The adipogenic transcriptional cofactor ZNF638 interacts with splicing regulators and influences alternative splicing

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Abstract Increasing evidence indicates that transcription and alternative splicing are coordinated processes; however, our knowledge of specific factors implicated in both functions during the process of adipocyte differentiation is limited. We have previously demonstrated that the zinc finger protein ZNF638 plays a role as a transcriptional coregulator of adipocyte differentiation via induction of PPARγ in cooperation with CCAAT/enhancer binding proteins (C/EBPs). Here we provide new evidence that ZNF638 is localized in nuclear bodies enriched with splicing factors, and through biochemical purification of ZNF638’s interacting proteins in adipocytes and mass spectrometry analysis, we show that ZNF638 interacts with splicing regulators. Functional analysis of the effects of ectopic zincfinger expression on a minigene reporter demonstrated that ZNF638 is sufficient to promote alternative splicing, a function enhanced through its recruitment to the minigene promoter at C/EBP responsive elements via C/EBP proteins. Structure-function analysis revealed that the arginine/serine-rich motif and the C-terminal zinc finger domain required for speckle localization are necessary for the adipocyte differentiation function of ZNF638 and for the regulation of the levels of alternatively spliced isoforms of lipin1 and nuclear receptor co-repressor 1. Overall, our data demonstrate that ZNF638 participates in splicing decisions and that it may control adipogenesis through regulation of the relative amounts of differentiation-specific isoforms.

Supplementary key words transcriptional coactivator • minigene reporter • nuclear speckles • adipocyte differentiation

The process of adipocyte differentiation is initiated and controlled by a number of transcription factors, including CCAAT/enhancer binding protein (C/EBP) β and C/EBPδ, and by cofactors (1), such as the recently characterized zinc finger protein ZNF638, which regulates the expression of the master controller of adipogenesis, PPARγ, in conjunction with C/EBP proteins (2). The gene expression program occurring during differentiation includes the engagement and cooperation of these tissue-selective factors and cofactors with components of the RNA polymerase II machinery at active transcription sites, initiation of transcription followed by mRNA processing, through RNA capping, polyadenylation, and removal of noncoding intronic sequences, prior to protein translation (3). The process of splicing gives rise to the mature mRNA though the sequential succession of several complexes, starting from the precomplex in which sites of splicing are chosen, to the catalytic removal of introns executed by complex C components (4). During differentiation and development, tissue-specific enrichment of splicing factors ensures that alternative splicing is achieved to generate tissue-specific, temporally and developmentally regulated isoforms required to confer the specific phenotype (4–6). It has been shown that the relative abundance or activity of splicing regulators that have opposing effects determines the use of competing splice sites, ultimately controlling the exon composition of tissue-specific isoforms (4, 5). In adipocytes, alternatively spliced isoforms such as those of the insulin receptor (7), lipin (8), mitochondrial oxodicarboxylate carrier (9), nuclear receptor co-repressor 1 (NCoR) (10), preadipocyte factor 1 (11), and mechanistic target of rapamycin (12) have been shown to play a role in the differentiation process.

Abbreviations: AR, acidic repeat; C/EBP, CCAAT/enhancer binding protein; CTD, C-terminal domain; DBD, DNA binding domain; EI, exon inclusion; ES, exon skipping; GFP, green fluorescent protein; GST, Glutathione S-transferase; HNRNP, heterogeneous nuclear ribonucleoprotein; NCoR, nuclear receptor co-repressor 1; NONO, non-POU domain-containing octamer binding protein; PABP1, polyadenylate binding protein 1; PGC1α, PPAR gamma coactivator 1-alpha; RRM, RNA recognition motif; RS, arginine/serine rich; ASF/SF2, pre-mRNA-splicing factor 2/alternative splicing factor; SR, serine/arginine rich; ZF, zinc finger; ZNF638, zinc finger protein 638.

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Sérine/arginine-rich (SR) proteins are a major class of splicing regulators. These proteins contain RNA recognition motifs (RRMs) at the amino terminus and arginine/serine-rich (RS) repeats at the carboxy terminus (13). SR proteins reside in discrete subnuclear regions, which represent storage areas for splicing factors serving nearby active transcription sites (14, 15). Furthermore, SR proteins bind to exonic splicing enhancer sequences and recruit spliceosomal ribonucleoproteins (RNPs) and non-RNPs to the pre-mRNA (16), thereby affecting the selection of splice sites already during the process of transcription. In addition to SR proteins, heterogeneous nuclear ribonucleoproteins (HNRNPs) bind to pre-mRNA transcripts and influence alternative splicing (17–19). It has been shown that pre-mRNA splicing is coupled to transcription both by the function of the C-terminal domain (CTD) of RNA polymerase II (3) and by a number of cofactors involved in both processes (20–23), as demonstrated for the metabolic coactivator PPAR γ, a coactivator of PPAR γ, and its dotted nuclear localization pattern in adipocytes (2), led us to hypothesize that ZNF638 may also participate in alternative splicing, as previously demonstrated for the coactivator PGC1α (23). In this study, we tested this hypothesis using several experimental approaches, including confocal microscopy, mutagenesis, mass spectrometry analysis, and functional studies.

MATERIALS AND METHODS

Plasmids

Full-length murine ZNF638.pCR3.1 plasmid (2) was used as a template to generate eight deletion mutants by whole vector mutagenesis (Genewiz). ZNF638-ΔZF1 contains a deletion from amino acids 424 to 456, eliminating the N-terminal ZF motif. The ΔAR motif lacks amino acids 1,876 to 1,908, eliminating the C-terminal ZF domain. The ΔRS plasmid served as a template to generate the ΔRS/ZF2 mutant lacking both the RS and ZF2 motifs, with two deletions, one from amino acids 470 to 573 and one from 1,876 to 1,908. The primer sequences to generate the previous mutants are listed in Table 1. The empty vector backbone pCR3.1 served as control. Glutathione S-transferase (GST)-ZNF638ΔRRM1 and green fluorescent protein (GFP)-ZNF638ΔRRM2 constructs expressing only the carboxy-terminal region of ZNF638, from amino acids 1,773 to 1,926, containing the C-terminal ZF domain were previously described (2). The fibronectin minigene reporter 7iBi89 plasmid (26) was obtained from Addgene (14065). This minigene contains exons 24 through 28 of the rat fibronectin gene including introns 24-25 and 25-26 flanking the alternatively spliced exon 25. The gene cassette is under the control of a human β-actin promoter and contains a human growth hormone polyadenylation signal. To generate a fibronectin minigene reporter containing C/EBPα responsive elements, a 46 bp sequence of the PPARγ2 promoter containing C/EBPα responsive elements (5′-TTTTACTGCAATTTTAAAAAGCAATCAATATTGAACAATCTCT-GCT-3′) (27) was inserted between the β-actin promoter and the beginning of the fibronectin minigene cassette. The C/EBPα responsive element sequence was synthesized including XhoI and BstGI restriction sites and cloned into the minigene plasmid at those sites (Genewiz). C/EBPβ and C/EBPδ plasmids were obtained from Addgene, and the C/EBPα construct was a gift of Kai Ge (National Institutes of Health).

Antibodies

Rabbit anti-ZNF638 antibody (Bethyl, A301-548A) was used to detect ZNF638. Mouse anti-pre-mRNA-splicing factor 2 (SF2) and alternative splicing factor (ASF) antibody (Santa Cruz, sc-636), anti-mouse-HRP antibody (Santa Cruz, sc-636), anti-GFP antibody (Invitrogen, sc-136296), anti-polyadenylate binding protein 1 (PABP1) antibody (Abcam, ab31645), anti-HNRNPLL antibody (Cell Signaling, A-11122), anti-HNRNPA1 antibody (Abcam, ab35392), anti-HNRNPA2B1 antibody (Abcam, ab31736), anti-HNRNPL antibody (Santa Cruz, sc-132712), anti-non-POU domain-containing octamer binding protein (NONO) antibody (Santa Cruz, sc-156296), anti-polyadenylate binding protein 1 (PAPB1) antibody (Cell Signaling, 4992), anti-C/EBPα antibody (Santa Cruz, sc-2054), anti-C/EBPβ antibody (Santa Cruz, sc-9762), anti-C/EBPδ antibody (Santa Cruz, sc-636), anti-mouse-HRP antibody (Santa Cruz, sc-2055), anti-rabbit-HRP antibody (Santa Cruz, sc-2054), and anti-goat-HRP antibody (Santa Cruz, sc-2020) were used for Western blotting.

| Table 1. Primers used to generate ZNF638 deletion mutants |
|-----------------|-----------------|
| ZNF638 Mutants | Primer Sequences (5′ → 3′) |
| ΔZF1 | Forward: GAAATTATCCACAAATACCTCCATGTTTGGAG |
| ΔR | Forward: CTACCTGGATCCCTTTGACAAAGCTAGTCTC |
| ΔRRM1 | Forward: AAAAGGGCCAGAGAAAGAAAAGTTGGAG |
| ΔRRM2 | Forward: GAATGGAAGGAGATGAGGACAGCTTC |
| ΔRRM1-3 | Forward: CTTAAGGAAAGAGGACAGCTTC |
| ΔDBD | Forward: GAGGGGCGCCATACCAAGAGAGGACAGCTTC |
| ΔAR | Forward: GAATAGTTCCACAAATACCTCCATGTTTGGAG |
| ΔZF2 | Forward: GCCAAGCAAAAGAAGGAGGAAGGAG |

ZNF638’s role in alternative splicing 1887
Cell culture and transfections

The 3T3-L1, 10T1/2, U2OS, and HEK-293 cells (ATCC), were cultured in high-glucose DMEM medium (Invitrogen), supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Mediatech) at 37°C and 5% CO2. The 3T3-L1 cells were cultured and differentiated as previously described (2). For minigene assays and transient transfections, cells were transfected with Xtreme Gene HP (Roche) and analyzed 24 h later.

Determination assays

The 10T1/2 cells were electroporated (Amaza) with either control, full-length ZNF638, or mutant plasmids, induced to differentiate in culture medium supplemented with 5 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), and 10 µM troglitazone for 2 days and subsequently maintained in culture medium supplemented with 5 µg/ml insulin (28). Gene expression was assayed by real-time PCR 3 days after induction of differentiation, following RNA extraction (TRizol) and reverse transcription (Roche). The sequences of the primers used were the following: for 36B4, forward 5′-GGTCCCCCAGCCCCAGTCCTT-3′; for Pparβ, forward 5′-GGGCCCTGCCTCTTC-3′; for p53, forward 5′-ACACCGAGATTTGCTCAACTG-3′; for CD36, forward 5′-TTTTGAGTGGTATGAAAAAGGGC-3′; reverse 5′-TGACATCAGGGCATCATGATG-3′; for Ppary2, forward 5′-GATGCAATGCTGCTATGACCCTTC-3′; reverse 5′-AGAGGTCACAGACTGATTGTC-3′; for ZNF638, forward 5′-TCCAGTTGAGATGGAACC-3′, reverse 5′-TGATGAGATCCGCTCTTGTTG-3′; for Lipin1α, forward 5′-GGTCCCCACAGCCCCAGTCCT-3′, reverse 5′-GCGCGCTGTTGGCAATTCAG-3′; for Lipin1β, forward 5′-CAGGGTGGGATGCA-3′, reverse 5′-AGAGCCTGCAATTCAGA-3′; for NCoRα, forward 5′-CTGCCAAGGCTGCTACTACAGCAAG-3′, reverse 5′-AACTGCTGAGACTGTGCTAGC-3′; for NCoRβ, forward 5′-CTGTCCTGCTATTCTGCTGTCTACAG-3′, reverse 5′-CTGTCCTGATCAGGCTCTACAG-3′. To quantify the extent of lipid accumulation, cells were stained with Oil Red O at day 6 of differentiation, and the dye was extracted with isopropanol and absorbance measured at 520 nm, as described previously (29, 30).

Immunofluorescence

U2OS cells were plated on chamber slides (Labtek) and transfected with either vector, full-length ZNF638, or one of the eight ZNF638 deletion mutants. Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% (v/v) Triton X-100 in PBS, blocked with 1% nonfat dry milk, and incubated with primary antibody for 1 h at room temperature or overnight at 4°C and with secondary antibodies for 1 h at room temperature or overnight at 4°C and subsequently exposed to secondary HRP-conjugated antibodies for 1 h. Blots were incubated with ECL substrate (Pierce) and exposed to film (Kodak).

Minigene splicing assays

HEK-293 cells were plated on 12-well plates and transfected at 70–80% confluency with 1 µg of fibronectin minigene reporter plasmid and either 5 µg of ZNF638.pCR3.1 or pCR3.1 vector. For C/EBP coexpression studies, cells were cotransfected with 1 µg fibronectin minigene plasmid containing C/EBP responsive elements and with 1 µg of C/EBPα, C/EBPβ, or C/EBPδ plasmids or vector, and 3 µg of vector control or ZNF638. Twenty-four hours after transfection, cells were harvested, and the extracted mRNA (RNeasy, Qiagen) was reverse transcribed using anchored oligo dT primer (Transcriptor First Strand cDNA Synthesis Kit, Roche). Ratio of exon inclusion (EI) to exon skipping (ES) was assessed by real-time PCR. The EI primers specifically amplified the longer isoform including the alternatively spliced exon 25.

Biochemical purification of ZNF638 interacting proteins present in adipocytes and mass spectrometry analysis

Purification of the GST-ZNF638p2 fusion protein from BL21 bacterial cells and preparation of nuclear extracts from differentiating 3T3-L1 cells 2 days after induction with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 µM dexamethasone (Fluka), and 5 µg/ml insulin (Sigma) were performed as previously described (2). ZNF638 interacting proteins purified from nuclear lysates obtained from differentiating 3T3-L1 adipocytes were separated by SDS-PAGE and stained with Coomassie blue. Prominent bands were excised and subjected to mass spectrometry analysis (Taplin Biological Mass Spectrometry Facility, Harvard Medical School, Boston, MA).

Immunoprecipitation assays

HEK-293 cells were transfected with GFP-ZNF638p2.pAcGFP or pAcGFP vector. Immunoprecipitation assays were performed using GFP-trap agarose beads (Chromotek, Allele), according to the manufacturer’s instructions. Protein lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). After blocking in TBS with Tween containing 2% nonfat dry milk, membranes were incubated with primary antibodies for 1 h at room temperature or overnight at 4°C and subsequently exposed to secondary HRP-conjugated antibodies for 1 h. Blots were incubated with ECL substrate (Pierce) and exposed to film (Kodak).

Fig. 1. ZNF638 colocalizes with the splicing factor ASF/SF2 in nuclear speckles. Subcellular localization of transiently expressed ZNF638 in U2OS cells detected by indirect immunofluorescence and by overlay with DAPI as nuclear counterstaining (A). Double immunofluorescence staining of transiently expressed ZNF638 and by overlay with endogenous ASF/SF2, a marker for nuclear speckles (B). A, B: Confocal imaging; scale bars: 10 µm.
and the ES primers amplified the short isoform, skipping exon 25, as previously described (31). The EI and ES primers utilized were the following: EI forward 5′-CCGTCATCCAGAGGTGCCC-3′, EI reverse 5′-GGAGGGACGGCCGTGGCTGTG-3′; and ES forward 5′-CCCCTATCTCTGATACCGTCATCCC-3′, ES reverse 5′-GTTCGTACACGCTGGAGACACTGAC-3′. The following real-time PCR conditions were used: 10 min 95°C, 35 cycles of 10 s at 95°C, 30 s at 67°C, and 30 s at 72°C. PCR products corresponding to EI or ES were further confirmed by sequencing (Genewiz).

Statistical analysis was performed by the two-sided Student’s t-test with unequal variance on three replicates as indicated. A P value < 0.05 was considered statistically significant.

RESULTS

The adipogenic cofactor ZNF638 localizes in nuclear bodies enriched in splicing factors

We previously identified ZNF638 as a transcriptional cofactor implicated in the regulation of PPARγ expression and adipocyte differentiation through interaction with the C/EBP family of transcription factors. In addition, we showed that ZNF638 is localized in the nucleus of differentiating 3T3-L1 cells in a punctate pattern (2). To better characterize the identity of the nuclear bodies in which ZNF638 resides, we performed immunofluorescence staining using antibodies against ZNF638 and the splicing factor ASF/SF2, which served as a marker for nuclear speckles (32). Confocal imaging analysis of U2OS cells transiently expressing ZNF638 revealed that ZNF638 colocalizes with endogenous ASF/SF2 in nuclear speckles (Fig. 1A, B).

The RS and the C-terminal ZF domains are required for ZNF638’s localization to nuclear speckles

To investigate the contribution of the domains present in ZNF638 to the speckled localization observed, we generated deletion mutants, as shown in the schematic representation in Fig. 2A. Indirect immunofluorescence staining of the ectopically expressed full-length ZNF638 and mutants followed by confocal imaging demonstrated that the ablation of either the RS domain (ZNF638-ΔRS) or the C-terminal ZF domain (ZNF638-ΔZF2), or of both domains (ZNF638-ΔRS/ZF2), abolished ZNF638’s localization to speckles (Fig. 2B). These findings indicate that the RS domain and the C-terminal ZF domain are required for ZNF638’s localization to nuclear speckles.

ZNF638 interacts with regulators of pre-mRNA splicing present in adipocytes

Given the punctate nuclear localization of ZNF638 and the evidence that speckles are nuclear bodies enriched in pre-mRNA splicing factors (14), we hypothesized that ZNF638 may complex with spliceosomal proteins. To assess this, we performed biochemical purification of ZNF638 interacting proteins from differentiating 3T3-L1 adipocytes using a GST fusion protein expressing the region containing the ZF domain present at the carboxy terminus (GST-ZNF638 ZF2 ) required for speckled localization. Mass spectrometry analysis identified 172 novel ZNF638 interactors (Fig. 3A), and their clustering according to function revealed that 38% of these interacting proteins have been previously shown to be either associated with constitutive and alternative splicing, reported in interchromatin granule clusters or present in early or late spliceosomal complexes (33–36) (Fig. 3A, Table 2). Mass spectrometry analysis also revealed that the novel ZNF638 interactors identified are involved in transcription, translation, nucleic acid binding, and metabolism, according to their annotation in the Uniprot database (Fig. 3A). Furthermore, we detected ZNF638 peptide sequences, which were not part of the ZNF638-GST fusion protein used for affinity purification (data not shown). To validate the results obtained via mass spectrometry analysis, we performed coimmunoprecipitation assays. As shown in Fig. 3B, ectopically expressed GFP-ZNF638 ZF2 was able to immunoprecipitate endogenous HNRNPA1, HNRNPA2B1, HNRNPLL, NONO, and PABP1. Taken together, our data
we assessed the effect of ZNF638 on splicing of the fibronectin minigene when a C/EBP responsive element cassette was introduced in the fibronectin minigene reporter (Fig. 4D). As shown in Fig. 4E, F, low amounts of ectopically expressed ZNF638 were able to increase the ratio of EI to ES only when C/EBPα, C/EBPB, or C/EBPβ were coexpressed, suggesting that loading of ZNF638 on promoters enhances its action on splicing. These data indicate that ZNF638 is able to influence alternative splicing of a minigene reporter and that this process is facilitated in the presence of C/EBP responsive elements and C/EBP proteins.

The RS motif and the C-terminal ZF domain are necessary for the proadipogenic function of ZNF638

Given our previous characterization of ZNF638 as a co-regulator of adipocyte differentiation, we assessed the requirement of each domain of ZNF638 for this process. We therefore ectopically expressed either vector, full-length ZNF638, or each deletion mutant in the mesenchymal cell line 10T1/2 and induced their differentiation. As shown in Fig. 5A, B, while 10T1/2 cells expressing full-length ZNF638 showed increased lipid accumulation and induced classic adipocyte markers, cells expressing ZNF638 mutants lacking either the RS, the C-terminal ZF, or both domains showed a decreased ability to induce adipocyte differentiation compared with full-length ZNF638, even though these mutants were expressed at the same levels as full-length ZNF638.

Given that alternatively spliced isoforms of lipin1 and NCoR have been previously shown to be differentially regulated during the adipogenic process (8, 10), we determined whether their levels were altered in 10T1/2 cells expressing ZNF638 or its deletion mutants. As shown in Fig. 5C, we observed an altered ratio of lipin1β/lipin1α and of NcoRα/NcoRβ in cells expressing the mutants lacking the RS or the C-terminal ZF domains compared with cells expressing full-length ZNF638. Overall, these data demonstrate that the RS and the C-terminal ZF domains are necessary for the proadipogenic function of ZNF638 and for the regulation of alternatively spliced isoforms present in adipocytes.

DISCUSSION

The ZF protein ZNF638 is a multidomain protein initially cloned in the mid-1990s whose function has remained unknown for more than a decade. We have recently characterized ZNF638 as a transcriptional cofactor involved in adipocyte differentiation acting in cooperation with C/EBPβ and C/EBPδ to regulate the expression of the nuclear receptor PPARγ in adipocytes (2). In the present study, we have provided novel evidence that ZNF638 localizes in nuclear regions enriched in splicing factors, that it interacts with splicing regulatory proteins present in adipocytes, and that it participates in the modulation of alternative splicing of a pre-mRNA transcript derived from a minigene reporter.
TABLE 2. ZNF638 interacting proteins implicated in pre-mRNA splicing identified by mass spectrometry analysis

| Gene Symbol | Protein Name                                                                 | Uniprot Accession Number | Number of Peptide Matches | References |
|-------------|------------------------------------------------------------------------------|--------------------------|---------------------------|------------|
| Cpsf6       | Cleavage and polyadenylation specificity factor subunit 6                      | Q6NVF9                   | 1 (36)                    |            |
| Ddx1        | ATP-dependent RNA helicase DDX1                                               | Q91VR5                   | 30 (36)                   |            |
| Ddx17       | Probable ATP-dependent RNA helicase DDX17                                    | Q501J6                   | 10 (33, 36)               |            |
| Ddx5x       | ATP-dependent RNA helicase DDX5x                                              | Q62167                   | 4 (33, 36)                |            |
| Ddx46       | Probable ATP-dependent RNA helicase DDX46                                    | Q56925                   | 3 (33, 34, 36, 53)        |            |
| Ddx5        | Probable ATP-dependent RNA helicase DDX5                                      | Q61656                   | 20 (33–36, 54)            |            |
| Ddx30       | ATP-dependent RNA helicase DDX30                                              | Q99MJ9                   | 3 (36)                    |            |
| Ddx15       | Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DDX15            | O32856                   | 6 (33, 36, 55)            |            |
| Ddx30       | Putative ATP-dependent RNA helicase DDX30                                     | Q99PU8                   | 4 (36)                    |            |
| Ddx9        | ATP-dependent RNA helicase A                                                  | O70133                   | 5 (33, 34, 36)            |            |
| Elavl1      | ELAV-like protein 1                                                           | P70372                   | 2 (34, 36)                |            |
| Ewsr1       | RNA binding protein EWS                                                        | Q16145                   | 10 (33, 36)               |            |
| Fbl         | RNA 2′-O-methyltransferase fibrillarin                                         | P35550                   | 2 (33)                    |            |
| Fus         | RNA binding protein FUS                                                        | P56959                   | 18 (33, 36)               |            |
| Gpacth1     | G patch domain-containing protein 1                                           | Q9DBM1                   | 1 (35)                    |            |
| H1b1c       | Histone H1.2                                                                  | P18564                   | 2 (33)                    |            |
| Hnrnp0      | Heterogeneous nuclear ribonucleoprotein A0                                    | Q8CX86                   | 1 (33, 36)                |            |
| Hnrnp1      | Heterogeneous nuclear ribonucleoprotein A1                                    | P49312                   | 14 (33–38)                |            |
| Hnrnp2b1    | Heterogeneous nuclear ribonucleoproteins A2/B1                                | Q8BG05                   | 15 (33–36)                |            |
| Hnrnp3      | Heterogeneous nuclear ribonucleoprotein A3                                    | Q9AO200                  | 8 (33–36)                 |            |
| Hnrnp4d     | Heterogeneous nuclear ribonucleoprotein D0                                     | Q86D686                  | 4 (33, 36)                |            |
| Hnrnp5l     | Heterogeneous nuclear ribonucleoprotein L                                      | Q8R081                   | 6 (33, 35, 36, 56)        |            |
| Hnrnp6m     | Heterogeneous nuclear ribonucleoprotein M                                      | Q90DE01                  | 4 (33, 35, 36, 57)        |            |
| Hnrnp10     | Putative uncharacterized protein                                               | Q5UZ01                   | 10 (36)                   |            |
| Hnrnp5u     | Heterogeneous nuclear ribonucleoprotein U                                     | Q8VEK3                   | 49 (33–36)                |            |
| Hnrpfl      | Heterogeneous nuclear ribonucleoprotein L-like                                | Q921F4                   | 1 (41, 42)                |            |
| Hspa5       | 78 kDa glucose-regulated protein                                               | P29029                   | 19 (33)                   |            |
| Hspa8       | Heat shock cognate 71 kDa protein                                             | P65017                   | 3 (36)                    |            |
| Igl2b3      | Insulin-like growth factor 2 mRNA binding protein 3                            | Q9CPN8                   | 10 (36)                   |            |
| Iif3        | Interleukin enhancer binding factor 3                                          | Q9Z1 × 4                 | 3 (33, 34)                |            |
| Matr3       | Matrin-3                                                                      | Q8K310                   | 1 (33, 36)                |            |
| Ncbp1       | Nuclear cap binding protein subunit 1                                          | Q3U3Y9                   | 1 (33–36, 58)             |            |
| Ncl         | Nucleolin                                                                     | P90405                   | 23 (36)                   |            |
| Nono        | Non-POU domain-containing octamer binding protein                             | Q99K48                   | 1 (33, 36, 57, 59, 60)    |            |
| Nop56       | Nucleolar protein 56                                                           | Q9D6Z1                   | 3 (33)                    |            |
| Nop58       | Nucleolar protein 58                                                           | Q6DFW4                   | 1 (33)                    |            |
| Npm1        | Nucleophosmin                                                                  | Q61957                   | 2 (36)                    |            |
| Nsf1        | Nuclear RNA export factor 1                                                    | Q89JX7                   | 1 (33, 36)                |            |
| Pabpc1      | Polyadenylate binding protein, cytoplasmic 1                                   | P29541                   | 4 (35, 61)                |            |
| Pppl1ca     | Serine/threonine-protein phosphatase PP1α catalytic subunit                    | P62137                   | 2 (36)                    |            |
| Ppp1r10     | Serine/threonine-protein phosphatase 1 regulatory subunit 10                   | Q80W00                   | 12 (33)                   |            |
| Rbm15       | Putative uncharacterized protein                                               | Q3THK4                   | 1 (36)                    |            |
| Rbm39       | RNA binding protein 39                                                         | Q8VH51                   | 2 (21, 33, 34)            |            |
| Rmxa        | Heterogeneous nuclear ribonucleoprotein G                                     | Q35479                   | 1 (33, 35, 36, 62)        |            |
| Rpl7a       | 60S ribosomal protein L                                                       | P12970                   | 4 (33)                    |            |
| Rpl8p       | 60S acidic ribosomal protein P0                                                | P18600                   | 4 (33)                    |            |
| Rps2        | 40S ribosomal protein S2                                                       | P25444                   | 2 (33)                    |            |
| Rps3a       | 40S ribosomal protein S3a                                                      | Q97351                   | 3 (33)                    |            |
| Sa6b2       | Scaffold attachment factor B2                                                  | Q80YR5                   | 7 (36)                    |            |
| Sf1         | Splicing factor 1                                                               | Q64213                   | 2 (33, 36, 63)            |            |
| Sf3b1       | Splicing factor 3β subunit 1                                                    | Q99NB9                   | 1 (33–36, 64)             |            |
| Sfnpq       | Splicing factor, proline and glutamine rich                                   | Q8VJ16                   | 34 (33, 36, 65)           |            |
| Slm         | SAFB-like transcription modulator                                              | Q8CH25                   | 1 (33)                    |            |
| Srpk1       | Serine/threonine-protein kinase SRPK1                                          | O70551                   | 1 (66)                    |            |
| Srsf4       | Serine/arginine-rich splicing factor 4                                         | Q8VE97                   | 1 (33, 36, 67)            |            |
| Srsf7       | Serine/arginine-rich splicing factor 7                                         | Q8BL97                   | 1 (33, 34, 36, 68)        |            |
| Syncrip      | Heterogeneous nuclear ribonucleoprotein Q                                     | Q7TMK9                   | 23 (35, 36, 69)           |            |
| Tafl5       | TAF15 RNA polymerase II, TATA box binding protein-associated factor           | Q8BQ46                   | 3 (36)                    |            |
| Tdrd3       | Tudor domain-containing protein 3                                              | Q91W18                   | 2 (34)                    |            |
| Timpo       | Lamina-associated polypeptide 2, isoforms α/zeta                              | Q61053                   | 1 (33)                    |            |
| U2surp      | U2 snRNP-associated SURF motif-containing protein                              | Q6NV83                   | 1 (33, 34)                |            |
| Wbp11       | WW domain binding protein 11                                                   | Q923D5                   | 1 (36, 70)                |            |
| Xmn2        | 5′-3′ exorNase 2                                                               | Q9DBR1                   | 2 (36)                    |            |
| Zhr         | Zinc finger RNA binding protein                                                | O85352                   | 6 (33, 36)                |            |
| Znf638      | Zinc finger protein 638                                                        | Q91404                   | 27 (33)                   |            |
Our systematic analysis of ZNF638 interactors in adipocytes revealed that ZNF638 complexes with 16 factors shown to be part of the early prespliceosomal complex, including U2SURP, SRSF7, TDRD3, SF3B1, FUS, DDX46, DDX5, DHX9, ELAVL1, ILF3, NCBP1, RBM39, and the HNRNP proteins A1, A2/B1, A3, and U (34). These interactions suggest an early association of ZNF638 with the splicing machinery and its participation in splice site selection during prespliceosomal assembly (4). Particularly noteworthy is the interaction of ZNF638 with HNRNPA1, HNRNPA2B1, and HNRNPPL1, which have been critically implicated in alternative splicing (37–42). Given that HNRNPA1 functions by antagonizing the splicing factor ASF/SF2 on splice site selection (37), it is possible that ZNF638 may affect the relative local abundance of HNRNPA1 through binding and sequestration, shifting the cellular balance in favor of the splicing factor SP2, as proposed in Fig. 6A.

Our mass spectrometry analysis has also revealed that the C-terminal portion of ZNF638 interacts with other ZNF638 proteins present in adipocytes, suggesting that either multiple ZNF638 molecules may be recruited to protein complexes formed by ZNF638 or that ZNF638 may be able to di- or multimerize. Direct verification of the functional significance of these findings in adipocyte differentiation will require further investigation.

The present data indicating that ZNF638 affects alternative splicing of a minigene containing C/EBP binding sites combined with the previous reported function of ZNF638 in transcription through interaction with C/EBP proteins at C/EBP responsive elements (2) suggest that ZNF638 may act as a dual function regulator. This double role is consistent with emerging evidence indicating that cofactors can participate in both transcription and splicing, as described for p52, RNA binding motif protein 39, RNA binding motif protein 14, and PGC1α (20–23). It is plausible that when loaded on a promoter through C/EBP responsive elements, ZNF638 could facilitate the recruitment of the splicing factor(s) required for splice choice to the CTD of the largest subunit of RNA polymerase II (3, 43) and to the proximity of the nascent pre-mRNA (Fig. 6B).
Fig. 5. Requirements of ZNF638 domains in adipocyte differentiation. Quantification of lipid accumulation in 10T1/2 cells expressing vector, full-length ZNF638, or deletion mutants at day 6 after induction of differentiation measured through quantification of Oil Red O extracted from stained cells (A). mRNA levels of adipocyte markers in 10T1/2 cells expressing either vector, full-length ZNF638, or deletion mutants, after 3 days of differentiation (B). Ratio of alternatively spliced isoforms during adipocyte differentiation in 10T1/2 cells expressing either vector, full-length ZNF638, or deletion mutants, after 3 days of induction of differentiation (C). A, B, C: Mean ± SEM. Full-length ZNF638 compared with vector control: # \( P < 0.05 \), ## \( P < 0.01 \). Mutants compared with full-length ZNF638: * \( P < 0.05 \), ** \( P < 0.01 \).

This model is supported by the identification through our mass spectrometry analysis of SRSF4, a splicing factor previously shown to be involved in alternative splice site selection during pre-mRNA processing (44, 45), as a novel ZNF638 interactor. This putative dual function of ZNF638 is reminiscent of the splicing role played by the coactivator PGC1α when loaded on the promoter of its target genes (23).
Through mutagenesis and confocal microscopy analysis, we showed that the RS domain and the C-terminal ZF motif are required for ZNF638’s localization in nuclear bodies enriched in splicing factors. While it has been previously recognized that RS domains can confer speckle targeting (46–48), our data indicate that also the U1/matrin-like ZF (C-X7–2–C-X12,16–H-X5–H) (49) present at the C terminus of ZNF638 is required for speckle localization. Interestingly, this type of ZF motif has been previously identified in other proteins present in nuclear speckles and shown to be required for their localization, as in the case of SF3A2 and SF3A3 (50). These observations suggest a possible contribution of matrin-like ZFs in targeting proteins to nuclear speckles.

Our structure-function analysis has revealed that the RS and the C-terminal ZF domains required for speckle localization are also necessary for the differentiation function of ZNF638. While it is well established that RS domains play a role in splicing (51), the requirement of SR domains in adipocyte differentiation has been only recently identified through the analysis of the RS domain in the function of the CDC-like kinase 1) kinase in adipocyte differentiation (52). Further studies will determine whether the RS and C-terminal ZF domains present in ZNF638 are necessary for the generation of adipocyte-specific isoforms through their function as splicing activation domains via interactions with spliceosomal components or whether their role in adipocyte differentiation is through the recruitment of cofactors involved in transcription.

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