**Therapeutics, Targets, and Chemical Biology**

**ZIC1 Overexpression Is Oncogenic in Liposarcoma**

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**Abstract**

Liposarcomas are aggressive mesenchymal cancers with poor outcomes that exhibit remarkable histologic diversity (there are five recognized subtypes). Currently, the mainstay of therapy for liposarcoma is surgical excision because liposarcomas are often resistant to traditional chemotherapy. In light of the high mortality associated with liposarcoma and the lack of effective systemic therapy, we sought novel genomic alterations driving liposarcomagenesis that might serve as therapeutic targets. **ZIC1**, a critical transcription factor for neuronal development, is overexpressed in all five subtypes of liposarcoma compared with normal fat, and in liposarcoma cell lines compared with adipose-derived stem cells. Here, we show that **ZIC1** contributes to the pathogenesis of liposarcoma. **ZIC1** knockdown inhibits proliferation, reduces invasion, and induces apoptosis in dedifferentiated and myxoid/round cell liposarcoma cell lines, but not in either adipose-derived stem cells or in a lung cancer cell line with low **ZIC1** expression. **ZIC1** knockdown is associated with increased nuclear expression of p27 proteins and the downregulation of prosurvival target genes BCL2L13, Jund, Fam57A, and EIF3M. Our results show that **ZIC1** expression is essential for liposarcomagenesis and that targeting **ZIC1** or its downstream targets might lead to novel therapy for liposarcoma. Cancer Res; 70(17): 6891–901. ©2010 AACR.

**Introduction**

Liposarcoma is the most common type of soft tissue sarcoma, accounting for at least 20% of adult sarcomas (1). Based on morphologic features and cytogenetic aberrations, liposarcomas are classified into three biological types encompassing five subtypes: (a) well-differentiated/dedifferentiated, (b) myxoid/round cell (RC), and (c) pleomorphic. The histologic subtype of liposarcoma remains the most important prognostic factor for survival, although tumor location, size, and completeness of resection improve outcome prediction for the individual patient (2, 3). Although diagnosis and clinical management have improved over the past decade, 40% of newly diagnosed patients will eventually die of the disease and new treatments are urgently needed for patients (~1,250 in the United States each year) who will die from inoperable liposarcoma. Our group has been conducting a genome-wide molecular genetic analysis of liposarcoma. The goal is to discover the “driver” genetic alterations necessary for liposarcomagenesis, with the expectation that identifying such genes will lead to the discovery of novel therapeutic targets, improve the diagnosis of liposarcoma subtypes, and improve outcome prediction for individual patients.

**ZIC1**, one of five **ZIC** family genes (4), is a transcription factor notable for its involvement in multiple developmental processes, including neurogenesis, myogenesis, and left-right axis establishment (5). In the normal adult, **ZIC1** expression has been detected only in neural tissues, typically restricted to the cerebellum. The coordinated expression of **ZIC1** during neural tube development is required for proper cerebellar development (6). Heterozygous **ZIC1** deletion is associated with Dandy-Walker malformation (7).

All five **ZIC** family members share five highly conserved tandem repeats known as C2H2 zinc finger motifs. Similar zinc finger domains have been described in the Gli family proteins, which can interact with **ZIC** family members in both antagonistic and synergistic fashions (8). Surprisingly, given its importance in cerebellar development, little is known about how **ZIC1** is regulated or how it controls the expression of downstream targets. **ZIC1** induces the expression of several wnt genes in *Xenopus* (9) and strongly activates the human apolipoprotein E gene (10). **ZIC1** expression is regulated by bone morphogenetic protein–related signals (11, 12).

In neurons, aberrant regulation of **ZIC1** has a well-defined oncogenic role. **ZIC1** is commonly expressed in medulloblastoma (13). **ZIC1** overexpression in mice decreases neuronal differentiation and expands neural progenitors (6), functions that could underlie a role in tumor initiation and progression. Also, **ZIC1** overexpression in chick neural tubes blocks...
differentiation (14), and when ZIC1 was aberrantly expressed in the ventral spinal cord (where it is normally absent), the cells failed to express markers normally seen in differentiated neuronal cells. However, in humans, aberrant ZIC1 expression is also seen in other solid tumors (15), including endometrial cancer (16) and desmoid tumors (15). However, neither the importance of ZIC1 nor its oncogenic role in these diseases has been clearly elucidated.

Here, we show that not only is ZIC1 overexpressed in all five liposarcoma subtypes, but also in additional genetically complex sarcoma types. We further show that the proliferation and survival of liposarcoma cell lines depends on ZIC1 overexpression. We also show that the lethal effects of ZIC1 knockdown are selective for liposarcoma cells, but not for cells that do not overexpress ZIC1. Furthermore, we show that the antiproliferative effects of ZIC1 knockdown in liposarcoma cells might be, in part, due to increased expression and activation of p27 protein.

### Materials and Methods

#### ZIC1 gene expression profiling

Affymetrix U133A microarrays were used to compile gene expression profiles to compare patients' liposarcomas [dedifferentiated (*n* = 51), well-differentiated (*n* = 51), myxoid (*n* = 14), myxoid/RC (*n* = 12), and pleomorphic (*n* = 22)] with subcutaneous or retroperitoneal normal fat (*n* = 13). This comparison was expanded to include other soft tissue sarcomas, including leiomyosarcoma (*n* = 23), malignant fibrous histiocytoma (*n* = 5), gastrointestinal stromal tumors (*n* = 99), and myxofibrosarcoma (*n* = 36). We then combined our microarray data with data from the Novartis Gene Expression Atlas (http://biogps.gnf.org) and various GEO sets (http://www.ncbi.nlm.nih.gov/geo) to determine the relative expression of ZIC1 in soft tissue sarcoma and normal fat to other human tissues, including central nervous system (CNS) tissues, and to epithelial-derived cancers, such as colorectal and breast cancers (17). All data were analyzed with BioConductor packages for the R statistical programming system. Quantitation and normalization were done with the gcRMA package, and differential gene expression was computed with the limma package. The Benjamini and Hochberg false discovery rate (FDR) method was used to correct for multiple testing.

#### ZIC1 immunofluorescence on tissue microarray

Tissue microarray slides were deparaffinized with xylene, followed by antigen retrieval by microwaving in sodium citrate buffer (pH 6.0) for 5 to 10 minutes. Samples were permeabilized with 10% triton/1× PBS for 20 minutes. Rabbit anti-human/mouse Zic-1 (ab72694, Abcam) was applied at 1:50 dilution, and incubated at room temperature for 4 hours, then goat anti-rabbit Alexa-594 (Invitrogen), was added at 5 μg/mL and incubated at room temperature for 1.5 hours. The cells were then stained with 4′,6-diamidino-2-phenylindole (Molecular Probes), and mounted with vectorshield (Vector). The results were viewed under Zeiss wide-field microscope and the images analyzed with Axiovision 4.6.

### Cell culture and treatment

DDLS8817, LPS141, and ML2308 liposarcoma cell lines were established from dedifferentiated and myxoid/RC liposarcoma samples obtained from patients who signed informed consent. The dedifferentiated (DDLS8817 and LPS141) cell lines were confirmed by cytogenetic analysis and by DNA copy number arrays (Agilent 244K) to harbor 12q amplification. The myxoid/RC cell line (ML2308) was found to contain the TLS-CHOP fusion transcript variant type 8-2 by reverse transcription PCR (RT-PCR). Primary human adipocyte-derived stem cells (ASC) were isolated as previously described (18) using subcutaneous fat tissue samples from consenting patients. Cell lines were maintained in DMEM HG/F12 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). The lung adenocarcinoma cell line, A549 (American Type Culture Collection) was maintained in F12K with 1.5 g/L sodium bicarbonate and 10% fetal bovine serum.

### Quantitative real-time RT-PCR

RNA was extracted from macrodissected cryomolds of tissue samples and reverse transcription was performed as previously described (19) to detect ZIC1 (Hs00602749_m1) and 18s RNA (Hs99999901_s1). Relative expression of ZIC1 was calculated by normalizing its expression to that of 18s rRNA. Statistical significance for all RT-PCR reactions was determined as previously described (20).

### Immunofluorescence for ZIC1 in cell lines

Cells were seeded and grown overnight on chamber slides to ~75% confluence and fixed in 4% paraformaldehyde. Cells were permeabilized, blocked, and incubated overnight with anti-ZIC1 (ab72694, Abcam) or anti-p27 (sc-528, Santa Cruz Biotechnology) at a 1:100 dilution in 2% bovine serum albumin/1× PBS. Slides were developed with goat anti-rabbit Alexa Fluor 488 (A11034, Invitrogen) at 1:1,000 dilution in 2% bovine serum albumin/1× PBS for 1.5 hours in a humidified chamber then mounted with 4′,6-diamidino-2-phenylindole medium (sc-24941, Santa Cruz Biotechnology). Fluorescent images were captured using an Olympus IX71 microscope.

### Short-hairpin RNA Infection

Four short-hairpin RNAs (shRNA) in the pLKO.1 vector (Sigma-Aldrich) were tested. The greatest knockdown of ZIC1 gene expression was achieved by ZIC1 Z1 "CGAGCCGCAAGCCCCATCTCTT" and ZIC1 Z4 "GCCCATTTGACTCTGAGAAA." The negative control was a scramble (Scr) sequence not targeting any known human genes, "CAACAGAAGGAGCACAA." These constructs were produced in a lentivirus system by the transient cotransfection of 293T cells (American Type Culture Collection) using LipofectAMINE 2000 (Invitrogen) with 10 μg of lentiviral plasmid, 9 μg of packaging plasmid psPAX2, and 1 μg of envelope plasmid pMD2.G. Viral supernatants (in HG-DMEM + 10% fetal bovine serum + 1.1 g/100 mL bovine serum albumin + 1× penicillin/streptomycin) were collected at 48, 72, and 96 hours after transfection, pooled, and concentrated by centrifugation.
using an Amicon Ultra-15 100K cutoff filter device (Millipore). Cells infected with lentivirus were selected with 1 μg/mL of puromycin. Knockdown of ZIC1 gene expression was confirmed by RT-PCR and immunofluorescence (IF).

**Proliferation assays**

DNA content was estimated and data analyzed as previously described (19), except that cells were plated in 96-well plates at a density of 10^5 cells per well and collected for 4 days.

**Bromodeoxyuridine uptake**

Cells (3 x 10^5) were plated in 10 cm culture plates. Adherent cells were incubated with 10 μmol/L of bromodeoxyuridine (BrdUrd; Sigma-Aldrich) and then fixed and analyzed using standard methods.

**Annexin V/7-AAD apoptosis assay**

Apoptosis was evaluated as previously described (19).

**Cleaved caspase-3 immunohistochemistry**

Cleaved caspase-3 was detected by immunohistochemistry using standard methods.

**Matrigel invasion assay**

The Matrigel invasion membrane (BD BioCoat Matrigel Invasion Chamber; BD Biosciences) was rehydrated, and 2.5 x 10^5 cells were added to a control or Matrigel invasion chamber in triplicate and placed in a humidified incubator for 22 hours. Noninvading cells were removed, and the membranes containing the invading cells were stained using the Diff-Quik staining kit (Dade Behring). Cells were counted in three separate fields at 40× magnification and the percentage of invasion was calculated as invasion through the Matrigel membrane relative to migration through the control membrane.

**Immunoblot**

Cell lysates containing 30 μg of protein were resolved by SDS-PAGE, transferred onto Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad), and probed with antibody for p27 (sc-528, Santa Cruz Biotechnology).

**Microarray analysis of ZIC1 shRNA infected cells**

DDLS8817 and LPS141 cells were infected with ZIC1 Z1 and Scr constructs in triplicate. RNA was isolated and following confirmation of ZIC1 knockdown was submitted for Illumina gene expression analysis (Illumina, Inc.).

**Results**

**ZIC1 expression in diverse human tissue types and soft tissue sarcomas**

We determined ZIC1 expression levels from global gene expression analysis of normal fat and of 364 human tumors, including all five liposarcoma subtypes and other soft tissue sarcoma types. Well-differentiated, dedifferentiated, myxoid, RC, and pleomorphic liposarcoma tissue samples had 10.8- to 130.5-fold greater mean ZIC1 expression than did corresponding normal fat samples (FDR < 2.7E-05 in all pair-wise comparisons; Fig. 1A). Leiomyosarcoma, malignant fibrous histiocytoma, and myxofibrosarcomas also showed 12.2- to 24.5-fold increase in ZIC1 expression compared with normal fat (FDR < 0.005). Gastrointestinal stromal tumor, however, did not have an increase (fold change, 1.2; FDR = 0.80). When compared with publically available gene expression data for other tissue types, the fold change of ZIC1 expression in the CNS compared with normal fat ranged from 110.0 (FDR = 0.0004) in the spinal cord to 2,598 (FDR = 4.8E-10) in the cerebellum. Thus, for some liposarcoma subtypes, ZIC1 expression seems to be restored to the levels observed in noncerebellar CNS tissues (Fig. 1A). Unlike liposarcomas, epithelial-derived breast and colorectal cancers did not show significantly increased ZIC1 expression compared with normal fat (fold changes of 2.4 and ~1.19, respectively; FDR > 0.1; Fig. 1A).

To determine whether ZIC1 overexpression in these diverse sarcoma subtypes resulted from genomic amplification, we performed high-resolution DNA copy number profiling on 241 tumors across these subtypes with both Affymetrix 250K single nucleotide polymorphism arrays and Agilent 244K array-based comparative genomic hybridization. We found little evidence of recurrent, focal, or high-level amplification of the ZIC1 locus (3q24). Additionally, we sequenced the coding exons and adjacent splice junctions of ZIC1 in 51 dedifferentiated, 4 well-differentiated, 18 myxoid, and 23 RC liposarcoma samples and just found 3 synonymous mutations, which are likely single nucleotide polymorphisms.

**ZIC1 is overexpressed in all liposarcoma subtypes**

To validate the overexpression of ZIC1 at the protein level in the five liposarcoma subtypes, we performed immunofluorescence for ZIC1 on a liposarcoma tissue microarray. The tissue microarray contained 10 to 15 representative tissue samples of each liposarcoma subtype. The fluorescent nuclear signal for ZIC1 was much stronger in all liposarcoma subtypes than in the normal fat tissue samples (Fig. 1B). Therefore, both ZIC1 transcript and protein expression are greater in liposarcomas than in normal fat.

**ZIC1 is overexpressed in liposarcoma cell lines**

To establish a laboratory model to study ZIC1 overexpression, we compared the relative expression of ZIC1 by RT-PCR in two dedifferentiated cell lines (DDLS8817 and LPS141), a myxoid/RC cell line (ML2308), a lung adenocarcinoma cell line (A549), and ASCs (Fig. 1C). ZIC1 expression, relative to expression in ASCs, was 4.1 to 6.0 times higher (P < 0.05, determined as previously described; ref. 20) in the liposarcoma cell lines and 0.03 times as high in A549 cells (P < 0.05). We next performed immunofluorescence for ZIC1 in the same cell lines. This confirmed that the ZIC1 protein expression was higher in the liposarcoma cell lines than in ASCs and A549 cells (Fig. ID), consistent with the U133A transcriptional results.

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**Invasion assays**

To establish a laboratory model to study ZIC1 overexpression, we compared the relative expression of ZIC1 by RT-PCR in two dedifferentiated cell lines (DDLS8817 and LPS141), a myxoid/RC cell line (ML2308), a lung adenocarcinoma cell line (A549), and ASCs (Fig. 1C). ZIC1 expression, relative to expression in ASCs, was 4.1 to 6.0 times higher (P < 0.05, determined as previously described; ref. 20) in the liposarcoma cell lines and 0.03 times as high in A549 cells (P < 0.05). We next performed immunofluorescence for ZIC1 in the same cell lines. This confirmed that the ZIC1 protein expression was higher in the liposarcoma cell lines than in ASCs and A549 cells (Fig. ID), consistent with the U133A transcriptional results.
A, box plot of ZIC1 expression as determined from Affymetrix U133A chip data in combination with data from the Novartis Gene Expression Atlas and various GEO sets. The expression data comes from 21 normal tissue samples and 364 human tumors samples: 150 liposarcoma, 163 non-liposarcoma soft tissue sarcoma, and 51 epithelial tumors. The whiskers (dotted lines) show the data extremes. The boxes represent the interquartile range (from 25th to 75th percentile of the expression), with the dark bars representing the medians. The width of the notches approximate the 95% confidence interval for the difference in two medians.

B, ZIC1 immunofluorescence on liposarcoma tissue microarray. The images shown are representative of the ZIC1 staining pattern in all samples of that tissue type studied.

C, ZIC1 transcripts quantified by RT-PCR in ASCs, dedifferentiated liposarcoma cell lines (DDLS8817 and LPS141), a myxoid/RC cell line (ML2308), and a lung cancer cell line (A549).

D, immunofluorescence for ZIC1 in the same cell lines.
shRNA knockdown of ZIC1

To study the dependence of liposarcoma cell survival on ZIC1 expression, we reduced its expression using an shRNA lentiviral system. We initially tested four different constructs and determined by RT-PCR that two constructs, labeled Z1 and Z4, were most effective in reducing ZIC1 mRNA levels (Fig. 2A). This article describes the results using construct Z1, but all functional studies were also conducted using Z4, with similar results (available on request). Z1 knocked down ZIC1 mRNA levels in all liposarcoma cell lines to ∼30% of levels seen in control cells transduced with scramble shRNA by 4 days postinfection (P < 0.05; Fig. 2B). ZIC1 immunofluorescence 8 days postinfection with the Z1 lentivirus indicated fewer nuclear signals than in the scramble controls in the liposarcoma cell lines. Knockdown with Z1 resulted in similarly reduced ZIC1 expression in ASCs and in the A549 lung cancer cell line.

ZIC1 knockdown inhibits the proliferation of liposarcoma cells

Beginning 7 days after lentiviral infection, a CyQuant DNA quantification assay (Fig. 3A) showed a reduction in cell proliferation in the ZIC1 knockdown liposarcoma cells, compared with the scramble and no-virus controls. Ten days after knockdown, the number of ZIC1 knockdown cells relative to scramble controls was reduced 50% (P < 0.001), 20% (P < 0.001), and 40% (P < 0.001) in ML2308, DDLS8817, and LPS141 cells, respectively. Thus, ZIC1 knockdown significantly reduced cell proliferation in all three liposarcoma cell lines.

To confirm the reduced proliferation of liposarcoma cells following ZIC1 knockdown, we determined the percentage of liposarcoma cells that incorporated BrdUrd, indicating active DNA replication. ZIC1 knockdown was compared with scramble controls starting 2 days postinfection (Fig. 3B). In both ML2308 and LPS141, BrdUrd uptake was reduced 30% beginning 4 days postinfection, and this peaked on day 6 in ML2308 and on day 10 in LPS141 with a 50% (P < 0.001) reduction in the BrdUrd uptake in the ZIC1 knockdown versus scramble controls. The results in DDLS8817 were similar, but the decrease in BrdUrd uptake began 8 days postinfection and peaked at 10 days with a 28% (P < 0.05) and 43% (P < 0.05) reduction, respectively. In each liposarcoma cell line studied, the reduction in BrdUrd uptake in ZIC1 knockdown compared with scramble controls was significant. Thus, ZIC1 knockdown resulted in reduced DNA replication and thus reduced proliferation in liposarcoma cells.

ZIC1 knockdown induces apoptosis in liposarcoma cells

We measured apoptosis with Annexin V and 7-AAD staining and found that the myoid/RC cell line (ML2308) showed the earliest effects of decreased ZIC1 transcription with a 2.0-fold increase in Annexin V(+), 7-AAD(−) staining in the ZIC1 knockdowns compared with the scramble controls measured 6 days postinfection (P < 0.001; Fig. 3C). This effect peaked at day 8 with a 2.3-fold increase in Annexin V(+), 7-AAD(−) staining (P < 0.01). For DDLS8817, maximum Annexin V(+), 7-AAD(−) staining occurred 9 days postinfection, with a 1.9-fold increase in staining in the ZIC1 knockdowns compared with the scramble controls (P < 0.001). In LPS141, Annexin V staining plateaued on day 12 with a 10.9-fold increase in Annexin V(+), 7-AAD(−) staining (P < 0.001). Thus, depending on the liposarcoma cell line, ZIC1 knockdown induced a 2- to 11-fold increase in apoptosis compared with scramble controls, which peaked 8 to 12 days postinfection.

To confirm the Annexin V/7-AAD apoptosis results, immunohistochemistry for cleaved caspase-3 was performed 8 days after lentivirus infection. ZIC1 knockdown increased cleaved caspase-3 staining: 3.0 ± 0.3-fold in ML2308, 2.0 ± 0.7-fold in DDLS8817, and 2.4 ± 0.8-fold in LPS141 compared with the Scr controls (Supplementary Fig. S2).

To determine if the induction of apoptosis following ZIC1 knockdown was specific to liposarcoma cells that overexpressed ZIC1, the same shRNA lentiviral system was used to knock down ZIC1 expression in ASCs and in A549 lung cancer cells, both of which have low levels of ZIC1 expression (Fig. 4A). Ten days postinfection, Annexin V(+), 7-AAD(−) staining in these two cell types was compared with that in the three liposarcoma cell lines (Fig. 4B). In the liposarcoma cell lines, the ZIC1 knockdowns resulted in a 3- to 11-fold increase in apoptosis compared with the scramble controls (P < 0.05). In contrast, in the nontransformed ASCs and in the non-ZIC1 A549 cell line, apoptosis was not significantly increased in the ZIC1 knockdowns compared with controls. These results suggest that ZIC1 knockdown is specifically lethal to liposarcoma cells that overexpress ZIC1.

ZIC1 knockdown inhibits liposarcoma cell invasion

To determine whether ZIC1 overexpression contributes to the invasiveness of liposarcoma cells, we seeded ZIC1 knockdown, scramble control liposarcoma, and A549 cells 6 days after knockdown into Matrigel invasion chambers. Twenty-two hours after seeding, ML2308, DDLS8817, and LPS141 ZIC1 knockdown cells showed 21% to 40% invasion, which was lower than in scramble controls (55–82% invasion, respectively; P < 0.01; Fig. 4C). Thus, the percentage of invading cells was decreased after reducing ZIC1 expression in all three liposarcoma cell lines. There was no significant difference in invasion between the scramble and ZIC1 knockdown A549 cells.

Knockdown of ZIC1 is associated with an increase in p27 expression and activation

Previous studies indicated that ZIC1 mutant mice have increased p27 protein expression, and that ZIC1 overexpression increases cell proliferation, inhibits neuronal differentiation, and decreases p27 expression in the cerebellum (6). Based on these findings, we postulated that the functional effects of ZIC1 overexpression in liposarcoma, driving proliferation and inhibiting differentiation, were partially mediated through p27 downregulation. To determine the effects of ZIC1 knockdown on p27 protein expression, we performed immunoblots of p27 for liposarcoma cells following ZIC1 knockdown and compared these with scramble controls. All three liposarcoma cell lines with ZIC1 knockdown showed an increase in p27 protein expression as early as 2 days postinfection compared with
Figure 2. A, extent of knockdown of ZIC1 transcription with four different ZIC1 constructs, compared with no-virus (NV) and scramble (Scr) controls. B, ZIC1 immunofluorescence 8 d postinfection in the ZIC1 knockdown liposarcoma cells.
scramble controls (Fig. 5). The immunoblot results were validated by immunofluorescence, which identified not only an increase in p27 protein expression, but also increased nuclear localization of p27 in the ZIC1 knockdowns compared with controls in the three liposarcoma cell lines (Fig. 5). These results suggest that ZIC1 knockdown is associated with increased levels of p27 in the nucleus, where it can act to inhibit cell proliferation.

**Target genes of ZIC1 knockdown in dedifferentiated liposarcoma cell lines**

To further address the prosurvival function of ZIC1, we investigated which genes were differentially expressed in a manner specific to ZIC1 knockdown. We performed expression array analysis using both LPS141 ZIC1 and DDLS8817 ZIC1 knockdown and compared the results with scramble controls at 4 and 8 days following shRNA transfection, the time when apoptotic indices began to increase. Several genes were 2- to 4-fold downregulated in both liposarcoma cell lines compared with scramble controls (Table 1; Supplementary Table 1A–D) and were therefore considered putative ZIC1 target genes. For four of these genes which are novel putative ZIC1 targets, we confirmed ZIC1-dependent expression by RT-PCR (Supplementary Fig. S1). Both the JunD proto-oncogene and BCL2L13 may protect cells from apoptosis depending on the cellular context. FAM57A, a RAF/PI3K/AKT pathway activator, and EIF3M, an essential gene required for global protein synthesis, are known drivers of proliferation and survival. Thus, all four of these validated putative ZIC1 target genes are significantly reduced with ZIC1 knockdown in liposarcoma cell lines and are likely to mediate the reduced proliferation and enhanced apoptosis observed following ZIC1 knockdown.

**Discussion**

Genetically, liposarcomas fall into two broad categories. Those with simple near-diploid karyotypes bear few
chromosomal rearrangements, such as translocations in myxoid/round-cell liposarcoma [t(12;16)(q13;p11), t(12;22)(q13;q12)]. In contrast, liposarcomas with complex karyotypes, including well-differentiated/dedifferentiated and pleomorphic liposarcoma, have varying degrees of chromosomal instability (21). Most of these complex liposarcoma types have frequent abnormalities in the Rb, p53, and specific growth factor signaling pathways, including amplification of the 12q14 chromosome region encompassing the \textit{MDM2} and \textit{CDK4} loci in dedifferentiated liposarcoma (22), but many of the key driver genes critical to liposarcomagenesis remain to be discovered. Here, we show that \textit{ZIC1} is significantly overexpressed in all five liposarcoma subtypes compared with normal fat and that both genetically simple and complex liposarcoma types depend on \textit{ZIC1} overexpression for proliferation, invasion, and survival. We also discovered \textit{ZIC1} overexpression in other types of soft tissue sarcoma, including leiomyosarcoma, malignant fibrous histiocytoma, and myxofibrosarcoma. Gastrointestinal stromal tumor is the only sarcoma type examined that did not overexpress \textit{ZIC1}. As gastrointestinal stromal tumorigenesis is primarily characterized by mutations in \textit{c-KIT} and \textit{PDGFRA}, lesions not characteristic of \textit{ZIC1}-overexpressing subtypes, it is likely the pathogenesis of the latter is distinct in the requirement for \textit{ZIC1} overexpression (23–25). The overexpression of \textit{ZIC1} in eight of nine soft tissue sarcoma and liposarcoma subtypes studied here makes this gene a promising biomarker associated with mesenchymal transformation, and a potential selective therapeutic target due to the low or absent \textit{ZIC1} expression in adult tissues outside of the CNS.

In the CNS, \textit{ZIC1} inhibits premature neuronal differentiation and increases cell proliferation in neural development. However, the underlying molecular actions of \textit{ZIC1} protein remain a mystery. Some studies have raised the possibility that \textit{ZIC} proteins could act as transcriptional cofactors and modulate the hedgehog signaling pathway, and that \textit{ZIC} proteins inhibit neuronal differentiation by activating Notch signals (5). Although the role of \textit{ZIC1} in the CNS has been investigated, the functional effects of \textit{ZIC1} overexpression in soft tissue sarcoma have, until now, not been examined.

Three different liposarcoma cell lines were used to model the potential proliferative role of \textit{ZIC1} overexpression in the liposarcoma human tumors. The functional effects of \textit{ZIC1} knockdown were consistent: decreases in proliferation, BrdUrd uptake, and Matrigel invasion and increase in apoptosis. The specificity of these responses are supported by \textit{ZIC1} knockdown having no effect on apoptosis or invasion in normal ASCs, which are neither immortal nor transformed, or in the A549 cell line, which does not overexpress \textit{ZIC1} by RT-PCR and immunofluorescence. Thus, the overexpression of \textit{ZIC1} is essential for survival and proliferation in these liposarcoma cell lines and not in the normal progenitors or in proliferating cells in general.

The majority of \textit{ZIC1} studies have investigated its role in the CNS, and as previously mentioned, murine \textit{ZIC1} mutants were shown to express elevated p27 and Wnt7a, but decreased cyclin D1 (6). Although we did not observe a correlation between \textit{ZIC1} expression and Wnt7A and/or cyclin D1, we did show a relationship between \textit{ZIC1} knockdown and an increase in p27 protein expression \textit{in vitro}. As previously reported, increases in p27 expression have been shown to reduce proliferation (26), reduce BrdUrd uptake (27), increase...
Annexin V staining (28), and decrease Matrigel invasion (29). Immunoblots showed an increase in p27 protein expression in the ZIC1 knockdowns as early as 2 days after lentivirus infection, and reached its maximal level 6 days postinfection. In addition, immunofluorescence showed that ZIC1 knockdown also seemed to activate p27 by its nuclear localization. Thus, the increase in p27 expression in all three liposarcoma cell lines following ZIC1 knockdown explains many of the observed early functional effects on cell proliferation and invasion. However, the observed maximal increases in p27 after 6 days does not account for the marked increase in cell death observed following 8 days of ZIC1 knockdown, suggesting that additional ZIC1 target genes may be mediating liposarcoma cell survival.

To discover putative ZIC1 target genes, we then performed expression array analyses 4 and 8 days following ZIC1 knockdown and found that BCL2L13 was 4-fold and 2-fold downregulated in DDLS and LPS141, respectively. BCL2L13 shows overall structural homology to the antiapoptotic Bcl-2 family of proteins, but contains a COOH-terminal membrane anchor region that is preceded by a unique 250-amino acid insertion containing two tandem repeats. BCL2L13 induces significant apoptosis with caspase-3 activation when transfected into 293T cells (30). BCL2L13 overexpression has been associated with l-asparaginase resistance and unfavorable clinical outcome in childhood acute lymphoblastic leukemia (31). This finding suggests that BCL2L13 might have a different apoptotic role in primary leukemic cells and in our dedifferentiated liposarcoma cell lines compared with the 293T cell lines, initially used to describe its apoptotic role. Four days following ZIC1 knockdown, JunD was 2.4-fold and 2.1-fold downregulated in DDLS8817 and LPS141, respectively. The JunD proto-oncogene is a functional component of the activator protein-1 transcription factor complex and exhibits a cell-dependent role in apoptosis. For example, JunD expression prevents cell death in adult mouse heart cells (32).

![Figure 5](image-url)

Figure 5. Left, immunoblots for p27 protein expression in ZIC1 knockdown and Scr control cells 2 to 8 d after lentivirus infection. Right, p27 immunofluorescence.
Table 1. Potential prosurvival target genes of ZIC1

<table>
<thead>
<tr>
<th>ZIC1 target gene</th>
<th>Expression fold change in DDLS days 4 and 8, respectively, after ZIC1 knockdown</th>
<th>Expression fold change in LPS141 days 4 and 8, respectively, after ZIC1 knockdown</th>
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<tr>
<td>BCL2L13</td>
<td>-4.0, -4.4</td>
<td>-2.0, NS</td>
</tr>
<tr>
<td>JunD</td>
<td>-2.4, -2.3</td>
<td>-2.1, -2.1</td>
</tr>
<tr>
<td>Fam57A (CT120)</td>
<td>-2.6, -2.2</td>
<td>-2.5, -2.4</td>
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<tr>
<td>EIF3M</td>
<td>-3.1, -3.1</td>
<td>-2.7, -2.7</td>
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</table>

NOTE: Expression fold change in potential prosurvival ZIC1 target genes discovered by expression array analysis of ZIC1 knockdown compared with scramble control dedifferentiated liposarcoma cells (DDLS8817 and LS141) at 4 and 8 d following shRNA transfection.

and in UV/H2O2-stressed mouse embryonic fibroblasts (33). JunD has been proposed to protect cells from p53-dependent senescence and apoptosis (34) and its downregulation upon ZIC1 knockdown might account for the induction of apoptosis in our liposarcoma cell lines. Fam57A (CT120), a membrane-associated gene important for amino acid transport (35), was found to be 2.6- and 2.5-fold downregulated in DDLS8817 and LPS141, respectively. CT120 has been implicated in lung carcinogenesis and its ectopic expression in NIH3T3 cells has been associated with Raf/Mek/Erk and PI3K/Akt signaling pathways driving cell proliferation, cell survival, and antiapoptotic pathways (36). EIF3M, an essential gene for global cellular protein synthesis and polysome formation and seems to be associated with the bulk of cellular mRNAs (37). The ectopic expression of the other EIF3 subunits, -3a, -3b, -3c, -3h, or -3i in stably transfected NIH3T3 cells has been shown to induce oncogenic transformation, enhance proliferation, and attenuate apoptosis (38). These findings suggest that the 3-fold downregulation of EIF3M following ZIC1 knockdown would inhibit protein synthesis and proliferation in liposarcoma cells.

We present here evidence that ZIC1 expression is necessary for liposarcoma proliferation, invasion, and survival and have identified several ZIC1 target genes that might mediate its functional effects. The overexpression of ZIC1 in both liposarcomas and, more broadly, other soft tissue sarcoma types and not in normal adult tissues outside of the CNS, makes this gene or its downstream targets a promising therapeutic target in these tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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