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 ZNF638 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.**—Du, C., X. Ma, S. Meruvu, L. Hugendubler, and E. Mueller. **The adipogenic transcriptional cofactor ZNF638 interacts with splicing regulators and influences alternative splicing.** ***J. Lipid Res.*** **2014.** 55: **1886–1896.**

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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.

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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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Serine/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 gamma coactivator 1-alpha (PGC1α), which exerts a dual function when recruited at promoters containing direct repeat-1 responsive elements (23).

We have recently identified ZNF638 as a novel coactivator with domain homology to PGC1α and shown that ZNF638 functions as a transcriptional cofactor regulating the early stages of adipocyte differentiation through protein-protein interaction with C/EBP family members at the PPARγ promoter (2). ZNF638 is composed of RS domains and RRMs, in addition to a homology region to the matrix-bound protein matrin 3 (MH1), two C_2H_2 zinc finger (ZF) motifs (ZF1 and ZF2), a putative DNA binding domain (DBD), and a region with acidic repeats (ARs) (24, 25). This characteristic domain composition of ZNF638, reminiscent of RNA binding proteins and of splicing regulators, 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 acid 424 to amino acid 456, eliminating the N-terminal ZF motif, the ΔRS construct lacks the RS motif present from amino acids 470 to 573, the ΔRRM1 has a deletion of the RRM1 motif from amino acids 676 to 751, ΔRRM2 has a deletion of the RRM2 motif from amino acids 901 to 981, and ΔRRM1-3 has a deletion encompassing all three RRM motifs, from amino acids 676 to 1,077. The ZNF638-ΔDBD construct has a deletion of the DBD, encompassing amino acids 1,353 to 1,482, the ΔAR contains a deletion of the ARs from amino acids 1,538 to 1,770, and ΔZF2 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)-ZNF638$_{ZF2}$ and green fluorescent protein (GFP)-ZNF638$_{ZF2}$ 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 construct 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/EBP responsive elements, a 46 bp sequence of the PPARγ2 promoter containing C/EBP responsive elements (5′-TTTTACTGCAATTTTAAAAAGCAATCAATATTGAACAATCTCT-GCT-3′) (27) was inserted between the β-actin promoter and the beginning of the fibronectin minigene cassette. The C/EBP responsive element sequence was synthesized including *Xho*I and *Bsr*GI 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)/alternative splicing factor (ASF) antibody (Invitrogen, 32-4600), Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, A-11008), and Alexa Fluor 594 goat anti-mouse antibody (Invitrogen, A-11005) were used for immunofluorescence staining. Mouse anti-β-actin antibody (Sigma, A5316), anti-GFP antibody (Invitrogen, A-11122), anti-HNRNPA1 antibody (Abcam, ab5832), anti-HNRNPA2B1 antibody (Abcam, ab31645), anti-HNRNPLL antibody (Santa Cruz, sc-132712), anti-non-POU domain-containing octamer binding protein (NONO) antibody (Santa Cruz, sc-136296), anti-polyadenylate binding protein 1 (PABP1) antibody (Cell Signaling, 4992), anti-C/EBPα antibody (Santa Cruz, sc-61), anti-C/EBPβ antibody (Santa Cruz, sc-7962), 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: GAATATTTCCACAACAATACCCTGATTG
	Reverse: GGTATTGTTGTGGAAATATTCTTGGAG
ΔRS	Forward: CCTCCCATCCGATAGGAAAAAGGCATTAG
	Reverse: CTTTTTCCTATCGGATGGGAGGATCTC
ΔRRM1	Forward: AAAAAGCCACAGAACAAAGAAATGAAG
	Reverse: GTTGGAACTCAAAGACTGATCCTTC
ΔRRM2	Forward: GAATCGGAGGAAGATGAGGAAGCTCTC
	Reverse: CTTCCTCATCTTCCTCCGATTCCTTGTTC
ΔRRM1-3	Forward: CCAAAGACTGACTCAGAGGTTCAAAG
	Reverse: GTTGGAACTCAAAGACTGATCCTTCTG
ΔDBD	Forward: GAGAAAAGCCAATAACAAAACAGTCTC
	Reverse: GTTTTGTTATTGGCTTTTCTCTTATTG
ΔAR	Forward: GACAATGATTCAAAAGTTGAGTTAG
	Reverse: TTCATCCAAATTAAATGTAAATAATG
ΔZF2	Forward: GCCAAGCAAAGAAAGGAAAAGGAGC
	Reverse: TCCAGCCTTCGGAACAAGAAAGTCC

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% CO_2. 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.

Differentiation assays

The 10T1/2 cells were electroporated (Amaxa) 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, 10 μM dexamethasone, 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′-GCTTCATTGTGGGAGCAGAC-3′, reverse 5′-ATGGTGTTCTTGCCCATCAG-3′; for CD36, forward 5′-TTTGGAGTGGTAGTAAAAAGGGC-3′, reverse 5′-TGACATCAGGGACTCAGAGTAG-3′; for aP2, forward 5′-ACACCGAGATTTCCTTCAAACTG-3′, reverse 5′-CCATCTAGGGTTATGATGCTCTTC-3′; for Pparγ2, forward 5′-GATGCACTGCCTATGAGCACTT-3′, reverse 5′-AGAGGTCCACAGAGCTGATTC-3′; for ZNF638, forward 5′-TCCCAGTTGAGAGTGGAACC-3′, reverse 5′-TGTGAGATCCGCTCTTGTTG-3′; for Lipin1α, forward 5′-GGTCCCCCAGCCCCAGTCCTT-3′, reverse 5′-GCAGCCTGTGGCAATTCA-3′; for Lipin1β, forward 5′-CAGCCTGGTAGATTGCCAGA-3′, reverse 5′-GCAGCCTGTGGCAATTCA-3′; for NCoR, forward 5′-CTGACAGGCCTCAAGAAAGG-3′, reverse 5′-AACCTGTTCCAGACGTGGTC-3′; and for NCoRω, forward 5′-CTGGCTGCTCTTGTGGATGCT-3′,reverse5′-CTGTCCCATTCCCTCTGACTG-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% (w/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. DAPI (4',6-diamidino-2-phenylindole, Invitrogen, D1306) was used for nuclear counterstaining. Confocal imaging was performed with an LSM 5 live DuoScan (Zeiss) laser scanning microscope with a 63× oil objective, using ZEN 2009 acquisition software (Zeiss).

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

Purification of the GST-ZNF638$_{ZF2}$ 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-ZNF638$_{ZF2}$.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).

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,

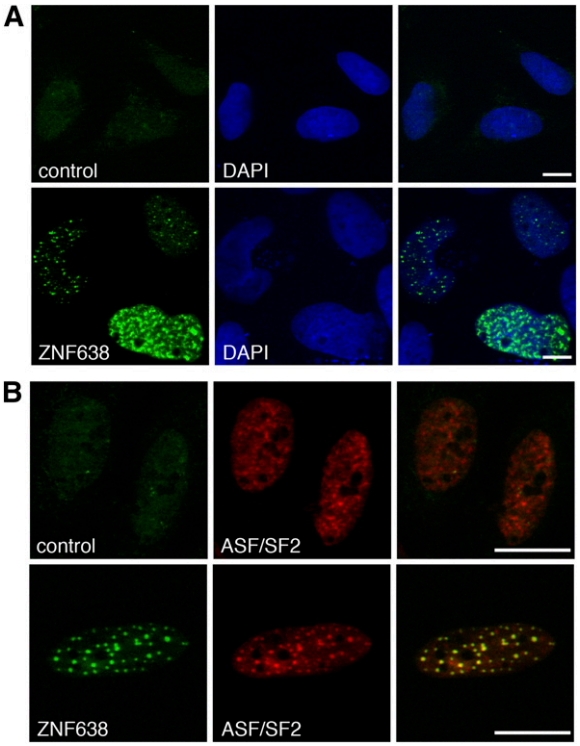

Fig. 1. ZNF638 colocalizes with the splicing factor ASF/SF2 in nuclear speckles. Subcellular localization of transiently expressed ZNF638 in U2OS cells detected by indirect immunofluorescence and by overlay with DAPI as nuclear counterstaining (A). Double immunofluorescence staining of transiently expressed ZNF638 in U2OS cells and overlay with endogenous ASF/SF2, a marker for nuclear speckles (B). A, B: Confocal imaging; scale bars: 10 μm.

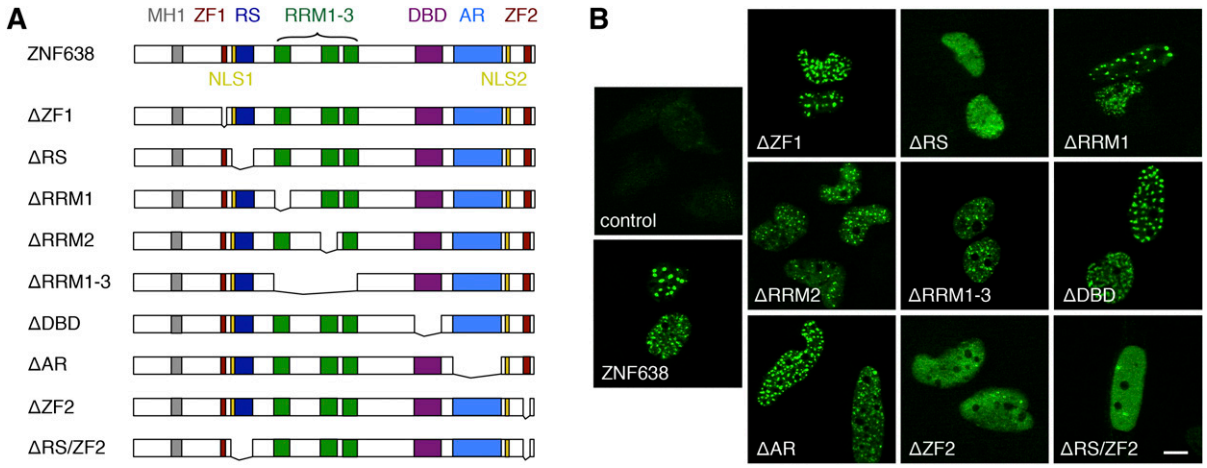

Fig. 2. The RS domain and the C-terminal ZF motif are required for ZNF638 localization to nuclear speckles. Schematic illustration of the domains present in full-length ZNF638 and schematic representation of ZNF638 deletion mutants analyzed in this study (A). NLS, nuclear localization signal. Subcellular localization of ectopically expressed full-length ZNF638 and ZNF638 deletion mutants in U2OS cells, detected by indirect immunofluorescence staining and confocal imaging (B). Scale bar, 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′-CCGTCATCCCAGAGGTGCCCCA-3′, EI reverse 5′-GGAGGGACGGCCGTTTGCTGTG-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 immuofluorescence 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 interacting proteins 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

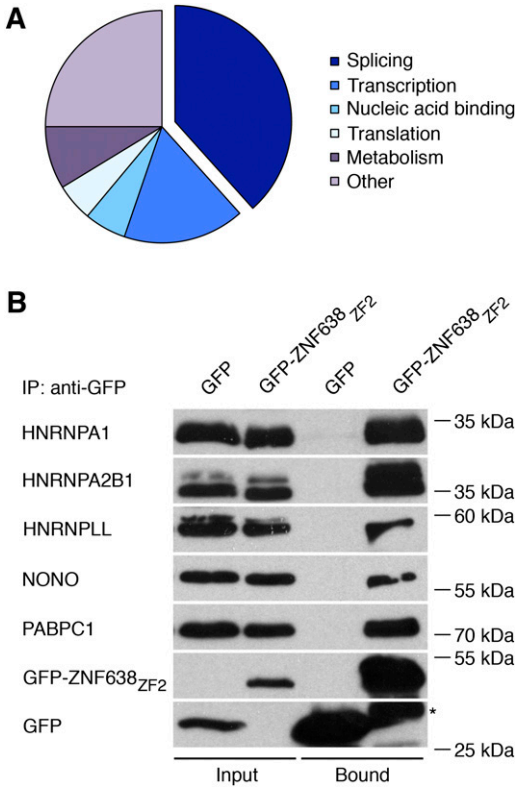

Fig. 3. ZNF638 interacts with factors involved in pre-mRNA splicing present in differentiating adipocytes. Graphical representation of the clustering of the 172 novel ZNF638 interacting proteins present in differentiating adipocytes identified by mass spectrometry analysis (A). Interacting proteins purified from nuclear extracts obtained from differentiating 3T3-L1 cells using a GST-ZNF638$_{ZF2}$ fusion protein were resolved by SDS-PAGE and subjected to mass spectrometry analysis. Coimmunoprecipitation assays were performed to validate mass spectrometry data (B). GFP-ZNF638$_{ZF2}$ fusion protein was expressed in HEK-293 cells, and endogenous interacting proteins were detected by Western blot after GFP immunoprecipitation. Asterisk indicates nonspecific band.

indicate that ZNF638 can complex with factors implicated in alternative splicing expressed in differentiating adipocytes.

ZNF638 modulates alternative splicing decisions on a minigene reporter

In order to test the hypothesis that ZNF638 may participate in the regulation of pre-mRNA splicing, we investigated the effects of coexpressing ZNF638 with a minigene reporter containing a genomic exon cassette derived from the rat fibronectin gene. This reporter system is used for the quantification of alternative splicing through measurements of an alternatively spliced exon (**Fig. 4A**) (26). When ZNF638 was coexpressed with the minigene reporter, we observed an increase in the ratio of EI to ES, as compared with the ratio observed in control cells (Fig. 4B, C). Exon inclusion and exclusion were further confirmed by direct sequencing of PCR products (data not shown).

Given our previously published data demonstrating a function for ZNF638 as a coactivator of C/EBP proteins at C/EBP responsive elements present in the PPARγ promoter, 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/EBPβ, or C/EBPδ 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 coregulator 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 NcoRω/NcoR 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)
Ddx3×	ATP-dependent RNA helicase DDX3×	Q62167	4	(33, 36)
Ddx46	Probable ATP-dependent RNA helicase DDX46	Q569Z5	3	(33, 34, 36, 53)
Ddx5	Probable ATP-dependent RNA helicase DDX5	Q61656	20	(33–36, 54)
Ddx50	ATP-dependent RNA helicase DDX50	Q99MJ9	3	(36)
Dhx15	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	O35286	6	(33, 36, 55)
Dhx30	Putative ATP-dependent RNA helicase DHX30	Q99PU8	4	(36)
Dhx9	ATP-dependent RNA helicase A	O70133	5	(33, 34, 36)
Elavl1	ELAV-like protein 1	P70372	2	(34, 36)
Ewsr1	RNA binding protein EWS	Q61545	10	(33, 36)
Fbl	rRNA 2'-*O*-methyltransferase fibrillarin	P35550	2	(33)
Fus	RNA binding protein FUS	P56959	18	(33, 34, 36)
Gpatch1	G patch domain-containing protein 1	Q9DBM1	1	(35)
Hist1 h1c	Histone H1.2	P15864	2	(33)
Hnrnpa0	Heterogeneous nuclear ribonucleoprotein A0	Q9CX86	1	(33, 36)
Hnrnpa1	Heterogeneous nuclear ribonucleoprotein A1	P49312	14	(33–38)
Hnrnpa2b1	Heterogeneous nuclear ribonucleoproteins A2/B1	O88569	25	(33–36, 39, 40)
Hnrnpa3	Heterogeneous nuclear ribonucleoprotein A3	Q8BG05	15	(33–36)
Hnrnpab	Heterogeneous nuclear ribonucleoprotein A/B	Q99020	8	(36)
Hnrnpd	Heterogeneous nuclear ribonucleoprotein D0	Q60668	4	(33, 36)
Hnrnpl	Heterogeneous nuclear ribonucleoprotein L	Q8R081	6	(33, 35, 36, 56)
Hnrnpm	Heterogeneous nuclear ribonucleoprotein M	Q9D0E1	4	(33, 35, 36, 57)
Hnrnpr	Putative uncharacterized protein	Q3UZI0	10	(36)
Hnrnpu	Heterogeneous nuclear ribonucleoprotein U	Q8VEK3	49	(33–36)
Hnrpll	Heterogeneous nuclear ribonucleoprotein L-like	Q921F4	1	(41, 42)
Hspa5	78 kDa glucose-regulated protein	P20029	19	(33)
Hspa8	Heat shock cognate 71 kDa protein	P63017	3	(36)
Igf2bp3	Insulin-like growth factor 2 mRNA binding protein 3	Q9CPN8	10	(36)
Ilf3	Interleukin enhancer binding factor 3	Q9Z1 × 4	3	(33, 34, 36)
Matr3	Matrin-3	Q8K310	1	(33, 36)
Ncbp1	Nuclear cap binding protein subunit 1	Q3UYV9	1	(33–36, 58)
Ncl	Nucleolin	P09405	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	Q61937	2	(36)
Nxf1	Nuclear RNA export factor 1	Q99JX7	1	(33, 36)
Pabpc1	Polyadenylate binding protein, cytoplasmic 1	P29341	4	(35, 61)
Ppp1ca	Serine/threonine-protein phosphatase PP1-α 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)
Rbmx	Heterogeneous nuclear ribonucleoprotein G	O35479	1	(33, 35, 36, 62)
Rpl7a	60S ribosomal protein L7a	P12970	4	(33)
Rplp0	60S acidic ribosomal protein P0	P14869	4	(33)
Rps2	40S ribosomal protein S2	P25444	2	(33)
Rps3a	40S ribosomal protein S3a	P97351	3	(33)
Safb2	Scaffold attachment factor B2	Q80YR5	7	(36)
Sf1	Splicing factor 1	Q64213	2	(33, 36, 63)
Sf3b1	Splicing factor 3B subunit 1	Q99NB9	1	(33–36, 64)
Sfpq	Splicing factor, proline and glutamine rich	Q8VIJ6	34	(33, 36, 65)
Sltm	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)
Taf15	TAF15 RNA polymerase II, TATA box binding protein-associated factor	Q8BQ46	3	(36)
Tdrd3	Tudor domain-containing protein 3	Q91W18	2	(34)
Tmpo	Lamina-associated polypeptide 2, isoforms α/zeta	Q61033	1	(33)
U2surp	U2 snRNP-associated SURP motif-containing protein	Q6NV83	1	(33, 34)
Wbp11	WW domain binding protein 11	Q923D5	1	(36, 70)
Xrn2	5′-3′ exoRNase 2	Q9DBR1	2	(36)
Zfr	Zinc finger RNA binding protein	O88532	6	(33, 36)
Znf638	Zinc finger protein 638	Q61464	27	(33)

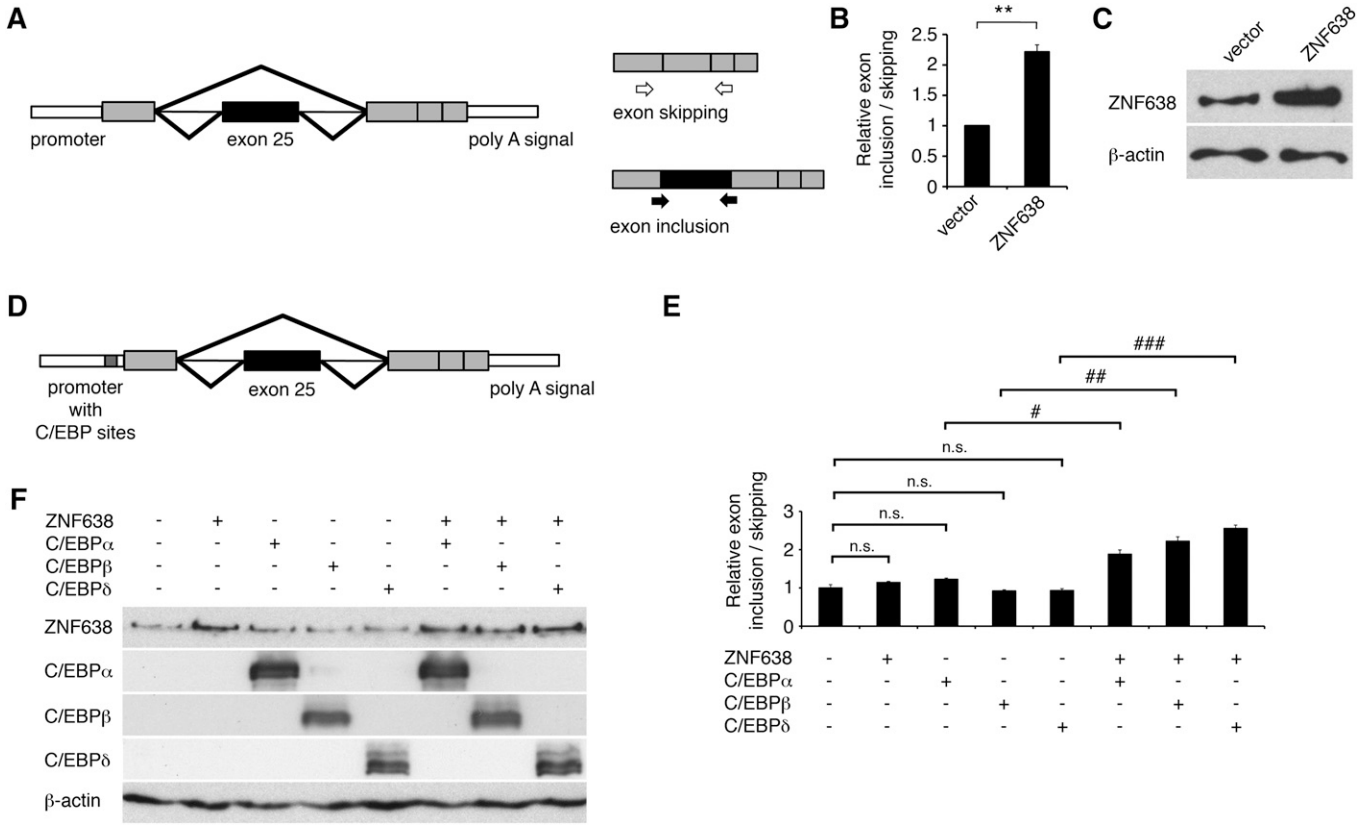

Fig. 4. ZNF638 modifies splicing of minigene reporter transcripts. Schematic representation of the fibronectin minigene reporter and of the assay performed (A). EI-specific primers (filled arrows) and ES primers (empty arrows) were used to quantify the mRNA isoforms retaining the alternative spliced exon 25 (black boxes) and the short isoform skipping the alternatively spliced exon. Gray boxes indicate constitutively retained exons; thin lines, introns; and thick lines, spliced isoforms. Quantification of the ratio of EI to ES in HEK-293 cells transiently expressing the minigene plasmid with ZNF638 or vector control (B). Mean ± SEM. Statistical analysis was performed on three independent experiments. ** $P < 0.01$. Western blot of ZNF638 expression levels from a representative experiment (C). β-actin was used as loading control. Schematic representation of the fibronectin minigene containing C/EBP responsive elements in the promoter (D). Ratio of EI over ES in HEK-293 cells transiently expressing the minigene containing C/EBP binding sites, in the presence or absence of ZNF638 and C/EBP factors (E). Experiments were repeated at least three times. A representative experiment is shown. Mean ± SEM; n.s., not statistically significant; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$. Western blot analysis to assess protein levels of transiently expressed ZNF638, C/EBPα, C/EBPβ, and C/EBPδ in a representative experiment. β-actin was used as loading control (F).

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 HNRNPLL, 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 SF2, 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).

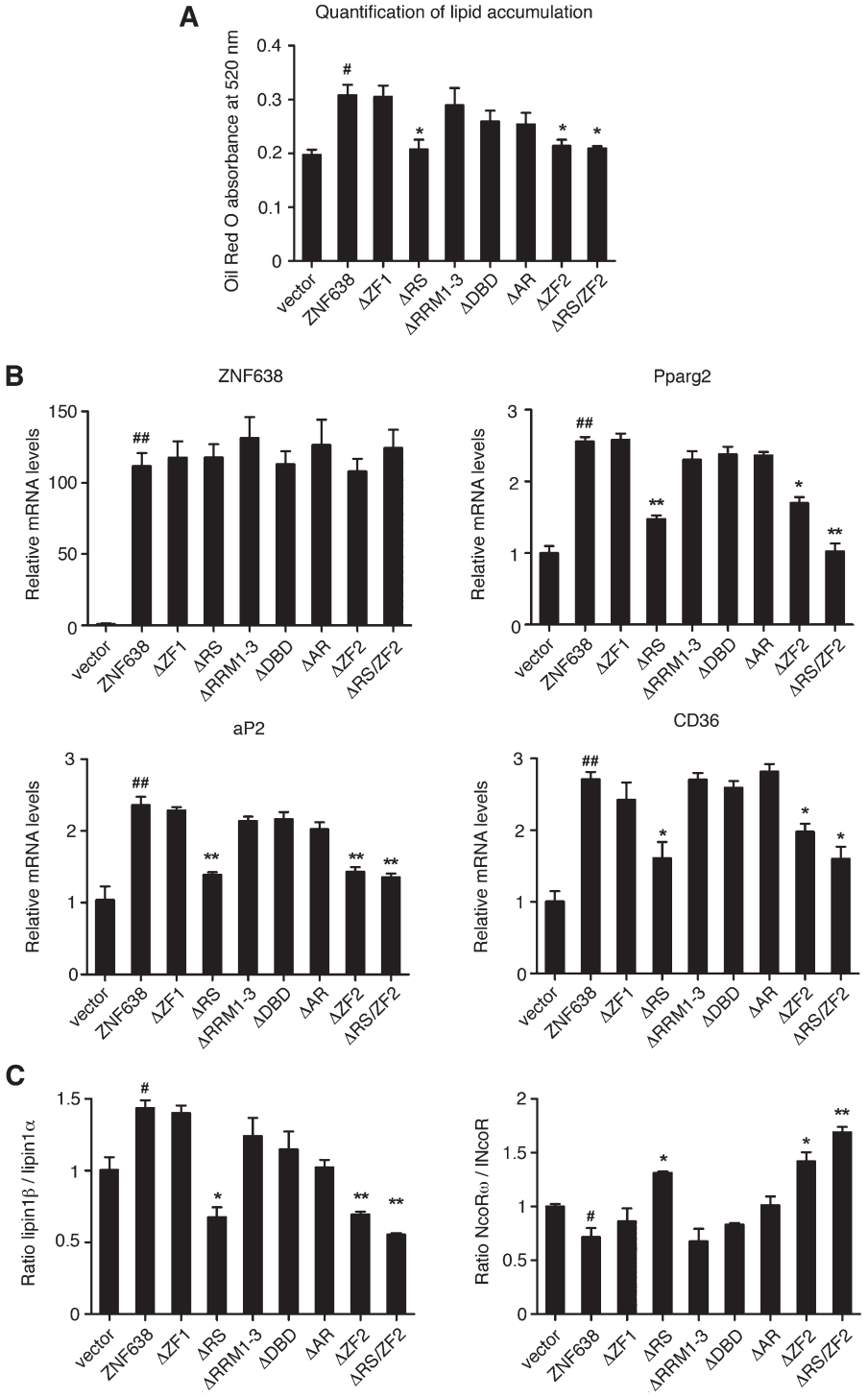

Fig. 5. Requirements of ZNF638 domains in adipocyte differentiation. Quantification of lipid accumulation in 10T1/2 cells expressing vector, full-length ZNF638, or deletion mutants at day 6 after induction of differentiation measured through quantification of Oil Red O extracted from stained cells (A). 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).

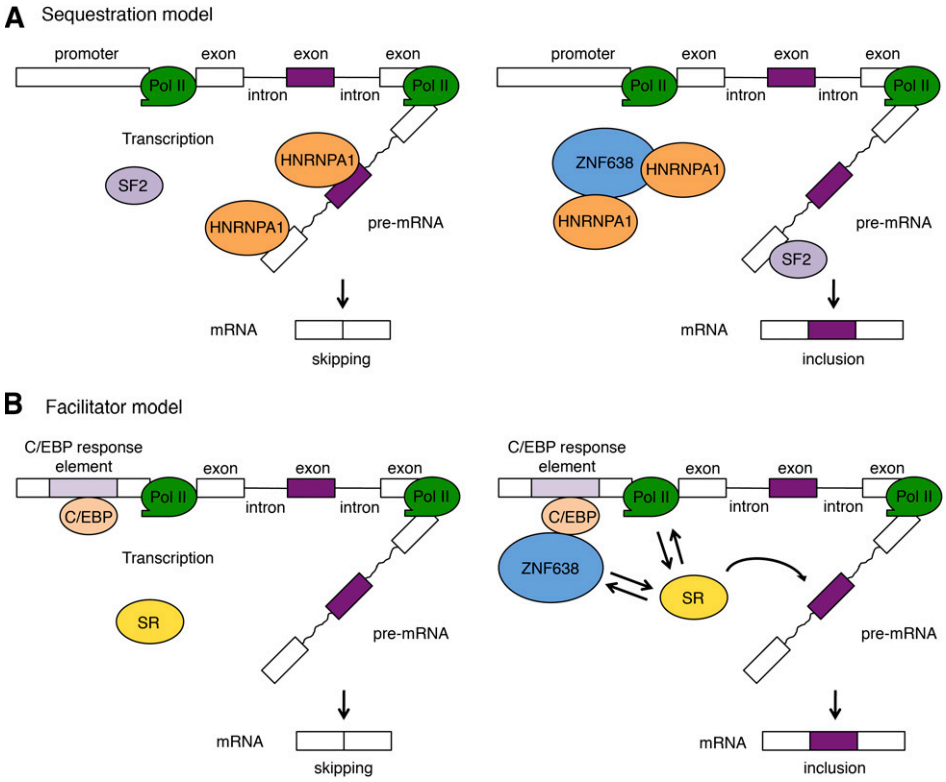

Fig. 6. Proposed models of ZNF638's mode of action on alternative splicing. Sequestration model (A). ZNF638 may affect splicing decisions by altering the balance between available inhibitory and activating splicing factors competing for their binding to the pre-mRNA. Via sequestration of HNRNP proteins like HNRPA1, ZNF638 may prevent their binding to splice sites on the pre-mRNA, increasing the availability of activating splicing factors, such as SF2. Facilitator model (B). The loading of ZNF638 on the promoters of its target genes via C/EBP responsive elements may facilitate the recruitment of SR proteins to the CTD of the polymerase II and to the nearby nascent pre-mRNA. A, B: Purple exon is an alternatively spliced exon. Pol II, RNA polymerase II.

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 ($C\text{-}X_2\text{-}C\text{-}X_{(12,16)}\text{-}H\text{-}X_5\text{-}H$) (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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