ewing sarcoma cell of origin. The two proposed cells of origin for Ewing sarcoma are a neural crest cell or a mesenchymal stem cell. Neural crest cells can give rise to mesenchymal stem cells during normal development. EWS/FLI has been shown to repress mesenchymal features and induce neural crest and neuronal features. When Ewing sarcoma cells lose EWS/FLI expression they adopt mesenchymal gene expression profiles and the ability to differentiate along mesenchymal lineages. Neither neural crest cells nor mesenchymal stem cells are transformed by the expression of EWS/FLI.

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CHAPTER 2

RECENT ADVANCES IN THE MOLECULAR PATHOGENESIS OF EWING'S SARCOMA

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REVIEW

Recent advances in the molecular pathogenesis of Ewing's sarcoma

EC Toomey[1], JD Schiffman[1,2] and SL Lessnick[1,2]

[1]Department of Oncological Sciences and Center for Children's Cancer Research, Huntsman Cancer Institute, Salt Lake City, UT, USA and [2]Division of Pediatric Hematology/Oncology, University of Utah School of Medicine, Salt Lake City, UT, USA

Tumor development is a complex process resulting from interplay between mutations in oncogenes and tumor suppressors, host susceptibility factors, and cellular context. Great advances have been made by studying rare tumors with unique clinical, genetic, or molecular features. Ewing's sarcoma serves as an excellent paradigm for understanding tumorigenesis because it exhibits some very useful and important characteristics. For example, nearly all cases of Ewing's sarcoma contain the (11;22)(q24;q12) chromosomal translocation that encodes the EWS/FLI oncoprotein. Besides the t(11;22), however, many cases have otherwise simple karyotypes with no other demonstrable abnormalities. Furthermore, it seems that an underlying genetic susceptibility to Ewing's sarcoma, if it exists, must be rare. These two features suggest that EWS/FLI is the primary mutation that drives the development of this tumor. Finally, Ewing's sarcoma is an aggressive tumor that requires aggressive treatment. Thus, improved understanding of the pathogenesis of this tumor will not only be of academic interest, but may also lead to new therapeutic approaches for individuals afflicted with this disease. The purpose of this review is to highlight recent advances in understanding the molecular pathogenesis of Ewing's sarcoma, while considering the questions surrounding this disease that still remain and how this knowledge may be applied to developing new treatments for patients with this highly aggressive disease.
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Introduction

Ewing's sarcoma is a rare but important solid tumor in children and young adults (Arndt and Crist, 1999). Ewing's sarcoma most frequently arises in bone, but <10% of tumors originate in the soft tissues (Horowitz *et al*., 1997). Approximately 15–25% of patients present with overt metastasis (Terrier *et al*., 1996); those without clinically detectable metastases likely have micrometastases, because in the absence of systemic chemotherapy most will relapse with distant metastatic disease after surgical resection (Wang and Schulz, 1953; Dahlin *et al*., 1961). This propensity to spread contributes to the poor prognosis for Ewing's sarcoma patients and long-term cure rate of ~60% (Linabery and Ross, 2008). It is hoped that elucidation of the molecular mechanisms at play in these tumors will translate to improved patient outcomes.

Approximately 85% of Ewing's sarcoma tumor specimens harbor the t(11;22)(q24;q12) chromosomal abnormality (Turc-Carel *et al*., 1988). This translocation fuses the *EWSR1* gene on chromosome 22 to the *FLI1* gene on chromosome 11 and encodes the EWS/FLI fusion protein (Delattre *et al*., 1992). EWS/FLI contains the amino-terminus of EWS fused, in frame, to the carboxyl-terminus of FLI. EWS is a protein of uncertain function, whereas FLI is a member of the ETS family of transcription factors. In the context of this fusion, a strong transcriptional activation domain is contributed by EWS and an ETS-type DNA-binding domain is contributed by FLI (Delattre *et al*., 1992; May *et al*., 1993a, b; Lessnick *et al*., 1995). Both of these domains are required for the oncogenic function of EWS/FLI, supporting the notion that the fusion acts as an aberrant transcription factor (May *et al*., 1993a, b). Interestingly, in Ewing's sarcoma cases lacking EWS/FLI, alternate translocations are present that fuse EWS (or a highly similar protein, TLS/FUS) to other ETS family transcription factors, including, ERG, ETV1, ETV4, and FEV (Sorensen *et al*., 1994; Jeon *et al*., 1995; Kaneko *et al*., 1996; Peter *et al*., 1997; Shing *et al*., 2003; Ng *et al*., 2007), which all likely mimic EWS/FLI (Sorensen *et al*., 1994; Teitell *et al*., 1999; Thompson *et al*., 1999; Braunreiter *et al*., 2006) (Figure 1). Askin's tumors and peripheral primitive neuroectodermal tumors also harbor EWS/ETS fusions, and are now considered to be manifestations of Ewing's sarcoma. Some investigators have used the term 'Ewing's sarcoma family of tumors' to highlight this relationship. However, we prefer the simpler term 'Ewing's sarcoma' to refer to all of these genetically identical tumors.

EWS/FLI and the alternate fusions have been classified as oncogenes based on their ability to transform immortalized murine NIH3T3 cells (May *et al*., 1993a; Braunreiter *et al*., 2006). In contrast, EWS/FLI is not sufficient to transform any normal human cell type tested so far (Lessnick *et al*., 2002; Miyagawa *et al*.,

Correspondence: Dr SL Lessnick, Center for Children's Cancer Research, Huntsman Cancer Institute, 2000 Circle of Hope, Room 4242, Salt Lake City, UT 84112, USA.
E-mail: stephen.lessnick@hci.utah.edu
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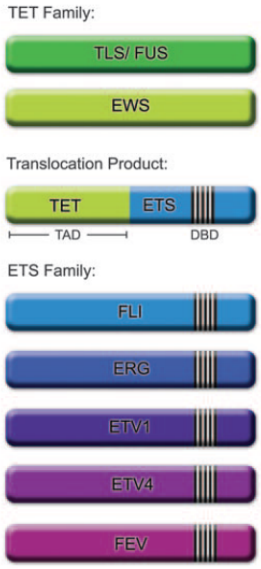

Figure 1 Ewing's sarcoma fusion protein organization. The Ewing's sarcoma translocation product is the result of a chromosomal rearrangement involving the N-terminal transcriptional activation domain (TAD) of a TET family member (either TLS/FUS or more commonly EWS) and the C-terminal portion of an ETS family member (FLI, ERG, ETV1, ETV4, or FEV), including the ETS DNA-binding domain (DBD).

2008; Riggi *et al*., 2008), suggesting that critical differences might exist between human and mouse cells in their abilities to respond to EWS/FLI. Nevertheless, ongoing EWS/FLI expression is required for the oncogenic phenotype of Ewing's sarcoma cells, further supporting the assertion that EWS/FLI is the critical oncoprotein in this disease (Chansky *et al*., 2004; Prieur *et al*., 2004; Kinsey *et al*., 2006; Owen and Lessnick, 2006; Smith *et al*., 2006; Stegmaier *et al*., 2007).

Ewing's sarcoma cell of origin

The cell of origin for Ewing's sarcoma is unknown. This lack of knowledge has hindered progress in the field by precluding the identification of an appropriate model system in which to study the development of the disease. Identification of a permissive cellular environment for the expression of EWS/FLI has also been problematic: expression of EWS/FLI in many primary cell lines results in cell death or growth arrest, whereas expression in more primitive cells or tumor cell lines causes differentiation defects, as reviewed in Kovar (2005). As stated, NIH3T3 cells provide a useful cellular context for EWS/FLI expression as they are transformed by the fusion (May *et al*., 1993a, 1997). However, concerns have recently been raised about how well the NIH3T3 model recapitulates the human disease. For example, recent comparative analyses have demonstrated poor correlation in gene expression patterns between the NIH3T3 model and other Ewing's sarcoma model and primary tumor data sets (Braunreiter *et al*., 2006; Hancock and Lessnick, 2008). Furthermore, some EWS/FLI gene targets that have been shown to be critical for oncogenic transformation in patient-derived Ewing's sarcoma cell lines, such as *NKX2.2* and *NR0B1*, are not induced by the fusion protein in NIH3T3 cells (Kinsey *et al*., 2006; Owen and Lessnick, 2006; Smith *et al*., 2006; Gangwal *et al*., 2008). These data suggest that the molecular pathways used in NIH3T3 mouse fibroblasts may be different from those used in *bona fide* Ewing's sarcoma.

Unlike other sarcomas, such as osteosarcoma and liposarcoma that show some lineage-specific differentiation, as reviewed in Charytonowicz *et al*. (2009), Ewing's sarcoma presents as an undifferentiated 'small round blue cell tumor' that reveals little insight into its cell of origin (Triche *et al*., 1987). When first described in 1921, Ewing (1921) proposed an endothelial origin. Since that time, numerous hypotheses have been put forth regarding the histogenesis of this tumor including hematopoietic (Kadin and Bensch, 1971), fibroblastic (Dickman *et al*., 1982), neural crest (Cavazzana *et al*., 1988), and mesenchymal progenitor/stem cells (Riggi *et al*., 2005; Tirode *et al*., 2007). The latter two cell types have been the focus of much investigation.

Several lines of evidence provide support for the neural crest cell of origin hypothesis. Early studies found that cell surface antigens associated with the neuroectodermal lineage were expressed on Ewing's sarcomas (Lipinski *et al*., 1986, 1987a, b). Consistent with this observation, both Ewing's sarcoma and peripheral primitive neuroectodermal tumor harbor the same t(11;22)(q24;q12) rearrangement. This suggests that these are the same tumor demonstrating differences in extent of neural differentiation (Turc-Carel *et al*., 1984; Whang-Peng *et al*., 1984; Kovar, 1998). A gene expression profiling study recently found that genes expressed in neural tissues or during neuronal differentiation are highly expressed in Ewing's sarcomas, and that Ewing's sarcomas clustered with fetal and adult brain tissue (Staege *et al*., 2004). This study also reported that EWS/FLI expression in bone marrow cells upregulated neural genes. This latter observation suggested an alternate hypothesis: that the Ewing's sarcoma neural phenotype is a result of EWS/FLI expression, rather than a reflection of the cell of origin. This hypothesis is further supported by work demonstrating that genes critical for neural crest development were upregulated when EWS/FLI was ectopically expressed in rhabdomyosarcoma, neuroblastoma, or human foreskin fibroblast cell lines (Lessnick *et al*., 2002; Rorie *et al*., 2004; Hu-Lieskovan *et al*., 2005).

There is a growing body of evidence that suggests Ewing's sarcoma is derived from a mesenchymal stem or progenitor cell. Early studies demonstrated that EWS/FLI and EWS/ERG blocked the differentiation of pluripotent murine bone marrow-derived mesenchymal progenitor cells (Torchia *et al*., 2003). Subsequent

studies established that introduction of EWS/FLI into unselected primary murine bone marrow cells or into murine mesenchymal progenitor cells allowed the transduced cells to form tumors in immunocompromised mice with a small round cell morphology (Castillero-Trejo *et al*., 2005; Riggi *et al*., 2005). Although it was suggested that these tumors also expressed CD99, a classical Ewing's sarcoma marker, there is some controversy as to whether the murine genome harbors a CD99 allele that is paralogous to the human version (Riggi *et al*., 2005; Kovar and Bernard, 2006).

A reciprocal approach complemented the gain-of-function data. Ewing's sarcoma cell lines in which EWS/FLI had been silenced with RNAi displayed a mesenchymal stem cell (MSC) gene expression profile, and these cells demonstrated the capacity to differentiate along both osteogenic and adipogenic lineages, consistent with an MSC phenotype (Tirode *et al*., 2007). Importantly, human MSCs also provide an appropriate cellular context for EWS/FLI expression. In contrast to other normal human cell types with forced EWS/FLI expression, human MSCs retain the ability to propagate in the presence of the fusion protein (Riggi *et al*., 2008). Gene expression profiles from these cells are similar to Ewing's sarcoma, but not to other bone and soft tissue tumors (Miyagawa *et al*., 2008; Riggi *et al*., 2008). As these cells were unable to form tumors when injected into immunocompromised mice (Riggi *et al*., 2008), it seems that EWS/FLI is necessary, but not sufficient, for oncogenic transformation of human MSCs.

Interestingly, it has been recently suggested that the proposed neural crest and MSC origins may not be mutually exclusive (Riggi *et al*., 2009). On the one hand, it was demonstrated that neural-derived MSCs are present in the bone marrow of developing mice (Takashima *et al*., 2007). Conversely, it has also been shown that neural crest stem cells contain some mesenchymal lineage plasticity (Lee *et al*., 2007). Taken together, it is possible that Ewing's sarcoma might arise from a neural-derived MSC or from a neural crest stem cell that harbors mesenchymal potential.

There are no known precursor lesions for Ewing's sarcoma, preventing the definitive identification of the cell of origin through analysis of early stage precancerous cells. An alternate approach towards the identification of the cell of origin could be the development of a mouse model for the disease through conditional EWS/FLI expression in the correct progenitor cell. Such an approach has been effective for the identification of cells of origin of other sarcomas, including alveolar rhabdomyosarcoma (Keller *et al*., 2004a, b), synovial sarcoma (Haldar *et al*., 2007), and osteosarcoma (Walkley *et al*., 2008). The development of genetically engineered mouse models of Ewing's sarcoma remains a challenge. Early attempts to express EWS/FLI, or equivalent translocations, in mice induced leukemia, and not sarcoma (Codrington *et al*., 2005; Torchia *et al*., 2007). Forced expression of EWS/FLI in mesenchymal cells of the developing limb bud in mice resulted in abnormalities of limb development without tumor formation (Lin *et al*., 2008). In this latter study, however, EWS/FLI accelerated the tumor development typically seen with p53 deletion, and changed the predominant tumor type from osteosarcoma to an 'undifferentiated' sarcoma. Whether this undifferentiated sarcoma represents a mouse equivalent of Ewing's sarcoma is unclear, and further study will be required to better define these tumors. Interestingly, it has been recently suggested that genomic differences in microsatellite content near critical EWS/FLI target genes may prohibit the development of a mouse model (see below; Gangwal and Lessnick, 2008; Gangwal *et al*., 2008).

EWS/FLI transcriptional regulation

It is well appreciated that EWS/FLI acts as an aberrant transcription factor and that several of its downstream targets contribute to tumorigenesis. Although early studies classified EWS/FLI as a strong transcriptional activator (Ohno *et al*., 1993; May *et al*., 1993b; Bailly *et al*., 1994; Lessnick *et al*., 1995), later studies suggested that it also functions as a transcriptional repressor at some gene targets (Hahm *et al*., 1999; Nakatani *et al*., 2003). The repressive capacity of EWS/FLI was further supported by expression profiling studies using patient-derived Ewing's sarcoma cell lines (Prieur *et al*., 2004; Smith *et al*., 2006). These microarray analyses identified over 1000 EWS/FLI-regulated genes (which are comprised of direct and indirect target genes) in A673 cells; surprisingly, downregulated targets comprised 80% or more of the dysregulated genes. Subsequent comprehensive transcriptional analyses comparing multiple Ewing's sarcoma cell lines and tumor samples revealed a more equal representation of EWS/FLI induced and repressed genes (Kinsey *et al*., 2006; Hancock and Lessnick, 2008; Kauer *et al*., 2009). Although the exact magnitude of transcriptional repression induced by EWS/FLI remains to be clarified, studies demonstrating the importance of target gene repression in tumorigenic phenotype suggest that this may be a critical EWS/FLI-mediated activity (Hahm *et al*., 1999; Prieur *et al*., 2004).

ETS family members (including EWS/FLI) bind to sequences containing a GGAA 'core' motif surrounded by bases that provide affinity and specificity to the interaction (Szymczyna and Arrowsmith, 2000; Sharrocks, 2001; Seth and Watson, 2005). *In vitro* binding site selection approaches identified ACCGGAAGTG as a site that binds wild-type FLI and EWS/FLI (and other ETS family members) with high affinity (Mao *et al*., 1994; Sharrocks, 2001; Seth and Watson, 2005). Whole genome localization analysis (ChIP-chip) demonstrated that EWS/FLI binds to this same sequence element *in vivo* (Gangwal *et al*., 2008). This latter study also identified GGAA-containing microsatellite sequences as EWS/FLI-binding sites associated with a number of genes involved in oncogenic transformation in Ewing's sarcoma. This result was confirmed by a ChIP-sequencing approach as well (Guillon *et al*., 2009). These findings were surprising because microsatellite sequences have been described as 'junk

DNA' without biologic function (Gangwal and Lessnick, 2008). The identification of microsatellites as cancer-relevant EWS/FLI-binding sites suggests that such elements have important roles in the development of Ewing's sarcoma. Follow-up studies have begun to unravel the mechanistic basis for EWS/FLI binding to microsatellite sequences and have suggested that similar mechanisms might also be used by other cancer-relevant ETS family members (Gangwal *et al*., 2010).

EWS/FLI binding to GGAA microsatellites was associated solely with gene activation. Other mechanisms of transcriptional activation and repression by EWS/FLI are slowly becoming defined. Protein–protein interactions and post-translational modifications seem to take part in both activating and repressing functions of EWS/FLI. For example, EWS/FLI has been shown to be phosphorylated in Ewing's sarcoma cell lines (Klevernic *et al*., 2009), and phosphorylation modulates DNA binding and transcriptional activity in a heterologous system (Olsen and Hinrichs, 2001), suggesting that this modification may be relevant to the oncogenic function of EWS/FLI. It was also recently reported that EWS/FLI undergoes O-GlcNAcylation (Bachmaier *et al*., 2009). This may be involved directly in EWS/FLI-mediated transcriptional activation, or alternately, may indirectly modulate EWS/FLI transcriptional function by altering the protein's intracellular half-life.

Full-length EWS directly associates with the general transcriptional machinery, including RNA polymerase II and TFIID, as reviewed in Tan and Manley (2009). In contrast, the EWS/FLI fusion does not seem to be in stable association with the RNA polymerase II complex (Bertolotti *et al*., 1998), but does interact with the RNA polymerase II subunit RPB7 (Petermann *et al*., 1998). In addition to the basal machinery, EWS/FLI interacts with other transcriptionally relevant proteins, such as RNA helicase A (Toretsky *et al*., 2006). Inhibition of this association with small molecules reduced tumor growth in xenograft mouse models, suggesting that this interaction is biologically relevant (Erkizan *et al*., 2009). Although the use of reporter assays indicate that the RNA helicase A-EWS/FLI interaction increases EWS/FLI transcriptional activity, the presence of RNA helicase A at the promoters of EWS/FLI-repressed targets complicates the interpretation of these findings (Toretsky *et al*., 2006).

Progress has also been made recently in understanding the mechanistic basis for transcriptional repression by EWS/FLI. For example, interaction between EWS/FLI and p300 serves to inhibit the latter protein's histone acetyl-transferase activity, blocking its transcriptional activation and resulting in transcriptional repression (Nakatani *et al*., 2003). A similar effect has been reported for wild-type EWS and its highly related family member TLS/FUS (Wang *et al*., 2008). In addition to direct transcriptional repression, it should be noted that EWS/FLI also upregulates the expression of transcriptional repressors, such as NKX2.2, corepressors, such as NR0B1, and polycomb-family repressors, such as EZH2, that seem to account for some of the down-regulated signature (Owen *et al*., 2008; Riggi *et al*., 2008; Kinsey *et al*., 2009).

EWS/FLI targets and transformation pathways

Genomic approaches linking RNAi technologies with microarray analysis have identified thousands of genes that are dysregulated by EWS/FLI in Ewing's sarcoma cell lines (Prieur *et al*., 2004; Kinsey *et al*., 2006; Smith *et al*., 2006; Kauer *et al*., 2009). Although not all of these genes are actively involved in the transformation process, a small but growing subset of these have been shown to be critical for oncogenesis (for example *NKX2.2*, *NR0B1*, and *GLI1*; Kinsey *et al*., 2006, 2009; Smith *et al*., 2006; Owen *et al*., 2008; Zwerner *et al*., 2008; Beauchamp *et al*., 2009; Joo *et al*., 2009). Interestingly, these proteins all have a role in transcriptional regulation. However, the mechanisms by which these transcriptional regulators, or their downstream targets, mediate oncogenic transformation are not fully understood. In contrast, other EWS/FLI target genes are more readily connected to hallmarks necessary for tumor formation and progression (Hanahan and Weinberg, 2000). For example, many Ewing's sarcoma tumors and cell lines express human telomerase reverse transcriptase (Schuck *et al*., 2002; Amiel *et al*., 2003; Ohali *et al*., 2003) and vascular endothelial growth factor (Fuchs *et al*., 2004b). Although it has not been demonstrated in Ewing's sarcoma cell lines *per se*, these genes are induced by EWS/FLI in heterologous cellular backgrounds (Fuchs *et al*., 2004a, b). The expression of these two genes allows for tumor cell immortalization and vasculogenesis. Other EWS/FLI target genes contribute to additional cancer-specific phenotypes such as preventing differentiation to 'pirate' some qualities of stem cells, metastatic spread, and drug resistance as discussed further below.

Cancer stem cells (CSCs) were recently identified in Ewing's sarcoma (Suva *et al*., 2009). These are to be distinguished from the MSC tumor cell of origin hypothesis discussed earlier. CSCs have the ability to form tumors from a single cell, and may be more resistant to chemotherapy, suggesting that current approaches that reduce tumor bulk may leave CSCs behind, thereby allowing tumors to reform. The identification of CSCs in Ewing's sarcoma will now allow key aspects of the CSC model to be tested in this disease. As an interesting corollary to the identification of Ewing's sarcoma CSCs, other work has identified a role for 'stem cell genes' in Ewing's sarcoma. For example, polycomb repressor complexes have a central role in maintaining stemness and pluripotency (Bracken *et al*., 2006; Lee *et al*., 2006; Tolhuis *et al*., 2006). Members of these complexes, including EZH2 and BMI1, are expressed in Ewing's sarcoma (and in the case of EZH2, regulated by EWS/FLI), are required for Ewing's sarcoma oncogenic transformation, and seem to block normal differentiation pathways in this disease (Douglas *et al*., 2008; Riggi *et al*., 2008; Burdach *et al*., 2009). Thus, the polycomb group family seems critical to Ewing's sarcoma tumorigenesis.

The PI3K-AKT pathway can mediate both cellular proliferation and survival (Vivanco and Sawyers, 2002). The caveolin gene (*CAV1*) connects EWS/FLI to this pathway. The *CAV1* promoter contains a GGAA microsatellite that is occupied by EWS/FLI *in vivo* (Gangwal *et al*., 2008), is highly expressed in Ewing's sarcoma tumors, and promotes tumor growth in mouse xenograft models of Ewing's sarcoma (Tirado *et al*., 2006). The requirement of CAV1 for tumorigenicity was attributed to its ability to indirectly activate E-cadherin expression (Tirado *et al*., 2006). E-cadherin is also upregulated when Ewing's sarcoma cell lines are grown under anchorage-independent conditions, and its expression correlated with phosphorylation of the receptor tyrosine kinase ERBB4 and phosphorylation of AKT (Kang *et al*., 2007). Cell–cell contacts and signaling mediated by E-cadherin and ERBB4 may promote survival and suppress anoikis to facilitate metastatic spread in Ewing's sarcoma (Kang *et al*., 2007).

Despite improved understanding of transformation pathways operative in Ewing's sarcoma, adequate therapy remains a challenge. Patients often show promising responses to initial courses of chemotherapy, but later relapse with chemotherapy-resistant tumors and metastases (Meyers and Levy, 2000). Glutathione-S-transferases (GST) are detoxification enzymes that mediate solubility and excretion of both endogenous and exogenous reactive compounds (Comstock *et al*., 1994). Recently, *GSTM4* was identified as a GGAA-microsatellite-containing gene that was required for the transformed phenotype and modulates resistance to chemotherapeutic agents in Ewing's sarcoma cells (Luo *et al*., 2009). Consistent with this *in vitro* observation, GSTM4 protein levels in primary tumors inversely correlate with patient survival. Similar relationships between expression levels and patient outcome were recently reported for another GST enzyme, MGST1 (Scotlandi *et al*., 2009). This latter study demonstrated efficacy of a small-molecule inhibitor of GST enzymes, 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)-hexanol, against a number of Ewing's sarcoma cell lines *in vitro*, suggesting a new therapeutic approach to this disease.

Parallel pathways and cooperating mutations

EWS/FLI is indisputably the central player in Ewing's sarcoma pathogenesis. However, the fact that EWS/FLI has not been sufficient to transform any human cell type *in vitro* implies that there are parallel pathways and/or cooperating mutations working in conjunction with EWS/FLI. Work in this area has provided promising leads to what such EWS/FLI collaborators may be, yet we remain far from a complete understanding of exactly what molecular combination provides the 'Ewing's sarcoma oncogenic cocktail' (Figure 2).

Two decades ago, it was appreciated that Ewing's cell lines expressed both insulin-like growth factor 1 (IGF-1) and its receptor (IGF-1R), and that blocking antibodies targeting the receptor could slow cell growth in culture (Yee *et al*., 1990). Since that time, a variety of studies have supported the importance of that pathway in Ewing's sarcoma (Hamilton *et al*., 1991; van Valen *et al*., 1992; Scotlandi *et al*., 1996, 1998, 2002b; Toretsky *et al*., 1997). This work has important clinical implications, as both small molecule and antibody-mediated approaches to IGF pathway blockade have shown efficacy in preclinical models of the disease, and in patients in early phase clinical trials (Benini *et al*., 2001; Scotlandi *et al*., 2002a, 2005; Benjamin *et al*., 2007; Tolcher *et al*., 2007; Kolb *et al*., 2008; Kurmasheva *et al*.,

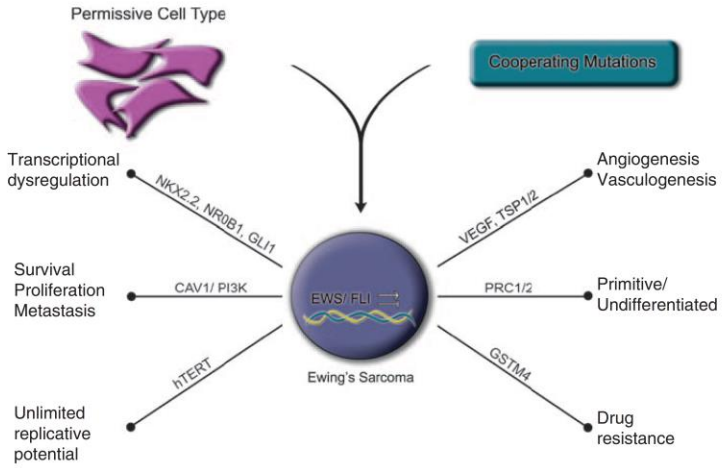

Figure 2 Ewing's sarcoma transformation requires several distinct events. The EWS/FLI (or other TET/ETS) translocation is the central mediator of this process, dysregulating a number of genes that contribute to oncogenesis and tumor progression. A permissive cell type for EWS/FLI expression is also required. Cooperating mutations such as those in the RB and p53 pathways, as well as growth factor signaling (including IGF) likely contribute to the fully transformed phenotype.

2009; Olmos *et al*., 2009; Manara *et al*., 2010). It should be noted that although the IGF pathway is often considered a 'parallel' pathway to EWS/FLI, it may in fact be modulated by the fusion protein. For example, the IGF-binding protein 3 gene (*IGFBP3*) is downregulated by EWS/FLI (Prieur *et al*., 2004), which might allow for increased IGF signaling. In addition, recent work suggests that EWS/FLI may regulate IGF1 itself (Cironi *et al*., 2008; Herrero-Martin *et al*., 2009).

CD99 (also called MIC2) is an integral transmembrane glycoprotein that is the most commonly used diagnostic marker in Ewing's sarcoma to distinguish Ewing's sarcoma from other small round blue cell tumors (Kovar *et al*., 1990; Ambros *et al*., 1991; Fellinger *et al*., 1991). Engagement of CD99 with monoclonal antibodies can induce apoptosis in Ewing's sarcoma cell lines (Sohn *et al*., 1998). This finding was extended to show that CD99 engagement slowed tumor formation in athymic mice and enhanced the growth inhibitory effects of doxorubicin and vincristine in cell culture experiments (Scotlandi *et al*., 2000). Recent data suggest that CD99 is not only a marker and therapeutic target for Ewing's sarcoma, but may also contribute to the disease phenotype. Knockdown of CD99 in Ewing's sarcoma cell lines resulted in decreased growth in tissue culture, diminished colony formation in soft agar assays, reduced cell motility, and smaller tumors with less metastasis in xenograft models (Rocchi *et al*., 2010). This study also suggested that CD99 inhibits full neuronal differentiation by decreasing the activity of the MAP kinase pathway.

Alterations in the RB and p53 pathways are likely cooperating mutations in Ewing's sarcoma. For example, expression of the cell cycle progression protein cyclin D1 (which phosphorylates and inactivates RB) is generally upregulated (Zhang *et al*., 2004; Fuchs *et al*., 2004b; Sanchez *et al*., 2008), whereas the cell cycle inhibitory proteins RB and p16INK4A are often mutated or deleted in Ewing's sarcoma (Kovar *et al*., 1997; Wei *et al*., 2000; Huang *et al*., 2005). Mutations in this pathway also have prognostic value, with patients harboring p53 or p16INK4A/ARF deletions having a worse prognosis than those without (Wei *et al*., 2000; de Alava *et al*., 2000; Huang *et al*., 2005). The biologic basis for these observations may be related to oncogenic stress mediated by the EWS/FLI fusion, as expression of EWS/FLI in primary fibroblasts leads to growth arrest or cell death (Deneen and Denny, 2001; Lessnick *et al*., 2002). Inhibition of the p53 and/or RB pathways in these models bypasses these effects, suggesting that there may be selective pressure *in vivo* for cooperating mutations in these pathways.

Copy number alterations in Ewing's sarcoma

Genome-wide approaches offer the opportunity to identify cooperative pathways more efficiently. Several studies in the past decade have used early generation array comparative genomic hybridization technologies on Ewing's clinical samples (Armengol *et al*., 1997; Maurici *et al*., 1998; Tarkkanen *et al*., 1999; Brisset *et al*., 2001; Ozaki *et al*., 2001; Hattinger *et al*., 2002; Shing *et al*., 2002; Selvarajah *et al*., 2007; Ferreira *et al*., 2008; Savola *et al*., 2009). Despite the small numbers of tumors and relatively low resolution offered by early generation array comparative genomic hybridization, many of these studies have described overlapping regions of copy number gains or losses (collectively referred to as copy number alterations; Table 1). Deletion of 9p21.3, containing the *CDKN2A* gene that encodes p16INK4A/ARF, was deleted in 14–67% of Ewing's tumors and cell lines (Brownhill *et al*., 2007; Neale *et al*., 2008). Conversely, gain of the specific region of 1q22 has been reported to occur in 18–63% of Ewing's samples or cell lines (Armengol *et al*., 1997; Tarkkanen *et al*., 1999; Shing *et al*., 2002; Savola *et al*., 2009), suggesting the presence of a possible oncogene in this region.

Copy number studies may also have prognostic value. For example, gains of the entire chromosome 8 have been reported by nearly every comparative genomic hybridization study and occur in as many as 23–80% of Ewing's samples (Armengol *et al*., 1997; Maurici *et al*., 1998; Brisset *et al*., 2001; Ozaki *et al*., 2001; Hattinger *et al*., 2002; Ferreira *et al*., 2008; Roberts *et al*., 2008; Savola *et al*., 2009), with the highest frequency found in metastatic and relapsed tumors. In addition, gain of 1q or independent loss of 16q was found to predict worse overall and event-free survival, regardless of localized or metastatic disease at diagnosis (Hattinger *et al*., 2002). This same study showed chromosome 12 gain to be associated with adverse event-free survival in patients with localized disease at diagnosis. Deletion of 6p has also been associated with worse outcome in diagnostic Ewing's sarcoma samples (Tarkkanen *et al*., 1999).

Total ploidy and absolute number of copy number alterations have also been shown to correlate with clinical outcome in Ewing's sarcoma. Patients with primary Ewing's tumors with $\leqslant 3$ copy number alterations were reported to do better than patients with >3 copy number changes in two different studies (Ferreira *et al*., 2008; Savola *et al*., 2009), whereas another group found that five copy number alterations could stratify patients (Ozaki *et al*., 2001). Taken together, it seems that increasing genomic instability in Ewing's sarcoma is associated with worse outcome.

Summary and conclusions

Ewing's sarcoma is a complex disease that requires the coordination of many events to arise. The enigmatic cell of origin that provides a permissive environment for the complex transcriptional dysregulation mediated by EWS/FLI, combined with cooperation of parallel pathways that may differ from case to case make research and treatment of this disease a challenge. Better insight into the cell of origin and the CSC potential in Ewing's sarcoma will provide a model in which to study this malignancy. This should afford researchers with a more

Table 1 Copy number alterations (CNAs) reported in Ewing's sarcoma

Deletion	*Gain*	*Frequency (%)*	*Sample type*	*Technology*	*Study*
	1q	12/41 (29%)	ES samples (primary, metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
		14/83 (17%)	ES samples (primary, localized)	Karyotyping and CGH	Hattinger *et al.* (2002)
		6/22 (27%)	ES samples (metastatic)	CGH	Brisset *et al.* (2001)
		7/28 (25%)	ES samples (metastatic)	Karyotyping (G-band)	Roberts *et al.* (2008)
		8/45 (18%)	ES samples (localized)	Karyotyping (G-band)	Roberts *et al.* (2008)
		9/52 (17%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	1q 21–q22	5/20 (25%)	ES samples (17 patients, two specimens from same patient in 3 cases)	CGH	Armengol *et al.* (1997)
	1q21–22	5/28 (18%)	ES samples	CGH	Tarkkanen *et al.* (1999)
	1q21–q32	5/8 (63%)	Cell line	CGH	Shing *et al.* (2002)
	1q22–qter	10/31 (32%)	ES samples	CGH	Savola *et al.* (2009)
	2	11/100 (11%)	ES samples (primary, localized + metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
		5/21 (24%)	ES samples (localized)	CGH	Brisset *et al.* (2001)
		9/31 (29%)	ES samples	CGH	Savola *et al.* (2009)
	2q	4/10 (40%)	ES samples (relapse)	CGH	Ozaki *et al.* (2001)
	4p	4/10 (40%)	ES samples (relapse)	CGH	Ozaki *et al.* (2001)
	4q	3/20 (15%)	ES samples (17 patients, two specimens from same patient in 3 cases)	CGH	Armengol *et al.* (1997)
	5	12/100 (12%)	ES samples (primary, localized + metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
	5p	5/25 (20%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
	7	10/100 (10%)	ES samples (primary, localized + metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
	7p21.1–p11.2	2/9 (22%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	7q	5/28 (18%)	ES samples	CGH	Tarkkanen *et al.* (1999)
	8	10/28 (36%)	ES samples	CGH	Tarkkanen *et al.* (1999)
		10/28 (36%)	ES samples (metastatic)	Karyotyping (G-band)	Roberts *et al.* (2008)
		14/25 (56%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
		20/45 (44%)	ES samples (localized)	Karyotyping (G-band)	Roberts *et al.* (2008)
		21/31 (67%)	ES samples	CGH	Savola *et al.* (2009)
		24/52 (46%)	ES samples (1°, Mets, recurrence)	FISH (24) or karyotyping (24)	Maurici *et al.* (1998)
		25/42 (60%)	ES samples (primary, metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
		5/22 (23%)	ES samples (metastatic)	CGH	Brisset *et al.* (2001)
		43/89 (48%)	ES samples (primary, localized)	Karyotyping and CGH	Hattinger *et al.* (2002)
		7/20 (35%)	ES samples (17 patients, two specimens from same patient in 3 cases)	CGH	Armengol *et al.* (1997)
		8/10 (80%)	ES samples (relapse)	CGH	Ozaki *et al.* (2001)
		8/21 (38%)	ES samples (localized)	CGH	Brisset *et al.* (2001)
	8p	22/52 (42%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	8q	24/52 (46%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	8q11.21–q22.3	6/9 (67%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	8q11.2–q22	7/8 (88%)	6 Cell line, 1 primary culture	CGH	Shing *et al.* (2002)
	8q23–q24.1	5/8 (63%)	4 Cell line, 1 primary culture	CGH	Shing *et al.* (2002)
	8q24.11–q24.21	7/9 (78%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	12	13/43 (30%)	ES samples (primary, metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
		17/52 (33%)	ES samples (1°, Mets, recurrence)	FISH (24) or karyotyping (24)	Maurici *et al.* (1998)
		23/88 (26%)	ES samples (primary, localized)	Karyotyping and CGH	Hattinger *et al.* (2002)
		3/28 (11%)	ES samples	CGH	Tarkkanen *et al.* (1999)
		3/28 (11%)	ES samples (metastatic)	Karyotyping (G-band)	Roberts *et al.* (2008)
		4/22 (18%)	ES samples (metastatic)	CGH	Brisset *et al.* (2001)
		5/20 (25%)	ES samples (17 patients, two specimens from same patient in 3 cases)	CGH	Armengol *et al.* (1997)
		5/25 (20%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
		6/21 (29%)	ES samples (localized)	CGH	Brisset *et al.* (2001)
		7/45 (16%)	ES samples (localized)	Karyotyping (G-band)	Roberts *et al.* (2008)
		9/31 (29%)	ES samples	CGH	Savola *et al.* (2009)
	12p	9/52 (17%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	12q	11/52 (21%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	12q12–q15	6/8 (75%)	Cell line	CGH	Shing *et al.* (2002)
	12q13.2–q14.1	9/31 (29%)	ES samples	CGH	Savola *et al.* (2009)
	12q14.1–q15	2/9 (22%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	14	9/100 (9%)	ES samples (primary, localized + metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)

Table 1 Continued

Deletion	*Gain*	*Frequency (%)*	*Sample type*	*Technology*	*Study*
	14q	3/20 (15%)	ES samples (17 patients, two specimens from same patient in 3 cases)	CGH	Armengol *et al.* (1997)
	14q11.2	2/9 (22%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	17q21.31–q25.3	6/9 (67%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	18	3/25 (12%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
	20	12/92 (13%)	ES samples (primary, localized + metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
		3/25 (12%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
		5/28 (18%)	ES samples (metastatic)	Karyotyping (G-band)	Roberts *et al.* (2008)
		5/45 (11%)	ES samples (localized)	Karyotyping (G-band)	Roberts *et al.* (2008)
		6/21 (29%)	ES samples (localized)	CGH	Brisset *et al.* (2001)
		8/52 (15%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	20p	10/52 (19%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	20q	10/52 (19%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
	20q11.23–q13.33	2/9 (22%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	21q22.3	2/9 (22%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
	22q11.21	2/9 (22%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
1p		3/10 (30%)	ES samples (relapse)	CGH	Ozaki *et al.* (2001)
		4/42 (10%)	ES samples (primary, metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
		6/80 (8%)	ES samples (primary, localized)	Karyotyping and CGH	Hattinger *et al.* (2002)
1p13–1p36.3		10/125 (8%)	ES samples (primary, localized + metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
1p36.32–p36.11		2/9 (22%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
3p		3/8 (38%)	Cell line	CGH	Shing *et al.* (2002)
7q11.2		5/25 (20%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
9p		7/31 (23%)	ES samples	CGH	Savola *et al.* (2009)
9p21		6/42 (14%)	ES samples (primary)	MLPA	Brownhill *et al.* (2007)
		6/9 (67%)	ES cell lines	MLPA	Brownhill *et al.* (2007)
		5/9 (56%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
10		4/25 (16%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
		7/52 (13%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
16q		10/31 (32%)	ES samples	CGH	Savola *et al.* (2009)
		11/52 (21%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
		12/78 (15%)	ES samples (primary, localized)	Karyotyping and CGH	Hattinger *et al.* (2002)
		13/41 (32%)	ES samples (primary, metastatic)	Karyotyping and CGH	Hattinger *et al.* (2002)
		3/28 (11%)	ES samples	CGH	Tarkkanen *et al.* (1999)
		4/25 (16%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
		5/8 (63%)	4 Cell line, 1 primary culture	CGH	Shing *et al.* (2002)
		8/45 (18%)	ES samples (localized)	Karyotyping (G-band)	Roberts *et al.* (2008)
16q22.3		5/9 (56%)	ES samples vs cell line xenografts	SNP microarray (Affy 100 K)	Neale *et al.* (2008)
17p		8/52 (15%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
17p13–p11.2		4/8 (50%)	3 Cell line, 1 primary culture	CGH	Shing *et al.* (2002)
19		4/25 (16%)	ES samples (23 primary, 2 relapse)	CGH	Ferreira *et al.* (2008)
19p		6/52 (12%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
19q		10/52 (19%)	ES samples (primary, includes 20 metastatic)	CGH	Ozaki *et al.* (2001)
Y		3/5, Males (60%)	Cell line	CGH	Shing *et al.* (2002)

Abbreviations: CGH, comparative genomic hybridization; ES, Ewing's sarcoma; FISH, fluorescence *in situ* hybridization; SNP, single nucleotide polymorphism.

comprehensive list of proteins necessary for Ewing's sarcoma tumorigenesis and an opportunity to understand the contribution of each. The mechanism by which EWS/FLI up- or downregulates these critical genes remains an important question. Finally, new technology will allow for the identification of mutations that cooperate with EWS/FLI and its targets to bypass the many mechanisms in place to prevent uncontrolled proliferation and other cancerous phenotypes. Research in all of these areas will lead to a more comprehensive model of Ewing's sarcoma genesis and disease progression that may inform the study of other cancers caused by ETS protein dysregulation. This improved molecular insight can also be incorporated into clinical trials that will eventually provide more targeted treatment for this disease.

Conflict of interest

The authors declare no conflict of interest.

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CHAPTER 3

BCL11B IS UP-REGULATED BY EWS/FLI AND CONTRIBUTES TO THE TRANSFORMED PHENOTYPE IN EWING SARCOMA

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BCL11B Is Up-Regulated by EWS/FLI and Contributes to the Transformed Phenotype in Ewing Sarcoma

Elizabeth T. Wiles[1], Bianca Lui-Sargent[2], Russell Bell[2], Stephen L. Lessnick[1,2,3]*

1 Department of Oncological Sciences, University of Utah, Salt Lake City, Utah, United States of America, **2** Center for Children's Cancer Research, Huntsman Cancer Institute, Salt Lake City, Utah, United States of America, **3** Division of Pediatric Hematology/Oncology, University of Utah, Salt Lake City, Utah, United States of America

Abstract

The EWS/FLI translocation product is the causative oncogene in Ewing sarcoma and acts as an aberrant transcription factor. EWS/FLI dysregulates gene expression during tumorigenesis by abnormally activating or repressing genes. The expression levels of thousands of genes are affected in Ewing sarcoma, however, it is unknown which of these genes contribute to the transformed phenotype. Here we characterize BCL11B as an up-regulated EWS/FLI target that is necessary for the maintenance of transformation in patient derived Ewing sarcoma cells lines. BCL11B, a zinc finger transcription factor, acts as a transcriptional repressor in Ewing's sarcoma and contributes to the EWS/FLI repressed gene signature. BCL11B repressive activity is mediated by the NuRD co-repressor complex. We further demonstrate that re-expression of SPRY1, a repressed target of BCL11B, limits the transformation capacity of Ewing sarcoma cells. These data define a new pathway downstream of EWS/FLI required for oncogenic maintenance in Ewing sarcoma.

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* E-mail: stephen.lessnick@hci.utah.edu

Introduction

Ewing sarcoma is an aggressive tumor that occurs in the bone and soft tissue of the pediatric and young adult population [1]. This tumor is characterized by a chromosomal translocation that fuses the 5′ portion of the *EWSR1* gene (encoding the EWS protein) on chromosome 22, in frame, to the 3′ portion of the *FLI1* gene (encoding the FLI protein) on chromosome 11 [2]. The resulting fusion protein, EWS/FLI, retains the ETS-type DNA binding domain from FLI as well as the transcriptional activating and repressing domains from EWS, and thus acts as an aberrant transcription factor. EWS/FLI is present in ~85% of Ewing sarcoma cases while the remaining 15% express a fusion between EWS and another member of the ETS family (ERG, ETV1, ETV4, or FEV) [3]. While the cell of origin is unknown, this fusion is thought to occur in a primitive cell type where it prevents terminal differentiation. Initial mutational studies suggest that Ewing sarcoma has a relatively low frequency of alterations in known tumor suppressors or oncogenes, supporting the concept that EWS/FLI, and the genes that it regulates, are largely responsible for oncogenesis and tumor maintenance [4,5]. Indeed, approaches that reduce the levels of EWS/FLI in Ewing sarcoma cells block the transformed phenotype of these cells [6–8].

Microarray analysis of gene expression in Ewing sarcoma cell lines compared to those with reduced EWS/FLI expression reveal significant alterations in the transcriptional signatures. EWS/FLI has been shown to decrease the expression of ~3000 genes while increasing the mRNA levels of ~500 genes [9]. It is important to determine which of these dysregulated genes contribute to the transformation process. Several EWS/FLI target genes have previously been identified that contribute to oncogenic processes in Ewing's sarcoma: *NKX2.2* [7], *NR0B1* [6], *GLI1* [10], *TGFBR2* [11], among others. In this work we characterize B-cell chronic lymphocytic leukemia/lymphoma 11B (*BCL11B*, also known as *CTIP2*), encoding a zinc finger transcription factor [12], as an important target gene up-regulated by EWS/FLI.

BCL11B expression in Ewing sarcoma has been noted previously. *BCL11B* was identified as a gene that was highly expressed in Ewing sarcoma, but only expressed in a restricted subset of normal tissues [13]. More recent microarray studies, including our own, have revealed that BCL11B is induced by EWS/FLI in Ewing sarcoma tumor samples and cell lines [9,14], as well as two of the proposed cells of origin, mesenchymal stem cells [15] and neural crest stem cells [16]. Interestingly, EWS/FLI does not modulate BCL11B expression in HEK293 cells [13] or NIH3T3 cells [17] (ETW unpublished observation), suggesting that a permissive cellular background is necessary for EWS/FLI to up-regulate BCL11B expression. Chromatin accessibility and transcription factor/co-factor availability may be elements that contribute to these cell specific differences in BCL11B regulation.

Bcl11b knock-out mice are initially viable, but die on post-natal day 1 [18]. Phenotypes described in this mouse reveal developmental defects in the skin [19], teeth [20], central nervous system (CNS) [21,22], and hematopoietic lineage [18,23]. For example, *Bcl11b* null murine thymocytes fail to undergo T-cell differentiation [23]. In the CNS, Bcl11b is necessary for the connection of corticospinal motor neurons to the spinal cord [22] and for neurogenesis in the dentate gyrus [24]. These observations identify

Bcl11b as a pivotal developmental transcription factor involved in fate specification decisions in multiple cell types.

BCL11B has also been studied in the context of malignancies, where it has been described as a haploinsufficient tumor suppressor in T-cell acute lymphoblastic leukemia (T-ALL). *BCL11B* was found to be mutated in 9–16% of human T-ALL samples. [25,26]. In addition, mouse models of both TLX1 driven T-ALL and gamma-ray induced thymic lymphomas had spontaneous deletions and mutations in *Bcl11b* [26,27]. These findings, which characterize BCL11B's tumor inhibitory function, present a conundrum: why does EWS/FLI induce the expression of a postulated tumor suppressor? We therefore sought to define the role of BCL11B in Ewing sarcoma. Surprisingly, in contrast to its tumor suppressive function in leukemia and lymphoma, we now show that BCL11B positively contributes to the transformed phenotype in Ewing sarcoma.

Materials and Methods

Constructs and Retroviruses

CHD4 short hairpin RNA (shRNA), EWS/FLI shRNA (EF-2 RNAi), EWS/FLI cDNA and mutants were previously described [7,28–31]. BCL11B shRNA constructs were designed targeting the 3′UTR and cloned into a pMKO.1puro vector [32]. Oligonucleotide sequences for previously unpublished shRNAs are provided in Supplemental Data (Table S1). BCL11B (variant 2) cDNA was sub-cloned from pMIGR [33] into pQCXIN (Clontech). SPRY1 cDNA (Thermo Scientific) was PCR amplified with an amino-terminal 3xFLAG-tag and cloned into pMSCVneo (Clontech).

Cell Culture

The A673 cell line (American Type Culture Collection) was grown as previously described [34] and TC71 cells (from Timothy Triche, Children's Hospital Los Angeles) [35] were grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/glutamine. Following retroviral infection, cells were selected with the appropriate antibiotic, resulting in polyclonal infected populations. Small interfering RNA (siRNA) transfections (siBCL11B: A673 6.25 nM and TC71 25 nM; siNCOR1: A673 25 nM) were carried out according the manufacturer's instructions (ThermoFisher). *In vitro* transformation assays were performed by plating 1×10^5 cells in 2% methylcellulose mixed 1:1 with cell growth media containing the appropriate antibiotic selection. Endpoint was dictated by the ability to see colonies on control plate (approximately 2–4 weeks; e.g., A673 form colonies faster than TC71), and was internally consistent for all experiments. Plates were sectioned into quadrants, and colonies were counted by eye using an Olympus SZ61 stereomicroscope and manual counter. Growth curves were generated by counting total cells and re-plating 5×10^5 cells every third day. Student's T-test was performed on the average three day population difference over fifteen days for each condition. A673 cells were treated with the indicated concentration of the histone deacetylase (HDAC) inhibitor, vorinostat (ChemieTek)or the lysine specific demethylase 1 (LSD1) inhibitor, HCI-2509 [31] for 48 h. A673 cells were treated with the indicated concentration of chaetocin (Sigma-Aldrich), a fungal metabolite that specifically inhibits SUV39H1, or Ex-527, a small molecule that inhibits SIRT1, (Sigma-Aldrich) for 24 h. For ERK detection, A673 cells were infected with empty vector or SPRY1 cDNA and selected with neomycin under normal tissue culture growth conditions. Cells were then trypsinized and plated into tissue culture plates (adherent) or into ultra-low attachment plates (suspension) for 24 h before protein extraction.

Quantitative Reverse-transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted with an RNAeasy kit (Qiagen). mRNA (15 ng) was quantitated by SYBR green (Bio-Rad) using one-step qRT-PCR with gene specific primers (Table S1). Messenger RNA was reverse-transcribed at 50°C for 10 minutes followed by a 5 minute denaturation at 95°C and then 45 cycles of PCR (95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds). Fold change was determined using the ΔC_t method comparing all samples to the control after normalizing to GAPDH. Student's T-test was performed using threshold cycle values.

Immunodetection

Western blots were performed with the following antibodies: FLI1 (Abcam 15289), BCL11B (Abcam 28448), tubulin (Calbiochem CP06), FLAG M2 (Sigma-Aldrich A8592), P-ERK1/2 (Cell Signaling 9106), total ERK1/2 (Cell Signaling 9102).

RNA Sequencing

RNA from A673 cells transfected with siBCL11B-3, siBCL11B-10 or siControl was extracted with the RNAeasy kit (Qiagen) and treated with DNAse 48 h post-transfection. Libraries for high-throughput sequencing were prepared according to the manufacturer's instructions (Illumina) and sequenced on the Illumina Hi-Seq with 50 cycles of single end reads. Sequences were aligned to the human genome build hg19. Raw sequence reads can be found in the NCBI SRA #058854. Differential gene expression was determined using the publically available USeq package (useq.-sourceforge.net). Significance parameters were set at an FDR of 10% and two-fold change.

Venn diagrams were created using the VennMaster program and comparing BCL11B regulated genes to EWS/FLI repressed genes [9]. GSEA was performed by creating a rank ordered list (most up-regulated genes to most repressed genes) using EWS/FLI knock-down microarray data from A673 cells and TC71 cells [6] and comparing to BCL11B RNAseq data using the GseaPreranked program from the Broad Institute [36].

Results

BCL11B Expression is Induced by EWS/FLI

BCL11B is highly expressed in Ewing sarcoma cell lines and tumor samples and has been identified as an EWS/FLI up-regulated target in numerous microarray studies [13,14,37]. However, the microarray data have never been validated nor has the functional significance of BCL11B in Ewing sarcoma been investigated. Using a knock-down/rescue approach in two different Ewing sarcoma cell lines (A673 and TC71), we found that reduction of EWS/FLI levels via a retroviral shRNA causes a significant reduction in BCL11B RNA and protein expression levels (Figure 1A, B). Furthermore, BCL11B RNA and protein expression are restored when an EWS/FLI cDNA (that is resistant to the RNAi effect) is re-expressed (Figure 1A, B).

EWS/FLI is a modular protein, with transcriptional regulatory functions contributed by the amino-terminus of EWS, and DNA binding contributed by the ETS domain of the FLI portion. We investigated the necessity of these regions in BCL11B regulation by using an EWS deletion construct which retains only the first six amino acids of EWS (Δ22), or a DNA binding double point mutant (R2L2) of EWS/FLI as the rescue construct, respectively (Figure 1C, D; [31]). Neither mutant construct rescued the expression of BCL11B after EWS/FLI knock-down indicating that both the EWS portion (which harbors a strong transcriptional

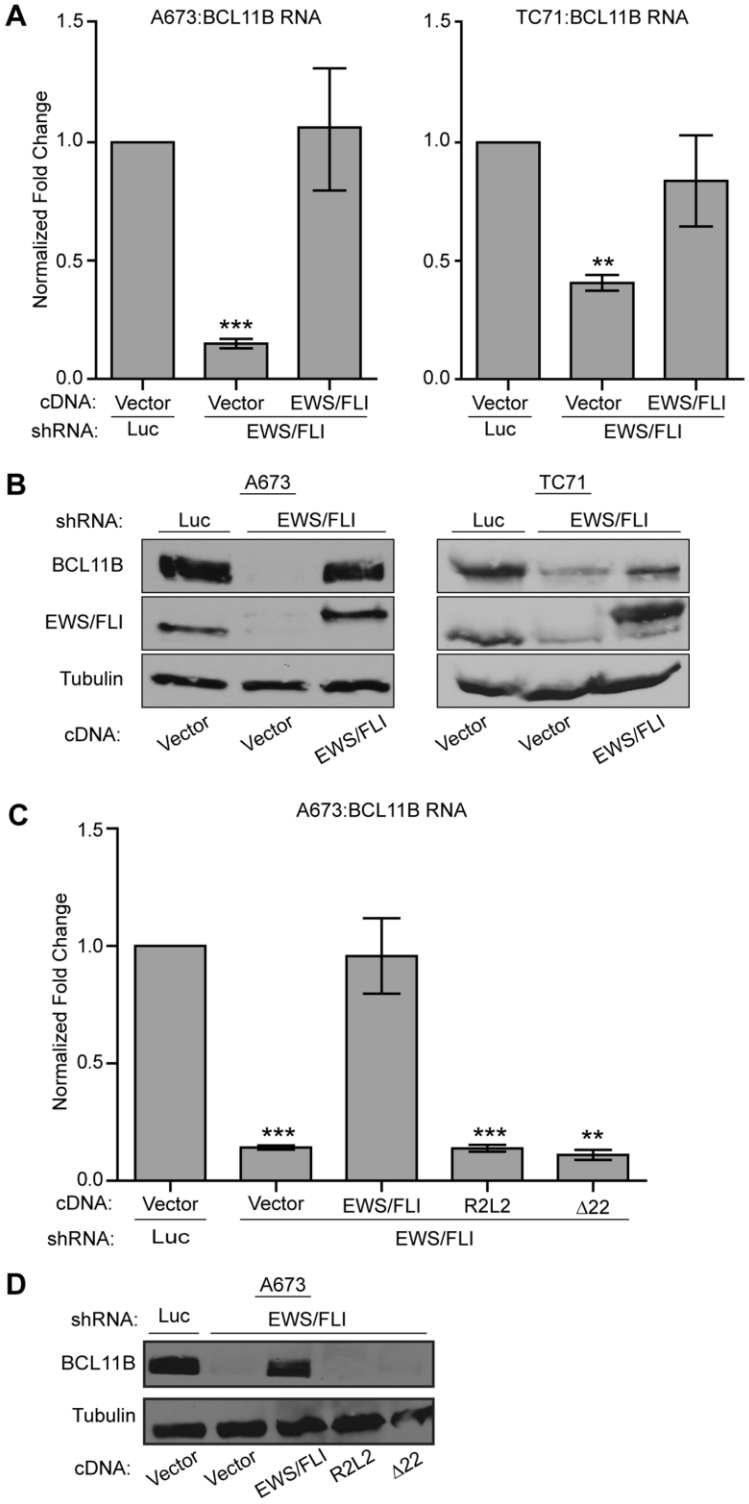
A
A673:BCL11B RNA
TC71:BCL11B RNA
Normalized Fold Change
1.5
1.0
0.5
0.0
***
**
cDNA:
shRNA:
Vector
Luc
EWS/FLI
B
A673
TC71
shRNA:
BCL11B
EWS/FLI
Tubulin
cDNA:
C
A673:BCL11B RNA
R2L2
Δ22
D
A673

Figure 1. BCL11B is up-regulated by EWS/FLI in Ewing sarcoma cells. A. A673 and TC71 Ewing sarcoma cells were infected with a control shRNA (Luc) or an shRNA targeting EWS/FLI followed by rescue with an empty vector or EWS/FLI cDNA. BCL11B RNA levels were determined by qRT-PCR. Error bars represent standard deviation (SD) of three technical replicates. P-values were determined using a Student's T-test comparing all conditions to control (Vector+Luc shRNA) (** for $p \leq 0.01$, *** for $p \leq 0.001$). B. A673 and TC71 cells were infected as in panel A, and protein extracts were Western blotted for BCL11B and EWS/FLI. Tubulin was used as a loading control. C. A673 cells were infected with a control shRNA or shRNA targeting EWS/FLI followed by rescue with EWS/FLI cDNA, or mutants of EWS/FLI containing a DNA biding mutation (R2L2) or an EWS deletion (Δ22). Error bars represent SD of three technical replicates. P-values were determined using a Student's T-test comparing all conditions to control (Vector+Luc shRNA) (** for $p \leq 0.01$, *** for $p \leq 0.001$). D. Western blot analysis of BCL11B levels in the same samples shown in panel C. Tubulin was used as a loading control.
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activation domain [38]) and the ETS DNA binding domain are required for the activation of BCL11B.

BCL11B Expression is Necessary for Maintenance of Transformation

We next took a loss of function approach to determine the involvement of BCL11B in maintenance of transformation in Ewing sarcoma. We used anchorage independent growth in methylcellulose as an *in vitro* measure of transformation. A673 and TC71 cells were infected with two different shRNA constructs targeting the 3′UTR of BCL11B (BCL11B-4 and BCL11B-6 shRNA). These shRNAs significantly decreased BCL11B protein levels (Figure 2A). Ewing sarcoma cells with reduced BCL11B expression grew in tissue culture with a slightly reduced growth rate (Figure 2B). However, when these cells were grown in methylcellulose under anchorage-independent conditions, significantly fewer colonies formed as compared to a control knockdown targeting luciferase (Luc shRNA), and correlated with the level of knockdown achieved by each construct (Figure 2C). These results demonstrate that BCL11B is necessary for maintaining the transformed phenotype of Ewing sarcoma cell lines.

BCL11B Contributes to the EWS/FLI Repressed Gene Signature

As BCL11B is a transcription factor, we sought to identify the genes it regulates in Ewing sarcoma to gain insight into its function in sustaining tumorigenicity. We performed an RNA-seq experiment comparing transcripts from A673 cells transfected with a control siRNA (siControl) or two different siRNAs targeting BCL11B (siBCL11B-3 and siBCL11B-10) (Figure 3A). We used the Useq package to identify differentially expressed genes with significance cutoffs set to a two-fold change and a false discovery rate (FDR) of 10%. This analysis identified 118 genes down-regulated by BCL11B and 26 genes up-regulated by BCL11B (Table S2). While this analysis does not distinguish direct from indirect targets, this is consistent with BCL11B's previous characterization as a transcriptional repressor [39].

Venn overlap analysis using genes we found to be repressed by BCL11B and those repressed by EWS/FLI revealed that a significant number of genes were present on both lists. Of the 118 genes repressed by BCL11B, 55 genes were also repressed by EWS/FLI ($p = 2.55 \times 10^{-24}$; Figure 3B). This indicates that the increase in expression of BCL11B mediated by EWS/FLI contributes to the 2133 gene EWS/FLI down-regulated signature. To further establish the relationship between EWS/FLI and BCL11B repressed genes, we used a different unbiased statistical approach by performing Gene Set Enrichment Analysis (GSEA). For this analysis we rank-ordered genes regulated by EWS/FLI from most up-regulated to the most down-regulated, and then identified where on this list the BCL11B repressed genes fell. GSEA produced a significant negative enrichment score (NES) in both A673 and TC71 cells indicating that genes repressed by BCL11B are well-correlated with those most repressed by EWS/FLI (A673 $NES = -2.123$, $p < 0.001$; TC71 $NES = -1.452$, $p = 0.005$; Fig. 3C). In contrast to the down-regulated genes, only two genes from the BCL11B up-regulated gene set overlapped with EWS/FLI up-regulated genes, and were not statistically significant. We therefore focused on the BCL11B repressed genes for the subsequent analyses.

Neutral cholesterol ester hydrolase 1 (*NCEH1*), Sprouty1 (*SPRY1*), adenosine A1 receptor (*ADORA1*), and transforming growth factor beta receptor 1 (*TGFBR1*) were found to be repressed by both EWS/FLI and BCL11B in our genome-wide studies. We chose these four genes from the list of fifty-five overlapping genes to validate our findings. We first confirmed our RNA-seq analysis in biologic replicates of A673 cells, as well as TC71 cells, by evaluating these genes with qRT-PCR. We found that when BCL11B levels are reduced by siRNAs, the transcript level of each of the genes increases in both A673 and in TC71 Ewing sarcoma cells (Figure 4A). We then validated the results from the EWS/FLI microarray by knocking down EWS/FLI in A673 cells and again observing an increased expression of these four genes (Figure 4B).

We next wanted to determine if the up-regulation of BCL11B by EWS/FLI was solely responsible for this repression. To test this, we used shRNA targeting EWS/FLI in A673 cells (which decreases BCL11B expression) and re-expressed the BCL11B cDNA. We found that BCL11B expression was able to partially rescue the increase in expression of SPRY1 and ADORA1, but not NCEH1 and TGFBR1 (Figure 4B). This suggests that BCL11B has some repressive activity against SPRY1 and ADORA1 independent of EWS/FLI; however, full repression of these genes, as well as repression of NCEH1 and TGFBR1, requires more complex regulation, likely including other genes regulated by EWS/FLI.

BCL11B Repression is Mediated by the NuRD Complex

BCL11B has been characterized as a transcriptional repressor in many cellular contexts. This repression is mediated by interactions with various co-repressors including the nucleosome remodeling and histone deacetylase complex (NuRD) [40,41], suppressor of variegation 3–9 homolog 1 (SUV39H1) [42,43], and sirtuin1 (SIRT1) [44]. We tested the necessity of these three co-repressors in BCL11B-mediated repression in Ewing sarcoma cells by shRNA knock-down for chomodomain helicase DNA binding protein 4 (CHD4), the core component of the NuRD complex, or chemical inhibitors targeting SUV39H1 or SIRT1. We found that the small molecule inhibitor of SIRT1, Ex-527, had no effect on the expression of NCEH1, SPRY1, ADORA1, or TGFBR1 (Figure S1A). Chaetocin, a fungal metabolite that specifically inhibits SUV39H1, was cytotoxic to A673 cells at concentrations lower than the IC50 (800 nM) for inhibition of SUV39H1 (data not shown). Even at these lower concentrations, it significantly reduced the expression of BCL11B (Figure S1B), and so prevented us from drawing any conclusions about the involvement of SUV39H1 in BCL11B mediated repression in Ewing sarcoma.

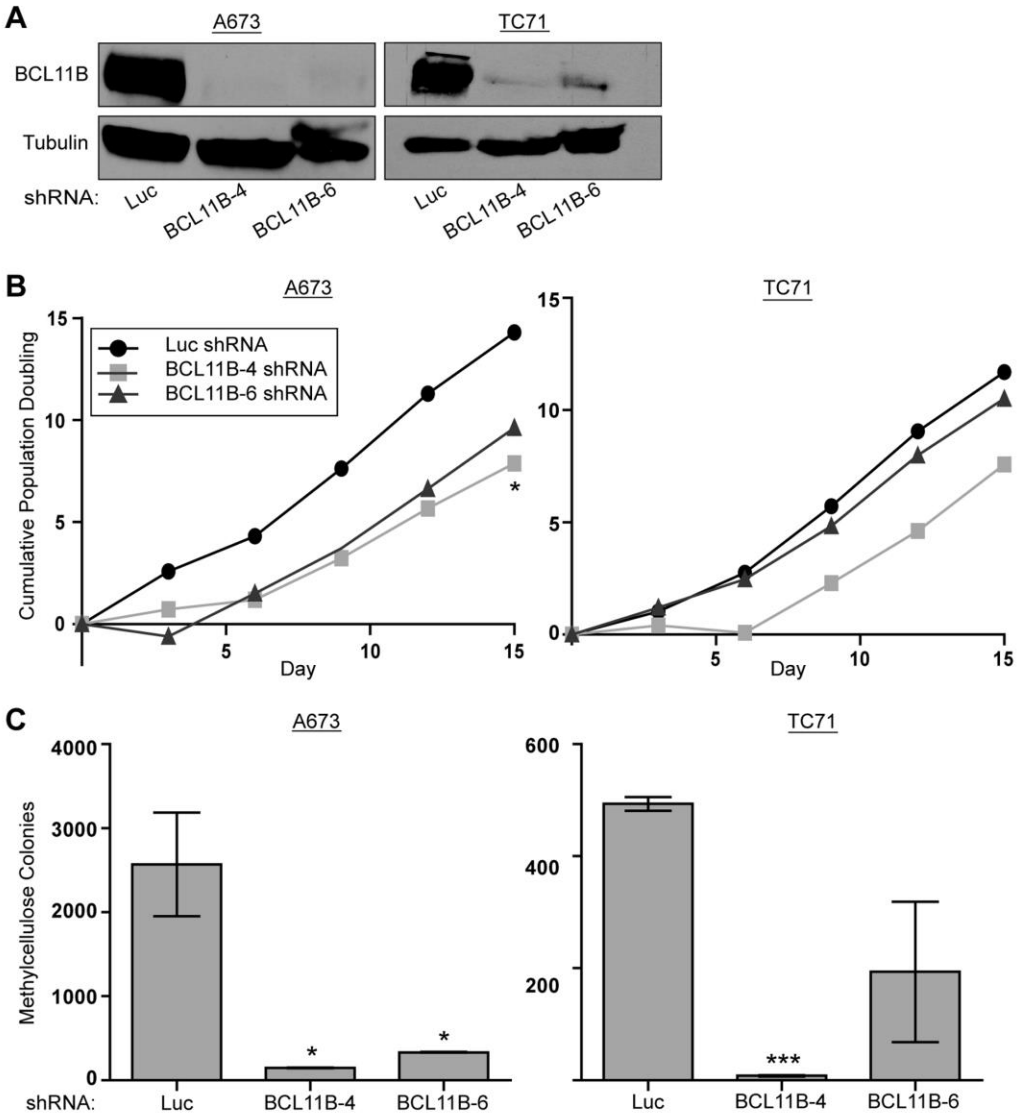

Figure 2. BCL11B is necessary for the maintenance of transformation in Ewing sarcoma cells. A. A673 and TC71 Ewing sarcoma cells were infected with retroviral shRNA constructs targeting BCL11B (BCL11B-4 and BCL11B-6 shRNA), or luciferase (Luc) as a control. BCL11B levels were determined by western blot. Tubulin was used as a loading control. B. Growth rates of A673 and TC71 cells harboring the indicated shRNA retroviral constructs were determined using a 3T5 assay [34]. P-values were determined using a Student's T-test comparing all conditions to control (Luc shRNA) (* for $p \leq 0.05$). C. Anchorage independent growth of control (Luc shRNA) and BCL11B (BCL11B-4 and BCL11B-6 shRNA) knock-down A673 and TC71 cells was assessed by the ability to form colonies in methylcellulose. Error bars represent SD of two technical replicates. P-values were determined using a Student's T-test comparing all conditions to control (Luc shRNA) (* for $p \leq 0.05$, *** for $p \leq 0.001$).
doi:10.1371/journal.pone.0059369.g002

In contrast to SIRT1 and SUV39H1, we found that targeting of the core NuRD component CHD4, using a retroviral shRNA in A673 cells, increased expression of NCEH1, SPRY1, ADORA1, and TGFBR1 (Figure 5A), while levels of BCL11B were unchanged. In addition to its nucleosome remodeling activity, the NuRD complex also contains class I histone deacetylases (HDACs). To test the necessity of HDACs in this repression, A673 cells were treated with the HDAC inhibitor vorinostat. After 48 hours of treatment, all four of the BCL11B target genes we identified were derepressed in a dose-dependent manner (Figure 5B). In addition, a more recently identified member of the NuRD complex, lysine specific demethylase 1 (LSD1) [45], was tested for its involvement in this repression. When A673 cells were treated with a recently-described small molecule inhibitor of LSD1, HCI-2509, the expression of these four genes was increased with a dose of 1 uM, the IC50 for this compound. It must be noted

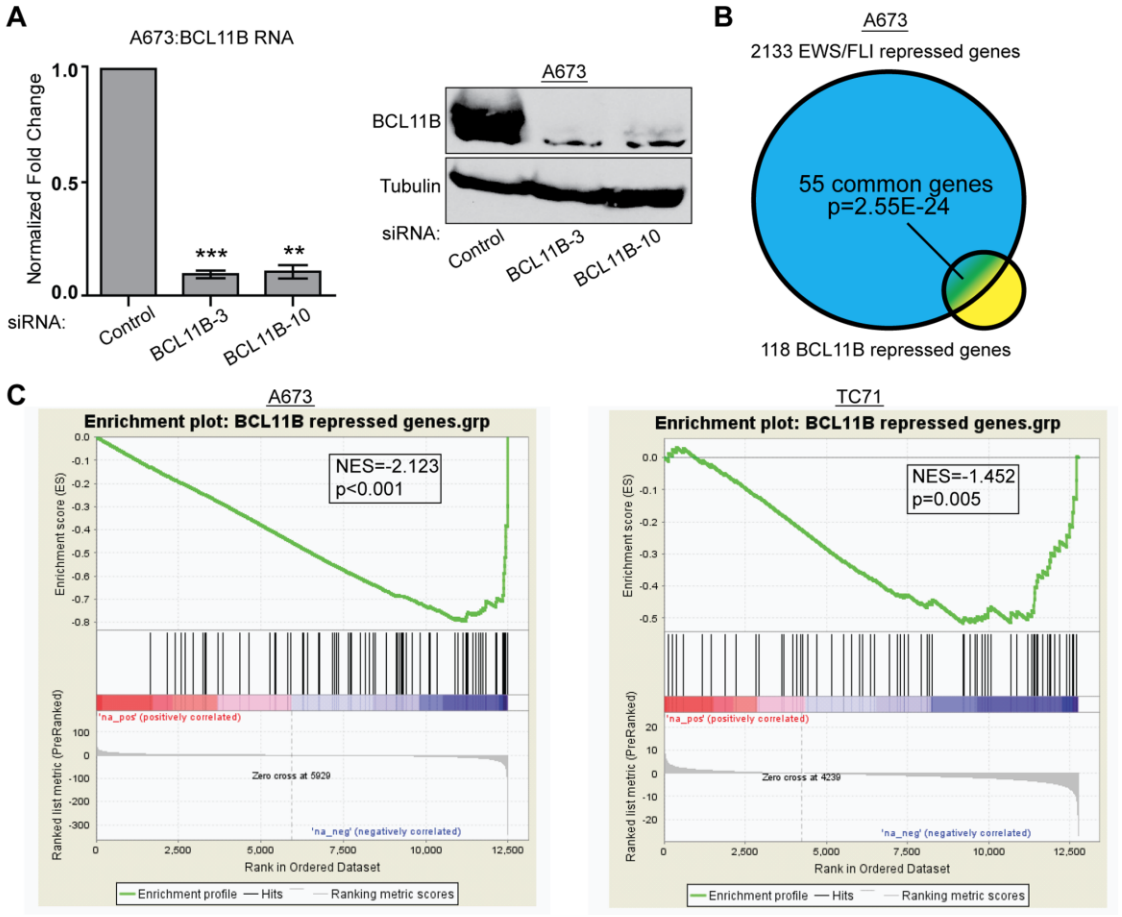

Figure 3. BCL11B represses genes that are part of the EWS/FLI repressed signature. A. qRT-PCR (left) and western blot analysis (right) of A673 cells 48 hours post-transfection with a control siRNA (siControl) or siRNAs targeting BCL11B (siBCL11B-3 and siBCL11B-10). Error bars represent SD of three technical replicates. P-values were determined using a Student's T-test comparing all conditions to control (** for p≤0.01, *** for p≤0.001). B. Venn diagram analysis shows significant overlap of the 118 gene BCL11B repressed gene set and the 2133 gene EWS/FLI repressed gene set. P-value determined by Chi square analysis. C. GSEA analysis comparing a rank-ordered EWS/FLI gene set (up-regulated genes to the left, down-regulated genes to the right) in A673 and TC71 cells to the BCL11B repressed gene set. NES indicates normalized enrichment scores. P-value determined by permutation testing.
doi:10.1371/journal.pone.0059369.g003

that HCI-2509 also decreased the expression of BCL11B at this dose (Figure 5C), rendering interpretation of this result difficult. As a control, we used siRNA targeting NCOR1, a co-repressor that also interacts with HDACs and has not been shown to effect BCL11B-mediated repression. Knock-down of NCOR1 had no effect on the BCL11B repressed genes (Figure S1C).

Re-expression of SPRY1 Limits Transformation in Ewing Sarcoma Cells

To further investigate the involvement of BCL11B mediated repression in the maintenance of *in vitro* transformation, we asked whether re-expression of one such BCL11B-repressed gene, *SPRY1*, could affect colony growth in methylcellulose. SPRY1 has multiple potential functions, but is best known as a negative regulator of the RAS/MAPK signaling pathway [46]. Because RAS/MAPK signaling contributes to cell growth and proliferation, and is active in Ewing sarcoma cells [47], it seemed plausible that re-expression of an inhibitor of this pathway, SPRY1, could reduce cell proliferation or transformation. We tested this by expressing a 3xFLAG-tagged SPRY1 cDNA in the A673 Ewing sarcoma cell line (Figure 6A) and performing growth curves and *in vitro* transformation assays. We found that SPRY1 expression had a minor effect on cell proliferation in tissue culture (Figure 6B). However, it significantly limited the ability of these cells to form colonies under anchorage independent growth conditions (Figure 6C). Surprisingly, this effect did not seem to be mediated via inhibition of RAS/MAPK signaling, as phosphorylation of the downstream effectors, ERK1/2, remained unchanged in cells re-expressing SPRY1 (Figure 6D). When cells were grown in ultra-low attachment plates for 24 h, P-ERK1/2 levels drastically decreased irrespective of SPRY1 expression (Figure 6D). This suggests that SPRY1

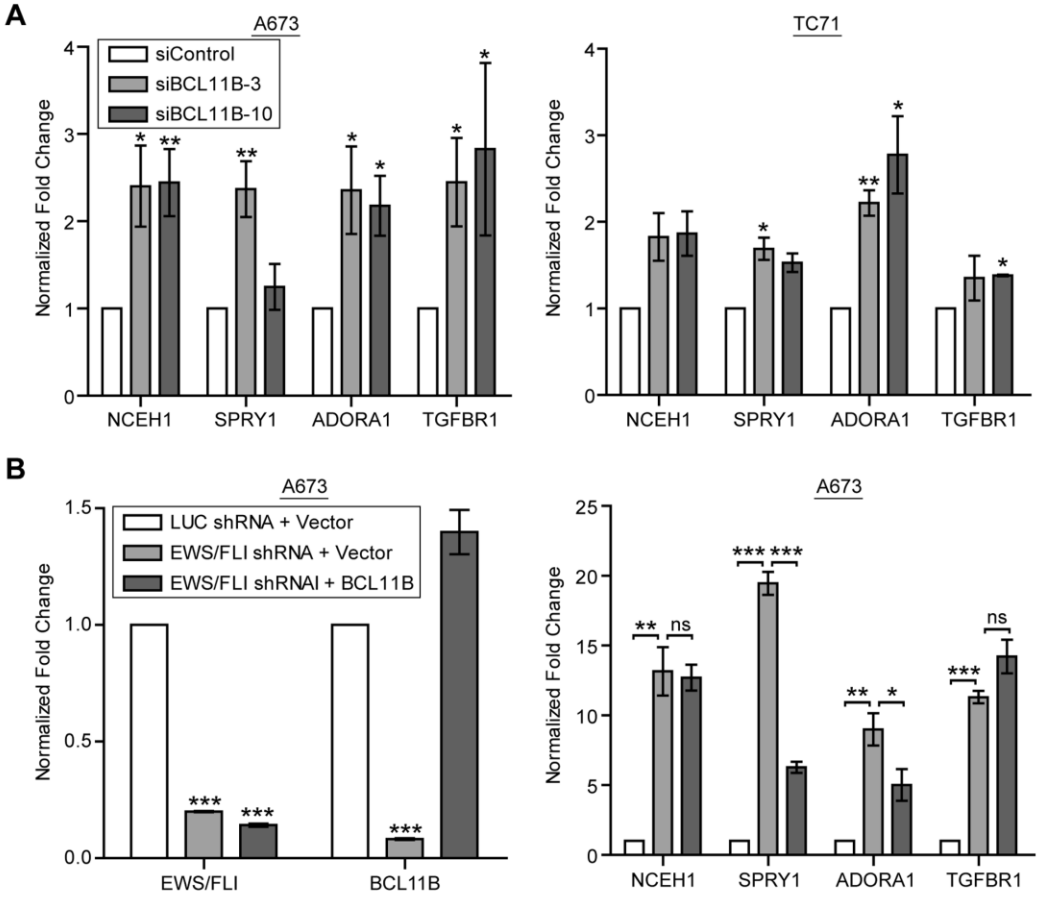

Figure 4. BCL11B mediates repression in Ewing sarcoma cells. A. BCL11B RNA-seq results were confirmed by performing qRT-PCR for the indicated mRNAs on control or BCL11B siRNA transfected A673 cells 48 hours post-transfection. P-values were determined using a Student's T-test comparing all conditions to control (* for p≤0.05, ** for p≤0.01). B. EWS/FLI was knocked-down using shRNA and rescued with an empty vector or BCL11B cDNA. mRNA levels of EWS/FLI and BCL11B were confirmed by qRT-PCR, and transcript levels of the indicated genes were assessed by qRT-PCR. Error bars represent SD of three technical replicates. P-values were determined using a Student's T-test comparing all conditions to control (Luc shRNA+Vector) (left panel) or comparing conditions as indicated (right panel) (ns for not significant, * for p≤0.05, ** for p≤0.01, *** for p≤0.001).
doi:10.1371/journal.pone.0059369.g004

mediates inhibition of anchorage-independent growth via an alternate, RAS/MAPK-independent, pathway.

Discussion

We have shown that EWS/FLI is responsible for the expression of BCL11B in Ewing sarcoma cells. The dramatic decrease in BCL11B expression levels with EWS/FLI knock-down suggests that BCL11B is not normally expressed in the Ewing sarcoma cell of origin. BCL11B is, however, necessary for the proper development of many organs including the brain [21,22,24], skin [19], and teeth [20], as well as for terminal differentiation of T-cells [18,23]. This allows for the possibility that in the likely primitive Ewing sarcoma cell of origin, the BCL11B promoter has an open chromatin structure that permits the strong activation of BCL11B following the formation of the translocation. At this time we consider BCL11B an indirect target of EWS/FLI as EWS/FLI did not show binding to the BCL11B promoter in our ChIP-chip data set [48]. Furthermore previously defined EWS/FLI binding sites – GGAA repeats and ETS consensus sites – are absent from the BCL11B promoter/enhancer region. There are however variant ETS sites in BCL11B's promoter/enhancer region that may allow for EWS/FLI binding and warrant further investigation.

This EWS/FLI-dependent up-regulation of BCL11B is necessary for the maintenance of the transformed phenotype in Ewing sarcoma cell lines *in vitro*. This oncogenic capacity is in stark contrast to its tumor inhibitory function in leukemia and lymphoma – the cancers in which BCL11B have mainly been studied [49]. Mutations or deletions of the *BCL11B* gene are found in 9–16% of human T-ALL [25,26] where it is thought to be a haploinsufficient tumor suppressor. Loss of one allele via the involvement of BCL11B in translocations in this malignancy as well as distinct mutations often found in the zinc finger region [25] may contribute to the oncogenic process in part by preventing differentiation. Moreover in a mouse model of thymic lymphoma,

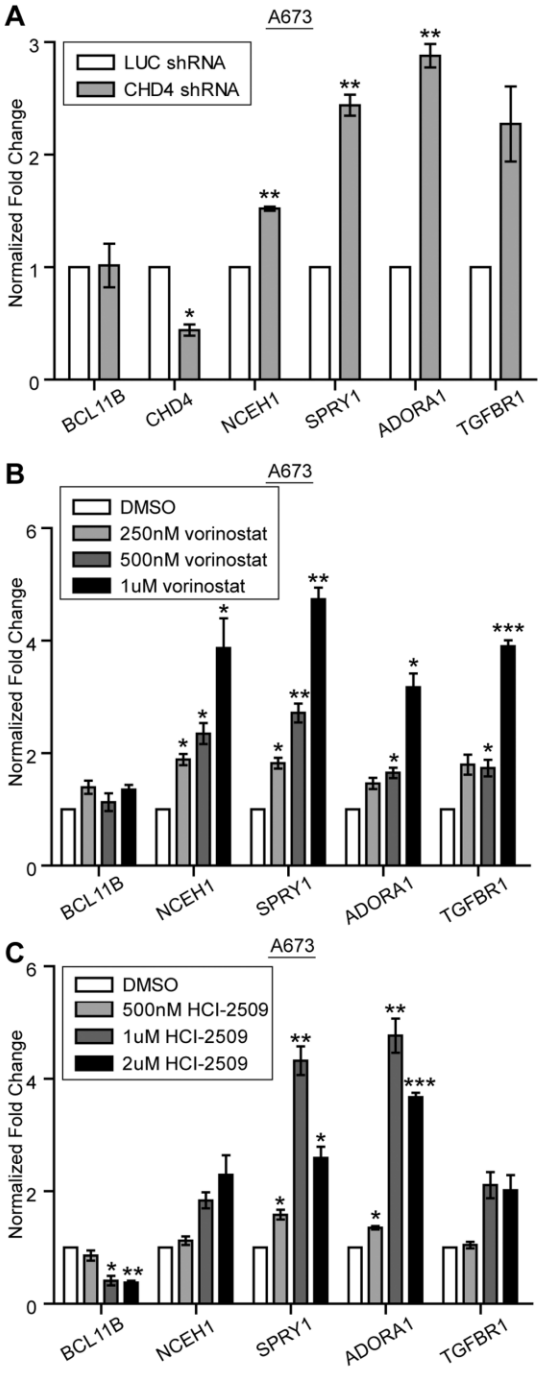

Figure 5. BCL11B mediates transcriptional repression via the NuRD complex. A. shRNA knock-down of CHD4 in A673 cells results in the up-regulation of BCL11B repressed genes as measured by qRT-PCR. B. A673 cells treated with the indicated dose of vorinostat for 48 hours results in the dose-dependent increase of the indicated BCL11B repressed genes as measured by qRT-PCR. C. qRT-PCR of A673 cells treated with the indicated dose of the LSD1 inhibitor, HCI-2509, for 48 hours. Error bars represent SD of three technical replicates. P-values were determined using a Student's T-test comparing all conditions to control (Luc shRNA (A) or DMSO (B,C)) (* for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).
doi:10.1371/journal.pone.0059369.g005

spontaneous homozygous deletions and point mutations occurred in Bcl11b. This group also found that ectopic expression of Bcl11b in HeLa cells suppressed cell growth [27]. BCL11B has not been widely studied in the context of solid malignancies and is not expressed in many [50].

The differing function of BCL11B in Ewing sarcoma does not appear to be due to a unique transcriptional activity in this tumor: BCL11B acts mainly as a transcriptional repressor in Ewing sarcoma, and thus acts similarly to what has been demonstrated in other cellular contexts. However, BCL11B regulates a unique set of genes in the A673 cellular background in comparison to other cell types in which it has been studied. For example, BCL11B has been implicated in cell cycle progression by directly repressing the cell cycle inhibitors p21WAF1 [43] and p57KIP2 [41] in microglial cells and SK-N-MC (which were originally characterized as a neuroblastoma cell line, but are in fact a Ewing cell line) cells, respectively. We were able to confirm downregulation of p57KIP2 transcript levels by BCL11B in SK-N-MC cells by qRT-PCR; however this was not observed in A673 or TC71 cells (ETW unpublished observation). At this time, we do not understand the differences among these cellular contexts that account for this discrepancy. Nonetheless, this discrepancy across Ewing sarcoma cell lines suggests that inhibition of p57KIP2 is not a central feature of BCL11B function in this tumor type. In developing T-cells BCL11B represses genes that allow for a more primitive state thus contributing to the differentiation process [23]. These classes of genes were not observed in our genome wide analysis of BCL11B regulated genes in A673 cells. Our data suggests that BCL11B contributes to the overall EWS/FLI repressed gene signature in Ewing sarcoma, and that the repression of a subset of these genes may be necessary for the transformed phenotype. EWS/FLI directly represses approximately 5% of the total EWS/FLI repressed genes [31]. This allows for a model where EWS/FLI up-regulates the expression of transcriptional repressors, such as BCL11B, which then indirectly account for the repression of the remaining 95% of the EWS/FLI down-regulated genes.

BCL11B facilitates transcriptional repression by recruiting a variety of chromatin modifying enzymes to the promoters of genes. BCL11B physically interacts with the histone methyltransferase SUV39H1 [42,43], the histone demethylase LSD1 [51], histone deacetylases HDAC1 and HDAC2 [42], the class III histone deacetylase, SIRT1 [44], as well as the NuRD co-repressor complex [40,41]. BCL11B likely utilizes these interactions to mediate repression in both a cell-type specific and a promoter-specific fashion. We have shown that the chromatin remodeling activity of the NuRD co-repressor complex participates in the repression of the four BCL11B repressed genes investigated in this study. Our data further suggest that vorinostat-repressible HDAC activity is involved in this repression. These data are consistent with vorinostat inhibition of NuRD-associated HDACs, but inhibition of non-NuRD associated HDACs may also play a role. We have also demonstrated that the LSD1 inhibitor, HCI-2509, increases the expression of these genes. The mechanism for HCI-2509 is unclear due to the fact that BCL11B levels are also somewhat reduced. This small molecule has recently been shown to de-repress some EWS/FLI directly repressed target genes and show specific toxicity for Ewing sarcoma cells [31]. The data presented here further demonstrate that HCI-2509 is able to

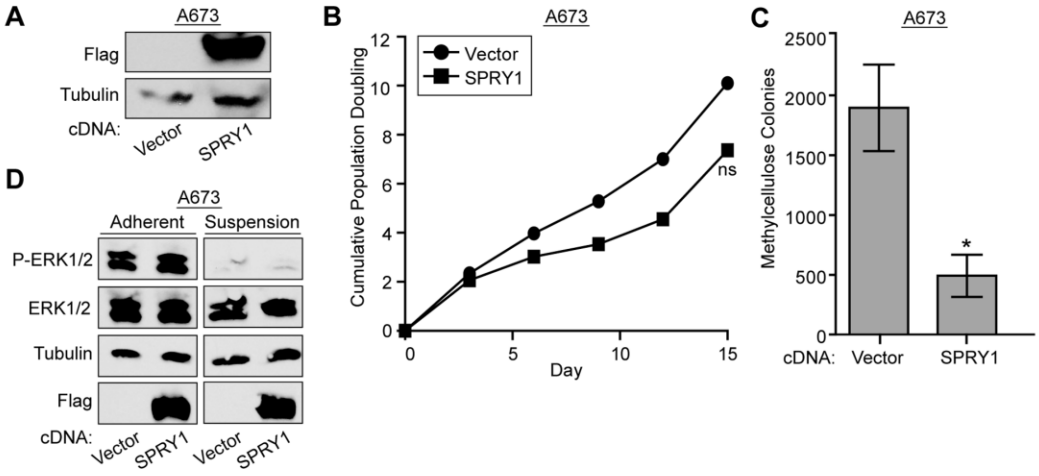

Figure 6. Re-expression of SPRY1 limits the transformation potential of Ewing sarcoma cells. A. Western blot shows expression of 3xFLAG SPRY1 construct in A673 cells. B. Growth rates of A673 cells expressing 3xFLAG SPRY1 were determined using a 3T5 assay. P-values were determined using a Student's T-test (ns for not significant).C. Anchorage independent growth of A673 cells expressing 3xFLAG SPRY1 was assessed by the ability to grow in methylcellulose. Error bars represent SD of two technical repeats. P-values were determined using a Student's T-test (* for $p \leq 0.05$). D. Western blot shows levels of phosphorylated and total ERK1/2 when A673 cells are grown under adherent or suspension conditions in the presence or absence of 3xFLAG SPRY1 cDNA. Tubulin is used as a loading control and flag shows 3xFLAG SPRY1 expression.
doi:10.1371/journal.pone.0059369.g006

reverse at least a portion of the EWS/FLI regulated gene signature which provides a possible mechanism for the toxicity to Ewing sarcoma cells.

We have not investigated the direct or indirect nature of this BCL11B mediated repression. *In vitro* studies have identified a GC-rich BCL11B binding site [39]; however, a genome-wide analysis of BCL11B binding *in vivo* [52] failed to confirm the *in vitro* findings. Thus, a *bona fide* BCL11B consensus site remains elusive. Without this information we were unable to inspect the promoters of our BCL11B regulated genes to identify potential direct targets. We did however perform motif enrichment analysis using the MEME suite [53] on the BCL11B repressed gene list to identify any enriched sequence motifs. This analysis failed to show any significant sequence enrichment (ETW, unpublished observations). This may be expected due to the mixed set of directly and indirectly regulated genes.

We have shown that the re-expression of the BCL11B repressed gene, *SPRY1*, reduces the transformation potential of Ewing sarcoma cells. This further implicates BCL11B mediated repression as an important contributor to the repressed gene signature in Ewing sarcoma cells. At this time the mechanism involved in SPRY1's ability to reduce transformation remains unclear, although it does not appear to be acting in its classic role by inhibiting RAS/MAPK signaling. Spry1 is also known to inhibit phospholipase C (PLC) activation, and during *Xenopus* mesoderm development, *Xtsprouty* inhibits the PLC pathway while still allowing for RAS/ERK signaling. SPRY1 may be impinging on an alternate growth factor signaling pathway or perform a novel function in the context of a Ewing sarcoma cell.

A recurring theme in Ewing sarcoma is alteration in the expression levels of important developmental genes. Various pathways involved in proper development and differentiation have been disrupted by EWS/FLI in Ewing sarcoma – sonic hedgehog [10,54], transforming growth factor beta (TGFB) [11,31], and WNT [55], among others. The timely expression of BCL11B during the development of many cell types is crucial for proper differentiation. Here we have shown the aberrant expression of BCL11B in Ewing sarcoma cell lines represses a subset of the EWS/FLI repressed gene signature and contributes to the transformed phenotype.

Supporting Information

Figure S1 Investigating mechanisms of BCL11B mediated repression. A. qRT-PCR from A673 cells treated with the indicated dose of the SIRT1 inhibitor, Ex-527, for 24 hours. B. qRT-PCR data from A673 cells treated with the indicated dose of the SUV39H1 inhibitor, Chaetocin, for 24 hours. C. qRT-PCR data from A673 cells transfected with siRNA targeting NCOR1 (siNCOR1) or control (siControl) for 48 hours. Error bars represent SD of three technical replicates. P-values were determined using a Student's T-test comparing all conditions to control (DMSO (A,B) or siControl (C) (* for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$).
(TIF)

Table S1 Primer Sequences.
(DOCX)

Table S2 BCL11B RNAseq Data with FDR 10% and 2 fold change.
(XLSX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ETW SLL. Performed the experiments: ETW BLS RB. Analyzed the data: ETW BLS RB SLL. Contributed reagents/materials/analysis tools: ETW BLS RB. Wrote the paper: ETW SLL.

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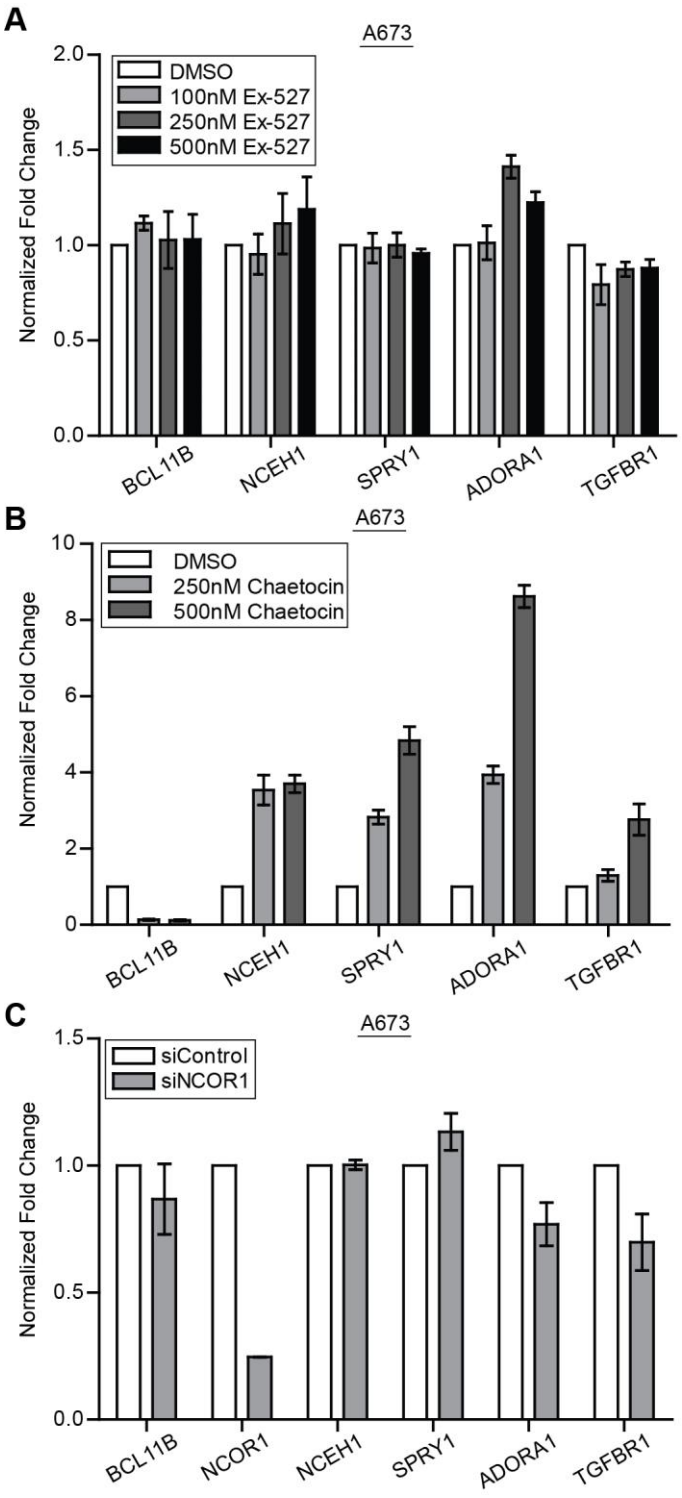


Figure 3.S1: Investigating mechanisms of BCL11B mediated repression.

CHAPTER 4

ZEB2 REPRESSES THE EPITHELIAL PHENOTYPE AND FACILITATES METASTASIS IN EWING SARCOMA

Elizabeth T. Wiles[1], Russell Bell[2] and Stephen L. Lessnick[1,2,3*]

[1]Department of Oncological Sciences; [2]Center for Children's Cancer Research, Huntsman Cancer Institute and [3]Division of Pediatric Hematology/Oncology, University of Utah, Salt Lake City, Utah, USA.

Abstract

The vast majority of cancer-related deaths are attributable to metastasis. Effective treatment of metastatic disease will be improved by a better understanding of the molecular mechanisms contributing to this phenomenon. Much of the work in this field has focused on metastasis of carcinomas, tumors of epithelial origin, while metastasis of sarcomas, tumors of mesenchymal origin, remains poorly understood. Experimental evidence from studies in carcinomas, coupled with clinical observations, highlights the importance of both epithelial and mesenchymal characteristics in these cancer cells that make them competent for metastasis. We set out to test if similar cellular plasticity contributed to sarcoma metastasis. We found that the transcription factor, ZEB2, repressed epithelial gene expression in Ewing sarcoma cells, and this, in turn, repressed the epithelial phenotype. When ZEB2 was experimentally reduced in these cells, epithelial characteristics including decreased migratory ability and cytoskeleton rearrangements were observed. Furthermore, ZEB2 reduction in Ewing sarcoma cells resulted in a decreased metastatic potential using a mouse metastasis model. Our data show that Ewing sarcoma cells may have more epithelial plasticity than previously appreciated. This coupled with previous data demonstrating Ewing sarcoma cells also have mesenchymal features primes these cells to successfully metastasize. This is clinically relevant for two important reasons. First, this may offer a therapeutic opportunity to induce characteristics of one cell type or the other depending on the stage of the disease. Second, and more broadly, this raises questions about the cell of origin in Ewing sarcoma and may inform future animal models of the disease.

Introduction

Cancer metastasis is a major clinical problem with > 90% of all cancer deaths attributed to metastatic disease (Gupta & Massague, 2006). Metastasis is a complex, multistep process that results in the dissemination of cells from the site of the primary tumor to distant organs where these cells are able to colonize and form a secondary lesion (Fidler, 2003). Recent experimental evidence suggests that successful metastasis requires a tumor cell to possess both epithelial and mesenchymal characteristics (Ocana *et al*, 2012; Tsai *et al*, 2012). Epithelial features promote cell growth at both the primary and secondary site, while mesenchymal features contribute a migratory capacity to these cells facilitating escape from the tumor, the ability to survive in the circulatory system, and extravasate at distant sites. The vast majority of our knowledge regarding the metastatic cascade comes from the study of carcinomas, tumors originating from the epithelial cells. In this setting, epithelial tumor cells are thought to undergo an epithelial-mesenchymal transition (EMT) in order to disseminate from the bulk tumor, and emerging evidence also supports the idea that a mesenchymal-epithelial transition (MET) is required for colonization of distant sites (Brabletz *et al*, 2001; Ocana *et al*, 2012; Thiery, 2002; Tsai *et al*, 2012).

Not all cancer metastases conform to this biological paradigm. Sarcomas, for example, are thought to arise from mesenchymal derived tissues such as bone, cartilage, and muscle (Helman & Meltzer, 2003), thus making it unlikely that an EMT-MET is a requirement in sarcoma metastasis. Several sarcomas are well differentiated and closely resemble what is thought to be the cell of origin (but could also be aberrantly differentiated), whereas others are poorly differentiated and the cell of origin remains uncertain (Taylor *et al*, 2011). A diverse group of sarcomas display features reminiscent

of an EMT-MET (Fitzgerald *et al*, 2011; Guo *et al*, 2007; Niinaka *et al*, 2010; Yang *et al*, 2010) suggesting that sarcomas may have some phenotypic plasticity.

Ewing sarcoma is an undifferentiated sarcoma characterized by the oncogenic fusion protein and transcription factor, EWS/FLI, and its small round blue cell histological presentation (Arndt & Crist, 1999). Due to its poorly differentiated nature, the cell of origin for this tumor is unknown. Neural crest stem cells (NCSC) (von Levetzow *et al*, 2011), mesenchymal stem cells (MSC) (Miyagawa *et al*, 2008) (Riggi *et al*, 2008), or perhaps neural crest-derived mesenchymal stem cells (Lee *et al*, 2007; Riggi *et al*, 2009; Takashima *et al*, 2007) are currently the most widely accepted cell of origin candidates. Further complicating the cellular histogenesis are the diverse locations including bone, most commonly the pelvis and long bones, and a variety of soft tissues such as kidney and pancreas, in which these tumors arise (Taylor *et al*, 2011). Patients generally present with this disease in the second decade of life and approximately 20-25% have detectable metastatic spread at diagnosis (Arndt & Crist, 1999). Patients lacking overt metastasis likely harbor micrometastases as indicated by the high rate of relapse at distant sites following surgical resection of the primary tumor in the absence of systemic chemotherapy (Dahlin *et al*, 1961; Wang & Schulz, 1953). The presence of metastatic disease comes with a poor prognosis, bringing the overall survival rate from 70-75% for localized disease to less than 20% for those with metastasis (Grier *et al*, 2003). The aim of this study is to better understand the cellular plasticity in Ewing sarcoma and how that contributes to the highly metastatic phenotype in this disease.

Results

ZEB2 is highly expressed in Ewing sarcoma

Metazoans are primarily composed of two cell types, epithelia and mesenchyme. Epithelial cells have an organized structure marked by intercellular adhesions while mesenchymal cells lack these cell-cell contacts and are characterized by their ability to migrate as single cells (Acloque *et al*, 2009). It is well established that Ewing sarcoma cells have mesenchymal features which become more pronounced upon EWS/FLI knock-down (Chaturvedi *et al*, 2012; Tirode *et al*, 2007). Histological examination has also demonstrated that a subset of Ewing sarcoma patient tumor samples display epithelial features (Collini *et al*, 2001; Gu *et al*, 2000; Schuetz *et al*, 2005). Despite retaining remnants of these two cell types, Ewing sarcoma cells are overwhelmingly considered to be undifferentiated. Experimental evidence has made clear that EWS/FLI prevents the mesenchymal phenotype in Ewing sarcoma (Chaturvedi *et al*, 2012; Tirode *et al*, 2007), but the factor(s) preventing an epithelial phenotype are unknown.

Using the Baird *et al* dataset that contains gene expression profiling of 19 Ewing sarcoma tumors (Baird *et al*, 2005), we confirmed variant expression of well accepted epithelial and mesenchymal markers (Kalluri & Weinberg, 2009). We next investigated the expression of the classic EMT-inducing transcription factors (EMT-TF) (Yang & Weinberg, 2008) (Figure 4.1A), reasoning that they represent good candidates for repressors of the epithelial phenotype in Ewing sarcoma. Strikingly, we found that zinc finger E-box binding homeobox 2 (ZEB2) (encoded by *ZFHX1B*, and also known as SMAD interacting protein 1, SIP1) was the only EMT-TF that was consistently expressed in Ewing sarcoma patient tumors (Figure 4.1A) and cell lines (Figure 4.1B). Many highly expressed genes in Ewing sarcoma are induced by EWS/FLI. However upon EWS/FLI

knock-down, ZEB2 expression levels do not change in the Ewing sarcoma cell lines tested (A673, SKNMC, TC71) (Figure 4.1C). This indicates that ZEB2 levels are not dependent on the translocation and thus ZEB2 may be expressed in the cell of origin. Accordingly, we show that ZEB2 is expressed in both proposed cells of origin, BM-MSC (bone marrow derived-MSC) and NCSC (Figure 4.1D) (von Levetzow *et al*, 2011). Much of the work in the Ewing sarcoma field has focused on EWS/FLI transcriptional targets, but a permissive cellular environment is crucial for EWS/FLI's oncogenic ability (Kovar, 2005), necessitating a better understanding of relevant gene expression in the cell before the translocation event.

ZEB2 represses epithelial gene expression in Ewing sarcoma

To determine if the expression of ZEB2 in Ewing sarcoma was indeed repressing epithelial gene expression, we performed genome-wide gene expression profiling using RNA-sequencing (RNA-seq) in A673 cells expressing a control siRNA (siControl) or two unique siRNAs targeting ZEB2 (siZEB2-5 or siZEB2-6) (Figure 4.2A). We found 46 genes were up-regulated and 124 genes were down-regulated by ZEB2 using significance cutoffs set at a two–fold change and a false discovery rate (FDR) of 10% (Supporting Information Table 4.S1). We validated the RNA-seq profile by qRT-PCR of select genes (Supporting Information Fig 4.1). To identify functional classes that were enriched in our ZEB2 repressed gene list, we used the DAVID analysis functional annotation clustering algorithm (Huang da *et al*, 2009a; Huang da *et al*, 2009b). The most enriched term representing this list was epithelial cell differentiation, followed by actin binding and cell junction supporting our hypothesis that ZEB2 is repressing epithelial gene expression in Ewing sarcoma cells. E-cadherin is notably absent from our repressed gene list, as its

expression does not increase even in the context of ZEB2 knock-down in Ewing sarcoma cells. E-cadherin is a hallmark of epithelial cells. However, its absence in 97% and 100% of tumor samples, respectively, in two independent studies (Machado *et al*, 2012; Schuetz *et al*, 2005), suggests that it may be in a more permanently repressed state, and Ewing sarcoma cells cannot achieve full epithelial status.

We compared the gene expression profile generated in Ewing sarcoma cells with reduced ZEB2 expression to gene expression data sets generated in cells undergoing EMT induced by different mechanisms. We first compared our ZEB2 gene expression profile to EMT gene expression profiles derived from human mammary luminal epithelial cells (HMLE) expressing TGF-β or EMT-TFs, Twist, Snail1, or Goosecoid (GSC). (Taube *et al*, 2010). As expected, heat maps display an inverse expression pattern in ZEB2 knock-down cells undergoing some degree of MET compared to the HMLE cells undergoing EMT. Unsupervised hierarchical clustering also clusters the EMT cells apart from the siZEB2 Ewing sarcoma cells (Figure 4.2C). We confirmed this finding using a distinct dataset that induced EMT in HMLE cells by suppressing E-cadherin expression with shRNA. In this study, HMLE cells expressing an shRNA targeting E-cadherin displayed increased migration and invasion *in vitro* and metastatic ability in mouse models. This increased metastatic potential was mediated, at least in part, by β-catenin. When cells were infected with shRNAs targeting both E-cadherin and β-catenin, they were no longer metastatic suggesting a more epithelial state (Gautier *et al*, 2004). Consistent with these experimentally verified cell states, Ewing sarcoma cells with reduced ZEB2 clustered with the double knock-down HMLE gene expression profile (epithelial), whereas the HMLEs that had transitioned to a mesenchymal status by

expressing only the shRNA targeting E-cadherin had an inverse gene expression profile and clustered separately (Figure 4.2D).

To validate these findings using a different cell type, we used an EMT gene expression profile derived from A549 lung adenocarcinoma cells stimulated with TGF-β and followed over a 72-hour time course (Keshamouni *et al*, 2009). In this model of EMT, western blot analysis shows that at 16 hours post-TGF-β treatment mesenchymal markers, vimentin and N-cadherin, increase in expression while E-cadherin expression begins to decrease indicating the start of the EMT (Keshamouni *et al*, 2006). Strikingly, our ZEB2 knock-down gene expression data displays an expression signature similar to the early time points (0.5 - 4 hours) which represents an epithelial cellular state. At 8 hours, the A549 gene expression profile starts to shift, and at 16 hours, a time point when the EMT has occurred based on the western blot analysis, the gene expression pattern is completely reversed (compared to our ZEB2 knock-down dataset) – representing a mesenchymal cellular state (Figure 4.2E). These transcriptional profiling comparisons demonstrate that Ewing sarcoma cells and epithelial cells regulate a similar panel of genes to achieve cellular plasticity.

ZEB2 represses the epithelial phenotype in Ewing sarcoma

To further study the gene expression changes seen by RNAseq, we designed a retroviral shRNA targeting the 3'UTR of ZEB2 to achieve stable knock-down in Ewing sarcoma cell lines. This construct reduced ZEB2 RNA and protein levels (Figure 4.3A,B). Using this shRNA, we examined the transcript changes of several genes identified as ZEB2 repressed targets. It has been suggested that total ZEB (ZEB1 and ZEB2) levels in a cell are interdependent and that there is some ability to compensate

between ZEB1 and ZEB2 (Park *et al*, 2008). In agreement with this hypothesis, we saw that knock-down of ZEB2 led to an increase in ZEB1 expression in three Ewing sarcoma cell lines (A673, SKNMC, and TC71). We also verified the ZEB2 mediated repression of several epithelial genes in A673 cells including desmosome components, desmoplakin (DSP) and plakophilin 2 (PKP2), an intermediate filament characteristic of simple epithelia, keratin 8 (KRT8), the tight junction protein, F11 receptor (F11R, also known as junctional adhesion molecule A, JAM-A) and a facilitator of cytoskeletal remodeling, Rho guanine nucleotide exchange factor 5 (ARHGEF5). ZEB2 repressed these genes to a lesser degree and not as consistently in two other Ewing sarcoma cell lines, SKNMC and TC71 (Figure 4.3A). KRT8 and DSP protein levels changed consistently with what was seen at the RNA level (Figure 4.3B). The concomitant increase in ZEB1 expression with ZEB2 knock-down may be responsible for the lack of expression of some epithelial genes in other cell lines – for example, KRT8 in SKNMC cells (see below, Figure 4.4B). Indeed, ZEB1 and ZEB2 can bind similar DNA sequences and have some redundant function (Postigo & Dean, 2000).

In vitro functional assays were performed to ask whether the increase in epithelial gene expression realized upon ZEB2 knock-down resulted in a more epithelial phenotype in Ewing sarcoma cell lines. Boyden chamber assays (Figure 4.3C) and wound-healing assays (Figure 4.3D) were used to define the migratory capacity of Ewing sarcoma cells. In both settings, Ewing sarcoma cells displayed reduced migratory ability upon ZEB2 knock-down. For the Boyden chamber assay cells were plated in serum free media in the upper chamber, and induced to migrate toward serum containing media in the bottom chamber. As a control for this assay, cells from each condition were plated in serum-free

media and counted 24 hours later to eliminate the possibility that differences in the cell's ability to survive under serum-free conditions contributed to the phenotype (Supporting Information Fig 4.2). The wound healing invasion pattern of cells with reduced ZEB2 shows a slower and more cohesive movement which is characteristic of epithelial cells. In contrast, A673 and SKNMC control cells demonstrate a more mesenchymal migration pattern marked by single cells invading the wounded area and faster wound closure.

Epithelial and mesenchymal cells have unique cytoskeletal arrangements. Using phalloidin to label F-actin, we show that A673 cells expressing an siRNA targeting ZEB2 traded a more diffuse actin cytoskeleton for more compact actin rings with a cobblestone morphology typical of epithelial cells. We also confirm the knock-down by the absence of ZEB2 nuclear staining in the siZEB2 transfected cells (Figure 4.3E). Taken together, the increase in epithelial gene expression upon ZEB2 reduction, allows for phenotypic and morphological changes consistent with an epithelial shift.

Ectopic expression of miR-200 family epithelializes Ewing sarcoma cells

The relationship between ZEB1 and ZEB2 and the miR-200 family of micro-RNAs (miRNA) (miR-200a, miR-200b, miR-200c, miR-141 and miR429) is well established (Gregory *et al*, 2008; Park *et al*, 2008). These miRNAs bind to the 3'UTR of ZEB1 and ZEB2 to repress translation; conversely, ZEB1 and ZEB2 can bind to the promoters of miR-200s to repress transcription. This double-negative feedback loop is thought to serve as a switch to regulate EMT – when ZEB levels are high and miR-200 levels are low, then cells are phenotypically mesenchymal, and when the reverse is true, then cells are more epithelial (Brabletz & Brabletz, 2010). We used this phenomenon to investigate whether Ewing sarcoma cells could be induced to epithelialize by a

mechanism that was distinct from RNAi methods previously tested, yet still dependent on ZEB2 levels.

We ectopically expressed the polycistronic miR-200 gene cluster from chromosome 1 (miR-200b, miR200a, and miR429) in A673 and SKNMC Ewing sarcoma cells (Figure 4.4A). As predicted, expression of miR-200 family members reduced the expression of ZEB2 protein (Figure 4.4B). Epithelial proteins, DSP and KRT8, were also increased in miR-200 expressing A673 and SKNMC cells. Notably, KRT8 expression was not observed when ZEB2 was specifically knocked-down in SKNMC cells (Figure 4.3B). This discrepancy may result from the repression of ZEB1 by the miR-200 family, in contrast to the compensation by ZEB1 when ZEB2 is specifically reduced. Furthermore Ewing sarcoma cells expressing miR-200s display a migration defect in wound-healing assays (Figure 4.4C) similar to that of cells with specific reduction of ZEB2 using shRNA (Figure 4.3D). The epithelial plasticity of Ewing sarcoma cells expressing miR-200 family members complements our findings implicating ZEB2 in the repression of the epithelial phenotype.

ZEB2 facilitates metastasis in Ewing sarcoma

We have established the ability of ZEB2 to repress epithelial features in Ewing sarcoma cells and the re-emergence of such features upon ZEB2 knock-down. We hypothesized that this innate epithelial repression primes Ewing sarcoma cells for metastasis, and thus if ZEB2 levels were reduced, Ewing sarcoma cells would lose the ability to metastasize. To test this hypothesis, we used an orthotopic mouse metastasis model whereby Ewing sarcoma cells are injected intratibially and cells spontaneously metastasize to the lung (Guan *et al*, 2008). This model accounts for all steps in the

metastatic cascade from escaping the primary tumor to colonizing the secondary site, making it superior to the tail vein injection metastasis model. Two doses of A673 cells (25K or 100K) infected with a retroviral shRNA targeting ZEB2 or control shRNA were injected into the right tibia of male NOD-SCID mice. Tumor growth was monitored weekly by measuring the right tibia using calipers. Mice were sacrificed at 6 weeks post-injection, and the lungs were harvested. ZEB2 knock-down had no effect on tumor growth (Figure 4.5A). Fewer mice injected with A673 cells expressing the ZEB2 shRNA showed macroscopic pulmonary metastases at both doses; however, this binary comparison did not yield statistical significance (Figure 4.5B). It was clear by gross observation that the metastatic burden was much greater in the control shRNA injected animals compared to the ZEB2 shRNA injected animals (Figure 4.5C). When the area of the lung with metastatic lesions was quantified, ZEB2 knock-down cells showed significantly less metastatic burden (Figure 4.5D and Supporting Information Fig 4.3). Importantly we saw no difference in tumor size and no correlation between tumor size and propensity to metastasize (Figure 4.5E) indicating ZEB2 does not play a role in primary tumor growth, but specifically contributes to the metastatic ability of Ewing sarcoma cells.

Discussion

Sarcoma metastasis is not well understood. Sarcomas are thought to arise from mesenchymal cells and thus do not have a baseline epithelial differentiation state that is seen in many carcinomas. This fact excludes sarcomas from the EMT-MET metastasis paradigm whereby tumor cells in carcinomas must lose their epithelial features to escape the primary tumor, but regain them in order to colonize the secondary site. However, it is

likely that the concept of cellular plasticity in metastasis can be applied to sarcomas. Undifferentiated Ewing sarcoma cells may innately have the right balance of epithelial and mesenchymal features that allow for successful growth at the primary and secondary sites, as well as a high propensity for metastasis. Providing support for this intrinsic ability, micrometastatic spread is nearly ubiquitous in Ewing sarcoma patients and occurs at early stages of the disease (Spraker *et al*, 2012) suggesting a parallel progression of tumor and metastases (Klein, 2009). This is in stark contrast to some carcinomas such as colorectal cancer where a slow progression and accumulation of mutations eventually leads to an invasive malignancy (Fearon & Vogelstein, 1990). A "passive metastasis" model has recently been proposed for Ewing sarcoma dissemination to contrast the deliberate steps necessary in carcinoma metastasis (Chaturvedi *et al*, 2012).

The passive metastasis model predicts that Ewing sarcoma cells have weak intercellular adhesions and ample access to the bone marrow and circulatory system due to their bone-associated locations. This puts tumor cells in a prime position to enter the blood stream and colonize the secondary site, most frequently lung and bone. This is consistent with our data showing that reduction of ZEB2 increases epithelial features and cell-cell adhesion. Ewing's tumors are then in a less passive state and therefore a decrease in metastatic potential is achieved. The fundamental ability of Ewing sarcoma cells to proliferate as well as metastasize may also contribute to the limited latency (72% of relapse occur within two years of diagnosis and 94% within five years) seen in Ewing sarcoma (Stahl *et al*, 2011). According to the EMT-MET metastasis model, colonization (the ability to form a macroscopic secondary lesion) is the limiting step in the multistep metastatic cascade. In this setting, circulating tumor cells are plentiful, and some are able

to seed the secondary site. However these cells are quiescent, marked by the absence of Ki-67 staining (Chambers *et al*, 2002; Muller *et al*, 2005). A subsequent event, the proposed MET, is then necessary for these cells to re-enter into the cell cycle. This has been used to explain the long latency often seen in breast cancer recurrence (Meng *et al*, 2004; Pantel *et al*, 2008).

Genetic Type II Metastasis was proposed by Brabletz to explain undifferentiated carcinoma metastases (Brabletz, 2012). In this model, poorly differentiated primary tumors that arise in primitive cell types give rise to undifferentiated metastases. These malignancies don't rely on EMT-MET for metastasis because their primitive state endows them with an intrinsic EMT phenotype. Other characteristics of cancers of this type include a high propensity for metastasis at early stages of the disease, limited latency, and the presence of a genetic alteration which contributes to a fairly fixed cell state that is compatible with tumor growth and metastasis. Ewing sarcoma metastasis conforms to this model. Ewing sarcoma is thought to occur in a primitive cell type where the genetic alteration, EWS/FLI, prevents mesenchymal differentiation and ZEB2 prevents epithelial differentiation, as demonstrated here. Metastasis is a frequent and early event in Ewing sarcoma compatible with the idea that EMT is not needed. The presence of overt metastasis at presentation coupled with the limited latency seen in this disease also supports a model where MET is not a limiting factor in colonization of the secondary site.

The cell of origin for Ewing sarcoma is unknown, but it is thought to be a NCSC or MSC. We show that ZEB2 is expressed in both of these primitive cells. It should be noted that these two cell types may not be mutually exclusive. Experimental evidence has

described the isolation of NCSCs from human embryonic stem cells which can be directed to differentiate along mesenchymal lineages in addition to neurons and Schwann cells (Lee *et al*, 2007). Studies show that experimental reduction of EWS/FLI, causes Ewing sarcoma cells to resemble MSCs both in gene expression and differentiation capacity (Tirode *et al*, 2007). This coupled with evidence that ectopic expression of EWS/FLI in human MSCs leads to a Ewing sarcoma gene expression signature has supported MSCs as the cell of origin. However, hMSCs expressing EWS/FLI are not transformed (Riggi *et al*, 2008). Ewing sarcomas have a relatively low frequency of mutations in known tumor suppressors and oncogenes supporting the concept that EWS/FLI is the main oncogenic driver in this malignancy (Huang *et al*, 2005; Shukla *et al*, 2012). If this is true, EWS/FLI alone should induce transformation if expressed in the correct cell of origin.

We propose an alternate, yet integrative, hypothesis for tumorigenesis in Ewing sarcoma: the EWS/FLI translocation occurs in a cell that is undergoing a normal developmental EMT. In this model, ZEB2 is expressed in a neural crest or neuroepithelia-derived progenitor cell to induce a developmental EMT. The EWS/FLI translocation can be acquired at various stages of this transition and blocks further mesenchymal differentiation, resulting in an undifferentiated Ewing sarcoma cell. Support for this model comes from the role of ZEB2 during development. ZEB2 is expressed in the developing neural crest and neural epithelium. The homozygous ZEB2 knock-out mouse is embryonic lethal due to defects in neural crest migration seen on embryonic day 8.5 (Van de Putte *et al*, 2003). This model is also consistent with the well characterized ability of EWS/FLI to prevent mesenchymal differentiation (Chaturvedi *et*

al, 2012; Tirode *et al*, 2007; Torchia *et al*, 2003), and may help to reconcile the observation that Ewing sarcoma cell lines can only differentiate along mesenchymal lineages in the absence of EWS/FLI (Tirode *et al*, 2007), while MSCs, being further along the differentiation spectrum than the Ewing sarcoma cell of origin, retain this ability even when expressing EWS/FLI (Riggi *et al*, 2008). Expression of EWS/FLI in human pediatric bone marrow derived MSCs (Riggi *et al*, 2010) as well as neural crest derived MSCs (von Levetzow *et al*, 2011) results in repression of MSC genes while increasing expression of NCSC genes which could reflect a de-differentiation, in line with our proposal that the cell of origin is a precursor to a MSC.

Strong evidence to support this concept also comes from the finding that neural crest cells and neuroepithelial cells are the initial source of MSCs from mouse embryonic stem cells *in vitro* (Takashima *et al*, 2007). This group went on to show that $P0^{+}$ neural crest cells and $Sox1^{+}$ neuroepithelial cells gave rise to MSCs during normal mouse development. Interestingly, in lineage tracing studies, these neural crest and neuroepithelial derived MSCs were present at low abundance in bone cell preparations from femoral and tibial bones of neonates, 4-week and 12-week-old-mice, and this population of cells decreased with age (Takashima *et al*, 2007). This places these cells in the proper space and time to serve as the cell of origin for Ewing sarcoma. Of relevance to the present study, desmosomes are abundant in neuroepithelium (Gallicano *et al*, 2001), and KRT8 is also expressed in early embryonic epithelial tissue (Dellagi *et al*, 1987; Jackson *et al*, 1981; Viebahn *et al*, 1995).

A mouse model for Ewing sarcoma has remained elusive, despite several attempts (Codrington *et al*, 2005; Lin *et al*, 2008; Torchia *et al*, 2007). One possible reason for this

is that EWS/FLI has not been expressed in the appropriate precursor cell. One prediction based on the model presented in this paper is that perhaps expression of EWS/FLI driven by the *Zeb2* promoter in conjunction with the *Sox1* promoter would allow for relevant spatial and temporal expression of this translocation. Based on the mouse expression studies of ZEB2 and its role as an EMT-TF, ZEB2 expression would mark the EMT of a neural crest cell or neuroepithelial cell and thus provide a cellular intermediate to a NCSC and a MSC.

Here we show that Ewing sarcoma cells express high levels of the EMT-TF, ZEB2, and that this transcription factor represses epithelial gene expression and epithelial phenotypes in Ewing sarcoma cells. Reduced ZEB2 expression results in the re-expression of genes involved in epithelial differentiation and the formation of intercellular junctions concomitant with a decrease in cell migration *in vitro* and decreased metastatic potential *in vivo*. We have proposed a model for Ewing's sarcomagenesis whereby the EWS/FLI translocation occurs in a cell undergoing a ZEB2 induced developmental EMT. This leaves the cell "stuck" in between an epithelial differentiation state (prevented by ZEB2 expression) and a mesenchymal differentiation state (prevented by EWS/FLI expression). This poorly differentiated cancer cell is now poised for successful growth in the primary and secondary sites, owing to its epithelial features, while at the same time made competent for metastasis by its mesenchymal features (see model Figure 4.6). In the future it will be interesting to investigate the ability of Ewing sarcoma cells to "toggle" expression of EWS/FLI and ZEB2 depending on their stage in the metastatic process to accentuate either epithelial or mesenchymal traits, reminiscent of what has been proposed to achieve EMT-MET in carcinoma

metastasis. This cellular plasticity may offer a therapeutic opportunity, if cells can be forced toward one lineage or the other depending on the stage of the disease.

Materials and methods

Constructs

shRNA targeting EWS/FLI (EF2 shRNA), or control (control shRNA) targeting luciferase or ERG (which is not expressed in these cells) have been previously described (Smith *et al* 2006). shRNA was designed targeting the 3'UTR of ZEB2 (Supporting Information Table 4.S2) and cloned into pMKO.1 puro retroviral vector (Masutomi *et al* 2003). ON-TARGETplus siRNA targeting the ORF of ZEB2 (siZEB2-5, siZEB2-6) and nontargeting siRNA (siControl) were purchased from Thermo Scientific. Lentiviral microRNA precursor constructs (miR-control, miR-200,a,b,429) were purchased from System Biosciences. These constructs were also modified by subcloning a CMV promoter and puromycin resistance cassette downstream of the miRNA precursor to allow for antibiotic selection.

Cell culture

Ewing sarcoma cell lines A673, SKNMC, (ATCC) and TC71 (a gift from Timothy Triche, Children's Hospital Los Angeles) were grown as previously described (Lessnick *et al*, 2002; Wiles *et al*, 2013). For retroviral plasmids, control shRNA (LUC or ERG) and ZEB2 shRNA, cells were infected and selected in the appropriate antibiotic selection media resulting in a polyclonal population. For lentiviral vectors, miR-control and miR-200 family, cells were infected and selected with the appropriate antibiotic selection media or sorted for GFP+ cells using a FACSAria (BD Biosciences).

Transfections with siRNA were preformed according to the manufacturer's instructions (Thermo Scientific) at final concentration of 25 nM.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted with an RNAeasy kit (Qiagen). mRNA (30 ng) was quantitated by SYBR green (BIO-RAD) using one-step qRT-PCR with gene specific primers. Messenger RNA was reverse-transcribed at 50°C for 10 minutes followed by a 5 minute denaturation at 95°C and then 45 cycles of PCR (95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds). For miRNA quantification, total RNA was extracted using the mirVana miRNA isolation kit (Ambion). RNA (10 ng) was reverse-transcribed using the Taqman Micro-RNA Reverse Transcription Kit and miRNA specific primers (Applied Biosystems) at 16°C for 30 minutes, 42°C for 30 minutes, followed by 5 minutes at 85°C in a BIO-RAD DNA engine Peltier thermal cycler. The reverse-transcribed DNA was then used for qPCR with Taqman small RNA assay miRNA specific probes (Applied Biosystems) with the following cycling parameters: 95°C 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fold change was determined using the ΔCt method comparing all samples to the control after normalizing to GAPDH (for mRNA) or snRNA U6 (for miRNA) or expression was determined as a percent of GAPDH expression, as indicated. qPCR and qRT-PCR were performed using the BIO-RAD MyiQ single color real-time PCR detection system.

RNA sequencing

RNA from A673 cells transfected with siZEB2-5, siZEB2-6 or siControl 72 hours post-transfection was extracted with the RNAeasy kit (Qiagen) and treated with DNAse.

Two biological replicates per condition were used to construct libraries for high-throughput sequencing according to the manufacturer's instructions (Illumina) and sequenced on the Illumina Hi-Seq with 50 cycles of single end reads. Sequences were aligned to the human genome build hg19. The RNAseq data from this publication has been submitted to the US National Center for Biotechnology Information-Sequence Read Archive (NCBI-SRA) and assigned the accession number SRP022361. Differential gene expression (siControl vs siZEB2-5,-6) was determined using the publically available USeq package (useq.sourceforge.net). Significance parameters were set at an FDR of 10% and two-fold change.

Comparison of RNA-seq with microarray data

Microarray data from Sartor *et al* (2009) (PMID 20007254, GEO accession GSE17708, platform hgu133plus2), Onder *et al* (2008) (PMID 18483246, GEO accession GSE9691, platform ht_hgu133a), and Taube *et al* (2010) (PMID 20713713, GEO accession GSE24202, platform ht_hgu133a), were downloaded as raw CEL files from GEO (www.ncibi.nlm.nih.gov/geo). Fluorescent intensities were extracted and normalized by RMA as implemented in the Bioconductor package affy (Gautier *et al*, 2004) and cdf and annotation packages appropriate to their platform. Differential gene expression (DGE) among published conditions (control vs treatment/cDNA/shRNA) was determined using the Bioconductor package limma and obtaining the $\log_2$ fold change ($\log_2$FC) from the top statistically significant differentially expressed genes. Genes from these sets were intersected with $\log_2$FC values of corresponding genes from the ZEB2 knock-down RNA-seq. The only genes allowed from the ZEB2 knock-down RNA-seq were those having a p-value for DGE less than 0.05. Final sets were derived by further

requiring that the absolute log_2FC DGE be greater than one for at least one of the conditions in the comparison. For the purposes of DGE comparison, heat maps were made from the results of standardizing column values followed by hierarchical clustering using Euclidean distance and complete linkage. Both row and column clustering were performed except in the case of Sartor *et al* where the sample order follows a time course.

Migration assays

Boyden chambers were performed using cell inserts with 8μm pores (BD Biosciences). Fifty-thousand cells were seeded in serum-free media in the upper compartment of a fibronectin (1μg/mL) coated chamber which was placed into a well containing media with 10% serum. Cells were allowed to migrate for 24 hours before fixation in methanol and staining with modified Giesma stain (1:10). Each condition was done in triplicate and five fields from each chamber were imaged at 10x using an Olympus IX81 microscope with CCD camera and DP Controller imaging software. Wound healing assays were performed by plating each condition in triplicate in six well plates and growing to ~90% confluence. Monolayer wound was created with a micropipette tip, washed 4x with PBS and grown in appropriate media containing 5% serum. Cells were washed twice with PBS and given fresh media before imaging. Phase contrast images were taken at 0, 24, 48, 72, and 96 hours on a Ziess Axiovert100 at 10x magnification with a Q imaging MicroPublisher 5.0 RTV camera using QCapture Pro7.0 imaging software. The area of the wound at each time point was measured using the magic wand tool in Adobe Photoshop.

Immunodetection

Western blots were performed using standard protocols with 4-15% gels, nitrocellulose membranes and developed with home-made enhanced chemiluminescence (ECL). The following antibodies were used: DSPI+II (Abcam 71690), KRT8 (Abcam 9023), Tubulin (Calbiochem CP06), ZEB2 (Active Motif 61095).

Immunofluorescence

A673 cells were plated on fibronectin (10μg/mL) coated coverslips 48 hours post transfection. Twenty-four hours after plating, cells were fixed in 3.7% formaldehyde. Fixed cells were blocked and permeablized with PBST-BSA (0.1% triton-x100 and 0.5% BSA). Cells were incubated for 1 hour at 37°C in a moist chamber with ZEB2 antibody (Active Motif 61095) (1:100), washed for 15 minutes in PBST-BSA, followed by incubation with AlexaFluor-568 secondary antibody (1:100), AlexaFluor-488 phalloidin (1:50) (Invitrogen) and DAPI (1μg/mL) for 1 hour at 37°C in a moist chamber. Cells were washed and mounted in FlouromountG (Southern Biotech). Cell images were captured using a Zeiss Axioskop2 mot plus microscope with a 40x plan NA 0.75 NeoFluor objective, Zeiss Axiocam MR camera, and Zeiss Axiovision v4.8.1 software (Carl Zeiss MicroImaging, Inc.). The following exposure times were used for the displayed images: 405 nm (DAPI) – 10 ms, 488 nm (Phalloidin) – 400 ms, 568 nm (ZEB2) – 600 ms.

Tibial injection metastasis model

All animal studies were performed in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee. Male NOD-SCID mice

(kindly provided by Alana Welm) were 6-7 weeks old at the time of injection. A673 cells were retrovirally infected with either a control shRNA or ZEB2 shRNA and selected in puromycin. Cells were counted and resuspended in growth factor reduced Matrigel matrix (BD Biosciences). After boring a hole in the tibia with a 26-gauge needle, 10 microliters of Matrigel containing 25K or 100K cells were injected into the right tibia using a glass Hamilton syringe. Tibial measurements using calipers were performed weekly to follow the growth of the tumors. Mice were sacrificed at 6 weeks and the lungs were harvested to evaluate for metastases. Mice that were sacrificed before the 6-week endpoint of the study due to a primary tumor size >2 cm in one direction and mice with no detectable primary tumor were excluded from the study.

Tissue processing

The lungs were washed in PBS and stored in 10% formalin immediately after harvesting. Samples were fixed in 10% formalin at 4°C for 24 hours. Lungs were then washed 3x with PBS and stored in 70% ethanol at 4°C. Whole lung images were acquired using an Olympus MVX10 dissecting microscope at 0.63x, Spot insight Firewire 4 camera, and Spot Alias imaging software. Area of whole lung and metastasis (front and back) was measured using ImageJ software.

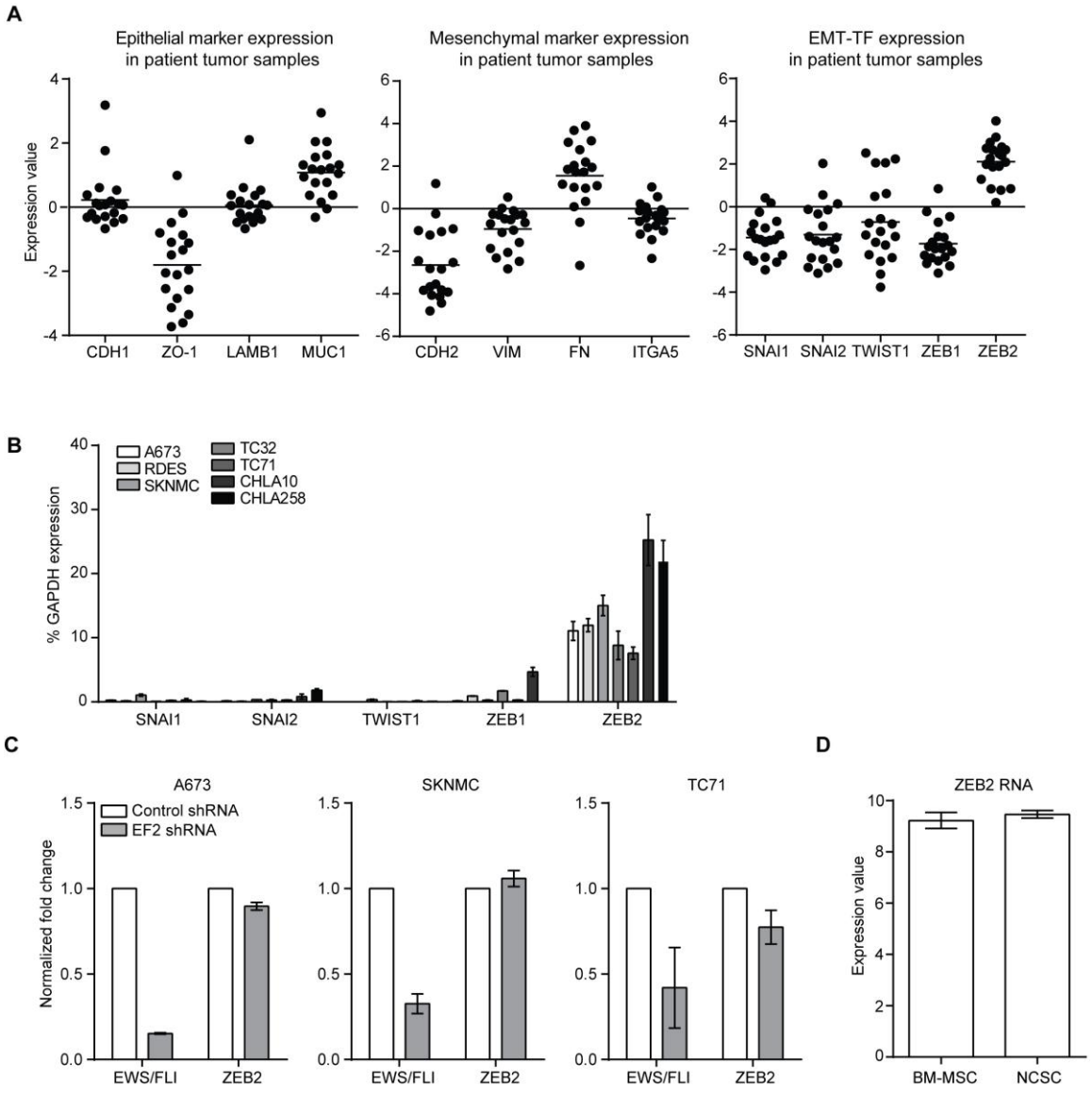


Figure 4.1: ZEB2 is expressed in Ewing sarcoma cells.
A. Gene expression data from Ewing sarcoma patient tumors from the Baird *et al* dataset showing the expression of epithelial markers, E-cadherin (CDH1), tight junction protein 1 (ZO-1), lamanin beta 1 (LAMB1), and mucin 1 (MUC1), mesenchymal markers, N-cadherin (CDH2), vimentin (VIM), fibronectin (FN), and integrin alpha 5 (ITGA5) , and EMT-TF, Snail1 (SNAI1), Snail2/Slug (SNAI2), TWIST1, ZEB1 and ZEB2. B. qRT-PCR data showing the expression of EMT-TF in a panel of Ewing sarcoma cell lines – A673, RDES, SKNMC, TC71, TC32, CHLA10 and CHLA258. C. qRT-PCR data showing EWS/FLI and ZEB2 expression levels in control shRNA and EWS/FLI shRNA infected Ewing sarcoma cell lines A673, SKNMC, and TC71. Errors bars represent standard deviation (SD) from three technical repeats. D. ZEB2 gene expression in hBM-MSC and hNCSC from the von Levetzow *et al* data set. Error bars represent SD.

Figure 4.2: ZEB2 represses epithelial gene expression in Ewing sarcoma cells. A. qRT-PCR data and western blot showing ZEB2 expression levels in A673 cells transfected with control siRNA or two different siRNAs targeting ZEB2 (siZEB2-5 and siZEB2-6). Tubulin is shown as a loading control. B. Graph showing the top ten enriched terms by DAVID analysis in the ZEB2 repressed gene set from our RNAseq analysis. Enrichment score equals –log(mean P-value). C. Heat map and hierarchical clustering of the ZEB2 RNAseq data with Taube *et al* EMT data in HMLE cells. D. Heat map and hierarchical clustering of the ZEB2 RNAseq dataset compared to the Onder *et al* EMT dataset in HMLE cells. E. Heat map of the ZEB2 RNAseq data with Keshamouni *et al* EMT time course data in A549 lung carcinoma cells. Color key represents $\log_2$FC.

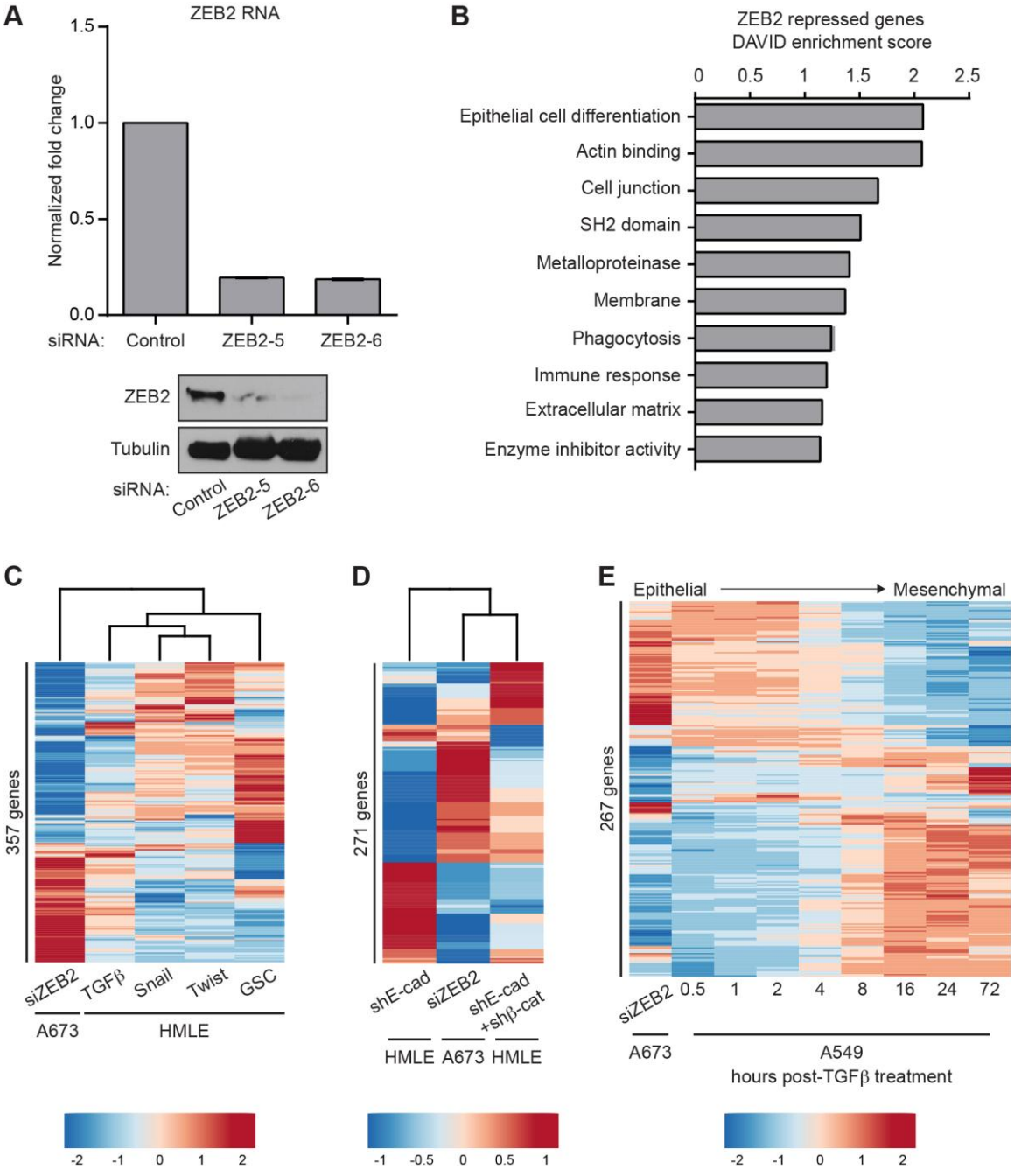

A
ZEB2 RNA
Normalized fold change
1.5
1.0
0.5
0.0
siRNA: Control ZEB2-5 ZEB2-6
ZEB2
Tubulin
siRNA: Control ZEB2-5 ZEB2-6
B
ZEB2 repressed genes
DAVID enrichment score
0 0.5 1 1.5 2 2.5
Epithelial cell differentiation
Actin binding
Cell junction
SH2 domain
Metalloproteinase
Membrane
Phagocytosis
Immune response
Extracellular matrix
Enzyme inhibitor activity
C
357 genes
siZEB2 TGFβ Snail Twist GSC
A673 HMLE
-2 -1 0 1 2
D
271 genes
shE-cad siZEB2 shE-cad +shβ-cat
HMLE A673 HMLE
-1 -0.5 0 0.5 1
E
Epithelial → Mesenchymal
267 genes
siZEB2 0.5 1 2 4 8 16 24 72
A673 A549
hours post-TGFβ treatment
-2 -1 0 1 2

Figure 4.3: ZEB2 represses the epithelial phenotype in Ewing sarcoma cells. A. qRT-PCR data showing ZEB2 knock-down in A673, SKNMC, and TC71 cells infected with control shRNA or shRNA targeting ZEB2 and the corresponding change of expression in the indicated genes. Error bars represent SD from technical triplicates. B. Western blot showing expression of ZEB2, KRT8, and DSP in A673, SKNMC, and TC71 cells expressing a control or ZEB2-targeting shRNA. Tubulin is shown as a loading control. C. Boyden chamber cell migration assays in A673, SKNMC, and TC71 cells infected with a control shRNA or ZEB2 targeting shRNA. Data are from two independent experiments each done in triplicate. Each point represents cells counted in one field – five fields were counted per chamber. Significance was determined by Student's T-test and the mean is shown. D. Representative images from three time points of a wound healing assay using control or ZEB2 shRNA infected A673 and SKNMC cells (left). Scale bar = 250μm. Quantification of the area of the wound healed measured every 24 hours (right). Data is the average of two independent experiments each done in triplicate (n=6). P-values indicated above each condition as determined by Student's T-test and error bars show SD. E. Immunofluorescence images showing ZEB2 expression levels (red - 568 nm) and actin cytoskeleton stained with phalloidin (green - 488 nm) in A673 cells transfected with control siRNA or siRNA targeting ZEB2 (siZEB2-5). Scale bar = 50μm.

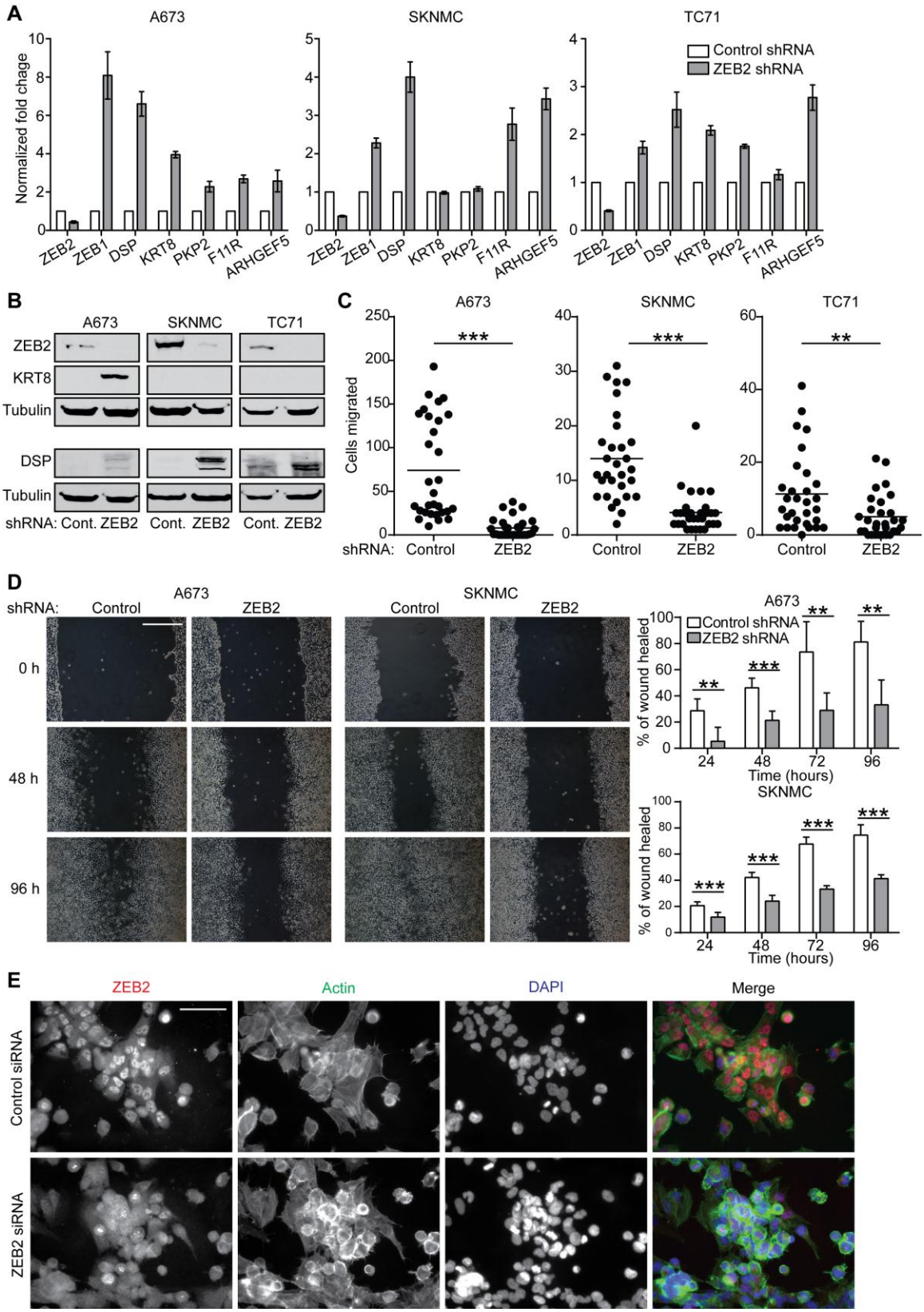

A
A673
SKNMC
TC71
Normalized fold chage
Control shRNA
ZEB2 shRNA
ZEB2
ZEB1
DSP
KRT8
PKP2
F11R
ARHGEF5
B
A673
SKNMC
TC71
ZEB2
KRT8
Tubulin
DSP
Tubulin
shRNA: Cont. ZEB2 Cont. ZEB2 Cont. ZEB2
C
A673
SKNMC
TC71
Cells migrated
***
**
shRNA: Control ZEB2
D
shRNA: Control ZEB2 Control ZEB2
A673
SKNMC
0 h
48 h
96 h
% of wound healed
Control shRNA
ZEB2 shRNA
Time (hours)
E
ZEB2
Actin
DAPI
Merge
Control siRNA
ZEB2 siRNA

Figure 4.4: Expression of miR-200 family members epithelializes Ewing sarcoma cells. A. qRT-PCR data showing the expression of the indicated miRNA in A673 and SKNMC cells infected with a lentivirus expressing a control miRNA or miR-200 family cluster from chromosome 1 (miR-200b, miR-200a, miR-429). Error bars represent SD from three technical repeats. B. Western blot showing expression of ZEB2, KRT8, and DSP in A673 and SKNMC cells expressing a control miRNA or miR-200 family cluster from chromosome 1 (miR-200b, miR-200a, miR-429). Tubulin is shown as a loading control. C. Representative images from three time points of a wound healing assay using control miRNA or miR-200 family cluster from chromosome 1 (miR-200b, miR-200a, miR-429) infected A673 and SKNMC cells (left). Scale bar = 250μm. Quantification of the area of the wound healed measured every 24 hours (right). Data are the average of two independent experiments each done in triplicate (n=6). P-values indicated above each condition as determined by Student's T-test and error bars show SD.

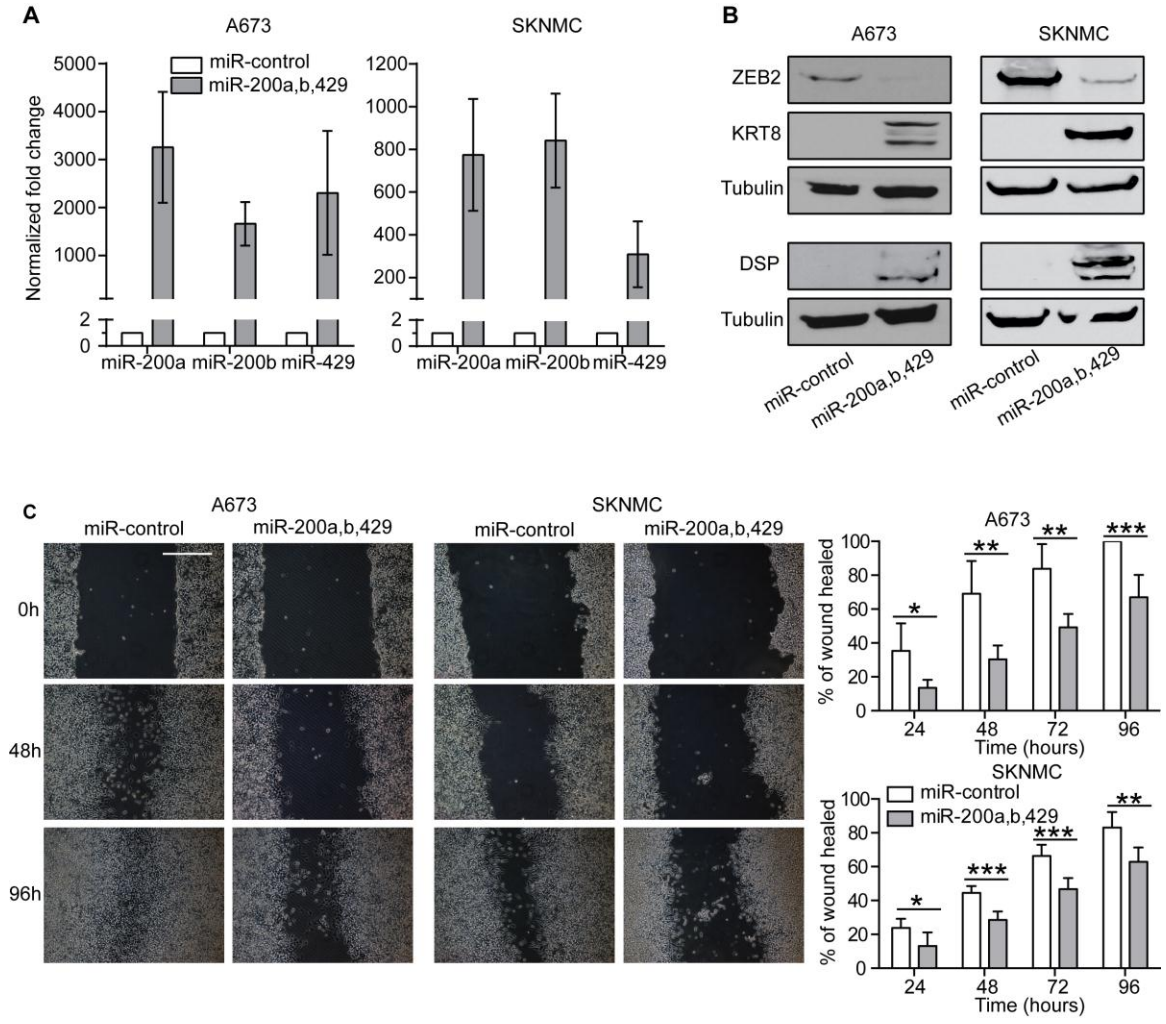
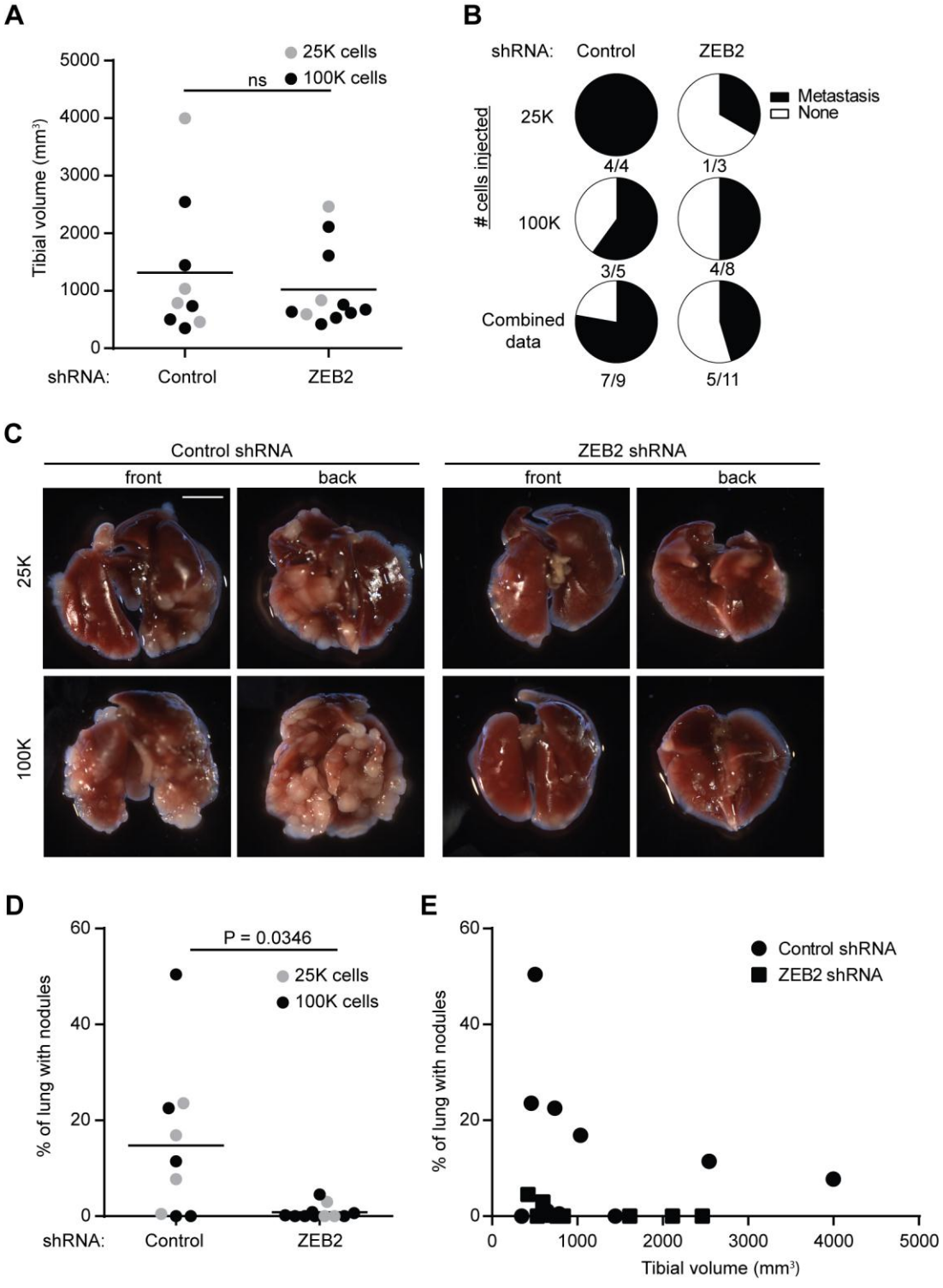

A
A673
SKNMC
miR-control
miR-200a,b,429
Normalized fold change
5000
4000
3000
2000
1000
2
0
1200
1000
800
600
400
200
miR-200a miR-200b miR-429
B
A673
SKNMC
ZEB2
KRT8
Tubulin
DSP
Tubulin
miR-control
miR-200a,b,429
C
A673
SKNMC
miR-control
miR-200a,b,429
0h
48h
96h
% of wound healed
Time (hours)
24
48
72
96
*
**
***

Figure 4.5: Reduction of ZEB2 decreases metastatic potential in Ewing sarcoma cells. A. Volume of the right tibia from mice 6 weeks postinjection with either 25K or 100K control or ZEB2 shRNA (as indicated) infected A673 cells. B. Pie charts showing the total number of mice with macroscopic metastatic lung lesions at the time of sacrifice (6 weeks postinjection). Combined data include both 25K and 100K conditions. C. Images of lungs (front and back) from the animal from each condition bearing the highest metastatic burden. Scale bar = 5mm. D. Graph showing the percent of the lung containing macroscopic pulmonary lesions in each condition, as indicated. P-value determined by Wilcoxon Mann Whitney test. E. Correlation of metastatic burden (y axis) and tumor size (x axis). Pearson's correlation coefficient for shControl = -0.2892 and shZEB2 = -0.4414.


A
25K cells
100K cells
ns
Tibial volume (mm³)
5000
4000
3000
2000
1000
0
shRNA:
Control
ZEB2
B
shRNA:
Control
ZEB2
Metastasis
None
cells injected
25K
100K
Combined data
4/4
1/3
3/5
4/8
7/9
5/11
C
Control shRNA
ZEB2 shRNA
front
back
front
back
25K
100K
D
P = 0.0346
25K cells
100K cells
% of lung with nodules
60
40
20
0
shRNA:
Control
ZEB2
E
Control shRNA
ZEB2 shRNA
% of lung with nodules
60
40
20
0
0
1000
2000
3000
4000
5000
Tibial volume (mm³)

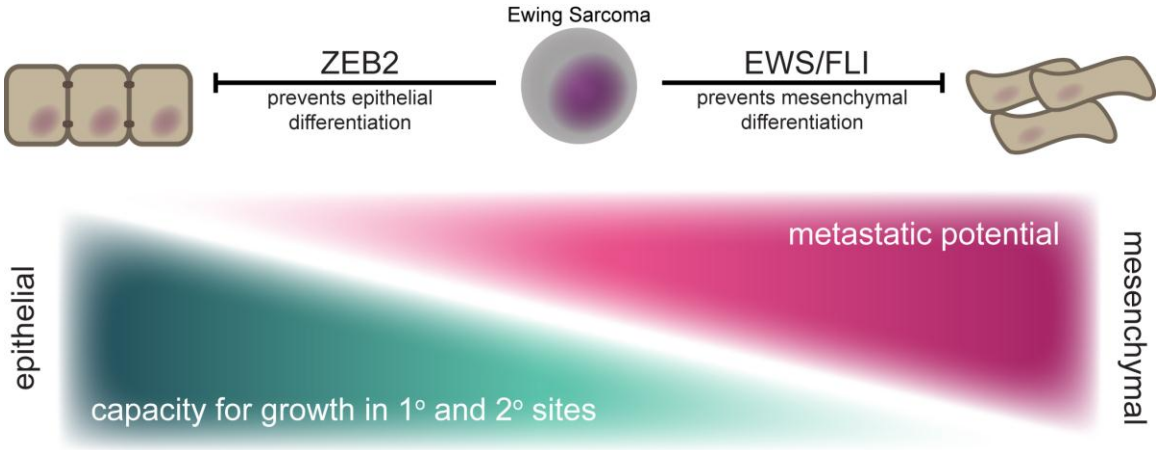


Figure 4.6: Model for ZEB2 in Ewing sarcoma.
ZEB2 prevents epithelial differentiation and EWS/FLI prevents mesenchymal differentiation. This leaves a Ewing sarcoma cell "stuck" between these two cell lineages where it is able to draw upon features from both cell types for successful growth and metastasis.

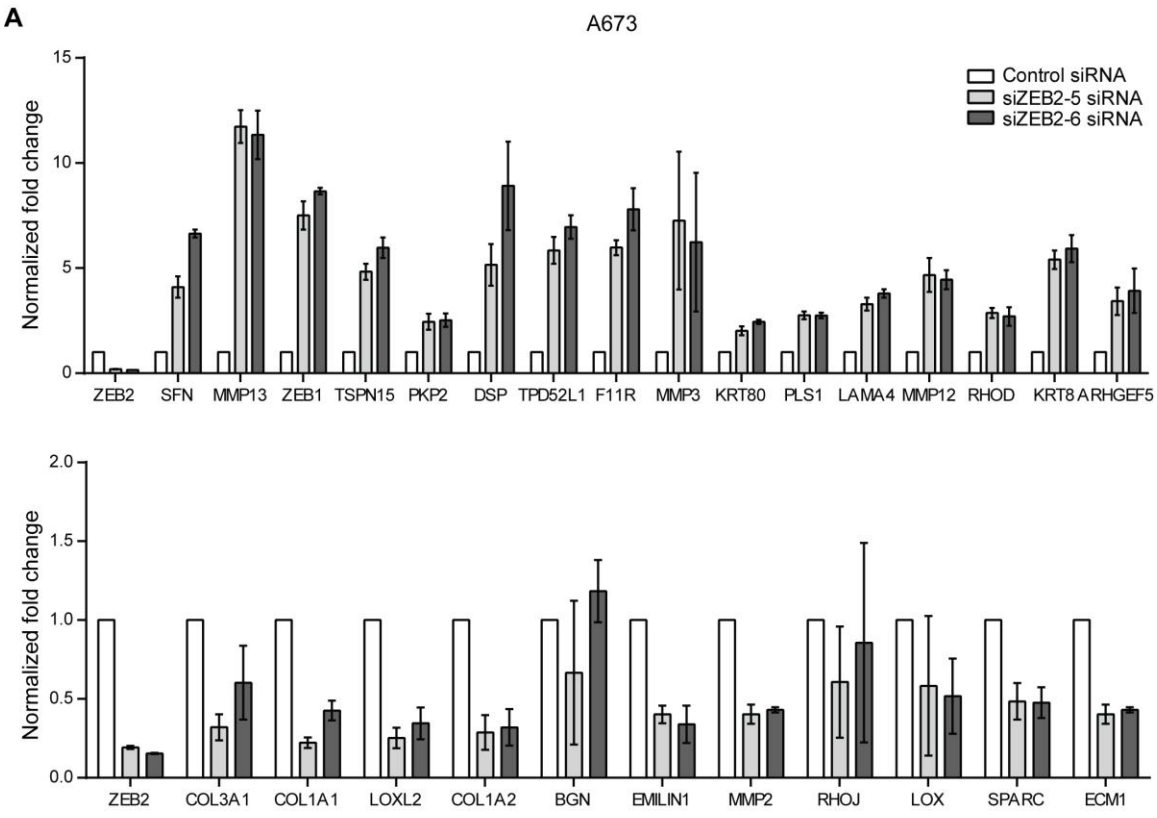


Figure 4.S1: RNA-seq validation
A. qRT-PCR data to validate the ZEB2 RNA-seq dataset: ZEB2 repressed genes (top) and ZEB2 up-regulated genes (bottom). Error bars represent SD from three technical repeats.

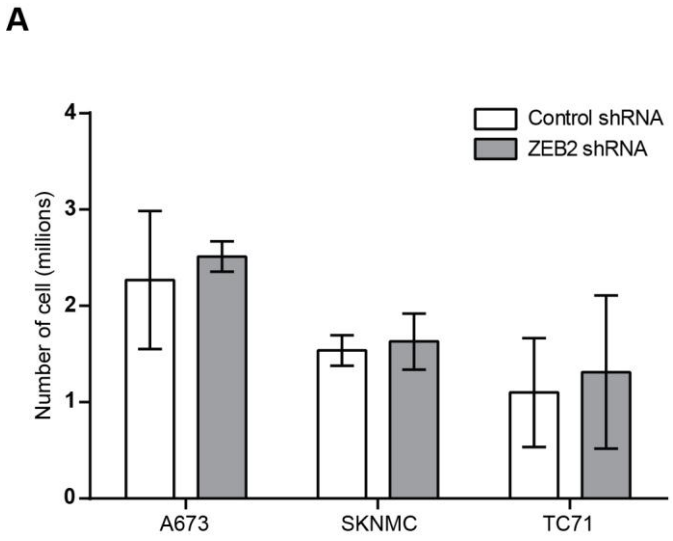
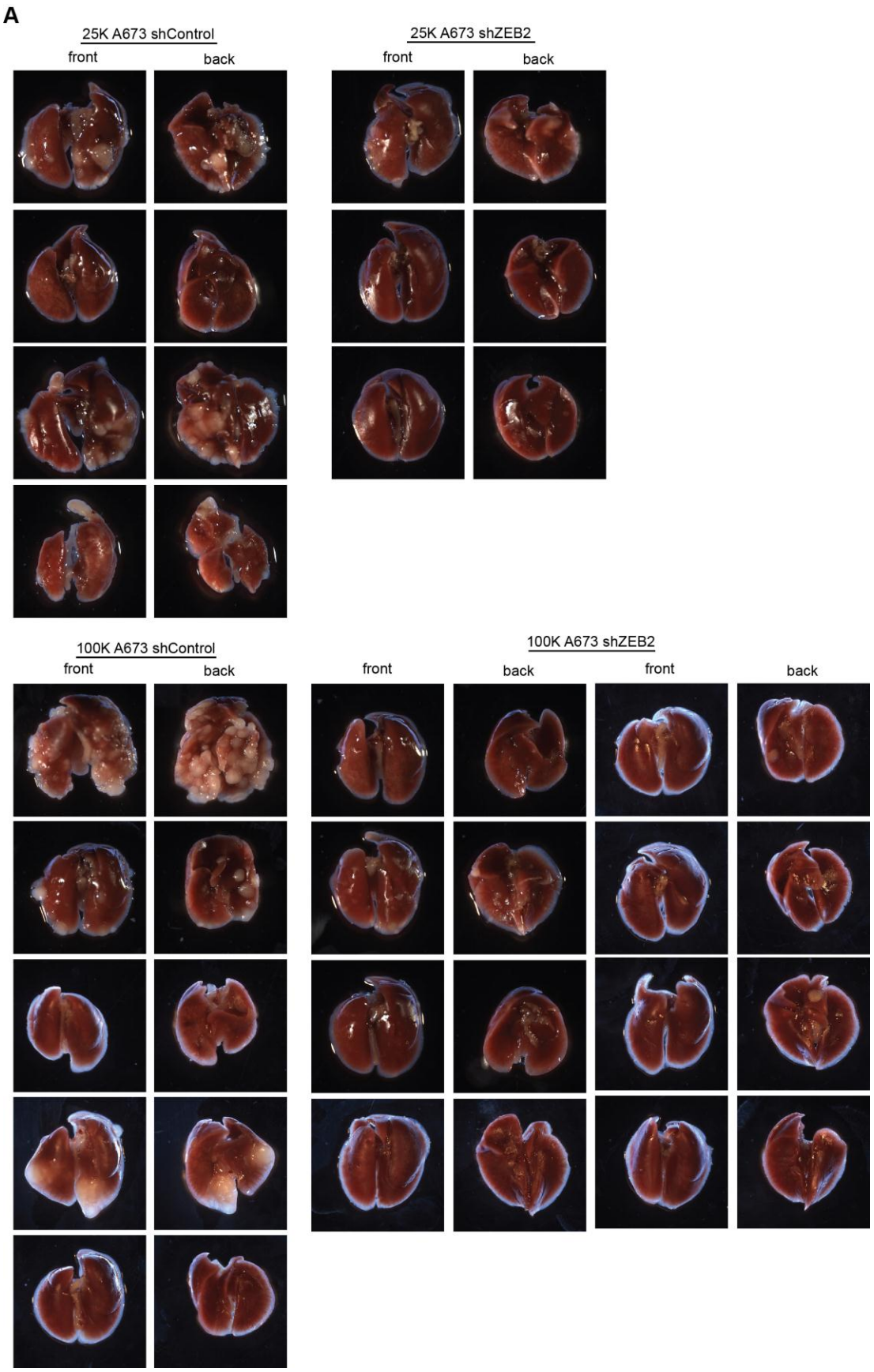


Figure 4.S2: No difference in cell viability in serum free media between control and ZEB2 knock-down cells.
A. Cell counts for A673, SKNMC, and TC71 cells infected with a control shRNA or shRNA targeting ZEB2. Cells ($2x10^6$) were plated in serum-free media and grown for 24 hours before counting in trypan blue. Graphs represent two independent experiments (n=2). Error bar represents SD.

Figure 4.S3: Pulmonary metastatic burden.
Images (front and back) of lungs from all conditions 6 weeks posttibial injections.


A
25K A673 shControl
front
back
25K A673 shZEB2
front
back
100K A673 shControl
front
back
100K A673 shZEB2
front
back
front
back

ZEB2 Repressed Genes			ZEB2 Upregulated Genes		
SFN	RND1	CALCA	COL3A1	MT1G	CAPN5
BC070061	PRKCZ	INADL	CMKLR1	OLFML2B	MT2A
KIAA1543	ABCC3	SLC16A14	COL1A1	CTHRC1	MT1E
CHI3L1	PHACTR2	HPGD	MRGPRF	NOTCH3	MMP2
GPR87	SCEL	MARVELD3	ACVRL1	KAAG1	COL6A1
F11R	RBM47	PLIN4	IGDCC4	ANGPTL2	C3AR1
TNS4	RBP7	SLC22A5	TLR7	NRXN2	CNRIP1
MMP13	MMP3	TMEM90B	CSPG4	TMEM55A	MT1F
SPINT2	KRT80	CORO2A	TMEM100	PCDH18	BX649164
MYO5B	LAMA4	PDE6A	GAP43	COL1A2	MT1X
DSP	PLS1	EPPK1	DCN	PGF	CDKL5
EXPH5	FBXO2	NIPAL3	DKK3	THY1	EMILIN1
NCF2	ITGA2	ID1	CRYAB	SRPX2	CSF1
AKR1B10	HEBP2	PPP1R1C	MT1H	SYT11	GPNMB
CEACAM5	TNK1	MCOLN3	LOXL2	CREB3L1	
ZEB1	RHOD	KIAA1244	ABCA1	EDEM2	
C10orf35	SPINT1	HLA-DMB			
TPD52L1	C3orf52	RAB11FIP4			
CLEC7A	SMR3B	CNTNAP2			
ARHGEF5	RPS6KA1	C11orf52			
ANXA3	MMP12	TMEM84			
SYNGR4	RNF125	IL2RG			
TC2N	FGFBP2	KCNE3			
C8orf47	LMTK3	GYLTL1B			
ARHGEF5L	CYP2S1	AHRR			
SH2D3A	CACNA1I	CBX7			
F2RL1	NIPAL1	ANKRD5			
LOC220930	MAP7D2	SHF			
TSPAN15	BAIAP2L1	SYT12			
CHMP4C	C19orf51	PKP2			
KIF21A	GRM2	GOLGA8B			
KRT8	TMEM144	TRA			
ELOVL7	SULT1E1	CD79A			
SMPDL3B	C3	PHKA1			
ALOX12E	LCP1	HIP1R			
COL17A1	HKDC1	LAMB3			
RAET1E	AF289551	PLCH1			
AGPAT9	LRAT	TXNRD1			
IL28RA	CDS1	BCAR3			
IRF6	RNF144B				
CDCP1	PAIP2B				

Table 4.S1: ZEB2 regulated genes as determined by RNAseq

GENE	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
ZEB2	CAGCACCAAATGCTAACCCAAGGAG	CACCACTGTGAATTCGCAGGTGTTC
SFN	GCGCATCATTGACTCAGCCCGG	GAGTCCTCGCTGAGGGTGTGC
MMP13	CCAGTTTGCAGAGCGCTACCTG	CACATCAGGAACCCCGCATCTTGG
ZEB1	GGAAGAGATCAAAGACATGTGACGC	GCTTATGTGTGAGCTATAGGAGCCAG
TSPN15	CCACCGTGTTCTGGCTGATTGG	CCCAAGGATGTACATGAATGCTTGG
PKP2	CGTGGGCAACGGAAATCTTCACC	GGCAGTTGTGCCAGCCTTTAGC
DSP	CGGCTACTGTCAAACCGGCACG	CTCCATACTTCAATTCAGGCTGCACG
TPD52L1	GCGCAGGCACAAGGTTTGTTGG	GCAGTGGTAGTCTGCATGTCATGC
F11R	GCGATCCTGTTGTGCTCCCTGG	CCGGTCCTCATAGGAAGCTGTG
MMP3	GGACAAAGGATACAACAGGGACC	CAGGGGGAGGTCCATAGAGGG
KRT80	CCATCTATGAGCAGGAGCTGAAGG	CCTGCTCCTCCAGCTGGCTC
PLS1	GCCAAGACATTAAGGACTCGAGAGC	GGATTGCCTGAAACCACATCTGCAG
LAMA4	GCTCAGGATACTGTGTGCACTGC	GGAGCACATCTTTCACAGTTAGGTC
MMP12	GCATTCAGTCCCTGTATGGAGACC	CCAGATGGCAAGGTTGGCCATAAGG
RHOD	GCCTTCCCCGAGAGCTACACC	CGACGATGATGGGTACCTTCTTGC
KRT8	GGCAGCTATATGAAGAGGAGATCCG	CAGCCAGGCTCTGCAGCTCC
ARHGEF5	CCTGAGGGCTCTTCAGATTCAAGAG	CGCTGCTGGCTCTGGATCTCC
COL3A1	GCCTCCAACTGCTCCTACTCGC	GGCCAGCTGGTCCAGGATAGC
COL1A1	GCCAAGACGAAGACATCCCACC	GGGTGACTCTGAGCCGTCGG
LOXL2	GCAAGGGAGAAGGGCCCATCTG	CGAATCCGAATGTCCTCCACCTG
COL1A2	CCTGGTCTCGGTGGGAACTTTGC	CCACGAGCACCCTGTGGTCC
BGN	GCACCTCTACGCCCTCGTCC	GGTTCCCGCCCATCTCGATGC
EMILIN1	CGCCACAGGAACTGGTGTGCC	GGACTCTCAGCACAGTCATCGC
MMP2	GGGAGAAGGCCAAGTGGTCCG	CCATGGTGAACAGGGCTTCATGG
RHOJ	GGGAACTGACAGCAGCTGCGG	GGTTGTAGTCCTCCTGTCCCGC
LOX	GCGACGACCCTTACAACC	GGACGCCTGGATGTAGTAGG
SPARC	GGCAGCCCCTCAGCAAGAAGC	GGTTCTGGCAGGGATTTTCCGC
ECM1	CCCAATGAACAGAAGGAAGGAACGC	GGAGTAGCTGGACTGTGGTAGG

shRNA	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
ZEB2	CGTAAAGAGAAGAGCATTTTTCAAGAGA AAATGCTCTTCTCTTTACG	CGTAAAGAGAAGAGCATTT TCTCTTGAA AAATGCTCTTCTCTTTACG

Table 4.S2: Primer sequences

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CHAPTER 5

DISCUSSION

Ewing sarcoma is a unique disease, but the developmental dysregulation so prevalent in this malignancy is a commonly used mechanism in tumorigenesis and progression. EWS/FLI is a powerful oncogenic transcription factor that interferes with many signaling pathways used to direct appropriate cell fate decisions. Of relevance, Ewing sarcoma occurs in a very specific age group – adolescents and young adults (Arndt & Crist, 1999) - perhaps indicating that the appropriate cell, responsive to the EWS/FLI translocation, is not present, is exceedingly rare, or becomes somehow altered in the body outside this age range. It seems EWS/FLI requires a very specific cellular context in which to target the appropriate genes that lead to transformation. The primitive cell type in which this transcriptional mayhem occurs has not been defined. However it surely makes significant contributions to oncogenic phenotypes in this tumor.

One EWS/FLI target gene, BCL11B, was characterized by the work presented within showing that its expression contributes to the maintenance of transformation in Ewing sarcoma cell lines. BCL11B is normally expressed in the developing CNS (Arlotta *et al*, 2005; Chen *et al*, 2008), skin (Golonzhka *et al*, 2009a), teeth (Golonzhka *et al*, 2009b) and T-cells (Li *et al*, 2010). In Ewing sarcoma cells, the expression of BCL11B is reduced to undetectable levels in the absence of EWS/FLI. This suggests that BCL11B is not normally expressed in the Ewing sarcoma cell of origin. The involvement of BCL11B in the development of tissue from several different lineages suggests that its genetic locus may be found in a euchromatic region of the genome until a cell differentiates (Gaspar-Maia *et al*, 2011). This would provide an accessible promoter region for EWS/FLI to induce high level gene expression. We have not tested whether BCL11B is a direct transcriptional target of EWS/FLI. However the presence of a variant ETS consensus

sequence in the BCL11B promoter region allows for this possibility and warrants future investigation.

BCL11B is a transcription factor and, as demonstrated, transcriptional regulation mediated by BCL11B contributes a significant number of genes to the EWS/FLI repressed gene signature. Aberrant regulation of other transcription factors and transcriptional regulators may be a more general mechanism used by EWS/FLI to broaden its transcriptional impact. In a similar way, EWS/FLI up-regulates, the homeobox transcriptional repressor, NKX2.2, which then is responsible for a large part of the EWS/FLI repressed genes (Owen *et al*, 2008). At this time several EWS/FLI target genes have been shown to contribute to the transformed phenotype. Perhaps the most interesting research avenue in this regard is the identification of the minimum set of EWS/FLI target genes that need to be expressed or repressed to recapitulate the disease. It is unlikely that one or two genes will be sufficient for this task (Kinsey *et al*, 2006), but by including transcriptional regulators, such as BCL11B and NKX2.2, that contribute a significant portion of EWS/FLI targets, and genes that disrupt relevant developmental signaling pathways, this might be feasible. This could be done in Ewing sarcoma cells with EWS/FLI expression reduced by RNAi. In this setting, the loss of transformation resulting from EWS/FLI knock-down would be rescued by re-expressing EWS/FLI up-regulated targets, and reducing the expression of EWS/FLI repressed targets. More impressive would be creating a Ewing sarcoma cell from the appropriate precursor cell in the absence of EWS/FLI.

The cell of origin in Ewing sarcoma remains elusive. Several different cell types have been proposed – endothelial cells were suggested by James Ewing in 1921 when he

first described the disease (Ewing, 1921). Since that time, myelogenous cells (Kadin & Bensch, 1971), neural crest cells (Cavazzana *et al*, 1988), and mesenchymal stem cells (Dickman *et al*, 1982), have all been posited as the cell of origin. One approach to identifying this enigmatic cell type is to place less emphasis on EWS/FLI and its target genes and more emphasis on genes that are expressed in this cell before the fusion occurs. We identified a gene that fit these criteria in our efforts to uncover a factor that could collaborate with EWS/FLI in keeping Ewing sarcoma cells in a poorly differentiated state. EWS/FLI's ability to prevent mesenchymal differentiation was well established (Tirode *et al*, 2007; Torchia *et al*, 2003), so we sought out a gene that could prevent epithelial differentiation. The leading candidates were EMT-TF because of their well described role in repressing the epithelial phenotype during developmental EMT. The two best studied examples of EMT occur during early embryonic development: gastrulation where the single epithelial cell layer internalizes to generate mesodermal and endodermal cell layers and in neural crest migration where cells of the neural tube undergo EMT to gain the migratory capacity necessary to travel throughout the embryo (Acloque *et al*, 2009).

These efforts led us to ZEB2, an EMT-TF that is highly expressed in Ewing sarcoma patient tumors and cell lines, but its expression is not dependent on EWS/FLI levels. Genome-wide gene expression analysis confirmed our hypothesis that ZEB2 repressed epithelial gene expression. *In vitro* functional assays demonstrated that when this ZEB2 enforced epithelial repression was released by reducing ZEB2 levels using RNAi, Ewing sarcoma cells adopted more epithelial traits. EMT-TFs contribute to metastasis in cancer, in addition to their natural role in development (Thiery *et al*, 2009),

suggesting ZEB2 may facilitate metastasis in Ewing sarcoma. We tested this hypothesis and found that the epithelial features in Ewing sarcoma cells realized upon reduction of ZEB2, led to a decreased metastatic potential of these cells in an orthotopic mouse metastasis model. These findings have promise in translating to the realm of patient care.

Metastatic disease is indicative of a very poor prognosis in Ewing sarcoma patients. Current therapy which includes aggressive chemotherapy, radiation and surgery achieves cure rates of 70% for patients with localized disease (McAllister & Lessnick, 2005). At this time, there is no effective therapy for patients with disseminated disease. In one large prospective clinical trial, patients with metastatic disease had a five-year overall survival just over 30% compared to over 70% for those with localized disease treated on the same protocols (Grier *et al*, 2003). ZEB2 may have potential to serve as a prognostic indicator. Patients with tumors expressing low levels of ZEB2 may have less chance for metastasis owing to the more epithelial nature of their tumor. ZEB2 expression in combination with other epithelial markers may predict which patients presenting with localized disease are most likely to have micrometastatic disease. Patients could then be stratified according to their risk and treated accordingly, thus limiting the toxic and long lasting effect of chemotherapy and radiation on these children and young adults.

The cellular plasticity that has been demonstrated in Ewing sarcoma may have therapeutic potential. Studies in carcinoma have suggested that epithelial characteristics of cancer cells endow them with a proliferative capacity at primary and secondary sites, while mesnchymal features are required to escape the primary tumor, survive in the blood stream, and arrive at the secondary site. These studies have led to some speculation about opportunity for differentiation therapy. A much better understanding of this cellular

plasticity and how tumor cells call upon aspects of these two cell types at certain stages of disease is needed before treatments are based upon the idea. Keeping this in mind, it is worth thinking about for the future. In Ewing sarcoma, if one could be truly certain a tumor had not disseminated, a treatment that could elicit epithelial characteristics, similar to the effects achieved by knocking-down ZEB2, might be beneficial. This may limit or prevent the tumor's metastatic potential. Due to the reality that metastasis is an early event in Ewing sarcoma, and in most cases the disease has already spread at the time of presentation, therapy nudging tumors toward the mesenchymal lineage may be a better approach. In this way, the cancer cells would lose proliferative capacity keeping the primary tumor from progressing and inhibiting outgrowth of micrometastases. This concept is supported by the observation that many Ewing sarcoma cell lines proliferate more slowly, or not at all, when EWS/FLI expression is reduced. While loss of EWS/FLI has many consequences, one that could contribute to this reduced growth is the shift toward the mesenchymal phenotype.

The importance of ZEB2 in Ewing sarcoma pathogenesis combined with a greater appreciation of the epithelial plasticity provides new insight into the cell of origin for this disease. Identifying a precursor cell has been a major challenge in the field and has severely limited study of disease development and progression and prevented the use of relevant animal models. The epithelial plasticity of Ewing sarcoma cells had not been experimentally demonstrated prior to this work. This finding warrants questioning of the generally accepted MSC cell of origin hypothesis. We prefer a model that integrates the long held belief that Ewing sarcoma originated from neural crest with the more recently favored MSC model. We propose that the cell of origin is an intermediate between neural

crest and an MSC. Two independent labs have demonstrated that neural crest can give rise to MSCs, and this is true for both mouse and human cells (Lee *et al*, 2007; Takashima *et al*, 2007).

Experimental investigation of ZEB2 in early embryonic development lends support for this model. *Zeb2* is expressed in the neural crest and neuroepithelium during mouse embryogenesis and is required for neural crest migration. Homozygous *Zeb2* knock-out mice die on embryonic day 8.5 due to neural crest migration defects. This highlights the role of ZEB2 in facilitating a developmental EMT in neural crest cells. By extension, ZEB2 expression in the human neural crest may induce EMT and during this transition the EWS/FLI translocation occurs, thus preventing the mesenchymal destination. This results in a poorly differentiated Ewing sarcoma cell that is "stuck" between epithelial and mesenchymal lineages, but has access to features of both cell types.

This alternative take on the cell of origin provides a new avenue for investigation in developing a mouse model for Ewing sarcoma. So far efforts to genetically engineer EWS/FLI expression in various mouse cell types have been fruitless. EWS/FLI expression in hematopoetic precursors gives rise to leukemias and lymphomas (Codrington *et al*, 2005; Torchia *et al*, 2007) and expression of the translocation in mesoderm derived limb bud did not induce tumor formation (Lin *et al*, 2008). In light of the hypothesis presented here, one might try to model this cancer in a neural crest cell undergoing EMT. To target this transitioning cell, EWS/FLI expression could be placed under dual control of a neural crest or neuroepithelium marker such as *Sox1* in addition to *Zeb2*, ensuring expression in a population of cells undergoing EMT. This work could be

complemented with *in vitro* modeling, which could be achieved by expressing EWS/FLI at various times following the culture systems described to generate MSCs via neural crest stem cells (Lee *et al*, 2007; Takashima *et al*, 2007). One advantage of the *in vitro* system is the use of human cells which notably have different requirements for transformation as compared to mouse cells (Rangarajan *et al*, 2004). This system could be further enhanced by using the newly described zinc finger and TALE nuclease technology to engineer targeted double strand breaks that result in the generation of translocations in the appropriate genomic context (Piganeau *et al*, 2013). In this way, heterozygousity and potential haploinsufficiency resulting from the translocation are accomplished.

A more comprehensive understanding of the contributions made by EWS/FLI and the appropriate cellular context in Ewing sarcomagenesis will provide useful knowledge about the disease that will translate to improved patient care. Identification of the cell of origin to enable modeling of the disease both in mice and human cells is a necessary intermediate in achieving this goal. Critical steps in tumor development including information regarding the time from the translocation event to initiation of transformation as well as disease penetrance can be elucidated. Mouse models will allow for study of the natural disease progression including monitoring metastasis in real time. Contributions of EWS/FLI target genes can also be better assessed using knock-ins and knock-outs of genes of interest rather than ectopic expression or RNAi-mediated approaches where the desired effect (ie gene knock-down) is often lost over the course of the study. These animals will also provide a relevant system in which to test new cancer therapies

including agents to target specific developmental pathways, differentiation therapy, and agents to improve the outcome of patients with metastatic disease.

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