Central to the development of obesity are the increases in number and size of adipocytes, according to nutrient availability (1, 2). Despite various therapies to limit weight gain and promote weight loss, it is surprising that none specifically target the adipocyte to limit its expansion or growth (1, 2). The complex transcriptional network and cellular processes that govern the differentiation of adipocyte progenitor cells contribute to the difficulty in targeting adipocytes therapeutically (1, 2). Protein phosphorylation is a key post-translational modification that determines the activation state, subcellular localization, and stability of adipogenic regulators (3–7). Furthermore, phosphorylation status also determines their interactions with molecular scaffold proteins, which aid in the coordination of complex transcriptional networks (3, 4).

We previously identified the molecular scaffold, 14-3-3ζ, as a critical regulator of glucose homeostasis and adipogenesis (4, 8, 9). Specific to the adipocyte, systemic deletion of 14-3-3ζ in mice significantly reduced visceral adiposity and impaired adipocyte differentiation, whereas transgenic overexpression of 14-3-3ζ exacerbated high-fat diet induced obesity (4). The hedgehog transcription factor, Gli3, was identified as a critical downstream effector in 14-3-3ζ-mediated adipogenesis (4), but the diversity of proteins in the 14-3-3ζ interactome suggests the possibility that other interacting proteins or pathways parallel to Gli3 may be also involved.

Unbiased approaches, such as proteomics and transcriptomics, can lead to the discovery of novel factors that drive adipogenesis, in addition to providing insight into physiological pathways influenced by adipogenic regulators like 14-3-3ζ (4, 10–13). All seven mammalian 14-3-3 isoforms have large, diverse interactomes (8, 12–15), and they are dynamic and change in response to various stimuli (10–13). Thus, inducing pre-adipocytes to differentiate may permit the identification of novel differentiation-specific factors within the 14-3-3ζ interactome and reveal pathways and biological processes that are essential to the development of a mature adipocyte.

To elucidate the 14-3-3ζ interactome during adipogenesis, we employed a proteomic-based discovery approach. Herein, we report that previously established factors required for adipogenesis, such as Ptrf/Cavin1 and Phb2 (Prohibitin-2), can be detected in the interactome, and novel factors, such as those involved in RNA splicing, are also enriched in the interactome during differentiation. To test for their roles in adipogenesis, siRNA knockdown approaches were used and revealed the requirement for RNA-splicing factors, such as Hnrpf, Sfpq, and Ddx6. Taken together, these findings demonstrate the usefulness of examining the interactome of 14-3-3 proteins in the...
context of a physiological process, such as adipocyte differentiation, and highlight the ability to find novel functional regulators through this approach. Understanding how the interactome is influenced by disease states, such as obesity, may lead to the identification of novel proteins that contribute to disease pathogenesis.

Results

Generation of TAP–14-3-3ζ mouse embryonic fibroblasts

To examine how adipocyte differentiation influences the 14-3-3ζ interactome, we generated mouse embryonic fibroblasts (MEFs)3 derived from transgenic mice that moderately overexpress a TAP-epitope–tagged human 14-3-3ζ molecule (TAP–14-3-3ζ MEFs) (4) (Fig. 1A). This approach was chosen to circumvent the variability in the expression of transiently expressed proteins and increased specificity of protein purification with epitope-tagged proteins (16). Differentiation of TAP–14-3-3ζ MEFs was induced with an established adipogenic mixture (MDI: insulin, dexamethasone, and isobutylm-

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3 The abbreviations used are: MEF, mouse embryonic fibroblast; siCon, control siRNA; TAP, tandem affinity purification.
### Table 1

Proteins with at least two unique peptides with a total spectral count in differentiated cells of ≥2 in comparison to undifferentiated cells

| Uniprot   | Description                                      | Gene name | Total spectrum IP1 | Total spectrum IP2 |
|-----------|--------------------------------------------------|-----------|--------------------|--------------------|
| Q8VD05    | Myosin-9                                         | Myh9      | 102                | 278                |
| Q4FK11    | Non-POU-domain-containing, octamer binding protein | Nono      | 16                 | 11                 |
| E9QMZ5    | Plectin                                          | Plectin   | 123                | 101                |
| E9QPE8    | Plectin                                          | Plectin   | 122                | 99                 |
| G5E8B8    | Anastellin                                       | Fn1       | 46                 | 60                 |
| Q6A7Y7    | Myosin-10                                        | Myh10     | 68                 | 107                |
| P9B7S5    | Ras GTPase-activating protein-binding protein 1   | G3bp1     | 20                 | 20                 |
| P61979    | Heterogeneous nuclear ribonucleoprotein K         | Hnrnpk    | 16                 | 35                 |
| Q9R002    | Interferon-activable protein 2                   | Iif202    | 13                 | 4                  |
| B7EAU9    | Filamin, a                                       | Flna      | 48                 | 65                 |
| Q6I033    | Lamina-associated peptide 2, isoforms α/ζ        | Tmpo      | 19                 | 13                 |
| B2RSN3    | MCG1395                                          |           |                    |                    |
| Q9I1V8    | ATP-dependent RNA helicase DDX1                  | Ddx1      | 29                 | 16                 |
| P49862    | ADP/ATP translocase 1                            | Scl25a1   | 14                 | 27                 |
| P51888    | ADP/ATP translocase 2                            | Scl25a5   | 12                 | 25                 |
| P90686    | Caprin-1                                         | Caprin1   | 19                 | 10                 |
| Q8BMK4    | Cytoskeleton-associated protein 4                 | Chk4      | 17                 | 33                 |
| B9J5G1    | Novel protein (2810405J04Rik)                     | F99a8     | 9                  | 8                  |
| Q61286    | Lamina-associated polypeptide 2, isoforms β/δ/ε/γ | Tmpo      | 14                 | 12                 |
| Q8VIJ6    | Splicing factor, proline- and glutamine-rich     | Sfpq      | 19                 | 7                  |
| P62702    | 40S ribosomal protein 54, X isoform              | Rps4x     | 15                 | 18                 |
| Q3TQX5    | DEA(D/H) (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked | Ddx3x | 19 | 14 |
| Q4VA29    | MCG140066                                        |           |                    |                    |
| P1I448    | 60S ribosomal protein L7                         | Rp7l      | 13                 | 19                 |
| Q3J0M1    | Tubulin, β                                        | Tub6b     | 16                 | 18                 |
| G3JUT7    | RNA-binding protein FUS (Fragment)               | Fus       | 12                 | 12                 |
| Q8VEM8    | Phosphate carrier protein, mitochondrial         | Scl25a3   | 7                  | 13                 |
| E9QPE7    | Myosin-11                                        | Myh11     | 18                 | 34                 |
| A2A547    | Ribosomal protein L19                           | Rpl19     | 6                  | 11                 |
| P63038    | 60-kDa heat shock protein, mitochondrial         | Hsp1d     | 13                 | 14                 |
| D32ZC3    | Protein Gm10119                                   | Gm10119   | 12                 | 16                 |
| Q9DB20    | ATP synthase subunit O, mitochondrial            | Atp5o     | 11                 | 22                 |
| O70475    | UDP-glucose 6-dehydrogenase                      | Ugdh      | 14                 | 17                 |
| A2APD4    | Small nuclear ribonucleoprotein-associated protein | SsrpB    | 5                  | 5                  |
| Q7C079    | Integrin β                                       | Itgb5     | 14                 | 7                  |
| G3UIZ2    | Heterogeneous nuclear ribonucleoprotein Q        | Syncrip   | 11                 | 9                  |
| D32ZU8    | Fragile X mental retardation protein 1 homolog   | Fmr1      | 15                 | 8                  |
| O35841    | Apoptosis inhibitor 5                            | Ap5a      | 11                 | 8                  |
| Q70309    | Integrin β                                       | Itgb5     | 14                 | 7                  |
| G3UIZ2    | Heterogeneous nuclear ribonucleoprotein Q        | Syncrip   | 11                 | 9                  |
| A2APD4    | Small nuclear ribonucleoprotein-associated protein | SsrpB    | 5                  | 5                  |
| O35841    | Apoptosis inhibitor 5                            | Ap5a      | 11                 | 8                  |
| A4FSU1    | MCG132443                                       | Rps16     | 12                 | 9                  |
| Q3TLJ4−5  | Isoform 5 of protein PRRC2C                      | Prc2c     | 11                 | 5                  |
| P1I448    | 60S ribosomal protein P0                         | Rplp0     | 8                   | 16                 |
| Q8QZY1    | Eukaryotic translation initiation factor 3 subunit L | Ezfl   | 5                  | 7                  |
| P36992    | Fragile X mental retardation protein 1 homolog   | Fmr1      | 15                 | 7                  |
| Q03265    | ATP synthase subunit α, mitochondrial            | Atp5α1    | 15                 | 14                 |
| P63017    | Heat shock cognate 71-kDa protein                | Hspa8     | 13                 | 9                  |
| P21981    | Protein-glutamine γ-glutamyltransferase 2        | Tgm2      | 8                   | 5                  |
| Q80UM7    | Mannosyl-oligosaccharide glucosidase             | Mogs      | 11                 | 3                  |
| P26349    | Splicing factor U2AF 65-kDa subunit              | U2af2     | 7                   | 7                  |
| A2AAMJ8   | MCG3738                                         | Sec61b    | 3                   | 3                  |
| P62412    | 40S ribosomal protein S8                         | Rps8      | 7                   | 12                 |
| P54823    | DEA(D/H) (Asp-Glu-Ala-Asp/His) box polypeptide 6 | Ddx6      | 8                   | 2                  |
| Q3TML6    | Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked | Eif2s3x | 6  | 7  |
| P26041    | Moesin                                           | Msn       | 13                 | 5                  |
| P62983    | Ubiquitin-40S ribosomal protein S27a             | Rsps27a   | 6                   | 5                  |
| P52480    | Pyruvate kinase isoforms M1/M2                   | Pkm2      | 4                   | 2                  |
| Q55UT0    | Ewing sarcoma breakpoint region 1               | Ewsr1     | 5                   | 6                  |
| E9Q2H5    | Uncharacterized protein                          | Gm8991    | 6                   | 3                  |
| Q8C5Q8    | ATP synthase γ chain                            | Atp5γ     | 17                 | 7                  |
| A2AMW0    | Capping protein (actin filament) muscle Z-line, β | Capzβ     | 7                   | 13                 |
| P08121    | Collagen α-1(III) chain                         | Col3a1    | 6                   | 5                  |
| P11087−2  | Isoform 2 of collagen α-1(III) chain             | Col1a1    | 10                  | 3                  |
| Q9Z2X1−2  | Isoform 2 of Heterogeneous nuclear ribonucleoprotein F | Hnrmpf2 | 3  | 4  |
| P11499    | Heat shock protein HSP 90β                       | Hsp90ab1  | 11                 | 6                  |
| P28301    | Protein-lysine 6-oxidase                        | Lox       | 4                   | 2                  |
| P84C08    | Caldesmon                                       | Cald1     | 13                 | 12                 |
| P27657    | 60S ribosomal protein L3                         | Rpl3      | 7                   | 9                  |
| O35737    | Heterogeneous nuclear ribonucleoprotein H        | Hnrnhp1   | 4                   | 3                  |
| A2ACG7    | Dolichyl-diphosphoglycerolcide–protein glycosyltransferase subunit 2 | Rpn2 | 7 | 3 |
| Q3TV18    | Pre-B-cell leukemia transcription factor-interacting protein 1 | Pbxip1 | 6 | 3 |
ethylxanthine), supplemented with rosiglitazone (Fig. 1, A and B), and confirmed by Oil Red-O staining and Pparg mRNA expression (Fig. 1, B and C).

**Differentiation of TAP–14-3-3ζ MEFs results in distinct changes in the interactome of 14-3-3ζ**

Although we previously identified the hedgehog signaling effector, Gli3, as a downstream regulator of 14-3-3ζ-dependent adipogenesis (4), we hypothesized that 14-3-3ζ may control other parallel processes underlying adipocyte differentiation. This is due in part to the large, diverse interactomes of 14-3-3 proteins (8, 12–15). Thus, we utilized affinity proteomics to identify interacting proteins that associate with 14-3-3ζ during adipocyte differentiation (Fig. 1A). The interactome of 14-3-3ζ at 24 h postinduction was examined because key signaling events underlying murine adipocyte differentiation occur during the first 24–48 h (2, 4). Over 100 proteins were identified by MS as 14-3-3ζ–interacting proteins (Table 1). Of these proteins, 56 have not been previously reported to interact with any member of the 14-3-3 protein family (Table 2)( 14).

**Table 1—continued**

| Uniprot | Description | Gene name | Total spectrum | Peptides | D | U | D | U |
|---------|-------------|------------|----------------|----------|-----|-----|-----|-----|
| F6QCI0  | Protein Taf15 (fragment) | Taf15      | 4              | 3         | 0   | 6   | 1   | 1   |
| O08569-3| Isoform 3 of heterogeneous nuclear ribonucleoproteins A2/B1 | Hnrnpa2b1 | 5              | 4         | 1   | 6   | 1   | 1   |
| O08583-2| Isoform 2 of TBO complex subunit 4 | Alyref     | 3              | 2         | 0   | 8   | 2   | 2   |
| O08573-2| Isoform short of galecin-9 | Lgals9     | 2              | 2         | 1   | 7   | 0   | 0   |
| Q564E8  | Ribosomal protein L4 | Rpl4       | 8              | 7         | 3   | 6   | 2   | 2   |
| B1ARA3  | 60S ribosomal protein L26 (fragment) | Rpl26      | 6              | 5         | 0   | 5   | 2   | 2   |
| O35129  | Prohibitin-2 | Phb2       | 4              | 4         | 0   | 7   | 3   | 3   |
| D3Y7C9  | 40S ribosomal protein S15 | Rps21      | 3              | 6         | 1   | 2   | 2   | 2   |
| Q6ZWX6  | Eukaryotic translation initiation factor 2 subunit 1 | Eif2s1     | 5              | 4         | 1   | 4   | 0   | 0   |
| D3Z9R1  | 60S ribosomal protein L36 | Gm5745     | 5              | 3         | 1   | 6   | 1   | 1   |
| P17427  | AP-2 complex subunit α2 | Ap2a2      | 8              | 3         | 1   | 7   | 2   | 2   |
| P64830  | ATP synthase subunit β, mitochondrial | Atp5b      | 9              | 2         | 1   | 8   | 2   | 2   |
| Q8CBM2  | Aspartate-β-hydroxylase | Ashp       | 8              | 4         | 0   | 6   | 3   | 3   |
| Q6NVF9  | Cleavage and polyadenylation specificity factor | Cpsf6      | 6              | 4         | 0   | 6   | 3   | 3   |
| Q672A9  | Nuclear phosphomin | Npm1       | 5              | 6         | 0   | 2   | 1   | 1   |
| Q672A9  | Constitutive coactivator of PPAR-γ-like protein 1 | Fam120a    | 5              | 2         | 0   | 4   | 0   | 0   |
| P14576  | Signal recognition particle 54-kDa protein | Srp54      | 4              | 2         | 0   | 4   | 0   | 0   |
| P63087  | Serine/threonine-protein phosphatase PPIy catalytic subunit | Ppp1cc     | 4              | 4         | 0   | 2   | 0   | 0   |
| P80315  | T-complex protein 1 subunit δ | Cct4       | 4              | 3         | 0   | 3   | 0   | 0   |
| P62960  | Nucleosome-sensitive element-binding protein 1 | Ybx1       | 3              | 2         | 0   | 5   | 1   | 1   |
| P97376  | Protein FRG1 | Frg1       | 3              | 2         | 0   | 5   | 1   | 1   |
| Q3U427  | High-density lipoprotein-binding protein, isofrom | Cdra       | 7              | 3         | 0   | 4   | 1   | 1   |
| B2R7B0  | MCG17262 | Pdap1      | 4              | 3         | 0   | 4   | 1   | 1   |
| P60335  | Poly(rC)-binding protein 1 | Pcbp1      | 4              | 3         | 0   | 4   | 1   | 1   |
| P47911  | 60S ribosomal protein L6 | Rpl6       | 6              | 8         | 4   | 2   | 0   | 0   |
| Q61990-2| Isoform 2 of poly(rC)-binding protein 2 | Pcbp2      | 4              | 2         | 1   | 6   | 1   | 1   |
| P62267  | 40S ribosomal protein S23 | Rps23      | 5              | 4         | 1   | 6   | 3   | 3   |
| D3Z148  | Caveolin (fragment) | Cav1       | 4              | 2         | 0   | 3   | 0   | 0   |
| P84084  | ADP-ribosylation factor 5 | Arf5       | 4              | 2         | 0   | 3   | 0   | 0   |
| O35273  | Polymerase I and transcript release factor | Pfrf       | 3              | 2         | 0   | 3   | 0   | 0   |
| E9Q132  | 60S ribosomal protein L24 | Rpl24      | 3              | 4         | 1   | 2   | 0   | 0   |
| O54890  | Integrin β3 | Itgb3      | 5              | 3         | 1   | 3   | 0   | 0   |
| O84377  | Insulin-like growth factor 2 mRNA-binding protein 1 | Ig2bp1     | 4              | 2         | 0   | 4   | 1   | 1   |
| P61730  | ADP-ribosylation factor 4 | Arf4       | 4              | 2         | 0   | 4   | 0   | 0   |
| Q9CR67  | Transmembrane protein 33 OS | Tmem33     | 3              | 2         | 0   | 4   | 1   | 1   |
| Q5XJ6E  | Ribosomal protein L10a | Rpl10a     | 7              | 6         | 3   | 2   | 0   | 0   |
| Q3THB3  | Heterogeneous nuclear ribonucleoprotein M | Hnrmnm     | 5              | 2         | 1   | 4   | 0   | 0   |
| Q6P5B5  | Fragile X mental retardation syndrome-related protein 2 | Fxr2       | 5              | 3         | 1   | 5   | 2   | 2   |
| D3Z6S1  | Uncharacterized protein | Tmem214     | 3              | 2         | 0   | 2   | 0   | 0   |
| P11152  | Lipoprotein lipase | Lpl        | 3              | 2         | 0   | 2   | 0   | 0   |
| Q6QCR2  | AP-3 complex subunit α1 | Ap3e1      | 3              | 2         | 0   | 2   | 0   | 0   |
| P59999  | Actin-related protein 2/3 complex subunit 4 | Arpc4       | 2              | 3         | 1   | 2   | 0   | 0   |
| P49312  | Heterogeneous nuclear ribonucleoprotein A1 | Hnrnpa1     | 5              | 3         | 1   | 3   | 1   | 1   |
| P61358  | 60S ribosomal protein L27 | Rpl27      | 4              | 3         | 1   | 3   | 1   | 1   |
| Q54734  | Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48-kDa subunit | Ddost       | 3              | 2         | 0   | 3   | 0   | 0   |
| Q07235  | Glia-derived nexin | Serpine2    | 6              | 2         | 0   | 4   | 2   | 2   |
| Q7TNV0  | Protein DEK | Dok        | 5              | 3         | 0   | 2   | 1   | 1   |
| Q922B1  | Aspartate–tRNA ligase, cytoplasmic | Dars       | 4              | 3         | 0   | 2   | 1   | 1   |
| P62320  | Small nuclear ribonucleoprotein Sm D3 | Snrpd3      | 3              | 2         | 1   | 5   | 2   | 2   |
| P15864  | Histone H1.2 | Hist1h1c    | 2              | 2         | 1   | 5   | 2   | 2   |
| Q8RW08 | Epiplatin | Eppk1       | 3              | 2         | 1   | 3   | 1   | 1   |
| Q62Q38  | Cullin-associated NEDD8-dissociated protein 1 | Cand1       | 4              | 2         | 1   | 2   | 1   | 1   |
**Table 2**

**Identification of novel interactors with 14-3-3 proteins**

The information in this table is compared to the data of Johnson et al. (14). There is a total of 56 novel interactors.

| Uniprot      | Description                                      | Gene name | Previously reported to interact with 14-3-3 |
|--------------|--------------------------------------------------|-----------|--------------------------------------------|
| Q8VDD5       | Myosin-9                                         | Myh9      | Yes                                        |
| Q4FK11       | Non-POU-domain-containing, octamer binding protein | Nono      | No                                         |
| E9QZC5       | Plectin                                          | Plect     | No                                         |
| E9QPE8       | Plectin                                          | Plect     | No                                         |
| G5E8B8       | Anastatin                                        | Fna1      | No                                         |
| Q61879       | Myosin-10                                        | Myh10     | Yes                                        |
| P97855       | Ras GTPase-activating protein-binding protein 1   | G3bp1     | Yes                                        |
| P61979       | Heterogeneous nuclear ribonucleoprotein K         | Hnrnpk    | Yes                                        |
| Q9B002       | Interferon-activable protein 202                 | Ifi202    | No                                         |
| R7FAU9       | Filamin, α                                       | Fna       | No                                         |
| Q61033       | Lamina-associated polypeptide 2, isoforms α/γ     | Tmpo      | Yes                                        |
| B2RSN3       | MCG1395                                          | Tubb2b    | Yes                                        |
| Q91VR5       | ATP-dependent RNA helicase DDx1                   | Ddx1      | Yes                                        |
| P49862       | ADP/ATP translocase 1                            | Scl2a4    | Yes                                        |
| P51881       | ADP/ATP translocase 2                            | Scl2a5a   | Yes                                        |
| Q60865       | Caprin-1                                         | Caprin1   | Yes                                        |
| Q8BMK4       | Cytoskeleton-associated protein 4                 | Cap4ψ     | Yes                                        |
| RBHGC1       | Novel protein (2R1D05504RiK)                     | Fapn      | No                                         |
| Q61029       | Lamina-associated polypeptide 2, isoforms β/β′/e/γ| Tmpo      | Yes                                        |
| Q8V1J6       | Splicing factor, proline- and glutamine-rich      | Sfpq      | Yes                                        |
| P67202       | 40S ribosomal protein 54, X isoform              | Rps4x     | Yes                                        |
| Q9TQO5       | DEAF1/D1 (Asp-Glu-Ala-Asp/His) box polypeptide X, X-linked | Ddx3x | No |
| Q4VA29       | MCG140066                                        | 2700060E02Rik | No |
| P14148       | 60S ribosomal protein L7                         | Rpl7      | Yes                                        |
| Q3UMM1       | Tubulin, β6                                      | Tubb6     | No                                         |
| G3UX7        | RNA-binding protein FUS (fragment)               | Fus       | No                                         |
| Q8VEM8       | Phosphate carrier protein, mitochondrial         | Scl2a3a   | Yes                                        |
| EQPE7        | Myosin-11                                        | Myh11     | No                                         |
| A2A547       | Ribosomal protein L19                            | Rpl19     | No                                         |
| P63038       | 60-kDa heat shock protein, mitochondrial          | Hspd1     | Yes                                        |
| D3Z6C3       | Protein Gm10119                                   | Gm10119   | No                                         |
| Q9DB20       | ATP synthase subunit O, mitochondrial             | Atp5o     | Yes                                        |
| O70475       | UDP-glucose 6-dehydrogenase                       | Ugdh      | No                                         |
| A2APD4       | Small nuclear ribonucleoprotein-associated protein| Snrpb     | No                                         |
| O7T039       | Integron β                                       | Itgb5     | No                                         |
| G3UZ1I2      | Heterogeneous nuclear ribonucleoprotein Q         | Syncrp    | No                                         |
| D3Z6G8       | Fragile X mental retardation protein 1 homolog   | Fmr1      | No                                         |
| O35841       | Apoptosis inhibitor 5                            | Ap5       | No                                         |
| A4FUS1       | MCG123443                                        | Rps16     | No                                         |
| Q3TLHA4-5    | Isoform 5 of protein PRRC2C                      | Prrc2c    | No                                         |
| P14869       | 60S acicid ribosomal protein P0                   | Rplp0     | Yes                                        |
| Q0QZY1       | Eukaryotic translation initiation factor 3 subunit L | Eif3l   | No                                         |
| P35922       | Fragile X mental retardation protein 1 homolog   | Fmr1      | Yes                                        |
| Q03265       | ATP synthase subunit α, mitochondrial             | Atp5a1    | Yes                                        |
| P63017       | Heat shock cognate 71-kDa protein                 | Hspd1     | Yes                                        |
| P21981       | Protein-glutamy-glutamytransferase 2             | Tgm2      | Yes                                        |
| Q80UM7       | Mannosyl-oligosaccharide glucosidase              | Manb      | No                                         |
| P26369       | Splicing factor U2AF 65-kDa subunit               | U2a2t     | No                                         |
| A2AJM8       | MCG7378                                          | Scc61b    | No                                         |
| P62242       | 40S ribosomal protein S8                          | Rps8      | Yes                                        |
| P54823       | DEAF1/D1 (Asp-Glu-Ala-Asp/His) box polypeptide 6  | Ddx6      | Yes                                        |
| Q3TML6       | Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked | Eif2s3x | No |
| P26041       | Moesin                                           | Msn       | Yes                                        |
| P62983       | Ubiquitin-40S ribosomal protein S27a              | Rps27a    | Yes                                        |
| P52480       | Pyruvate kinase isozymes M1/M2                    | Pkm2      | Yes                                        |
| Q5SSL0       | Ewing sarcoma breakpoint region 1                | Ewsr1     | No                                         |
| EQQ7H5       | Uncharacterized protein                           | Gm8991    | No                                         |
| Q9C2QN       | ATP synthase y chain                             | Atp5c1    | No                                         |
| A2AMW0       | Capping protein (actin filament) muscle Z-line, β | Cap2b     | No                                         |
| P08121       | Collagen α-1(Ill) chain                          | Col3a1    | Yes                                        |
| P11087-2     | Isoform 2 of collagen α-1(I) chain               | Col1a1    | Yes                                        |
| Q9Z2X1-2     | Isoform 2 of heterogeneous nuclear ribonucleoprotein F | Hnrnpf | Yes |
| P11499       | Heat shock protein HSP 90αβ                       | Hsp90b1   | Yes                                        |
| P28301       | Protein-lysine 6-oxidase                         | Lux       | Yes                                        |
| Q8VCQ8       | Caldesmon 1                                      | Cald1     | No                                         |
| P27659       | 60S ribosomal protein L3                          | Rpl3      | Yes                                        |
| O35737       | Heterogeneous nuclear ribonucleoprotein H         | Hnrphp1   | Yes                                        |
| A2ACG7       | Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 2 | Rps2n | No |
| Q3TVI8       | Pre-B-cell leukemia transcription factor-interacting protein 1 | Pbxip1 | No |
| FQCC00       | Protein Taf15 (fragment)                         | Taf15     | No                                         |
| O98869-3     | Isoform 3 of Heterogeneous nuclear ribonucleoproteins A2/B1 | Hnrnp2b1 | Yes |
| O98853-2     | Isoform 2 of THO complex subunit 4               | Alyref    | Yes                                        |
| O98573-2     | Isoform short of galectin-9                      | Lgaβg9    | Yes                                        |
| Q6S48E8      | Ribosomal protein L4                             | Rpl4      | No                                         |
| R1ARA3       | 60S ribosomal protein L26 (Fragment)             | Rps26     | No                                         |
| O35129       | Prohibitin-2                                      | Phb2      | Yes                                        |
| D3YTQ9       | 40S ribosomal protein S15                        | Rps15     | No                                         |
| Q6ZW3X6      | Eukaryotic translation initiation factor 2 subunit 1 | Eif2s1 | Yes |

Determining adipogenic factors in the 14-3-3-3ζ interactome

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protein transport, and nucleic acid transport was detected using gene ontology to define their biological processes (17) (Table 3). Thus, these proteomic data demonstrate the dynamic nature of the 14-3-3 protein interactome and suggest that 14-3-3 may regulate multiple processes, such as RNA processing, during adipocyte differentiation.

Identification of known regulators of adipocyte differentiation in the 14-3-3ζ interactome

We were able to detect proteins with known and purported roles in adipogenesis, such as Pthr/Cavin1, Phb2, Fragile-X mental retardation protein-1 (Fmr1), and Rpn2, through our proteomic analysis of the 14-3-3ζ interactome (Table 1 and Fig. 1D) (18–23). These proteins do not have any purported roles in RNA splicing. Using siRNA-mediated knockdown approaches, we examined their roles in adipocyte differentiation, as assessed by Oil Red-O incorporation and RNA splicing. Using gene ontology to define their biological processes (17) (Table 3). Thus, these proteomic data demonstrate the dynamic nature of the 14-3-3ζ protein interactome and suggest that 14-3-3ζ may regulate multiple processes, such as RNA processing, during adipocyte differentiation.

Table 2—continued

| Uniprot | Description                                                  | Gene name     | Previously reported to interact with 14-3-3ζ |
|---------|--------------------------------------------------------------|---------------|---------------------------------------------|
| D3Z3R1  | 60S ribosomal protein L36                                     | Gm5745        | No                                          |
| P17427  | AP-2 complex subunit σ2                                      | Apa2a         | No                                          |
| P56480  | ATP synthase subunit β, mitochondrial                         | Atp5b         | Yes                                         |
| Q8CRM2  | Aspartate-β-hydroxylase                                       | Asph          | No                                          |
| Q6NV9   | Cleavage and polyadenylation specificity factor subunit 6     | Cps6f         | Yes                                         |
| Q25Q90  | Nucleophosmin                                                | Npm1          | No                                          |
| Q6AA9   | Constitutive coactivator of PPARγ-like protein 1              | Fam120a       | Yes                                         |
| P14576  | Signal recognition particle 54-kDa protein                   | Srp54         | No                                          |
| P63087  | Serine/threonine-protein phosphatase PP1γ catalytic subunit   | Pp1cc         | Yes                                         |
| P80315  | T-complex protease 1 subunit δ                               | Cc4           | Yes                                         |
| P62960  | Nuclease-sensitive element-binding protein 1                  | Ybx1          | No                                          |
| P97376  | Protein FRG1                                                 | Frg1          | No                                          |
| Q3U4Z7  | High-density lipoprotein-binding protein, isoform CRA_d       | Hldbp         | No                                          |
| B2RTB6  | MCG17262                                                    | Pdap1         | No                                          |
| P60335  | Poly(rC)-binding protein 1                                    | Pcbp1         | Yes                                         |
| P47911  | 60S ribosomal protein L6                                      | Rpl6          | Yes                                         |
| Q61990-2| Isomorph 2 of Poly(rC)-binding protein 2                      | Pcbp2         | Yes                                         |
| P62367  | 60S ribosomal protein S23                                     | Rps23         | Yes                                         |
| D3Z148  | Caveolin (Fragment)                                          | Cav1          | No                                          |
| P84084  | ADP-ribosylation factor 5                                     | Arf5          | Yes                                         |
| O54724  | Polymerase I and transcript release factor                    | Ptf            | Yes                                         |
| E9Q132  | 60S ribosomal protein L24                                     | Rpl24         | No                                          |
| O54890  | Integrin β-3                                                | Itgb3         | No                                          |
| O88477  | Insulin-like growth factor 2 mRNA-binding protein 1           | Igl2bp1       | Yes                                         |
| P61750  | ADP-riboseylation factor 4                                    | Arf4          | Yes                                         |
| Q9CBR7  | Transmembrane protein 33 OS                                   | Tmem33        | Yes                                         |
| Q5XF6   | Ribosomal protein L10a                                       | Rpl10a        | No                                          |
| Q3THB3  | Heterogeneous nuclear ribonucleoprotein M                     | Hnrnpm        | No                                          |
| Q6P5B5  | Fragile X mental retardation syndrome-related protein 2       | Fxr2          | No                                          |
| D3Z6S1  | Uncharacterized protein                                       | Tmem214       | No                                          |
| P11152  | Lipoprotein lipase                                           | Lpl            | Yes                                         |
| Q9DRC2  | AP-3 complex subunit α1                                      | Apa3α         | No                                          |
| P59999  | Actin-related protein 2/3 complex subunit 4                   | Arpc3         | Yes                                         |
| P49312  | Heterogeneous nuclear ribonucleoprotein A                     | Hnrnpa1       | Yes                                         |
| P61358  | 60S ribosomal protein L27                                     | Rpl27         | Yes                                         |
| O54734  | Dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48-kDa subunit | Ddost       | Yes                                         |
| Q07235  | Gila-derived nexin                                           | Serpine2      | No                                          |
| Q9228B  | Protein DEK                                                  | Dek            | Yes                                         |
| Q7TV0   | Aspartate–tRNA ligase, cytoplasmic                            | Dars          | No                                          |
| P62320  | Small nuclear ribonucleoprotein 5/3 D3                       | Snrdp3        | Yes                                         |
| P15864  | Histone H1.2                                                | Hist1h1c      | Yes                                         |
| Q8RRW0  | Epipakin                                                     | Eppk1         | No                                          |
| Q62Q38  | Collin-associated NEDD8-dissociated protein 1                 | Cand1         | Yes                                         |

Requirement of RNA processing during adipogenesis

Because enrichments in RNA splicing proteins were detected in the 14-3-3ζ interactome during differentiation (Table 1), it suggested that 14-3-3ζ could influence pre-mRNA processing during adipogenesis. Splicing is mediated by the spliceosome complex, which removes intronic regions from pre-mRNA (constitutive) or facilitates alternative splicing of mRNA at regulatory regions enriched with splicing factors (24). Initially, the spliceosome inhibitor, madrasin, was used to examine the requirement of the spliceosome during adipocyte differentiation (25), and inhibition of the spliceosome blocked adipogenesis (Fig. 3, A and B). Pre-mRNA of the canonical adipogenic gene, Pparg, undergoes alternative splicing to yield Pparg1 and Pparg2 mRNAs, which are further translated into Pparγ isoforms, Pparγ1 and Pparγ2 (26–29). To examine whether the spliceosome is involved in processing of Pparg mRNA, we utilized quantitative PCR to measure mRNA levels of Pparg1, Pparg2, and a novel Pparg1 variant, Pparg1sv (29). Spliceosome inhibition significantly reduced the expression of the Pparg2 and Pparg1sv mRNA (Fig. 3B). Thus, the activity of the spliceosome is required for adipocyte differentiation.

Within the adipocyte differentiation-associated 14-3-3ζ interactome, U2AF, a component of the spliceosome, was
**Table 3**

Gene ontology classification of proteomic hits by biological process

| Annotation cluster 1 | mRNA processing | 2.80E-12 | 1.18E-09 | 4.33E-09 |
|----------------------|----------------|----------|----------|----------|
| GO:0006397           | RNA splicing   | 8.22E-12 | 2.31E-09 | 1.27E-08 |
| GO:0006396           | mRNA metabolic process | 2.70E-11 | 5.71E-09 | 4.18E-08 |
| GO:0006395           | RNA processing | 1.09E-09 | 1.84E-07 | 1.68E-06 |

| Annotation cluster 2 | Macromolecular complex assembly | 1.23E-04 | 0.01719154 | 0.19018503 |
|----------------------|--------------------------------|----------|-------------|------------|
| GO:00065003          | Protein complex assembly       | 1.87E-04 | 0.01956958 | 0.28880909 |
| GO:00070271          | Protein complex biogenesis     | 1.87E-04 | 0.01956958 | 0.28880909 |
| GO:0043933           | Macromolecular complex subunit organization | 2.40E-04 | 0.02229116 | 0.37052613 |

| Annotation cluster 3 | Nucleobase, nucleotide, and nucleic acid transport | 1.65E-04 | 0.01976438 | 0.25533903 |
|----------------------|---------------------------------------------------|----------|-------------|------------|
| GO:00059313          | Establishment of RNA localization                 | 0.001160158 | 0.09343294 | 1.7786077 |
| GO:00059653          | RNA transport                                     | 0.001160158 | 0.09343294 | 1.7786077 |
| GO:00059657          | Nucleic acid transport                            | 0.001160158 | 0.09343294 | 1.7786077 |
| GO:0006403           | RNA localization                                  | 0.001227278 | 0.09002225 | 1.88067318 |

| Annotation cluster 4 | ATP synthesis-coupled proton transport | 0.002165467 | 0.13143078 | 3.29598278 |
|----------------------|---------------------------------------|------------|-------------|------------|
| GO:0005986           | Energy-coupled proton transport, down electrochemical gradient | 0.002165467 | 0.13143078 | 3.29598278 |
| GO:00034220          | Ion transmembrane transport            | 0.00311983 | 0.20818502 | 6.20587966 |
| GO:0005992           | Proton transport                       | 0.005711473 | 0.26103289 | 8.4769156 |
| GO:0006818           | Hydrogen transport                     | 0.00623853 | 0.24696352 | 8.9124057 |
| GO:0006119           | Oxidative phosphorylation              | 0.007021577 | 0.25748192 | 10.3215008 |
| GO:0006754           | ATP biosynthetic process                | 0.019701699 | 0.53433002 | 2.6812786 |
| GO:00046034          | ATP metabolic process                   | 0.025117659 | 0.59166666 | 3.25160709 |
| GO:00092011          | Ribonucleoside triphosphate biosynthetic process | 0.027339487 | 0.59377318 | 3.8509638 |
| GO:00092061          | Purine ribonucleoside triphosphate biosynthetic process | 0.027339487 | 0.59377318 | 3.8509638 |
| GO:0009143           | Nucleoside triphosphate biosynthetic process | 0.02886954 | 0.5741125 | 3.6812786 |
| GO:0009142           | Nucleoside triphosphate metabolic process | 0.033742375 | 0.58749696 | 4.17971708 |
| GO:0009123           | Ribonucleoside triphosphate metabolic process | 0.034593574 | 0.58312761 | 4.19751399 |
| GO:0006091           | Generation of precursor metabolites and energy | 0.03610094 | 0.58839461 | 4.35977313 |
| GO:0009144           | Purine nucleoside triphosphate metabolic process | 0.038109124 | 0.58832535 | 4.5173304 |
| GO:0009152           | Purine ribonucleoside biosynthetic process    | 0.039015563 | 0.58726975 | 4.59509181 |
| GO:0005085           | Transmembrane transport                 | 0.042081945 | 0.60604395 | 4.8566327 |
| GO:0009206           | Ribonucleoside biosynthetic process       | 0.042750615 | 0.59362044 | 4.91090183 |
| GO:0009141           | Nucleoside triphosphate metabolic process | 0.046658895 | 0.60897427 | 5.2282486 |

| Annotation cluster 5 | Blood vessel development                   | 0.028163983 | 0.57774561 | 3.7041482 |
|----------------------|-------------------------------------------|------------|-------------|------------|
| GO:0001568           | Vasculature development                   | 0.030823763 | 0.58598984 | 3.8715132 |

Detected (Table 1) (24). This suggests that 14-3-3ζ may influence the activity of the spliceosome during adipogenesis through its interactions. Focusing on Ppar, we found that siRNA-mediated depletion of 14-3-3ζ significantly blocked the increase in total Ppar mRNA levels and attenuated the production of Ppar1, Ppar2, and Ppar1sv splice variants (Fig. 3C). Furthermore, significantly decreased abundance of Ppar1 and Ppar2 protein was detected in 14-3-3ζ-depleted cells (Fig. 3, D and E). When taken together, these findings demonstrate the importance of the spliceosome and suggest indirect actions of 14-3-3ζ in the splicing of key adipogenic mRNAs.

**Regulation of mRNA processing by 14-3-3ζ during adipocyte differentiation**

To gain a better understanding of the global effects of 14-3-3ζ depletion on mRNA splicing, we utilized our previous transcriptomic analysis from control and 14-3-3ζ-depleted 3T3-L1 cells undergoing differentiation (4). Differential exon usage (DEXSeq) was used as a surrogate measure of alternative splicing of mRNA (Fig. 4A) (30). Any changes in splice variant levels were not due to global effects of 14-3-3ζ depletion on RNA transcription because no gross differences in the incorporation of a uracil analog were detected (Fig. 4B). At 24 and 48 h post-differentiation, 163 and 172 unique genes, respectively, were found to undergo differential exon usage (Fig. 4C). Gene ontology analysis revealed that at each time point, distinct groups of genes were alternatively spliced (Table 4). The use of this approach to detect genes with DEXSeq was validated by the ability to detect alternative exon usage in Ppar after 48 h of differentiation (Fig. S2) (28). The effect of 14-3-3ζ depletion was assessed at each time point, and 78, 37, and 36 genes were alternatively spliced (Table 4). The use of this approach to detect genes with DEXSeq was validated by the ability to detect alternative exon usage in Ppar after 48 h of differentiation (Fig. S2) (28).
assembly (GO:0065003, \( p = 3.44 \times 10^{-3} \)), macromolecular complex subunit organization (GO:0043933, \( p = 7.56 \times 10^{-4} \)), and regulation of biological quality (GO:0065008, \( p = 9.51 \times 10^{-3} \)) be detected by gene ontology analysis. Collectively, these data demonstrate that adipogenesis promotes the alternative splicing of genes, and this process can be influenced by 14-3-3\( ^{\alpha} \).

Requirement of RNA-splicing factors in adipocyte differentiation

14-3-3\( ^{\alpha} \) is not a bona fide splicing factor, and it is likely that specific RNA-splicing factors within its interactome are responsible for the observed effects on differential exon usage (Fig. 4D). Transient transfection of siRNA in 3T3-L1 pre-adipocytes against eight splicing factors identified in our proteomic analysis of the 14-3-3\( ^{\alpha} \) interactome (Table 1) was performed to examine their roles in 3T3-L1 adipogenesis (Fig. 5 and Fig. S1B). They were chosen by the number of connections exhibited within each cluster of proteins (Fig. 1D) (17). Transcript levels of the chosen splicing factors, as determined by RNA-Seq, were generally unaffected by knockdown of 14-3-3\( ^{\alpha} \); however, some splicing factors were influenced by differentiation (Fig. S3) (GEO accession code GSE60745). Knockdown of Ddx6, Sfpq, Hnrpf, or Hnrpk was sufficient to impair 3T3-L1 differentiation, as assessed by Oil Red-O incorporation and total \( \text{Pparg} \) mRNA expression (Fig. 5 and Fig. S1B). Closely related proteins with similar roles, such as Ddx1, Nono, Hnrpm, and Syncrip (Hnrpq) were not required for 3T3-L1 adipogenesis (Fig. 5 and Fig. S1B).

To further explore the role of splicing factors within the 14-3-3\( ^{\alpha} \) interactome, we examined the impact of their depletion on \( \text{Pparg} \) mRNA splice variant formation and Ppar\( ^{\gamma} \) protein abundance. In undifferentiated cells, knockdown of Hnrnpf and Ddx6 had effects on the levels of \( \text{Pparg1} \) or \( \text{Pparg2} \) mRNA (Fig. 6A). However, in differentiating 3T3-L1 cells, only knockdown of \( \text{Hnrrpf} \) and Sfpq were found to significantly reduce \( \text{Pparg2} \) or \( \text{Pparg1sv} \) mRNA levels (Fig. 6A). Ppar\( ^{\gamma1} \) and Ppar\( ^{\gamma2} \) protein levels differed from what was observed with the pattern of \( \text{Pparg} \) mRNA variants. Ddx6-depleted cells exhibited significantly decreased Ppar\( ^{\gamma1} \) abundance, whereas all siRNA-transfected cells significantly reduced Ppar\( ^{\gamma2} \) (Fig. 6, B and C). Another adipogenic gene that undergoes alternative splicing is \( \text{Lpin1} \). This results in the generation of splice variants, Lipin-1\( ^{\alpha} \) and Lipin-1\( ^{\beta} \), which have differen-
Determining adipogenic factors in the 14-3-3ζ interactome

Figure 3. Inhibition of the spliceosome or depletion of 14-3-3ζ prevents the alternative splicing of Pparg mRNA. A, 3T3-L1 cells were incubated with 1 or 10 μM madrasin in the presence of the adipogenic differentiation mixture (MDI), followed by differentiation for 7 days. Adipogenesis was assessed by Oil Red-O incorporation (representative of n = 5 independent experiments). B, RNA was isolated from madrasin-treated cells induced to differentiate for 48 h, and quantitative PCR was used to measure Pparg splice variants (n = 5 per group; *, p < 0.05 when compared with undifferentiated cells; #, p < 0.05 when compared with 0 μM madrasin, differentiated cells; bar graphs represent means ± S.D.). C, 3T3-L1 cells were transfected with siRNA against 14-3-3ζ or siCon and differentiated for 48 h, followed by isolation of total RNA to measure Pparg splice variants by quantitative PCR (n = 4 per group; *, p < 0.05 when compared with undifferentiated siCon-transfected cells; #, p < 0.05 when compared with differentiated, siCon-transfected cells; bar graphs represent means ± S.D.). D and E, 3T3-L1 cells were transfected with siRNA against 14-3-3ζ or siCon and differentiated for up to 7 days, followed by isolation of protein to measure Pparg isoforms by immunoblotting (D). Protein abundance for each Pparg isoform was measured by densitometry (E) (n = 4 per group; *, p < 0.05 when compared with undifferentiated siCon-transfected cells; #, p < 0.05 when compared with differentiated, siCon-transfected cells; bar graphs represent means ± S.D.).

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In the present study, affinity proteomics was used to determine how adipogenesis influences the interactome of 14-3-3ζ. Surprisingly, the interactome was dynamic, because differentiation altered the landscape of proteins that interact with 14-3-3ζ. This approach permitted the identification of processes that may be regulated by 14-3-3ζ during adipocyte differentiation and led to the discovery of novel adipogenic factors within the 14-3-3ζ interactome that are required for adipocyte differentiation. Namely, an enrichment of proteins associated with RNA processing and splicing was detected, and the novel contributions of RNA splicing factors, such as Hnrpf, Ddx6, and Sfpq, in adipogenesis were identified. Future in-depth analysis of all 14-3-3ζ-interacting partners may reveal novel factors and pathways that facilitate adipocyte differentiation and may aid in the development of approaches to control adipogenesis as a means to treat obesity.

We previously identified an essential function of the hedgehog signaling effector Gli3 in 14-3-3ζ-regulated adipocyte differentiation (4). However, because of the large, diverse interactome of 14-3-3 proteins (10, 14), we hypothesized that it is unlikely that one protein would be solely responsible for 14-3-3ζ-mediated adipogenesis. It is known that the interactomes of 14-3-3 proteins are dynamic and change in response to various stimuli (8, 10–15). The functional significance of such changes in the interactome is not clear, but it suggests that 14-3-3 proteins may regulate biological processes critical for adipocyte development through their interactions. Using a gene ontology-based approach, we found that the 14-3-3ζ interactome is enriched with proteins involved in RNA binding and splicing during differentiation and identified its contribution to the alternative splicing of mRNAs. Because over 100 proteins were found to be unique to the 14-3-3ζ interactome during adipocyte differentiation roles on adipogenesis (32). To examine the effect of depletion of 14-3-3ζ, Hnrpf, Ddx6, Hnrpk, and Sfpq on Lpin1 splicing, 3T3-L1 cells were transiently transfected with siRNA, followed by the induction of differentiation. Gene silencing of 14-3-3ζ and led to the discovery of novel adipogenic factors within the 14-3-3ζ interactome that are required for adipocyte differentiation. Namely, an enrichment of proteins associated with RNA processing and splicing was detected, and the novel contributions of RNA splicing factors, such as Hnrpf, Ddx6, and Sfpq, in adipogenesis were identified. Future in-depth analysis of all 14-3-3ζ-interacting partners may reveal novel factors and pathways that facilitate adipocyte differentiation and may aid in the development of approaches to control adipogenesis as a means to treat obesity.

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differentiation, it suggests that 14-3-3 may also regulate other cellular processes required for adipocyte development. For example, we detected an interaction of 14-3-3 with the mitochondrial regulator, Phb2 (Prohibitin-2) (Table 1), which others have shown to be essential for the expansion of mitochondria mass and mitochondrial function during adipogenesis (18, 19). Further in-depth studies are required to assess whether 14-3-3 has regulatory roles in mitochondrial dynamics, but when taken together, it demonstrates the possibility of examining the individual contributions of interacting partners to elucidate key biological processes required for adipocyte differentiation.

The spliceosome is responsible for constitutive and alternative splicing of mRNA, whereby intronic regions of mRNA are removed or sections of mRNA enriched with splicing factors at regulatory elements are removed, respectively (24). Various splicing factors have been found to be important for adipogenesis (33, 34), but no studies have directly tested the role of the spliceosome in this process. To this end, we found that inhibition of the spliceosome with madrasin was sufficient to block 3T3-L1 adipogenesis and prevent the generation of various Pparg splice variants. In our analysis of the 14-3-3 interactome, we detected the interaction of 14-3-3 with U2AF, a component of the spliceosome. 14-3-3-associated interactions can modulate the activity of interacting partners (4, 35), suggesting that 14-3-3 could influence the activity of the spliceosome and interfere with processes associated with constitutive or alternative splicing. Although the approaches used in the present study were unable to measure effects on constitutive splicing, we were able to detect changes in alternative splicing at the level of Pparg and from whole transcriptome data (4). The exact mechanisms by which 14-3-3 is able to influence alternative splicing is not known, and 14-3-3 is likely dependent on the specific splicing factors that it interacts with during differentiation.

Through the use of a functional siRNA screen, we identified novel adipogenic roles of various RNA-splicing factors involved in alternative splicing. These include Hnrfp, Hnrfk, Ddx6, and Sfpq. Sfpq belongs to the DHBS (Drosophila behavior/human splicing) protein family and is required for transcriptional regulation (36, 37). Although a recent study by Wang et al. (38) found no effect of forced overexpression of Nono and Sfpq on adipogenesis, we report that Sfpq depletion impairs adipocyte differentiation. DHBS proteins may exhibit redundant, com-
Determining adipogenic factors in the 14-3-3ζ interactome

Table 4
Analysis of common and unique genes during the first 48 h of 3T3-L1 adipogenesis

| Comparison | GO biological process complete | Mus musculus: REFLIST (22221) | upload_1 |
|------------|--------------------------------|-------------------------------|---------|
|            |                                | 230                           | Expected| Over/under | Fold enrichment | p value |
| Common to all time points | Xenobiatic glucuronidation (GO:0052697) | 9 | 9 | 0.09 | + | 96.61 | 9.70E-12 |
|            | Flavonoid glucuronidation (GO:0052696) | 9 | 9 | 0.09 | + | 96.61 | 9.70E-12 |
|            | Flavonoid metabolic process (GO:0009812) | 11 | 9 | 0.11 | + | 79.05 | 5.80E-11 |
|            | Cellular glucuronidation (GO:002695) | 12 | 9 | 0.12 | + | 72.46 | 1.26E-10 |
|            | Uronic acid metabolic process (GO:0006063) | 13 | 9 | 0.13 | + | 66.89 | 2.56E-10 |
|            | Glucuronate metabolic process (GO:0019583) | 13 | 9 | 0.13 | + | 66.89 | 2.56E-10 |
|            | Cellular response to xenobiotic stimulus (GO:0071466) | 50 | 10 | 0.52 | + | 19.32 | 1.68E-06 |
|            | Xenobiotic metabolic process (GO:0006805) | 46 | 9 | 0.48 | + | 18.9 | 1.66E-05 |
|            | Response to xenobiotic stimulus (GO:0009410) | 56 | 10 | 0.58 | + | 17.25 | 4.95E-06 |
|            | Monosaccharide metabolic process (GO:0005996) | 152 | 12 | 1.57 | + | 7.63 | 7.67E-04 |
|            | Single-organism carbohydrate metabolic process (GO:0044723) | 301 | 15 | 3.12 | + | 4.81 | 6.68E-03 |
| Cell adhesion (GO:0007153) | 754 | 35 | 7.8 | + | 4.48 | 1.34E-09 |
| Biological adhesion (GO:0022610) | 764 | 35 | 7.9 | + | 4.43 | 1.39E-09 |
| Carbohydrate metabolic process (GO:0005975) | 385 | 17 | 3.98 | + | 4.27 | 3.63E-06 |
| Cell–cell signaling (GO:0007267) | 792 | 34 | 8.2 | + | 4.15 | 2.62E-08 |
| Nervous system development (GO:0007399) | 2086 | 50 | 21.59 | + | 2.32 | 1.40E-04 |
| Multicellular organism development (GO:0002275) | 4498 | 76 | 46.56 | + | 1.63 | 3.16E-02 |
| Single-organism developmental process (GO:0044767) | 5073 | 85 | 52.51 | + | 1.62 | 8.08E-03 |
| Developmental process (GO:0032502) | 5112 | 85 | 52.91 | + | 1.61 | 1.12E-02 |
| Primary metabolic process (GO:0044238) | 7337 | 113 | 75.94 | + | 1.49 | 2.66E-03 |
| Cellular metabolic process (GO:0044237) | 7109 | 109 | 73.58 | + | 1.48 | 7.04E-03 |
| Organic substance metabolic process (GO:0071704) | 7692 | 117 | 79.62 | + | 1.47 | 2.59E-03 |
| Metabolic process (GO:0006805) | 8159 | 122 | 84.45 | + | 1.44 | 2.85E-03 |
| Single-organism cellular process (GO:0044763) | 8646 | 129 | 89.49 | + | 1.44 | 8.63E-04 |
| Cellular process (GO:0009987) | 13906 | 182 | 141.76 | + | 1.28 | 8.17E-05 |
| G-protein–coupled receptor signaling pathway (GO:0007186) | 1803 | 3 | 18.66 | - | < 0.2 | 4.79E-02 |
| Unique to 24 h | Negative regulation of response to cytokine stimulus (GO:0006876) | 43 | 5 | 0.24 | + | 21.01 | 4.10E-02 |
| DNA repair (GO:0006281) | 400 | 12 | 2.21 | + | 5.42 | 2.22E-02 |
| Cellular response to DNA damage stimulus (GO:0006974) | 618 | 15 | 3.42 | + | 4.38 | 1.60E-02 |
| Cellular macromolecular complex assembly (GO:0034622) | 624 | 15 | 3.45 | + | 4.34 | 1.80E-02 |
| Cellular macromolecular metabolic process (GO:0044260) | 5396 | 60 | 29.87 | + | 2.01 | 2.97E-05 |
| Macromolecule metabolic process (GO:0043179) | 6116 | 66 | 33.84 | + | 1.95 | 7.53E-06 |
| Cellular nitrogen compound metabolic process (GO:0034641) | 4081 | 44 | 22.59 | + | 1.95 | 3.18E-02 |
| Primary metabolic process (GO:0044238) | 7337 | 78 | 40.61 | + | 1.92 | 4.49E-08 |
| Organic substance metabolic process (GO:0071704) | 7692 | 80 | 42.58 | + | 1.88 | 5.30E-08 |
| Nitrogen compound metabolic process (GO:0006807) | 6786 | 69 | 37.56 | + | 1.84 | 3.16E-05 |
| Cellular metabolic process (GO:0044237) | 7109 | 72 | 39.35 | + | 1.83 | 1.07E-05 |
| Metabolic process (GO:0006805) | 8139 | 80 | 45.16 | + | 1.77 | 1.51E-06 |
| Cellular process (GO:0009987) | 13906 | 101 | 75.81 | + | 1.33 | 6.09E-03 |
| Unique to 48 h | Positive regulation of molecular function (GO:0044093) | 1317 | 23 | 7.88 | + | 2.92 | 3.07E-02 |

penisatory functions (39), but given that only Sfpq depletion impaired 3T3-L1 adipogenesis, it suggests specific protein–protein or protein–nucleic acid interactions occur may with each DHBS member in the context of differentiation (37). We were also able to detect novel adiogenic roles of Hnrps and Hnrpκ, members of the heterogeneous nuclear ribonucleoproteins (Hnrps), which facilitate mRNA splicing (40, 41). Alternative splicing of mRNA is critical for maintaining genetic diversity and cell identity, in addition to the expression of key factors required for differentiation (42, 43). Specific to adipogenesis, differential promoter usage and alternative splicing are required for the expression of the canonical adiogenic transcription factor Ppary (26–28, 43). Other regulatory factors are also formed from alternative splicing, including nCOR1 and Lipin1 (33, 43, 44). In the present study, we identified distinct roles of each splicing factor in generating Pparg mRNA splice variants. Not all tested splicing factors had significant effects on Pparg mRNA or total Ppary protein levels, despite being required for differentiation. It is likely that they control the splicing of other genes, such as Lipin1, that are required for adipogenesis. Thus, future studies aimed at elucidating the generation of splice variants by each splicing factor would greatly increase the current knowledge of key factors required for adipocyte development.

Protein abundance of 14-3-3ζ and other isomers is increased in visceral adipose tissue from obese individuals (45, 46), and we have previously reported that systemic overexpression of 14-3-3ζ in mice is sufficient to potentiate weight gain and fat mass in mice fed a high-fat diet (4). With respect to the pancreatic β-cell, single-cell transcriptomic analysis revealed higher mRNA expression of YWHAZ in β-cells from subjects with type 2 diabetes (47), and we have found that systemic overexpression of 14-3-3ζ was sufficient to reduce β-cell secretory function in mice (9). The exact mechanisms of how changes in 14-3-3ζ function affect the development of obesity or β-cell dysfunction are not known, but in-depth examination of the interactome, in addition to how 14-3-3ζ may influence the generation of splice variants, in the context of both conditions may yield novel biological insight as to how 14-3-3ζ influences the development of either disease. This approach has already been useful in understanding how changes in 14-3-3ζ or 14-3-3σ expression promote the development of various forms of cancer and the identification of novel therapeutic targets (48, 49).
In conclusion, this study provides compelling evidence demonstrating the usefulness of elucidating the interactome of 14-3-3\textsuperscript{H9256} as a means to identify novel factors required for adipogenesis. Additionally, a systematic investigation of interacting partners may also provide insight as to which physiological processes are essential for 14-3-3\textsuperscript{H9256}–mediated adipocyte differentiation. Lastly, deciphering how various disease states influence the interactome of 14-3-3 proteins may also aid in the discovery of novel therapeutic targets for the treatment of chronic diseases, such as obesity and type 2 diabetes.

**Experimental procedures**

**Generation of TAP–14-3-3\textsuperscript{H9256} MEFs and cell culture**

All animal procedures were approved and conducted in accordance with guidelines set by the University of British Columbia Animal Care Council. Embryos at embryonic day 13.5 were harvested from pregnant transgenic mice overexpressing a TAP-epitope–tagged 14-3-3\textsuperscript{H9256} molecule (4), and MEFs were generated according to established protocols. 3T3-L1 cells (between passages 11 and 17) and MEFs were maintained in 25 mM glucose DMEM, supplemented with 10% newborn calf serum or fetal bovine serum, respectively, and 1% penicillin/streptomycin (ThermoFisher Scientific, Waltham, MA). Differentiation of MEFs and 3T3-L1 cells was induced with DMEM, supplemented with 10% fetal bovine serum, 172 nM insulin, 500 μM isobutylmethylxanthine, and 500 nM dexamethasone (MDI). Differentiation medium for MEFs was further supplemented with rosiglitazone (Sigma–Aldrich). Following incubation with differentiation medium for 2 days, the medium was replaced every 2 days with 25 mM glucose DMEM, supplemented with 10% fetal bovine serum and 172 nM insulin. Differentiation was assessed by Oil Red-O incorporation (Sigma–Aldrich), as previously described (4). To inhibit pre-mRNA processing, 3T3-L1 cells were incubated with the spliceosome inhibitor, madrasin (Sigma–Aldrich), during incubation with differentiation medium (25).

**Mass spectrometry**

Equal amounts of cell lysates from undifferentiated and differentiated TAP–14-3-3\textsuperscript{H9256} MEFs were subjected to an overnight incubation with IgG coupled to protein G beads (ThermoFisher Scientific) in radioimmune precipitation assay buffer. Bound proteins from each pulldown were eluted with 1/1000 SDS sample buffer without reducing agents and separated by SDS-PAGE prior to in-gel digestion (50). For each sample, peptides from three fractions (<50 kDa, >50 kDa, and IgG bands) were then purified on C-18 stage tips (51) and analyzed using a LTQ-Orbitrap Velos (ThermoFisher Scientific) as previ-
The data were processed with Proteome Discoverer v. 1.2 (ThermoFisher Scientific) followed by a Mascot analysis (2.3.0; Matrix Science, Boston, MA) using the Uniprot-Swissprot_mouse protein database (05302013, 540261 protein sequences). Only proteins with at least two peptides (false positive discovery rate $<0.05$) in one of the two samples were retained. Two independent pulldowns were used for MS and proteomic analysis. The proteins were analyzed with String-Db to categorize them based on their biological processes (17).

**siRNA-mediated knockdown, RNA isolation, and quantitative PCR**

3T3-L1 cells were seeded at a density of 75,000/well prior to transfection with control siRNA or two independent target-specific Silencer Select siRNAs (ThermoFisher Scientific). Transfection was performed using Lipofectamine RNAmax, as per manufacturer instructions (ThermoFisher Scientific), at a final siRNA concentration of 20 $\mu$M per well. Total RNA was isolated from 3T3-L1 adipocytes or MEFs with the RNEasy kit (Qiagen, Mississauga, Canada). Synthesis of cDNA was performed with the qScript cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, MD), and transcript levels were measured with SYBR green chemistry or TaqMan assays on a QuantStudio 6-flex real-time PCR system (ThermoFisher Scientific). All data were normalized to $Hprt$ by the $2^{-\Delta\Delta C_t}$ method, as previously described (4, 9, 35). All sequences of primers, TaqMan assays, and siRNAs can be found in Table S1. Confirmation that 14-3-3 knockdown had no effect of global RNA transcription was determined using the Click-iT RNA Alexa 488 imaging kit, as per the manufacturer’s instructions (ThermoFisher Scientific).

**Analysis of differential exon usage**

To understand how adipocyte differentiation and depletion of 14-3-3 knockdown affected alternative splicing of mRNA, differential exon usage via DEXSeq was used as a surrogate measurement (30). Our previous transcriptomic data (GEO accession code GSE60745) were aligned to the mouse genome (Ensembl NCBI M37) via TopHat (v. 2.1.1). The number of reads mapping to a particular exon were compared with the total number of exons in a given gene and expressed as fragments per kilobase

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Figure 6. siRNA-mediated knockdown of identified splicing factors in the 14-3-3 interactome alters the splicing of Pparg mRNA. A, 3T3-L1 pre-adipocytes were transfected with siCon or target-specific siRNAs, followed by differentiation (+ MDI) for 48 h. Total RNA was isolated, and quantitative PCR was used to measure Pparg mRNA splice variants ($n = 4$ per group; *, $p < 0.05$ when compared with undifferentiated siCon-transfected cells; #, $p < 0.05$ when compared with differentiated, siCon-transfected cells; $\bar{b}$ graphs represent means $\pm$ S.D.). B and C, 3T3-L1 pre-adipocytes were transfected with siCon or target-specific siRNAs, followed by differentiation (+ MDI) for up to 7 days. Following isolation of total cell lysates, immunoblotting was performed to measure Ppary 1 or 2 and Lipin-1 protein abundance (B). Densitometric analysis was utilized to assess the impact of target knockdown on Ppary 1 or 2 abundance (C) ($n = 4$ per group; *, $p < 0.05$ when compared with undifferentiated siRNA-transfected cells; #, $p < 0.05$ when compared with differentiated siCon-transfected cells; $\bar{b}$ graphs represent means $\pm$ S.D.).
per million mapped reads (30). A false discovery rate of 0.05 was used to filter results. This data set was also analyzed to examine how depletion of 14-3-3ζ or differentiation affects the expression profile of target genes. Genes identified by DEXSeq were subjected to gene ontology analysis to categorize genes by biological function (53). Analysis of Lpin1 splicing was performed by RT-PCR, as described previously (32). PCR products were resolved on an agarose gel, followed by densitometric analysis of splice variants by ImageJ (31). Analysis of Pparg splicing was measured by quantitative PCR, using previously reported primer sequences against Pparg1, Pparg2, and Pparg1sv (29).

**Immunoblotting**

The cells were lysed in radioimmune precipitation assay (RIPA) buffer, supplemented with protease and phosphatase inhibitors, as previously described (4). Immunoprecipitation was performed on whole cell lysates from 3T3-L1 adipocytes at different stages of differentiation with established protocols (35). Proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against 14-3-3ζ, Pparg, Lpin1, and β-actin (Cell Signaling Technology, Danvers, MA).

**Statistical analysis**

All data were analyzed by one- or two-way analysis of variance, followed by appropriate post hoc tests or by Student’s *t* test. The data were considered significant when *p* < 0.05 and when applicable displayed as means ± S.D.

**Author contributions**—Y. M. performed experiments, analyzed data, and wrote and reviewed the manuscript. M. S. and N. N. F. performed experiments and analyzed data. T. M. designed parts of the study and reviewed the manuscript. G. E. L. performed experiments, analyzed data, wrote the manuscript, and is responsible for the integrity of this work.

**Acknowledgments**—We thank François Harvey in the Bioinformatics platform at the Centre Hospitalier de l’Université de Montréal for bioinformatics support and Dr. James D. Johnson (University of British Columbia, Vancouver, Canada) for critical reading of this manuscript.

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