LOC66273 Isoform 2, a Novel Protein Highly Expressed in White Adipose Tissue, Induces Adipogenesis in 3T3-L1 Cells1,2

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1Supported in part by the Chinese Universities Scientific Fund (no. 2010JS004) and the National Transgenic Major Project (2009ZX08009-116B).
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Abstract

Obesity results in part from altered adipocyte metabolism and enhanced adipogenesis. However, the factors that influence insulin-independent differentiation of preadipocytes in response to excess intake of dietary energy remain poorly understood. Based on our recent finding that LOC66273 isoform 2 (LI2), a gene that encodes a novel Mth938 domain-containing protein, is highly expressed in white adipose tissues, we hypothesized that LI2 plays an important role in adipogenesis. Plasmid pcDNA3.1-LI2 was electroporated into 3T3-L1 preadipocytes to overexpress the LI2 protein. Synthetic siRNA was transfected into 3T3-L1 cells to knockdown endogenous LI2. Using constitutively active and potent siRNA against LI2, we determined cell morphology, cell viability, and adipocytic factors in 3T3-L1 preadipocytes. Our results indicated that LI2 was sufficient to drive preadipocyte differentiation via modulating the phosphorylation level and transcriptional activity of CREB, coincident with expression of several adipogenic regulators and mature adipocyte markers, without insulin treatment. In addition, overexpression of the LI2 protein inhibited preadipocyte growth, whereas knockdown of the LI2 protein resulted in preadipocyte apoptosis via caspase-3 activation during adipogenesis. These results indicated that LI2 might function to switch preadipocytes from proliferation to differentiation and to maintain the viability of preadipocytes during adipogenesis by regulating the caspase-3 pathway. Our findings highlight the importance of LI2 in the formation of new adipocytes, thus helping understand the mechanisms responsible for insulin-independent adipogenesis in mammals. J. Nutr. 142: 448–455, 2012.

Introduction

Epidemiological studies have indicated that obesity induced by a high-sugar and high-fat diet is a major risk factor for type II diabetes and other metabolic syndromes in humans (1,2). Given the alarming rise in obesity and diabetes worldwide, identifying nutritional and genetic factors that influence adipocyte metabolism and adipogenesis is of enormous importance for improving human health and well-being (3). It is known that excess intake of dietary energy can result in the generation of additional adipocytes and/or a large increase in body fat content (1,3,4). However, the factors that influence insulin-independent differentiation of preadipocytes remain poorly understood (5). Particularly, white adipose tissue plays a major role in the regulation of energy homeostasis (6). The available evidence shows that adiposity can be regulated by some functional nutrients (4–6). For example, dietary supplementation with arginine can effectively control body weight by reducing the percentage of white adipose tissue (7–9). Interestingly, individuals exhibit different responses to high-sugar and high-fat diets, possibly due to differences in expression of key regulatory genes related to glucose and lipid metabolism (10–13). Therefore, it is important to identify functional genes that regulate fat metabolism, thereby reducing the incidence of obesity and diabetes.

The cellular and molecular mechanisms responsible for adipocyte differentiation have been extensively studied using preadipocyte culture systems, such as 3T3-L1 cell lines (14,15). 3T3-L1 cells have a fibroblast-like morphology (14). Under appropriate conditions, these cells differentiate into an adipocyte-like phenotype and have an increased capacity for the synthesis and accumulation of TG (14). The 3T3-L1 cells are also sensitive to lipogenic and lipolytic hormones and drugs, including epinephrine, isoproterenol, and insulin (16). Therefore, the 3T3-L1 fibroblast is a useful cell model to study the function of genes in vitro during adipogenic induction (16,17).
LI2 (LOC66273 isof orm 2, previously identified as 1810020D17Rik, GI 66273) was first cloned in our laboratory, and our previous research indicated that LI2 was highly expressed at both mRNA and protein levels in white adipose tissues (18), implicating a possible role for LI2 in adipogenesis. Results of bioinformatics analyses revealed that LI2 is highly conserved from zebrafish to humans but shares no obvious homology to any known genes or proteins (19,20). To our knowledge, the function of the LI2 protein has not been determined. cAMP responsive element binding protein (CREB; GI 12912) is necessary and sufficient to drive adipogenic conversion of 3T3-L1 preadipocytes (21). Notably, CREB regulates transcription of the adipocyte-specific genes such as Pck1 (phosphoenolpyruvate carboxykinase 1, GI 18534) and Fas (fatty acid synthase, GI 14104) (21,22). In the present study, using a cell-based, high-throughput screening platform based on a quantitative CREB luciferase trans-reporting system, we identified that LI2 upregulated CREB transcription activity. In view of these findings, we hypothesized that LI2 may play an important role in the induction of preadipocyte differentiation and adipogenesis. Characterization of LI2’s functions and its mechanism is important for fully elucidating the molecular basis responsible for adipocyte proliferation and differentiation, which could have important implications for understanding the biology of obesity and other metabolic-related diseases.

Materials and Methods
Reagents. DMEM, TRizol, ThermoScript RT-PCR System, and FBS were procured from Invitrogen Life Technologies. Rabbit antibodies against β-actin (GI 11461), PPARY (GI 19016), CCAAT/enhancer binding protein α (c/EBPα, GI 12606), adipocyte-type fatty acid-binding protein (aFABP; GI 11770), FAS, preadipocyte factor 1 (PREF-1, GI 13386), growth arrest and DNA-damage-inducible protein 153 (GADD153, GI 13198), CREB, and P-CREB, as well as insulin, 3-isobutyl-methyl-xanthine, and dexamethasone, were purchased from Sigma-Aldrich. The antibody against caspase-3 (GI 12367) was a product of Transduction Laboratories (Becton Dickinson). Rabbit polyclonal antibodies against the LI2 protein were prepared as previously described (18).

cDNA cloning and cDNA library construction. A mouse function-unknown-gene cDNA library was constructed from the RefSeq database of MEDLINE using a mouse multiple-tissue cDNA library (Clontech) according to the published methods (23) with some modifications. Briefly, we selected candidate mouse sequences with “hypothetical,” “predicted,” “putative,” or “unknown” in their classification. Two-step PCR amplification was used to make the open reading frames of these genes. The purified PCR products were cloned into the mammalian expression vector pCDNA.3.1/myc-His (+) B (Invitrogen). LI2 (according to GenBank accession no. NM_183251.3) was amplified as previously described (18).

Dual-luciferase reporter assay. CREB luciferase activities were measured using trans-reporting systems, which were conducted as previously described (23) with some modifications. Approximately 50 ng each eukaryotic expression plasmid in our mouse cDNA library (including pCDNA3.1-LI2) was cotransfected with a dual-luciferase trans reporter system into 3T3-L1 preadipocytes, pCDNA3.1-cAMP-dependent protein kinase (GI 18747) served as the positive control. A Synergy4 Multifunction Microplate Reader (Bio-Tek Instruments) was used to detect both firefly and renilla luciferase activities. The relative luciferase activities were calculated to represent the transcriptional activities of CREB. The activities at all other time points were normalized to the values of the control.

Cell line and transfection. 3T3-L1 fibroblasts (American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO2. Routine cell culture procedures were strictly followed to maintain proper cell density. Transfection of 3T3-L1 cells was performed by electroporation as previously described (21). Untransfected preadipocytes were induced using MDI regents as the positive control.

Induction of preadipocyte differentiation. Before induction, 3T3-L1 fibroblasts were seeded into culture plates and maintained in DMEM supplemented with 10% FBS. After 2 d, cells were confluent and used to perform adipocytic induction using MDI regents (2 μmol/L insulin, 0.5 μmol/L 3-isobutyl-methyl-xanthine, and 1 μmol/L dexamethasone for 2 d, and then 2 μmol/L insulin alone for an additional 2 d) as previously described (21).

Oil Red O staining. Differentiation of preadipocytes to mature adipocytes was confirmed by Oil Red O staining of lipid vesicles (21), which were conducted on d 8 after electroporation (for overexpression) or d 4 after induction (for RNA interference). Briefly, cells were rinsed in PBS prior to fixing with 4% paraformaldehyde. Then the cells were incubated in propylene glycol, after which Oil Red O stock solution was applied. The nuclei were stained with hematoxylin. Photographs of cell morphology were made with an Axioskop-2 microscope (Olympus) and an Image-Processing System (VisiRon Systems).

RT-PCR and qPCR analysis. RNA was prepared using TRizol reagents and RT was performed with the ThermoScript RT-PCR System according to the manufacturer’s protocol. LI2 and Gapdh were amplified by PCR and visualized by ethidium bromide staining as previously described (18). qPCR analysis of LI2, Pparg (GI 19016) and Cebpa (GI 12606) was carried out using the TaqMan Sequence Detection System and the DNA double-strand–specific SYBR Green I dye (Roche) according to the manufacturer’s instructions. Data were normalized to 185 rRNA mRNA levels as previously described (24). The data at all other time points are expressed as relative abundance by normalizing to the values of the control (d 0).

Protein extraction and immunoblot analysis. 3T3-L1 cytoplasmic and nuclear proteins were extracted using the ProteoJET cytoplasmic and nuclear protein extraction kits (Fermentas), respectively, according to the manufacturers’ protocol. Immunoblot analysis was performed as previously described (17). The corresponding primary antibodies were used to incubate with the membranes and signals were detected using the LI-COR Infrared Imaging System (Odyssey).

Cell proliferation assay. Cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay (17). Briefly, cells were transfected with the indicated plasmid. At 24 h following transfection, 4000 cells/well were seeded in 96-well plates. At the indicated time points, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide solution (Sigma-Aldrich) was added, followed by a 4-h period of incubation. The formazan dye was then solubilized and the absorbance measured at 570 nm using a Synergy4 Multifunction Microplate Reader (Bio-Tek Instruments).

siRNA synthesis and transfection. Double-stranded siRNA (free of RNase contamination) were designed, chemically synthesized, and PAGE purified by Sigma-Aldrich as previously described (21). Sense sequences of siLI2-1 and siLI2-2 were 5'-AGUUGCAAGGCUUCUCAU-CUAA-3' and 5'-AUUCGAAGAUGGCGAAGG-3', respectively. The nonsilencing siRNA was 5'-UUUCGCGAGUGUCAGGU-3'. All siRNA were dissolved in buffer at a concentration of 20 μmol/L. Synthetic siLI2-1 and siLI2-2 were transfected into cells. The mRNA and protein levels of endogenous LI2 expression were assessed using RT-PCR analysis and immunoblot on d 2 after induction. The siLI2-1 and siLI2-2 were transfected into 3T3-L1 preadipocytes. After 2 d, the confluent transfected cells were induced to undergo differentiation using MDI regents. On d 2 and 4 following induction, percentages of cell apoptosis were analyzed by flow cytometry, relative caspase-3 activities were detected by Ac-DEVD-AMC protease analysis, and relative
luciferase activities were determined using a dual-luciferase reporter system.

**Fluorescence microscopy.** Cell transfection and fluorescence detection were performed as previously described (17). Briefly, cells were transiently transfected with siLI2-1 or siLI2-2, plated on glass cover slips, and treated with MDI regents to induce differentiation. After 4 d, cells were fixed, quenched, permeabilized, and stained with Hoechst33342. Representative fluorescent photomicrographs were made using an Axiostar-2 microscope (Olympus), standard filter sets (Leika MicroImaging), and the Metavue software (Visitron Systems).

**Flow cytometry analysis.** Cell apoptosis and cell cycle were analyzed using flow cytometry FACSCalibur (Becton Dickinson Biosience). Percentages of cell apoptosis (apoptosis rate) were analyzed by flow cytometry with Annexin-V-FITC and propidium iodide according to the manufacturer's protocol (Biosea Biotechnology) as previously described (17). Assays of the cell cycle were carried out 24 h after serum withdrawal for synchronization. Then, cells were incubated with DMEM containing 10% FBS for various hours. The cells (5 × 10⁵) were harvested by trypsinization, washed twice with PBS, fixed in 70% ethanol overnight, and stained with propidium iodide (20 mg/L final concentration)/Triton-X100 containing 10 g/L RNase (DNase free). After incubation at 37°C for 30 min, the samples were analyzed. The cells in the S phase and G2/M phase represent proliferating cells. Therefore, the percentage of cells in the S+G2/M phases was used as an indicator of cell growth.

**Ac-DEVD-AMC analysis for caspase-3 activity.** Caspase-3 activity was measured using the Ac-DEVD-AMC Protease Assay kit (BD Biosciences PharMingen) according to the manufacturer’s instructions. Briefly, cells were lysed and the protein concentration was measured using the BCA Protein kit (Pierce). Equal amounts of total cell lysates were mixed with the caspase-3 assay buffer containing a fluorogenic substrate Ac-DEVD-AMC (20 μmol/L). Caspase-3 mediated the cleavage of Ac-DEVD-AMC into free AMC and the resulting fluorescence was measured using a Synergy4 Multifunction Microplate Reader.

**Statistical analysis.** All data were analyzed using 1-way ANOVA in the SAS Statistical System Software (version 9; SAS Institute). Where appropriate, ANOVA for repeated measures was performed. For all datasets, the normality of variance was tested using the Levene’s test. Values are presented as means ± SEM. The Duncan’s test was used to identify differences among means for different time points (excluding cell proliferation and cell cycle assays) when the F-test was significant. P < 0.05 was considered significant.

**Results**

**Overexpression of the LI2 protein and CREB transcription activities.** A high-throughput, cell-based, functional screening platform based on a quantitative CREB luciferase trans-reporting system revealed several novel genes associated with CREB transcription activities (Fig. 1A), including the LI2 gene. Overexpression of LI2 increased the CREB reporter gene activity ~2.5-fold compared with the control (P < 0.05) (Fig. 1A). Note that in this assay, cAMP-dependent protein kinase served as the positive control (Fig. 1A).

**FIGURE 1** Screening and identification of the LI2 gene. Expression of LI2 was associated with CRE pathway activation, MDI-induced preadipocyte differentiation, and cell viability in 3T3-L1 cells. (A) Relative luciferase activities in 3T3-L1 preadipocytes on d 2 following transfection. The x axis indicates the serial numbers of gene clones in our library. Results of pcDNA3.1-LI2 and pcDNA3.1-PKA are means ± SEM, n = 3. *Different from the control (pcDNA3.1), P < 0.05. (B) Protein levels of LI2 and β-actin in 3T3-L1 preadipocytes on d 0, 2, 4, and 8 following differentiation induction. (C) Protein levels of LI2 and β-actin in 3T3-L1 preadipocytes on d 4 following transfection with pcDNA3.1 or pcDNA3.1-LI2. (D) Cell viability of LI2-transfected 3T3-L1 preadipocytes on d 0, 2, 4, 6, and 8 following differentiation induction. Results are means ± SEM, n = 8. *Different from the control group at each time point, P < 0.05. (E) Cell cycle analysis at 0, 24, and 36 h after induction. Results are means ± SEM, n = 3. *Different from the control group at each time point, P < 0.05. PKA, cAMP-dependent protein kinase.
Expression of LI2 during 3T3-L1 adipocyte differentiation.

Two days after adipocytic induction using MDI regents, LI2 mRNA levels increased in 3T3-L1 adipocytes (relative mRNA levels for LI2 were 9.2 ± 0.7 on d 2 and 1.0 ± 0.1 on d 0) ($P < 0.05$), and continued to increase during the 8-d differentiation period (10.4 ± 0.8 on d 4 and 13.5 ± 1.1 on d 8) ($P < 0.05$). Consistent with LI2 mRNA levels, the endogenous abundance of the LI2 protein was enhanced (Fig. 1B).

Overexpression of the LI2 protein and cell proliferation.

Abundance of the LI2 protein in 3T3-L1 cells transfected with pcDNA3.1-LI2 was higher than that in the control on d 4 (>4-fold; Fig. 1C) ($P < 0.05$). During an 8-d period, overexpression of LI2 in 3T3-L1 preadipocytes resulted in a remarkably lower rate of proliferation compared with the control or the cells transfected with a blank pcDNA3.1 vector at each time point (Fig. 1D) ($P < 0.05$). However, the apoptosis rate of 3T3-L1 preadipocytes transfected with pcDNA3.1-LI2 (6.6 ± 1.1% on d 2 and 11.4 ± 0.9% on d 4) did not differ from the control (7.1 ± 0.8% on d 2 and 10.5 ± 1.2% on d 4) or the cells transfected with pcDNA3.1 (7.4 ± 0.5% on d 2 and 12.5 ± 1.4% on d 4) at either d 2 or d 4. In addition, when transfected with a blank pcDNA3.1 vector, cells in the S phase and G2/M phase substantially increased compared with the unstimulated cells at either 24 or 36 h after serum stimulation ($P < 0.05$). Conversely, the percentage of LI2-overexpressed 3T3-L1 cells in the S phase and G2/M phase was virtually unchanged at 24 or 36 h after serum treatment (Fig. 1E).

Overexpression of the LI2 protein and preadipocyte differentiation.

Similar to the MDI-treated 3T3-L1 cells, numerous fat droplets were observed in LI2-overexpressing cells, whereas few lipid droplets appeared in the cells transfected with the blank pcDNA3.1 vector (Fig. 2A). In addition, mRNA levels for Pparg and Cebpα [important biomarkers for adipogenesis (25)] in 3T3-L1 fibroblasts transfected with pcDNA3.1-LI2 were higher compared with the control during adipogenesis (Table 1) ($P < 0.05$), though Pparg and Cebpα mRNA levels in any treatments increased over time ($P < 0.05$). Similarly, PPARγ and c/EBPα protein levels were elevated ($P < 0.05$) in LI2-overexpressed cells compared with the control, whereas levels for PPRE-1 and GADD153 proteins [adipogenesis suppressors (26,27)] decreased (Fig. 3B) ($P < 0.05$). In addition, overexpression of the LI2 protein enhanced the protein levels for αFABP and FAS [important proteins for fatty acid uptake, transport, and metabolism (28,29)] (Fig. 3B) ($P < 0.05$).

Effect of siLI2-1 or siLI2-2 on endogenous LI2 expression.

Nonsilencing siRNA or the 2 siRNA against LI2 (siLI2-1 and siLI2-2) were transfected into 3T3-L1 cells. LI2 mRNA levels were decreased in cells transfected with both siLI2-1 and siLI2-2 (Fig. 2B) ($P < 0.05$). In contrast, no effects were observed for the treatment with nonsilencing siRNA (Fig. 2B). Furthermore, the expression of endogenous LI2 protein was reduced by siLI2-1 or siLI2-2 (Fig. 2C) ($P < 0.05$).

LI2 protein knockdown and preadipocyte differentiation.

The potent siRNA against the LI2 gene was transfected into 3T3-L1 preadipocytes and after 2 d, the confluent transfected cells underwent differentiation in the presence of MDI regents. Similar to the previous reports (21,22), many fat droplets were observed in the cells transfected with nonsilencing RNA (Fig. 2D). However, few lipid droplets appeared in the cells transfected with either siLI2-1 or siLI2-2 (Fig. 2D). Additionally, mRNA (Table 2) and protein (Fig. 3C) levels for PPARγ or c/EBPα in 3T3-L1 cells transfected with either siLI2-1 or siLI2-2 were lower than those in the control during adipogenesis ($P < 0.05$). Furthermore, knockdown of LI2 protein resulted in decreased ($P < 0.05$) expression of αFABP and FAS but increased ($P < 0.05$) expression of PREF-1 and GADD153 (Fig. 3C).

Downregulation of the LI2 protein and 3T3-L1 cell apoptosis during differentiation induction.

The 3T3-L1 cells transfected with siLI2-1 or siLI2-2 exhibited many condensed, fragmented, brightly stained nuclei in culture, which are the hallmark of apoptosis, but no such changes were detected in nonsilencing siRNA transfected cells (Fig. 3A). In addition, 3T3-L1 cells transfected with either siLI2-1 or siLI2-2 had a higher rate of apoptosis compared with the control during adipocytic induction (Table 3), suggesting the translocation of phosphatidyl serine from the cytoplasmic surface of the cell membrane to the external cell surface ($P < 0.05$).

Impacts of LI2 protein knockdown on caspase-3 and CREB expression and apoptosis.

During adipogenesis, fluorescence intensity generated by the cleavage of Ac-DEVD-AMC into the free AMC was much higher in lysates from either siLI2-1 or siLI2-2 transfected cells compared with the negative control (Table 3) ($P < 0.05$). The level of the caspase-3 protein also increased in 3T3-L1 cells transfected with either siLI2-1 or siLI2-2 than that in the control group (Fig. 4A), suggesting that knockdown of the LI2 protein resulted in more cleavage of procaspase-3 when 3T3-L1 fibroblasts were at the stage of differentiation ($P < 0.05$). In addition, CREB transcription activities were reduced ($P < 0.05$) in cells transfected with either siLI2-1 or siLI2-2 (Table 3). Similarly, transfection with either siLI2-1 or siLI2-2 decreased the phosphorylation level of CREB during 3T3-L1 adipogenesis but not the protein level of CREB (Fig. 4A) ($P < 0.05$).

Discussion

To our knowledge, this study is the first report that LI2, a novel Mth938 domain-containing protein, plays a role in preadipocyte differentiation and adipogenesis without insulin treatment. LI2 is highly expressed in white adipose tissue at both the mRNA and protein levels (18), suggesting that LI2 plays an important role in adipogenesis. The following lines of evidence from the present study support this novel hypothesis. First, mRNA and protein levels of LI2 markedly increased in 3T3-L1 adipocytes during hormone-induced differentiation. Second, overexpression of the LI2 protein enhanced phosphorylation and transcription activity of CREB, which is consistent with the notion that CREB is necessary and sufficient to drive adipogenic conversion of 3T3-L1 preadipocytes (22). Third, overexpression of LI2 could promote adipogenesis, which was associated with enhanced expression of key genes regulating adipocyte differentiation and of markers for mature adipocyte. Finally, depletion of the LI2 gene through siRNA-induced silencing completely blocked the process of insulin-induced preadipocyte differentiation accompanied by decreased expression levels of adipocytic factors. Collectively, these data support the view that LI2 plays a key role in regulating insulin-independent differentiation of 3T3-L1 preadipocytes via modulating the transcription activity of CREB (Fig. 4B). Because 3T3-L1 cells were used as a model for studying adipogenesis, in vivo research will be needed to verify the physiological function of LI2.
There is growing interest in the biochemical properties of the LI2 protein. Encoded by the M9 genome, the hypothetical M938 protein crystallizes as a dimer, which is linked by one disulfide bond between each monomer, but the protein exists as a monomer in solution (20). The physiological function of the protein has not been determined (19,20). Our previous study indicated that overexpression of LI2 can activate the MAPK pathway, thereby increasing the transcriptional activity of ELK1 (GI 13712) and c-JUN (GI 16476) via modulating the ERK1/2 (GI 26413) and JNK1 (GI 26419) signaling pathway (18). The results of the present study indicate that the M938 domain is associated with differentiation and adipogenesis. Therefore, it can be hypothesized that the ERK1/2 and JNK1 signaling pathway may play an important role in LI2-induced adipogenic differentiation.

**FIGURE 2** Effects of LI2 overexpression or knockdown of endogenous LI2 on 3T3-L1 preadipocyte differentiation. (A) Representative phase-contrast photomicrographs of LI2-transfected 3T3-L1 cells on d 8. Red arrows indicate the cells with lipid droplets and lipid vesicles [upper portion, low-magnification (40×) images to reveal the whole well; lower portion, high-magnification (400×) images of a few representative cells]. The mRNA (B) and protein (C) levels of endogenous LI2 in 3T3-L1 preadipocytes transfected with siRNA on d 2 following induction. (D) Representative phase-contrast photomicrographs of 3T3-L1 cells after LI2-knockdown (d 8 following induction). Red arrows indicate the cells with lipid droplets and lipid vesicles [upper portion, low-magnification (40×) images to reveal the whole well; lower portion, high-magnification (400×) images of a few representative cells].

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Differentiation of preadipocytes into adipocytes requires the coordination of a complex network of transcription factors, cofactors, and signaling molecules (22,33,34). Results of our study indicate that depletion of Ll2 can completely block MDI-induced preadipocyte differentiation and reduce the phosphorylation level and transcription activity of CREB coincident with reduced abundances of the PPARγ, C/EBPα, α-FABP, and FAS proteins but increased abundances of the PREF-1 and GADD153 proteins. PPARγ and C/EBPα are master genes controlling the transcriptional regulation of adipogenesis (25). PREF-1 has been known as an inhibitor of preadipocyte differentiation via downregulation of the transcriptional activities of c/EBPβ (GI 12608) and c/EBPδ (GI 12609) (26,35). GADD153 acts as a dominant-negative inhibitor by forming heterodimers with other CEBP members and represses the activities of PPARγ (27). In addition, as a major marker for mature adipocytes, FAS catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA as well as the incorporation of palmitate into saturated long-chain fatty acid (29,36). The current consensus is that α-FABP modulates fatty acid metabolism by binding long-chain fatty acid and other hydrophobic ligands (28). Taken together, our data indicate that Ll2 is necessary and sufficient to induce adipogenesis via regulating the phosphorylation level and transcriptional activity of CREB. Such a biochemical change results in preadipocyte differentiation by upregulating the expression of PPARγ and C/EBPα as well as downregulating the expression of PREF-1 and GADD153. Subsequently, FAS and α-FABP can be upregulated to promote the uptake, transport, and synthesis of fatty acids as well as the generation of additional adipocytes (Fig. 4B). Further

![Image](LOC66273_isoform_2_and_adipogenesis)
research is needed to fully understand this process and its mechanism.

Another novel and important finding of this study is that LI2 plays an important role in preadipocyte viability and growth. Specifically, overexpression of LI2 in 3T3-L1 preadipocytes inhibited preadipocyte proliferation while inducing adipocytic differentiation. Because the increased expression of the LI2 protein had no effect on cell apoptosis, we hypothesize that LI2 plays a crucial role in switching preadipocytes from proliferation to differentiation during adipogenesis. On the contrary, knockdown of LI2 protein resulted in differentiation-induced apoptosis in 3T3-L1 cells, suggesting that LI2 protein is crucial to maintain cell viability during the process of preadipocyte differentiation. In addition, blocking the expression of LI2 in the early phases of adipogenesis led to induction of both procaspase-3 cleavage and caspase-3 activation. As one of the major events involved in the classic apoptosis of the mitochondrial pathway (37), caspase-3 participates in signal transduction pathways that regulate apoptosis (38). Particularly, overexpression of LI2 inhibited the abundance of GADD153 but increased the activated CRE activity of CREB. Consistent with our findings, available evidence shows that nonphosphorylated CREB contributes to p53-mediated apoptosis brought about by glucose deprivation (39). Additionally, GADD153 has been implicated in adipogenesis via mechanisms involving endoplasmic reticulum stress and caspase-3 activation (40). These data further support the view that LI2 plays a crucial role in switching proliferation to differentiation and maintaining cell viability. These effects of LI2 are likely associated with the CREB-mediated caspase-3 pathway (Fig. 4B).

In conclusion, the LI2 gene is upregulated during adipogenesis. Findings from experiments involving constitutively active and potent siRNA against LI2 support the notion that LI2 is required to drive preadipocyte differentiation via modulating the expression of adipogenic regulators. LI2 can enhance CREB transcriptional activity while inhibiting the caspase-3-mediated apoptosis. In addition, LI2 functions to switch preadipocytes from proliferation to differentiation and also maintain preadipocyte viability during adipogenesis. These data highlight the importance of LI2 in the insulin-independent formation of new adipocytes and help us to understand the complex mechanisms responsible for the generation of additional adipocytes and obesity in response to excess intake of dietary energy.

Acknowledgments
X.M., W.D., J.W., G.W., H.Z., J.Y., L.Z., and D.L. designed and conducted research; X.M., W.D., and H.Z. analyzed data; X.M., G.W., L.Z., and D.L. wrote the paper; and X.M. and D.L. had primary responsibility for the final content. All authors read and approved the final manuscript.

**TABLE 2** Relative mRNA levels for Pparg and Cebpa of 3T3-L1 preadipocytes transfected with siRNA on d 0, 4, and 8 following induction

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^a Values are means ± SEM, n = 3. *Different from control (non-siRNA), P < 0.05. Means in a column without a common letter differ, P < 0.05.

**TABLE 3** Percentages of cell apoptosis (apoptosis rate), relative caspase-3 activity, and relative luciferase activity in 3T3-L1 preadipocytes transfected with siRNA on d 2 and 4 following induction

<table>
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<th></th>
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<td>33.7 ± 2.5^*</td>
<td>34.5 ± 2.9^*</td>
</tr>
<tr>
<td>Relative caspase-3 activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fold of non-siRNA, d 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 2</td>
<td>1.0 ± 0.1^a</td>
<td>12.2 ± 0.8^*</td>
<td>12.8 ± 0.9^*</td>
</tr>
<tr>
<td>d 4</td>
<td>1.4 ± 0.2^a</td>
<td>13.2 ± 1.0^*</td>
<td>10.5 ± 1.2^*</td>
</tr>
<tr>
<td>Relative luciferase activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 2</td>
<td>1.00 ± 0.09^a</td>
<td>0.25 ± 0.04^*</td>
<td>0.19 ± 0.07^*</td>
</tr>
<tr>
<td>d 4</td>
<td>1.41 ± 0.11^a</td>
<td>0.28 ± 0.07^*</td>
<td>0.33 ± 0.09^*</td>
</tr>
</tbody>
</table>

^a Values are means ± SEM, n = 3. *Different from control (non-siRNA), P < 0.05. Means in a column without a common letter differ, P < 0.05.
Literature Cited


