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ZIC1 Overexpression Is Oncogenic in Liposarcoma

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Abstract

Liposarcomas are aggressive mesenchymal cancers with poor outcomes that exhibit remarkable histologic diversity, with five recognized subtypes. Currently, the mainstay of therapy for liposarcoma is surgical excision since 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 (ASC). 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 ASC or a lung cancer cell line with low *ZIC1* expression. *ZIC1* knockdown is associated with increased nuclear expression of p27 protein, and the down-regulation of pro-survival target genes: *BCL2L13*, *JunD*, *Fam57A*, and *EIF3M*. Our results demonstrate that *ZIC1* expression is essential for liposarcomagenesis and that targeting *ZIC1* or its downstream targets may lead to novel therapy for liposarcoma.

Keywords

Liposarcoma; oncogenesis; ZIC1

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: (1) well-differentiated/dedifferentiated, (2) myxoid/round cell (RC), and (3) pleomorphic. The histologic subtype of liposarcoma remains the most important prognostic factor for survival, while 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 (roughly 1,250 in the US 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 diagnosis of liposarcoma subtypes, and improve outcome prediction for the individual patient.

ZIC1, one of five *ZIC*-family genes (4), is a transcription factor notable for 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 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 (BMP)-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 on 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 demonstrate 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 demonstrate that the anti-proliferative effects of *ZIC1* knockdown in liposarcoma cells may 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), 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 (MFH) (n=5), gastrointestinal stromal tumors (GIST) (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 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 (TMA)

TMA 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/1X phosphate-buffered saline for 20 minutes. Rabbit anti-human/mouse *Zic-1* (ab72694, Abcam, Cambridge, MA) was applied at 1:50 dilution, and incubated at room temperature for 4 hours, then goat-anti-rabbit-Alexa-594 (Invitrogen, Carlsbad, CA), was added at 5µg/ml and incubated at room temperature for 1.5 hours. The cells were then stained with DAPI (Molecular Probes, Eugene, OR), and mounted with vectorshield (Vector, Burlingame, CA). The results were viewed under Zeiss widefield microscopy 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 RT-PCR. Primary human adipocyte-derived stem cells (ASCs) were isolated as 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 (FBS) and 1% penicillin/streptomycin (Invitrogen). The lung adenocarcinoma cell line, A549 (ATCC, Manassas, VA) was maintained in F12K with 1.5g/L sodium bicarbonate and 10% FBS.

Quantitative Real-Time Reverse Transcription PCR

RNA was extracted from macro-dissected cryomolds of tissue samples and reverse transcription was performed as described(19) to detect *ZIC1* (Hs00602749_m1) and 18s rRNA (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 described (20).

Immunofluorescence for ZIC1 in Cell Lines

Cells were seeded and grown overnight on chamber slides to approximately 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, Santa Cruz, CA) at a 1:100 dilution in 2% BSA/1XPBS in a humidified chamber at 4°C. Slides were developed with goat anti-rabbit Alexa Fluor 488 (A11034, Invitrogen) at 1:1000 dilution in 2% BSA/1XPBS for 1.5 hours in a humidified chamber then mounted with DAPI medium (sc-24941, Santa Cruz Biotechnology). Fluorescent images were captured using an Olympus IX71 microscope.

shRNA Infection

Four short-hairpin RNAs (shRNAs) in the pLKO.1 vector (Sigma-Aldrich, USA) were tested. Greatest knockdown of *ZIC1* gene expression was achieved by *ZIC1* Z1

“CGAGCGACAAGCCCTATCTTT” and *ZIC1* Z4 “GCCATATTTGACTCTGAGAAA”. The negative control was a scramble (Scr) sequence not targeting any known human genes, “CAACAAGATGAAGAGCACCAA”. These constructs were produced in a lentivirus system by the transient cotransfection of 293T cells (ATCC) 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% iFBS + 1.1g/100mL BSA + 1x Pen/Strep) were collected at 48, 72, and 96 h after transfection, pooled, and concentrated by centrifugation using an Amicon Ultra-15 100K cutoff filter device (Millipore, USA). Cells infected with lentivirus were selected for with 1 µg/ml puromycin. Knockdown of *ZIC1* gene expression was confirmed by RT-PCR and IF.

Proliferation Assays

DNA content was estimated and data analyzed as described(19), except cells were plated in 96-well plates at a density of 10^3 cells per well and collected for four days.

BrdU Uptake

Cells (3×10^5) were plated in 10-cm culture plates. Adherent cells were incubated with 10µM of bromodeoxyuridine (Sigma-Aldrich) and fixed and analyzed using standard methods.

Annexin V/7-AAD Apoptosis Assay

Apoptosis was evaluated as described (19).

Cleaved Caspase3 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 2.5×10^4 cells were added to a control or Matrigel invasion chamber in triplicate and placed in a humidified incubator for 22 hours. Non-invading cells were removed, and the membranes containing the invading cells were stained using the Diff-Quik™ staining kit (Dade Behring, U.S.). Cells were counted in three separate fields at 40X magnification and percent 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, Hercules, CA), 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., San Diego, CA).

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) (Figure 1A). Leiomyosarcoma, malignant fibrous histiocytoma (MFH), and myxofibrosarcomas also demonstrated 12.2- to 24.5- fold increase in *ZIC1* expression compared with normal fat (FDR<0.005). GIST, however, did not have an increase (fold change 1.2; FDR=0.80). When compared to 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 2598 (FDR=4.8E-10) in the cerebellum. Thus, for some liposarcoma subtypes, *ZIC1* expression appears to be restored to the levels observed in non-cerebellar CNS tissues (Figure 1A). Unlike liposarcomas, epithelial-derived breast and colorectal cancers did not demonstrate significantly increased *ZIC1* expression compared with normal fat (fold changes of 2.4 and -1.19, respectively [FDR>0.1]) (Figure 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 SNP arrays and Agilent 244K aCGH arrays. 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 three synonymous mutations, which are likely SNPs.

ZIC1 Is Overexpressed in All Liposarcoma Sub-Types

To validate the overexpression of *ZIC1* at the protein level in the five liposarcoma subtypes, we performed immunofluorescence for *ZIC1* on a liposarcoma TMA. The TMA 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 (Figure 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 (DDL8817 and LPS141), a myxoid/RC cell line (ML2308), a lung adenocarcinoma cell line (A549), and adipocyte-derived stem cells (ASCs) (Figure 1c). *ZIC1* expression, relative to expression in ASCs, was 4.1–6.0 times higher ($P<0.05$, determined as described (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 (Figure 1d), consistent with the U133A transcriptional results.

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 (Figure 2a). This paper describes the results using construct Z1, but all functional studies were also conducted with Z4, with similar results (available on request). Z1 knocked down *ZIC1* mRNA levels in all liposarcoma cell lines to approximately 30% of levels seen in control cells transduced with scramble shRNA by four days postinfection ($P<0.05$) (Figure 2b). *ZIC1* immunofluorescence 8 days postinfection with the Z1 lentivirus indicated less nuclear signal 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 Proliferation of Liposarcoma Cells

Beginning seven days after lentiviral infection, a CyQuant DNA quantification assay (Figure 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 3 liposarcoma cell lines.

To confirm the reduced proliferation of liposarcoma cells following *ZIC1* knockdown, we determined the percentage of liposarcoma cells that incorporated BrdU, indicating active DNA replication. *ZIC1* knockdowns were compared with scramble controls starting 2 days postinfection (Figure 3b). In both ML2308 and LPS141, BrdU 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 BrdU uptake in the *ZIC1* knockdowns versus scramble controls. The results in DDLS8817 were similar, but the decrease in BrdU 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 BrdU 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 the myxoid/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 ($P<0.001$) (Figure 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 8-12 days postinfection.

To confirm the Annexin V/7-AAD apoptosis results, immunohistochemistry for cleaved caspase 3 was performed eight 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 to the Scr controls (Supplemental Figure 2).

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 (Figure 4a). Ten days postinfection, Annexin V(+), 7-AAD(-) staining in these two cell types was compared with that in the three liposarcoma cells lines (Figure 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 non-transformed 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 six days after knockdown into Matrigel invasion chambers. Twenty-two hours after seeding, ML2308, DDLS8817, and LPS141 *ZIC1* knockdown cells demonstrated 21–40% invasion, which was lower than in scramble controls (55–82% invasion, respectively; $P < 0.01$; Figure 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 Increased 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 by as early as 2 days postinfection compared with scramble controls (Figure 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 (Figure 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 pro-survival 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- and DDLS8817-*ZIC1* knockdown and compared the results with scramble controls at four and eight days following shRNA transfection, the time when apoptotic indices began to rise. Several genes were 2- to 4-fold down-regulated in both liposarcoma cell lines compared to scramble controls (Table 1 and Supplementary Table 1A-D) and were therefore considered putative *ZIC1* target genes. For four of these genes that are novel as putative *ZIC1* targets, we confirmed *ZIC1*-dependent expression by RT-PCR (Supplemental Figure 1). 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 *MDM2* and *CDK4* loci in dedifferentiated liposarcoma (22), but many of the key driver genes critical to liposarcomagenesis remain to be discovered. Here we show that *ZIC1* is significantly overexpressed in all 5 liposarcoma subtypes compared with normal fat and that both genetically simple and complex liposarcoma types depend on *ZIC1* overexpression for proliferation, invasion, and survival. We also discovered *ZIC1* overexpression in other types of soft tissue sarcoma, including leiomyosarcoma, MFH, and myxofibrosarcoma. GIST is the only sarcoma type examined that did not overexpress *ZIC1*. As GIST tumorigenesis is primarily characterized by mutations in *c-KIT* and *PDGFRA*, lesions not characteristic of *ZIC1*-overexpressing subtypes, it is likely the pathogenesis of the latter is distinct in the requirement for *ZIC1* overexpression (23–25). The overexpression of *ZIC1* in eight of nine soft tissue sarcoma and liposarcoma subtypes studied here make this gene a promising biomarker associated with mesenchymal transformation, and a potential selective therapeutic target due to the low or absent *ZIC1* expression in adult tissues outside of the CNS.

In the CNS *ZIC1* inhibits premature neuronal differentiation and increases cell proliferation in neural development. However, the underlying molecular actions of *ZIC1* protein remain a mystery. Some studies have raised the possibility that *ZIC* proteins can act as transcriptional cofactors and modulate the hedgehog-signaling pathway and that *ZIC* proteins inhibit neuronal differentiation by activating Notch signals (5). While the role of *ZIC1* in the CNS has been investigated, the functional effects of *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 *ZIC1* overexpression in the liposarcoma human tumors. The functional effects of *ZIC1* knockdown were consistent: decreases in proliferation, BrdU uptake, and Matrigel invasion and increase in apoptosis. Specificity of these responses are supported by *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 *ZIC1* by RT-PCR and immunofluorescence. Thus, the overexpression of *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 *ZIC1* studies have investigated its role in the CNS, and as previously mentioned, murine *ZIC1* mutants were shown to express elevated p27 and Wnt7a, but decreased cyclin D1 (6). While we did not observe a correlation between *ZIC1* expression and Wnt7A and/or Cyclin D1, we did demonstrate a relationship between *ZIC1* knockdown and an increase in p27 protein expression in vitro. As previously reported, increases in p27 expression have been shown to reduce proliferation (26), reduce BrdU uptake (27), increase Annexin V staining (28), and decrease Matrigel invasion (29). Immunoblots demonstrated 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 appeared to activate p27 by its nuclear localization. Thus, the increase in p27 expression in all 3 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 by 6 days does not account for 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 analysis 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 anti-apoptotic Bcl-2 family of proteins, but contains a C-terminal membrane anchor region that is preceded by a unique 250-amino acid insertion containing 2 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* may 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 AP1 transcription factor complex and exhibits a cell-dependent role in apoptosis. For example, JunD expression prevents cell death in adult mouse heart cells (32) 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 may 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 anti-apoptotic pathways (36). EIF3M, eukaryotic initiation factor 3 subunit M, is an essential gene for global cellular protein synthesis and polysome formation and appears 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 may 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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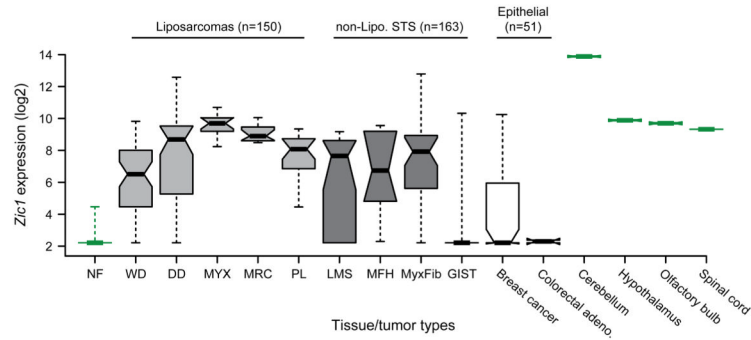
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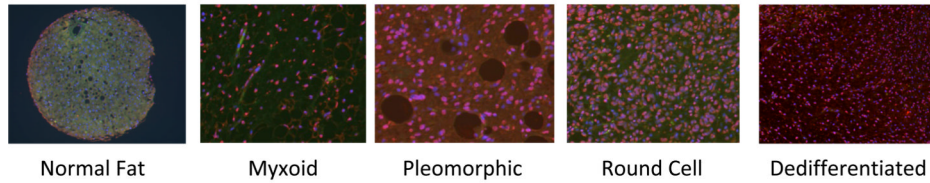
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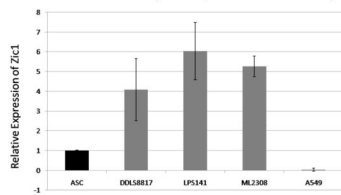
a. *ZIC1* Transcript Expression in Various Tissue Types



b. Liposarcoma and normal fat Tissue Microarray (TMA)



c. *ZIC1* Transcript Expression in Liposarcoma Cell Lines and Adipose-Derived Stem Cells



d. *ZIC1* Protein Expression in Liposarcoma Cell Lines and Adipose-Derived Stem Cells

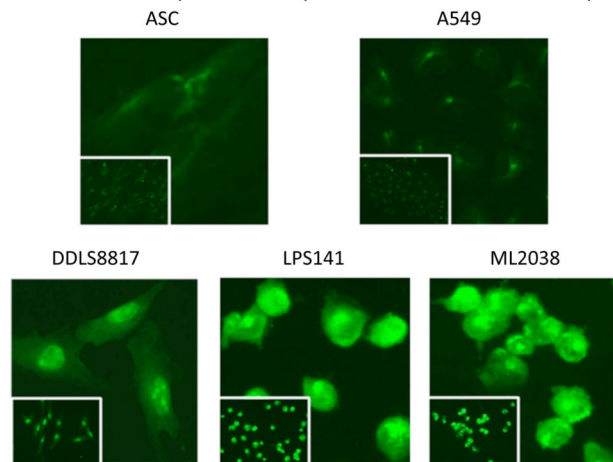
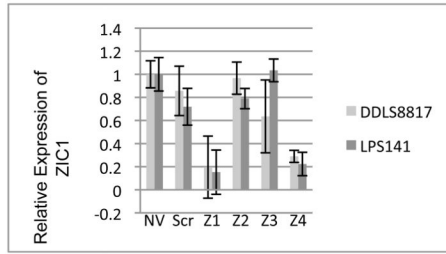


Figure 1.

(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 come 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. NF, normal fat; WD, well-differentiated; DD, dedifferentiated; MYX, myxoid; MRC, myxoid/RC; PL, pleomorphic; LMS, leiomyosarcoma; MyxFib, myxofibrosarcoma.

(b) ZIC1 immunofluorescence on liposarcoma TMA. 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 (DDL8817 and LPS141), a myxoid/RC cell line (ML2308), and a lung cancer cell line (A549). (d) Immunofluorescence for ZIC1 in the same cell lines.

a. Four different constructs tested for *ZIC1* Knockdown



b. *ZIC1* Z1 construct shRNA reduction in *ZIC1* transcription and protein expression.

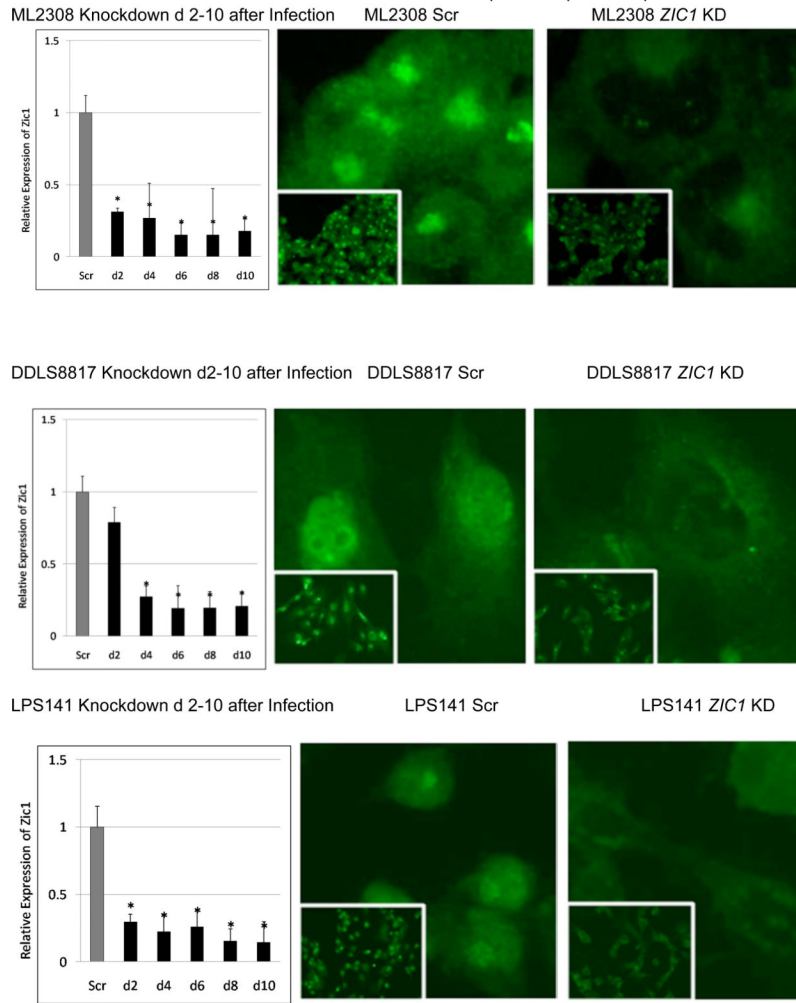


Figure 2.

(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 days postinfection in the *ZIC1* knockdown liposarcoma cells.

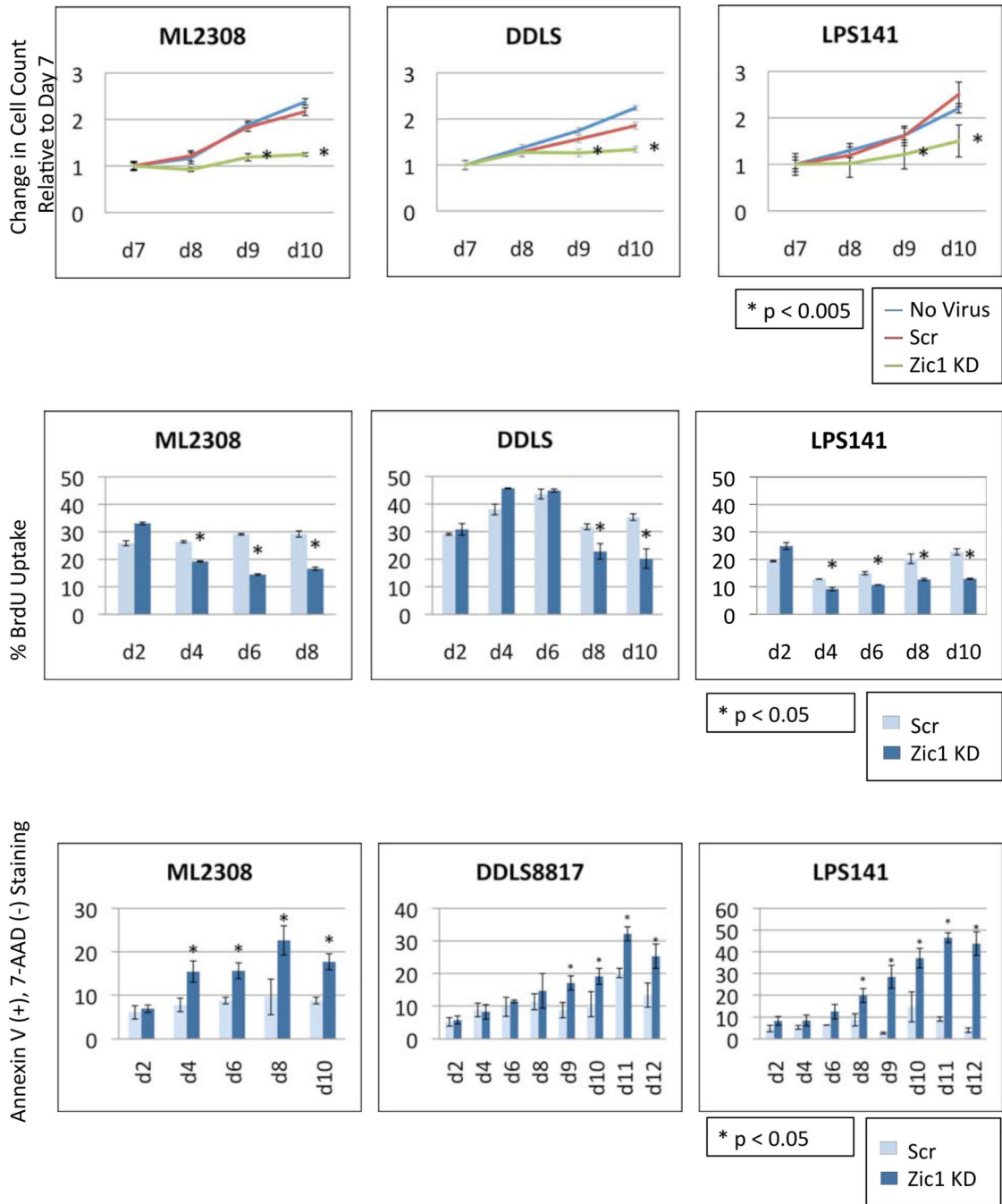
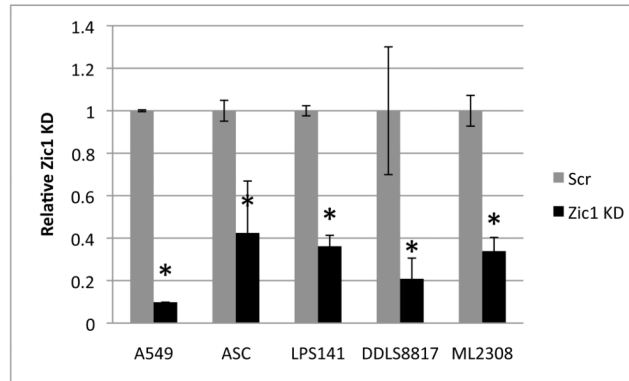


Figure 3.

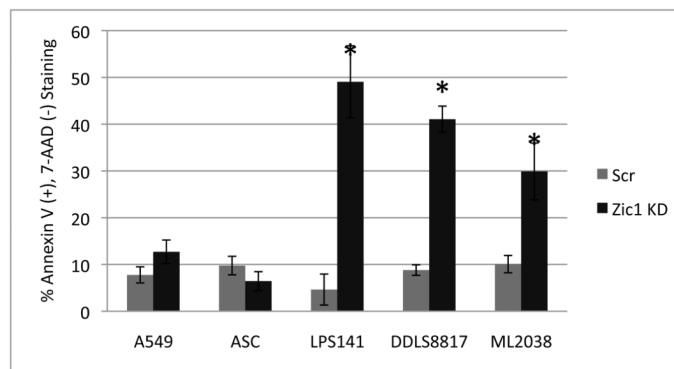
(a) Effects of ZIC1 knockdown on proliferation of liposarcoma cells. Cell proliferation postinfection with Z1 knockdown construct or Scr control and in no virus control was assessed by a DNA quantitation assay (CyQuant). Statistical differences between the ZIC1 knockdowns and the Scr control cells were analyzed using the 2-tailed unpaired *t*-test. (b) BrdU incorporation assays in liposarcoma cell lines postinfection with Z1 knockdown or Scr control constructs. The histograms show the percentage of cells actively synthesizing their DNA in S Phase. This experiment for ML2308 cells was stopped at day 8 because not enough viable cells remained at day 10. Statistical significance was assessed using the two-tailed unpaired *t*-test. (c) Early apoptosis, measured by Annexin V and 7-AAD staining.

Zic1 suppression is selectively lethal to liposarcoma cells

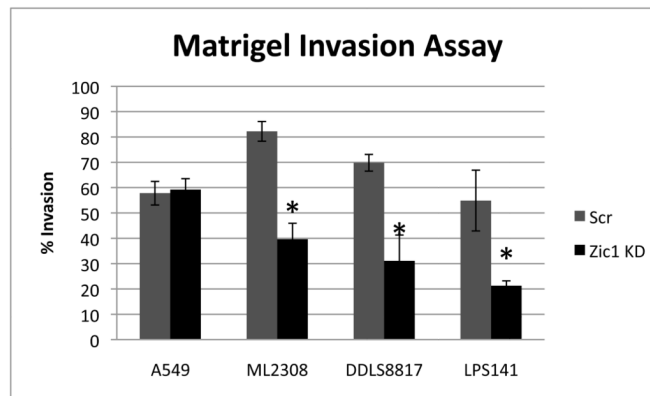
a. Zic1 KD ten days after lentivirus infection



b. Annexin V (+), 7-AAD (-) Staining ten days after lentivirus infection



c. Matrigel Invasion Assay six days after Lentivirus Infection

**Figure 4.**

Selective effects of *ZIC1* knockdown on apoptosis and invasion of liposarcoma cells compared to ASCs and A549 lung cancer cells. (a) Verification of *ZIC1* knockdown with the Z1 shRNA compared to Scr control by RT-PCR. (b) Annexin V and 7-AAD staining to measure apoptosis in the *ZIC1* knockdown cells compared with Scr controls. (c) Effects of *ZIC1* knockdown on the invasiveness of liposarcoma and lung cancer cells. At 6 days postinfection, cells were seeded into Matrigel invasion chambers, and invasion was assessed 22 hours later. Asterisks mark statistically significant differences ($P < 0.05$) between *ZIC1* knockdown and Scr control.

ZIC1 suppression by shRNA induces the expression of p27 in liposarcoma cell lines

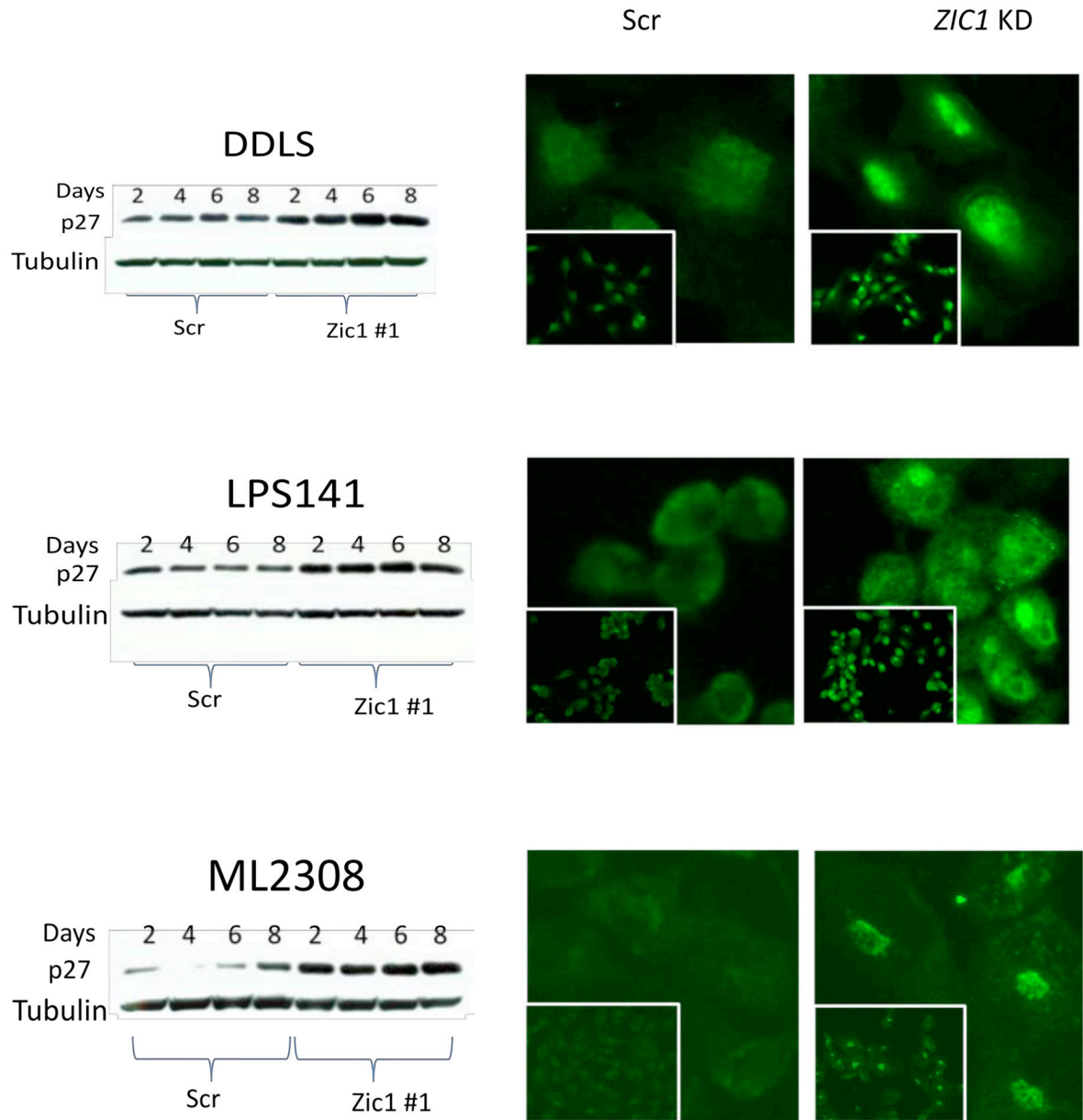


Figure 5.

Left, immunoblots for p27 protein expression in *ZIC1* knockdown and Scr control cells two to eight days after lentivirus infection. Right, p27 immunofluorescence.

Table 1
Potential Pro-Survival Target Genes of *ZIC1*

Expression fold-change in potential pro-survival *ZIC1* target genes discovered by expression array analysis of *ZIC1* knockdown compared to scramble control dedifferentiated liposarcoma cells (DDLS8817 and LS141) at four and eight days following shRNA transfection.

<i>ZIC1</i> Target Gene	Expression fold-change in DDLS days 4 and 8, respectively, after <i>ZIC1</i> knockdown	Expression fold-change in LPS141 days 4 and 8, respectively, after <i>ZIC1</i> knockdown
BCL2L13	-4.0; -4.4	-2.0; NS
JunD	-2.4; -2.3	-2.1; -2.1
Fam57A (CT120)	-2.6; -2.2	-2.5; -2.4
EIF3M	-3.1; -3.1	-2.7; -2.7