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RESEARCH PAPER

Itm2a silencing rescues lamin A mediated inhibition of 3T3-L1 adipocyte differentiation

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ABSTRACT

Dysregulation of adipose tissue metabolism is associated with multiple metabolic disorders. One such disease, known as Dunnigan-type familial partial lipodystrophy (FPLD2) is characterized by defective fat metabolism and storage. FPLD2 is caused by a specific subset of mutations in the LMNA gene. The mechanisms by which LMNA mutations lead to the adipose specific FPLD2 phenotype have yet to be determined in detail. We used RNA-Seq analysis to assess the effects of wild-type (WT) and mutant (R482W) lamin A on the expression profile of differentiating 3T3-L1 mouse preadipocytes and identified Itm2a as a gene that was upregulated at 36 h post differentiation induction in these cells. In this study we identify Itm2a as a novel modulator of adipogenesis and show that endogenous Itm2a expression is transiently downregulated during induction of 3T3-L1 differentiation. Itm2a overexpression was seen to moderately inhibit differentiation of 3T3-L1 preadipocytes while shRNA mediated knockdown of Itm2a significantly enhanced 3T3-L1 differentiation. Investigation of PPARγ levels indicate that this enhanced adipogenesis is mediated through the stabilization of the PPARγ protein at specific time points during differentiation. Finally, we demonstrate that Itm2a knockdown is sufficient to rescue the inhibitory effects of lamin A WT and R482W mutant overexpression on 3T3-L1 differentiation. This suggests that targeting of Itm2a or its related pathways, including autophagy, may have potential as a therapy for FPLD2.

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Introduction

Nuclear lamins are type V intermediate filament proteins that polymerise to form components of the nuclear lamina; a fibrous meshwork associated with the inner nuclear membrane.[1] The mammalian genome contains 3 lamin genes; LMNA, LMNB1 and LMNB2 that encode lamin A/C, lamin B1 and lamin B2/B3 respectively. The LMNA gene is alternatively spliced to produce 2 main isoforms, lamin A and lamin C, the expression of which is developmentally regulated.[2] Unlike B type lamins, which are ubiquitously expressed during development, A type lamins are not detected in undifferentiated cells but are expressed in most differentiated somatic cells.[3] A type lamins are found both at the nuclear periphery and, to a lesser extent, within the nucleoplasm.[4]

Lamins are important structural components of the nucleus and are essential for nuclear integrity; a deformed nuclear morphology is typically evident when the lamins are lost or mutated.[1] In addition to providing structural support, lamins play a role in regulating nuclear processes including DNA replication, gene transcription, cell proliferation and cell differentiation. Lamins interact with chromatin and have been shown to modulate chromatin organization in several different contexts.[4]

Mutations in the LMNA gene are responsible for multiple inherited disorders, collectively known as 'laminopathies'. Dunnigan-type familial partial lipodystrophy (FPLD2) is an autosomal dominant laminopathy characterized by defective fat metabolism and storage.[5] Symptoms of this disorder manifest at puberty and include the loss of peripheral, subcutaneous adipose tissue from the extremities (limbs, truncal and gluteal regions) with a build-up of visceral and nuchal adipose tissue. This is accompanied by a myriad of metabolic symptoms such as hepatic steatosis, atherosclerosis and

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insulin resistance, which leads to type II diabetes mellitus.[6] Defective energy storage is thought to be the primary pathogenic factor in such lipodystrophies, leading to the development of the characteristic metabolic disease state.[7]

FPLD2 is caused almost exclusively by heterozygous missense mutations in the 8th and 11th exons of the LMNA gene.[8-10] Mutations that result in lipodystrophies are mainly found in the immunoglobulin-like fold of the lamin A/C protein. They are not considered to alter the 3 dimensional structure of the protein, however the majority of these mutations generate amino acid substitutions that lead to a decrease in the surface positive charge of the immunoglobulin- like domain, which may affect protein-protein interactions.[11,12] Common mutations leading to FPLD2 occur at amino acid 482 of the lamin A protein, in which the positively charged arginine if often substituted with a neutral amino acid such as tryptophan (R482W) or glutamine (R482Q).[6,13]

Adipocyte terminal differentiation involves a transcriptional cascade in which the expression of pro-adipogenic factors is temporally induced alongside the downregulation of various anti-adipogenic factors.[14] The molecular mechanisms involved in this process are well studied and several of the key players, such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (CEBPα) have been characterized.[15] Recently the relationship between A type lamins and chromatin state has been described in human adipocyte stem cells (ASC) both pre and post terminal differentiation. In this context, A type lamins are reported to interact with thousands of promoters within the genome, and to modulate chromatin modifications at these sites. In addition, lamin A-promoter interactions are shown to be remodeled during the adipogenic program, with variable impact on the transcriptional outcome of the genes involved.[16]

The physiologic characteristics of FPLD2 support the hypothesis that LMNA mutations lead to altered or impaired lamin A function, which in turn influences the dynamic process of adipogenesis. Although the exact molecular mechanisms leading to the disease phenotype remain unclear, several factors have been implicated. The FPLD2 causing LMNA R482W mutation has been reported to diminish lamin A binding to the sterol response element binding protein 1 (SREBP1) transcription factors as well as fragile X-related protein 1 (FXR1P).[17-20] The SREBP1 transcription factors are master regulators of lipid metabolism and play a role in the induction of adipocyte terminal differentiation,[14,21] while FXR1P has only recently been described and a modulator of adipogenesis.[20] In both FPLD2 patient fibroblasts and LMNA R482W transduced human preadipocytes, the reduced lamin-SREBP1 interaction was associated with the upregulation of many SREBP1 targets, while FXR1P itself is also upregulated in both contexts.[19,20] In addition, previous studies have described mutant prelamin A accumulation in FPLD2 patient fibroblasts.[22,23] The accumulation of this unprocessed form of the lamin A protein was suggested to generate the lipodystrophic disease phenotype by sequestering SREBP1 at the nuclear membrane and preventing its action in adipocyte differentiation.[22] In contrast, a recent study has reported no prelamin A accumulation in fibroblasts carrying several FPLD2 LMNA mutations.[24]

In recent years several loss of function PPARγ mutations have been reported to generate a disease phenotype similar to that of FPLD2. Now classified as FPLD3, this genetically distinct form of partial lipodystrophy exhibits clinical features comparable to that of FPLD2, most notably the specific pattern of fat loss from the gluteal and limb regions, insulin resistance and type II diabetes mellitus.[25,26] The similarities observed in phenotype between FPLD2 and FPLD3 suggests that FPLD2 LMNA mutations may lead to reduced or impaired PPARγ function during adipogenesis.

The terminal differentiation of mouse preadipocytes has been studied extensively using the 3T3-L1 cell model. In this well-established system, the pre-adipocytes are grown to confluence and allowed to undergo growth arrest, after which point an adipogenic cocktail is applied to initiate differentiation. During the adipogenic program, PPARγ expression is upregulated approximately 24 to 48 h post induction.[27] In this context the overexpression of both wild-type and R482W mutant lamin A are reported to have a similar inhibitory effects on adipocyte differentiation.[28] We performed RNA-Seq analysis at 36 h post induction of 3T3-L1 pre-adipocytes to investigate the effects of R482W mutant and wild-type lamin A on gene expression profiles at this initial phase of terminal differentiation. Among the genes that were altered in response to lamin A we identified an increase in Integral membrane protein 2 A (ITM2A) expression in both wild type and R482W mutant transfected cells.

Itm2a was first identified as a novel marker for chondro-osteogenesis in a cDNA library screen generated from mouse mandibular condyle explant cultures.[29] This type II membrane protein belongs to a family of integral membrane proteins that includes ITM2B and ITM2C, all of which are part of a BRICHOS superfamily. ITM2 proteins are composed of 4 defined regions; a hydrophobic, linker, extracellular BRICHOS and the intracellular C-terminal domains. There is a high degree of conservation between the ITM2 proteins as well as between mammalian homologues.[30]

Although the exact function of Itm2a is unclear, several studies have described a regulatory role in chondrogenic and myogenic differentiation, as well as thymocyte development.[31-33] Itm2a expression is low in early C3H10T1/2 cell chondrogenesis and then strongly upregulated as the cells progress through the differentiation program. Ectopic Itm2a overexpression is seen to inhibit the chondrogenic differentiation of this cell line. In addition, Itm2a is differentially expressed between ASCs and mesenchymal stem cells (MSCs) with distinct chondrogenic potentials; ASCs have a reduced capacity to differentiate into chondrocytes when compared with MSCs, and display higher endogenous levels of Itm2a.[31,34]

Itm2a has been identified as a PAX3, GATA3 and PKA CREB target in diverse systems.[32,33,35] In the C2C12 myoblast cell line, endogenous Itm2a expression is increased during cell differentiation and overexpression leads to enhanced myotube formation. Itm2a expression is detected at sites of myogenesis in mice and PAX3 mutant embryos display reduced Itm2a.[32,36] Itm2a was described as a GATA3 target in mouse thymocytes and reported to be downregulated in GATA3 knockout thymocytes when compared with control.[33] Recently a novel role for ITM2A has been reported in autophagy. It is described as a PKA-CREB signaling target, and when overexpressed appears to interfere with autophagic flux, leading to the accumulation of autophagosomes and a block in the formation of autolysosomes. ITM2A silencing was also seen to obstruct autophagy, with the formation of large agglomerations within the cell.[35] These reports highlight the importance of Itm2a in the differentiation of several cell types, however the exact molecular function of the protein remains to be described. Itm2a knockout mice have been produced independently by 2 different groups[32,33] neither of which have reported a specific altered phenotype.

Here, we explore the relationship between Itm2a, adipogenesis and inhibition of adipogenesis by lamin A.

Results

Lamin A overexpression inhibits 3T3-L1 adipogenesis and increases Itm2a expression

Exogenous expression of human lamin A (WT and R482W mutant) has previously been shown to inhibit in vitro differentiation of mouse 3T3-L1 preadipocytes.[28] To investigate the effects of lamin A on the transcriptional profile of these cells during the early stages of terminal differentiation we stably transfected 3T3-L1 preadipocytes with human LMNA overexpression constructs (pCDNA3-LMNA-WT and pCDNA3-LMNA-R482W) and induced the cells to differentiate. At 36 h post application of the induction cocktail a single RNA-Seq analysis was performed. Altered expression of over 200 genes was detected in comparison to control and analysis of these data sets identified 76 common transcripts, affected by both WT and R482W mutant lamin A (Supplemental Table 1). Interestingly, overexpression of 2 of the top 3 upregulated genes, Ptprq and Wnt6, have previously been shown to reduced adipocyte differentiation[37,38] while a role for Itm2a in adipogenesis has not previously been reported.

Our RNA-Seq data indicates a 16 and 8-fold increase in Itm2a expression in response to WT lamin A and R482W mutant respectively. To confirm the effects of lamin A on Itm2a expression we stably transfected 3T3-L1 preadipocytes with human pCMV(PB)-Flag-LMNA-WT and pCMV(PB)-Flag-LMNA-R482W using a piggyBac transposable system.[65,66] These preadipocytes were then induced to differentiate, adipogenesis was assessed at day 8 through Oil Red O staining of intracellular lipid droplets (Fig. 1A) and lamin A overexpression was assessed at the mRNA level using quantitative real-time PCR (qPCR) and at the protein level by western blotting (Fig. 1B, C). Cells transfected with EV control differentiated well, while cells expressing both WT and R482W mutant lamin A accumulated significantly less lipid droplets, confirmed by Oil Red O quantification (Fig. 1A).

Previously, several reports have shown that lamin A overexpression reduces PPARγ2 expression in adipogenesis; overexpression of WT and mutant lamin A in 3T3-L1 cells[28] and in MSCs[39] produced this effect while reduction of PPARγ2 expression was also observed when lamin A overexpression was driven from an adipose specific promoter in transgenic mice.[40] To determine the effects of lamin A on specific adipogenic markers, PPARγ2 and CEBPα expression was measured at day 4 in differentiating 3T3-L1 cells expressing WT or R482W mutant lamin A and the expression of both markers was reduced in these cells in comparison with the control (Fig. 1D, F). Immunoblot analysis of PPARγ at day 4 of differentiation revealed the presence of 3 PPARγ isoforms of ~60, ~55 and ~45 kDa respectively (Fig. 1E). The 2 larger isoforms PPARγ2 and PPARγ1, are produced through the use of alternative promoters and have differential abilities to promote adipogenesis.[41] PPARγ2 has an additional 28 N-terminal amino acids and is exclusively expressed in adipose tissue, where it functions as a master regulator of adipogenesis.[41-43] A number of studies have identified a smaller PPARγ isoform, known as γORF4, which does not contain the ligand-binding domain of PPARγ1 and 2 and displays dominant negative activity toward PPARγ[41,44]. It is likely, although not confirmed, that the 45kDA protein detected here is the described previously γORF4.

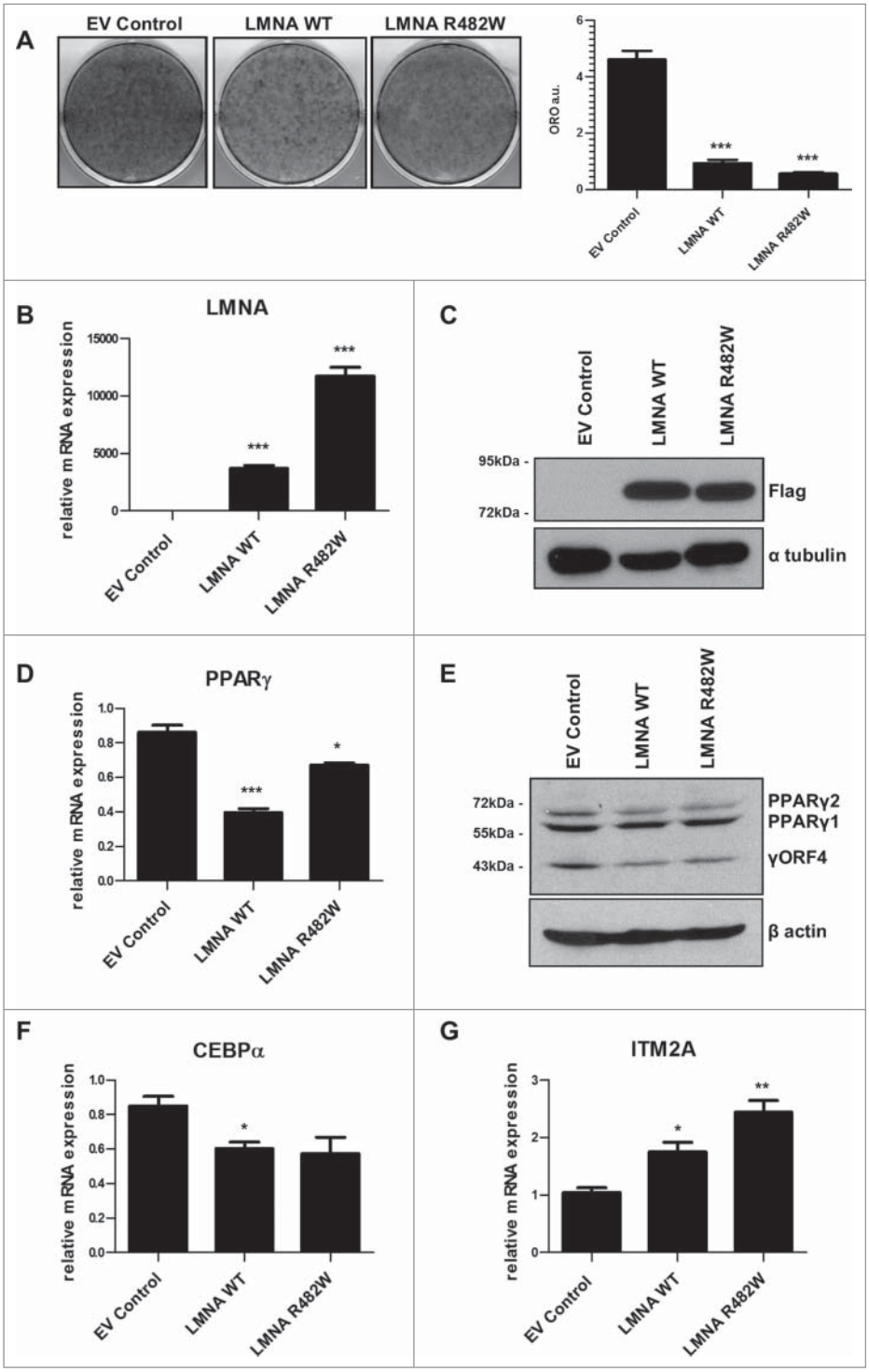

Figure 1. LMNA inhibits differentiation of 3T3-L1 cells and increases Itm2a mRNA expression. 3T3-L1 preadipocytes were stably transfected with pCMV(PB)-Flag-LMNA-WT, pCMV(PB)-Flag-LMNA-R482W or empty vector (EV) control pCMV(PB) and induced to differentiate into adipocytes. (A) Adipogenesis was assessed at day 8 post induction; cells were stained for lipid droplet accumulation with Oil Red O. Oil Red O quantification was performed using ImageJ and expressed as Oil red O absorbance units (ORO a.u.). (B) Total RNA was isolated at day −2 of differentiation from the stably transfected 3T3-L1 preadipocytes and LMNA expression measured by qPCR using primers specific for human LMNA. (C) Immunoblot analysis of flag-LMNA WT and R482W mutant at day −2. (D, F) Total RNA was isolated at day 4 post induction and the expression of PPARγ and CEBPα was analyzed by qPCR. (E) Immunoblot analysis of PPARγ at day 4 post induction. (G) Itm2a expression was analyzed at day −2 by qPCR. Student's *t*-test (2-tailed, assuming equal variance) was used to calculate statistical significance compared with empty vector control cells, indicated as follows: * = P < 0.05;** = P < 0.01; *** = P < 0.001.

Interestingly, ectopic expression of WT and R482W mutant lamin A appears not only to reduce PPARγ2 as expected, but also γORF4, while having little to no effect on PPARγ1 (Fig. 1E).

Having confirmed lamin A overexpression and the resulting inhibition of adipogenic differentiation, Itm2a expression levels were assessed in the stably transfected 3T3-L1 preadipocytes. Both WT and R482W mutant lamin A increased endogenous Itm2a expression levels (Fig. 1G).

Endogenous Itm2a expression and promoter activity in 3T3-L1 differentiation

Itm2a expression has previously been characterized in chondrogenic and myogenic differentiation where it is upregulated at distinct stages of each of the respective differentiation programmes.[31,32] Microarray analysis of gene expression in 3T3-L1 differentiation has reported relatively low levels of Itm2a expression throughout differentiation with little variation observed across the adipogenic program.[45] We profiled Itm2a expression during 3T3-L1 differentiation and similar to the microarray data described previously we observed relatively low expression of Itm2a, however we did detect a significant downregulation of gene expression at day 2 (Fig. 2A), approximately 48 h post application of the induction cocktail. This reduction in Itm2a expression was consistently observed at this specific stage of cell differentiation. Expression levels invariably reverted back to pre-induction levels by 96 h. Consistent with these results, our RNA-Seq data indicates that endogenous Itm2a is likely to be present at a very low level in 3T3-L1 cells, as FPKM values were approximately 3600 fold lower than GAPDH.

The in-vitro differentiation of 3T3-L1 preadipocytes involves the growth of these cells to confluence (day-2) where they undergo growth arrest. Induction of differentiation (day 0) is triggered by the application of an induction cocktail that activates insulin growth factor (Insulin), cAMP (IBMX) and glucocorticoid (dexamethasone) signaling pathways. Approximately 16 to 20 h post induction, the cells re-enter the cell cycle and undergo mitotic clonal expansion (MCE), after which point they exit the cell cycle and terminally differentiate. The adipogenic transcription factors CEBPα and PPARγ are upregulated post MCE and drive the adipogenic program.[27,46]

Figure 2A shows this characteristic induction of PPARγ expression in 3T3-L1 differentiation alongside the observed downregulation of Itm2a at day 2. It is unclear whether Itm2a is downregulated before or after PPARγ transcriptional activation, however the data does suggest that Itm2a expression is reduced in response to the addition of the induction cocktail (MDI) containing IBMX (M), dexamethasone (D) and Insulin (I).

To explore the relationship between these induction components and the observed reduction in Itm2a expression we constructed a series of piggyBac transposable luciferase reporter plasmids containing fragments of the mouse Itm2a promoter (2 kb and 0.5 kb) (Fig. 2B) and analyzed their activity in response to full (MDI) and sub-maximal (DI or D) induction cocktail. 3T3-L1 pre-adipocytes were stably transfected with the luciferase constructs, grown to confluence, and induced to differentiate. Oil-Red-O staining at day 8 showed reduced adiposity of cells treated with the sub-maximal induction cocktail (DI or D). Luciferase activity was measured at day 1 (24 h) and 2 (48 h) post induction of differentiation. Cells stably transfected with the 2 kb or 0.5 kb reporter construct displayed reduced luciferase activity in response to the full induction mix (MDI) in comparison with partial induction mixes DI or D (Fig. 2C, D) indicating that full induction of adipogenesis is associated with reduction of Itm2a promoter activity in the early stages of differentiation.

The luciferase data indicates that the M (IBMX) component of the MDI is critical for the observed reduction in Itm2a promoter activity. Forskolin like IBMX is known to raise cellular cAMP levels and activate the cAMP-PKA-CREB signaling pathway in adipogenesis and these 2 agents lead to similar levels of 3T3-L1 and MEF cell differentiation when used in an adipogenic induction cocktail.[47,48] However, in contrast to the situation in adipogenesis it has been reported that forskolin mediated PKA-CREB activation leads to increased human Itm2a promoter activity through a conserved CRE site in transfected HEK293 cells.[35] This indicates that regulation of the Itm2a promoter differs in different cell contexts with CREB signaling resulting in Itm2a downregulation during 3T3-L1 cell differentiation vs. upregulation in HEK293 cells.

The effect of lamin A on the Itm2a promoter

In an attempt to investigate the effects of LMNA expression on the Itm2a promoter we performed a series of transient co-transfections in both 3T3-L1 and 3T3-NIH cells. However, in all instances lamin A overexpression affected the expression of luciferase from the internal transfection control preventing data normalization. This phenomenon was observed with all 3 constitutive promoters commonly used in standard control luciferase constructs (CMV, SV40 and HSV-TK promoters).

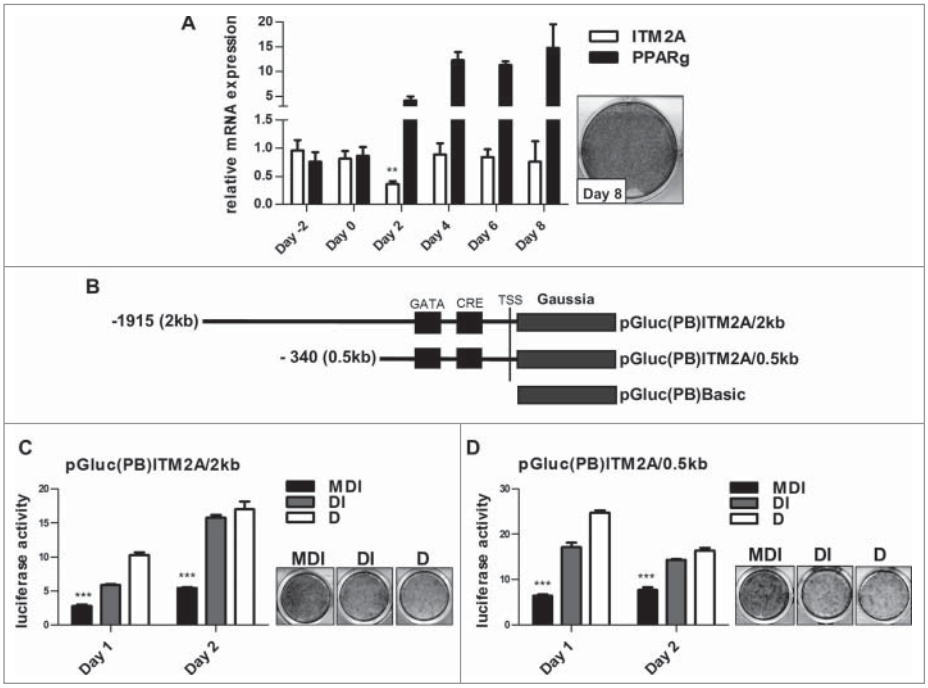

Figure 2. Endogenous Itm2a expression and promoter activity during 3T3-L1 differentiation (A) 3T3-L1 preadipocytes were grown to confluence (day-2) and 2 d later induction media was applied (day 0). A further 2 d later cells were supplemented with fresh media containing insulin (day 2). Following this, fresh media was applied every 2 d until day 8. Adipogenesis was assessed at day 8 by staining with Oil Red O. Total RNA was isolated at the indicated time points during 3T3-L1 differentiation. Itm2a and PPARγ expression was analyzed by qPCR. Transcript expression at the various time points is shown relative to expression at day 0. A Student's *t*-test (2-tailed, assuming equal variance) was used to calculate statistical significance at day 2 in comparison to transcript levels at day 0. (B) Schematic depiction of Itm2a promoter luciferase constructs bearing a 2 kb or 0.5 kb promoter fragment cloned upstream of *Gaussia* luciferase in pGluc(PB) basic vector. The distance (−1915 and −340) from the transcriptional start site (TSS) and previously predicted GATA and CRE binding sites are shown. (C, D) Luciferase activity (secreted) in 3T3-L1 cells stably transfected with pGluc(PB)ITM2A/2 kb and pGluc(PB) ITM2A/0.5 kb at day 1 and 2 of differentiation. Cells were induced to differentiate using full differentiation media (MDI– methylisobutylxanthine, dexamethasone and insulin), sub-maximal media (DI– dexamethasone and insulin) or D (dexamethasone) as indicated. Adipogenesis was assessed at day 8 by staining with Oil Red O. Luciferase activity is normalized to the pGluc(PB)basic empty vector control. Student's *t*-test (2-tailed, assuming equal variance) was used to calculate statistical significance compared with MDI induced cells, indicated as follows: $^{*} = P < 0.05$;$^{**} = P < 0.01$; $^{***} = P < 0.001$.

To circumvent this issue, we generated lamin A/Itm2a reporter stable 3T3-L1 lines using a 2-step transfection and selection approach; 3T3-L1 preadipocytes were stably transfected with the Itm2a reporter construct (G418 selection) and subsequently stably transfected with lamin A overexpression constructs (puromycin selection). However, results with these dual cell lines were highly variable and inconsistent and consequently this approach was not pursued.

Itm2a overexpression inhibits 3T3-L1 differentiation

To investigate the role of Itm2a in adipogenesis we stably transfected 3T3-L1 preadipocytes with a piggyBac transposable Itm2a overexpression construct (pMSCV(PB) Itm2a) or empty vector control (pMSCV(PB)) and induced the cells to differentiate. Oil Red O staining at day 8 showed that 3T3-L1 differentiation was inhibited to a moderate but significant degree in the Itm2a expressing cells in comparison to EV control (Fig. 3A). QPCR analysis of Itm2a expression at various time points in these differentiating cells confirmed maintenance of Itm2a overexpression throughout adipogenesis (Fig. 3B).

PPARγ appeared to be unaltered by increased Itm2a expression; qPCR analysis showed no significant difference in PPARγ expression throughout differentiation in comparison to EV control (Fig. 3C) and PPARγ protein isoforms were equally unaffected (Fig. 3D, E). CEBPα expression was also assessed through qPCR analysis, and although small variations were observed, overall no significant differences between Itm2a overexpressing cells and EV control were observed (data not shown).

Detection of ectopically expressed Itm2a protein in 3T3-L1 cells was attempted using both a Flag antibody and a commercially available Itm2a antibody, but was unsuccessful. The reason for our inability to detect Itm2a in the transfected 3T3-L1 cells was unclear. Similar

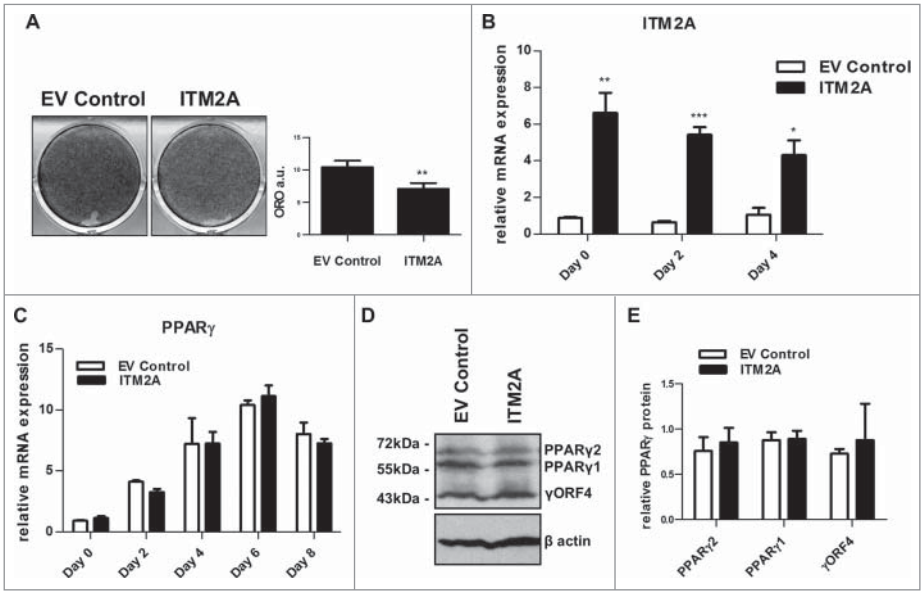

Figure 3. Itm2a overexpression in 3T3-L1 differentiation. 3T3-L1 preadipocytes were stably transfected with pMSCV(PB)-Itm2a or empty vector control pMSCV(PB) plasmid and induced to differentiate into adipocytes. (A) Adipogenesis was assessed at day 8 post induction by staining with Oil Red O and quantification was performed using ImageJ and expressed as Oil red O absorbance units (ORO a.u.). (B,C) qPCR analysis of Itm2a and PPARγ expression during differentiation of stably transfected 3T3-L1 cells. (D) Immunoblot analysis of PPARγ at day 4 post induction. (E) Quantification of PPARγ protein relative to β-actin, with mean and standard deviations determined by densitometry from 2 biologic replicates. A Student's *t*-test (2-tailed, assuming equal variance) was used to calculate statistical significance compared with empty vector control cells, indicated as follows: $^{*} = P < 0.05$; $^{**} = P < 0.01$; $^{***} = P < 0.001$.

results were reported in a previous study by Boeuf et al., 2009, where they were unable to detect a tagged version of ectopically expressed Itm2a protein in the bipotential C3H10T1/2 cell line. Boeuf et al., 2009 were equally unsuccessful in detecting endogenous Itm2a in this cell line, despite using commercial antibodies and 4 different anti-sera raised against 2 distinct Itm2a epitopes.

The mechanism underlying the difficulty in overexpressing Itm2a to high levels in 3T3-L1 cells is unclear. In the experiment described, the overall levels of Itm2a overexpression in 3T3-L1 cells were approximately 8-fold higher than control. We attempted to boost overexpression of Itm2a to a higher level by placing the gene under the control of a strong CMV promoter to see if adipogenesis could be inhibited further by higher Itm2a expression levels and to facilitate Itm2a protein detection. However, expression levels of Itm2a under the CMV promoter (pCMV(PB)Itm2a), were similar to the levels observed with the MSCV promoter. In comparison, lamin A overexpression driven from the same CMV promoter is approximately 5000–10000-fold higher than control transfected cells, as measured by qPCR (Fig. 1B).

Knockdown of Itm2a enhances 3T3-L1 adipogenesis

The role of endogenous Itm2a in 3T3-L1 cell differentiation was explored by silencing Itm2a expression. 3T3-L1 preadipocytes were stably transfected with a piggyBac transposable Itm2a shRNA knockdown construct, pRFP(PB).shItm2a or scramble control pRFP (PB).shControl and induced to differentiate. Knockdown of endogenous Itm2a appeared to enhance adipogenesis in comparison to control cells (Fig. 4A). The stably transfected cells were induced to differentiate using full induction media MDI, sub-maximal media DI or D alone. Enhanced adipogenesis was observed in response to the full MDI induction media while a significant difference was not observed with DI or D alone (Fig. 4A). QPCR analysis of Itm2a expression confirmed the knockdown of endogenous Itm2a at various time points across differentiation (Fig. 4B). A similar stimulatory effect on adipogenesis was observed with 3 (shItm2a-1, −2 and −3) out of 4 different shItm2a constructs assayed when compared with the scramble control (data not shown).

Similar to the difficulty encountered with detection of ectopically expressed Itm2a, detection of the endogenous Itm2a protein in 3T3-L1 cells using the commercial Itm2a antibody was unsuccessful despite repeated efforts. In contrast, Itm2a overexpression was detectible in transfected 3T3-NIH mouse fibroblasts. Thus, validation of shRNA mediated Itm2a knockdown at the protein level was performed in these cells. 3T3-NIH cells were transfected with pCMV.ITM2A and either pRFP(PB).shITM2A or scramble control pRFP(PB). shControl. Itm2a protein knockdown was analyzed by

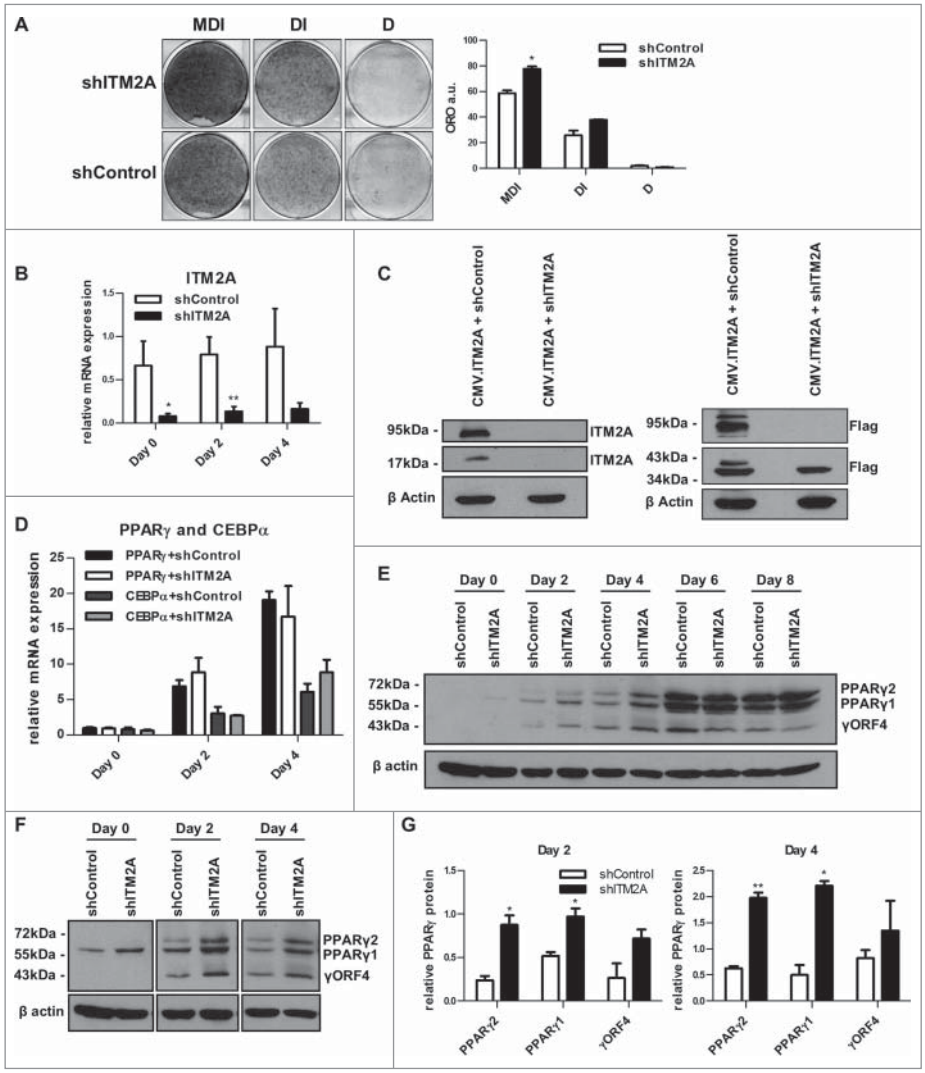

Figure 4. shRNA mediated knockdown of Itm2a enhances 3T3-L1 differentiation and increases PPARγ protein. 3T3-L1 preadipocytes were stably transfected with pRFP(PB).shItm2a or scramble control pRFP(PB).shControl and induced to differentiate using full induction media MDI (methylisobutylxanthine, dexamethasone and insulin), sub-maximal media DI (dexamethasone and insulin) or D (dexamethasone) as indicated. (A) Adipogenesis was assessed at day 8 post induction by staining with Oil Red O; quantification was performed using ImageJ and expressed as Oil Red O absorbance units (ORO a.u.). (B, D) qPCR analysis of Itm2a, PPARγ and CEBPα and immunoblot analysis of PPARγ (E,F) expression during differentiation of 3T3-L1 cells stably transfected with pRFP(PB).shItm2a or scramble control pRFP(PB). (C) Immunoblot analysis of ITM2A knockdown in 3T3-NIH cells dual transfected with pCMV.Itm2a and pRFP(PB).shItm2a or pRFP(PB).sh.Control. (G) Quantification of PPARγ protein isoforms relative to β-actin, with mean and standard deviations determined by densitometry from 2 biologic replicates. Statistical significance compared with scramble control cells indicated as follows: $^{*} = P < 0.05$; $^{**} = P < 0.01$; $^{***} = P < 0.001$.

immunoblot with Itm2a and Flag antibodies (Fig. 4C) and several Itm2a protein species were detected.

To date, there is a limited amount of data published on the murine Itm2a protein. The predicted molecular weight of the 263 amino acid protein is 30kDa,[29] however immunoblot detection has previously reported a 45kDa protein in mouse brain tissue and 2 protein species of 45kDa and 43kDa in the EL4 T lymphocyte cell line.[49,50] An N-linked glycosylation site is predicted at amino acid 166,[29] and protein de-glycosylation in the EL4 T lymphocytes was seen to convert the 45kDa and 43kDa proteins to a smaller 39kDa protein.[50] Proteolytic processing of the Itm2a related protein ITM2B (human) has been characterized in HEK293TR cells, and several cleavage events identified. Firstly, a propeptide is released from the C-terminal of the protein through furin-mediated cleavage, followed by shedding of the extracellular BRICHOS domain via ADAM10 processing. Finally, the residual N-terminal fragment undergoes intramembrane proteolysis by a signal peptide peptidase-like 2 (SPPL2) protease, to produce an intracellular domain.[51,52] As protein structure is highly conserved within the ITM2

family,[30] it is likely that Itm2a is also processed in a similar fashion.

In our analysis, several protein species were detected when Itm2a was overexpressed in 3T3-NIH cells. A 90kDa protein was detected using both Itm2a and FLAG antibodies (Fig. 4C) and its expression was inhibited by shItm2a. Although much larger than the predicted Itm2a size, it could represent a heavily glycosylated form of the protein, or a dimer. We consistently detected a 20kDa protein, which was eliminated by shItm2a expression. This protein could potentially represent an Itm2a N-terminal fraction produced by cleavage similar to that described for ITM2B.[51] Itm2a is Flag-tagged at the C-terminal and in support of our cleavage theory, the Flag antibody was unable to detect the 20kDa protein. A 40 kDa protein was also detected using the FLAG antibody which was partially knocked down by the shItm2a. Finally, the Itm2a antibody was seen to bind a 20kDa protein in the cell culture media, which could potentially represent the cleaved and secreted BRICHOS domain (data not shown).

PPARγ and CEBPα expression in transfected cells was analyzed by qPCR during differentiation. No alterations were observed in the expression of either transcription factor in response to Itm2a knockdown (Fig. 4D). Surprisingly, when PPARγ protein levels were measured in these cells, an increase in all 3 PPARγ isoforms was observed at day 2 and 4 of differentiation in comparison with control cells (Fig. 4E, F). Increased PPARγ1 protein was also observed at day 0 (Fig. 4E, F) before induction of the adipose specific PPARγ2 isoform. Densitometry performed across biologic replicates (Fig. 4G) confirmed a significant increase in PPARγ1 and PPARγ2 protein isoforms, while elevated γORF4 was less consistent. The observation that there was no difference in PPARγ mRNA expression between shItm2a and shControl transfected cells indicates that Itm2a knockdown is likely to influence PPARγ protein stability. An increase in PPARγ2 protein stability during adipogenic differentiation would be expected to promote adipogenesis and could account for the observed increase of lipid droplet accumulation in cells transfected with shItm2a.

Itm2a knockdown rescues lamin A inhibition of adipogenesis in 3T3-L1 differentiation

The observation that lamin A increases Itm2a expression and knockdown of Itm2a enhances adipogenesis led us to consider whether Itm2a knockdown could ameliorate lamin A inhibition of adipogenesis. To investigate this idea, we generated a series of 6 dual constructs that contained either LMNA WT, LMNA R482W or empty vector (EV) with shItm2a or shControl. The constructs created were pCMV(PB)-Flag-LMNA-WT.shItm2a, pCMV(PB)-Flag-LMNA-WT.shControl, pCMV(PB)-Flag-LMNA-R482W.shItm2a, pCMV(PB)-Flag-LMNA-R482W.shControl, pCMV(PB).shItm2a and pCMV(PB).shControl. 3T3-L1 preadipocytes were stably transfected with these constructs and induced to differentiate. Figure 5A shows that Itm2a knockdown rescued inhibition of adipogenesis mediated by both WT lamin A and R482W mutant. Moreover, Itm2a knockdown in the lamin A expressing 3T3-L1 cells enhanced adipogenesis to the same extent as Itm2a knockdown enhanced adipogenesis in the EV control cells. Lamin A overexpression was assessed at the mRNA and protein levels (Fig. 5B, C). Interestingly, increased amounts of lamin A protein were observed when Itm2a expression was reduced (Fig. 5C), lending support to the notion that Itm2a knockdown somehow influences protein stability in 3T3-L1 cells.

As previously mentioned Itm2a knockdown does not appear to alter PPARγ expression in comparison to shControl (Fig. 4D). Figure 5D reiterates this, with Itm2a knockdown having no effect on PPARγ mRNA levels at day 4 of differentiation when cells were stably transfected with pCMV(PB).shItm2a or pCMV(PB).shControl. Itm2a knockdown was however able to rescue the lamin A mediated reduction of PPARγ mRNA, and restore it to EV control expression levels. PPARγ was assessed at the protein level and as previously observed lamin A (WT and R482W) overexpression was seen to reduce PPARγ2 while Itm2a knockdown increased the protein levels of all 3 PPARγ isoforms (Fig. 5E). This significant increase in PPARγ2 protein, presumably mediated by increased stability, is most likely the driving force behind the enhanced adipogenesis observed in shItm2a transfected cells. The increase in PPARγ2 protein arising from Itm2a knockdown is able to enhance adipogenesis despite the lamin A mediated reduction in PPARγ transcription and expression. Thus, Itm2a knockdown not only rescues but also overrides lamin A inhibition of adipogenesis.

Itm2a expression in lamin A wild type and KO MEFs

To further elucidate the relationship between lamin A and Itm2a we investigated the endogenous levels of Itm2a expression in wild type and lamin A knockout mouse embryonic fibroblasts (MEFs). Previously generated LMNA −/− MEFS were grown as described and lamin A knockout was confirmed at the protein level in these cells (Fig. 6A). Measurement of Itm2a expression in the lamin A knockout MEFs showed that Itm2a was significantly reduced in these cells in comparison with

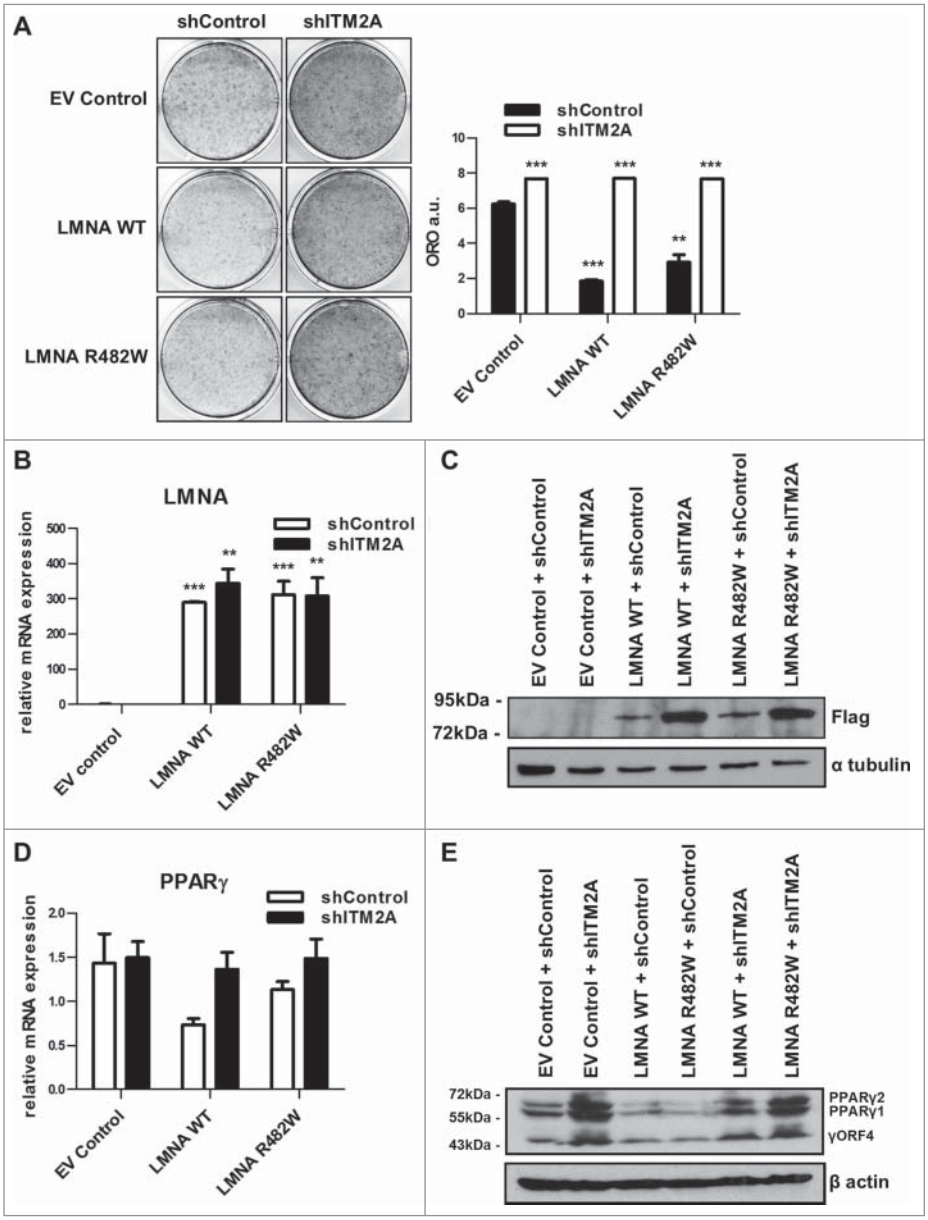

Figure 5. shRNA mediated knockdown of Itm2a rescues LMNA inhibition of 3T3-L1 differentiation. 3T3-L1 preadipocytes were stably transfected with pCMV(PB)-Flag-LMNA-WT.shItm2a, pCMV(PB)-Flag-LMNA-WT.shControl, pCMV(PB)-Flag-LMNA-R482W.shItm2a, pCMV (PB)-Flag-LMNA-R482W.shControl or empty vector control pCMV(PB).shItm2a and pCMV(PB).shControl dual constructs. The cells were induced to differentiate. (A) Adipogenesis was assessed at day 8 post induction by staining with Oil Red O; quantification was performed using ImageJ and expressed as Oil red O absorbance units (ORO a.u.). (B) Total RNA was isolated at day −2 of differentiation from the stably transfected 3T3-L1 preadipocytes and LMNA expression measured by qPCR using primers specific for human LMNA. (C) Immunoblot analysis of flag-LMNA WT and R482W mutant at day −2. (D) Total RNA was isolated at day 4 post induction and the expression of PPARγ was analyzed by qPCR. (E) Immunoblot analysis of PPARγ at day 4 post induction. Student's *t*-tests (2-tailed, assuming equal variance) were used to calculate statistical significance compared with empty vector control cells, indicated as follows: $^{*} = P < 0.05$; $^{**} = P < 0.01$; $^{***} = P < 0.001$.

wild type MEFs (Fig. 6B), supporting the theory that lamin A increases or modulates Itm2a expression in some way. These results are in keeping with our findings. Furthermore and consistent with our finding that Itm2a knock down enhances adipogenesis, lamin A knockout MEFs differentiate into adipocytes more readily than their WT counterparts.[28]

Discussion

While significant advances have been made in understanding the pathophysiology of FPLD2, the molecular etiology of the disease remains to be confirmed. Previous reports have described pleiotropic effects of lamin A on gene regulation and interaction of lamin A with multiple

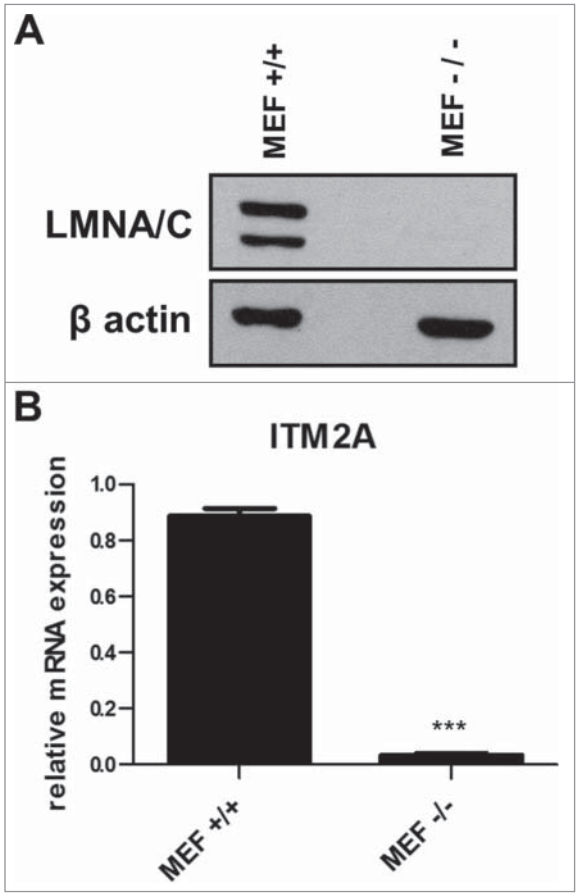

Figure 6. Itm2a expression is downregulated in LMNA KO MEFs. (A) Total RNA was isolated from LMNA wild type (+/+) and knockout (−/−) MEFs and the expression of Itm2a was analyzed by qPCR. (B) Immunoblot analysis of mouse LMNA in wildtype and KO MEFs. Student's *t*-test (2-tailed, assuming equal variance) was used to calculate statistical significance compared with LMNA wildtype control cells, indicated as follows: $^{*} = P < 0.05$; $^{**} = P < 0.01$; $^{***} = P < 0.001$.

proteins. Both of these aspects are altered by mutations in the LMNA gene.[17-20] In the case of FPLD2, there is a clear impact of lamin A mutations on adipogenesis and several studies have proposed altered lamin-SREBP1 interactions as the driving force of the disease phenotype.[17-19,53] Boguslavsky et al. (2006) originally reported that overexpression of both WT and R482W mutant lamin A inhibit 3T3-L1 differentiation. The fact that overexpression of wild-type as well as mutant lamin A has an effect on adipogenic conversion led to the suggestion that mutations responsible for FPLD2 may confer a 'gain of function' and result in higher binding affinity to a target. They suggest that overexpression of a wild-type lamin A may lead to 'gain of function' phenotype not because of an increased binding affinity to a target but due to increased number of molecules with a normal binding affinity competing for it. Here, we investigated the effect of lamin A in the early stages of adipogenesis, and our RNA-Seq data indicates that overexpression of both WT and R482W mutant lamin A cause altered expression of 76 genes in common, consistent in part with the proposal of Boguslavsky et al. (2006). Our analysis indicated that Itm2a regulation is altered by lamin A overexpression and led us to explore the relationship between Itm2a expression and adipogenesis as well as the modulatory relationship between lamin A and Itm2a within this context.

Itm2a in adipogenesis

Characterization of Itm2a activity during 3T3-L1 differentiation identified a distinct expression profile for this gene in adipogenesis and showed that endogenous Itm2a is transiently downregulated in early cell differentiation. This transient downregulation has not been reported previously even though global gene expression has been profiled during 3T3-L1 adipogenesis.[45] Our work here indicates that Itm2a is a lowly expressed gene/protein in 3T3-L1 cells and that it's expression is only altered for a specific period, around day 2 of adipogenesis. Investigation of cells stably transfected with a 2 kb or 0.5 kb Itm2a promoter reporter construct confirmed that Itm2a promoter activity is reduced in 3T3-L1 cells when adipogenesis is fully induced using MDI, while a reduction in promoter activity is not observed under limiting induction conditions with DI or D alone (Fig. 2C, D). Thus, full induction of adipogenesis is associated with reduced Itm2a promoter activity in the early stages of differentiation.

Investigation of the role of Itm2a in adipogenesis showed that ectopic expression of Itm2a moderately but significantly inhibited the differentiation of 3T3-L1 cells. However, the mechanism of this inhibition is unclear as there were no obvious effects on the mRNA or protein levels of the key adipogenic transcription factors PPARγ and CEBPα in Itm2a over-expressing cells. Knockdown of endogenous Itm2a had the opposite effect to overexpression and enhanced adipogenesis in comparison to control cells. Similar to Itm2a overexpression, knockdown of Itm2a did not effect PPARγ and CEBPα mRNA levels. In contrast, a significant increase in PPARγ1 and PPARγ2 protein isoforms was observed and although not significant, a similar trend was observed for the γORF4 isoform (Fig. 4D, E). These observations suggest that Itm2a knockdown enhances PPARγ protein stability. Such an enhancement could explain the effect of Itm2a knockdown on adipogenesis as an increase in PPARγ2 protein stability would be expected to promote adipogenesis.

While the mechanisms underlying enhanced PPARγ protein stability in Itm2a knock down cells are unclear, altered regulation of autophagy during cell differentiation

may be involved as a) autophagy is known to be upregulated during the early phase (day 0 to 4) of 3T3-L1 adipogenic differentiation,[54,55] and autophagic degradation of adipogenic inhibitors is considered necessary for cell differentiation,[56] b) inhibition of autophagy by genetic or chemical means inhibits 3T3-L1 and MEF cell differentiation into mature adipocytes,[57,58] c) autophagy activation is likely to stabilize PPARγ2 in 3T3-L1 differentiation through the repression of proteasome-dependent PPARγ2 degredation,[55] d) Itm2a is reported to regulate autophagic flux by interacting with specific v-ATPases, the proton pumps that mediate lysosome acidification,[35,59] and c) ITM2A overexpression is seen to interfere with autophagic flux in a similar way to BafA1, an autophagy inhibitor that blocks the formation of mature autolysosomes.[35]

Lamin A and Itm2a

Consistent with previous reports,[28,40] we observed that overexpression of WT and R482W mutant lamin A have an inhibitory effect on 3T3-L1 differentiation. Here we show that endogenous Itm2a expression is upregulated during adipogenesis in WT lamin A and R482W mutant cells (Fig. 1G), suggesting that lamin A mediated altered regulation of Itm2a may play a role in lamin A inhibition of adipogenesis. In agreement with this, we detected significantly lower levels of endogenous Itm2a expression in LMNA knockout vs. normal MEFs (Fig. 6B). Taken together, these data indicate that lamin A has a regulatory role in the control of Itm2a expression.

We attempted to determine if WT lamin A or mutant directly affected the expression of Itm2a using the 2 kb and 0.5 kb Itm2a promoter reporter constructs. However, data normalization proved problematic as transient lamin A overexpression had significant effects on the expression of firefly luciferase from all 3 constitutive promoters commonly used for normalization purposes in promoter reporter assays. This is not surprising given that lamin A is implicated in many different aspects of genome biology through extensive interactions with chromatin (Dechat et al., 2008) and with proteins of the nuclear lamina (Mendez lopez and Worman 2012). Efforts to circumvent this issue by using a 2-step stable transfection approach to generate lamin A/Itm2a reporter stable 3T3-L1 lines were also problematic as results were highly variable and thus it was not possible to demonstrate a direct effect of lamin A on the Itm2a promoter reporter constructs during differentiation. Alternative approaches will be required to determine the precise mechanism by which lamin A influences Itm2a expression.

Overall the data suggests that lamin A overexpression is likely to inhibit adipogenesis through a combination of mechanisms. In addition to previous work showing that lamin A alters SREBP1 and FXR1P function in adipogenesis,[19,20] and reduces PPARγ expression,[28] our work demonstrates an effect on Itm2a expression that is likely to have a functional effect on adipogenesis in 3T3-L1 preadipocytes.

It is not clear how lamin A alters Itm2a expression in this context. One plausible mechanism stems from the report by Lund et al., (2013) where they identified 4000 genes that disengage from lamin A during human ASC differentiation. Perusal of their data set shows that ITM2A is part of this gene set and suggests that lamin A disengagement from ITM2A may facilitate its transient downregulation in cell differentiation.

Lamin A and Autophagy

The observation that knockdown of Itm2a enhances adipogenesis prompted us to explore whether lamin A inhibition of adipogenesis could be rescued by knocking down Itm2a expression. Interestingly the lamin A inhibition was completely rescued by this approach (Fig. 5 A). Moreover, the rescue was effective despite the observation that the Itm2a knockdown enhanced the stability of lamin A. A likely explanation for this is that the increased stability of PPARγ in the Itm2a knockdown cells drives adipogenesis forward and its effects are dominant over the inhibitory effects of the increased lamin A.

The relationship between ITM2A and autophagy along with the demonstration that Itm2a knockdown can suppress lamin A inhibition of adipogenesis suggests a potential role for autophagy modulation in the treatment of lamin associated lipodystrophies. A recent study has described autophagy dysregulation in the LMNA H222P driven cardiomyopathy, where increased AKT-mTORC1 signaling is observed in human patients.[60] Treatment of LMNA H222P/H222P mice with the mTOR inhibitor temsirolimus was seen to reactivate autophagy and lead to improved cardiac function in these mice.[60] In addition, the treatment of Hutchinson-Gilford Progeria Syndrome (HGPS – premature aging laminopathy) patient primary fibroblasts with the mTOR inhibitor rapamycin, was seen to improve the disease phenotype and slow senescence of these cells by stimulating autophagy.[61] Thus, taken together our findings suggest that exploration of therapies that reactivate autophagy are warranted for treatment of FPLD2.

Materials and methods

Cell culture and transfections

3T3-L1 preadipocytes were from Zen-Bio. LMNA WT and KO MEF cells were kindly provided by Colin Stewart. 3T3 NIH and mouse embryonic fibroblasts (MEFs)

were maintained in standard growth medium; Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco – South American origin), 2 mM L-glutamine and 100 U/ml penicillin, 100 μg/ml streptomycin, at 37°C in a 5% CO2 incubator. For differentiation, 3T3-L1 cells were grown to confluence in standard growth medium (day −2). Two days post confluence (day 0) cells were induced with 0.5 mM 3-Isobutyl-1-methylxanthine, 10 μg/ml insulin and 1 μM dexamethasone (MDI), 10 μg/ml insulin and 1 μM dexamethasone (DI) or just 1 μM dexamethasone (D). Two days later (day 2) fresh medium containing 10 μg/ml insulin was applied. Fresh standard medium was applied every 2 d after that until day 8 when cells were fixed and stained with Oil Red O. Briefly, cells were washed with phosphate buffer saline (PBS) and fixed with 10% formaldehyde solution (Sigma-Aldrich) in PBS for 15–30 minutes at 37°C. Cells were washed with water and a working Oil Red O solution (0.5% in isopropanol – diluted 3:2 with ddH20, Sigma-Aldrich) applied overnight. Oil Red O quantification was performed using ImageJ as described previously[62] and expressed as Oil red O absorbance units (ORO a.u.). For all stable transfections, 3T3-L1 cells were grown to 50–70% confluency in 60 mm dishes and co-transfected with overexpression/shRNA/reporter constructs and piggyBac transposase using Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer's instructions. Between 24 and 48 h post transfection selection antibiotics were applied (800 μg/ml G418 or 0.75–1.5 ug/ml puromycin, Sigma Aldrich) and cells were selected for 1–2 weeks. 3T3 NIH transient transfections were performed with TurboFect *in vitro* transfection reagent (Thermo Scientific), according to the manufacturer's instructions. 48 h post transfection the cells were lysed and Itm2a protein analyzed by immunoblot.

RNA-Seq

3T3-L1 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 5% CO2 and 37°C. For transfection, cells were grown to 70% confluency and transfected with the overexpression vector pcDNA3.1 bearing the LMNA mutant (R482W) or wild type gene or with an empty vector control using Lipofectamine 2000 (Invitrogen). After 24 hrs post transfection, G418 was applied (800 μg/ml G418) for approximately 2 weeks to select for stable transfectants. For differentiation, 3T3-L1 cells were grown to confluence in standard growth medium (day −2). Two days post confluence (day 0) cells were induced to differentiate in fresh medium containing 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone (D) and 10 μg/ml insulin (I). 36 hrs post-induction total RNA was isolated. For RNA seq analysis a DNase-treated RNA (3 μg) sample of the mutant, wild-type and control RNA preparations were used to prepare RNA-sequencing libraries with the TruSeq RNA Sample Prep kit for RNA-sequencing. Libraries were prepared according to the Illumina protocol (Illumina Part #15008136 Rev. A) and sequenced on an Illumina GAII sequencer (Trinity Genome Sequencing Laboratory, Institute of Molecular Medicine, Trinity College Dublin, Ireland). The workflow to prepare the libraries consisted of purification and fragmentation of the mRNA, first strand cDNA synthesis, second strand cDNA synthesis, repair of fragmented ends into blunt ends, adenylate 3′ ends, ligation of adapters, PCR amplification and library validation. 60 bp paired end reads were obtained from an Illumina GAII in FASTQ format. Sequence ends were filter by quality after processing using the FASTQ quality trimmer from the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Sequence reads were aligned to the mouse reference genome (mm9) using Tophat.[63] FPKM values were analyzed for differentially expressed genes using the Cufflinks package and annotations from UCSC.[64]

With respect to the expression levels of the constructs, analysis of unmatched RNA-Seq reads not aligning to the mouse genome and aligning to the human LMNA gene was performed for first and second pair-end reads and normalized to total unmatched reads per sample and indicated that the expression level of WT LMNA was approximately 3.25 fold higher than the R482W mutant LMNA (615 vs 190 reads respectively).

PiggyBac transposable constructs

PiggyBac transposable constructs were generated as follows; piggyBac (PB) terminal repeats (TR) were amplified from pCyL50 and cloned into the overexpression vectors pIRES2-EGFP (Clontech), and pMSCVpuro (Clontech) and the knockdown vector (pRFP-C-RS (Origene) such that they flanked the promoter/MSC and mammalian antibiotic resistance markers. The 5′PBTR was amplified using the forward primer (FP) 5′GGTACCTCGCGCGACTTGGTTTGC3′, and the reverse primer (RP) 5′GCTAGCCAACAAGCTCGTC ATCGC3′. The 3′PBTR was amplified using 5′TTAA TTAACGAGAGCATAATATTGATAT3′ as a FP 5′GAG CTCGGTATTCACGACAGCAGG3′ an a RP. Gibson assembly cloning was used to insert the TR into each construct, and the primers described above contained additional 'overlap' sequence of approximately 15bp at the 5′ end, to complement each vector respectively and facilitate the assembly cloning. The 5′PBTR was inserted into the AseI site of pIRES2-EGFP, the NdeI sites of

pMSCVpuro and the EcoR1 site of pRFP-C-RS, while the 3′PBTR was inserted into the BsaI site of pIRES2-EGFP, the PciI sites of pMSCVpuro and the PciI site of pRFP-C-RS. In addition, piggyBaC-terminal repeats were cloned into the pGlucBasic (NEB) secreted luciferase backbone in the manner described above. The 5′PBTR was inserted into the AaT II site and the 3′PBTR was inserted into the BstZ17I site to produce pGluc(PB)basic. A human LMNA (WT and R482W mutant) cDNA plasmid was kindly provided by Howard J Worman. LMNA was amplified from this plasmid and inserted into the XhoI and EcoRI sites of pIRES(PB)-EGFP. For clarity, we will refer to these constructs as pCMV(PB)-Flag-LMNA-WT and pCMV(PB)-Flag-LMNA-R482W. Itm2a mouse cDNA was purchased from Origene (MR203468), amplified and inserted into the XhoI and EcoRI sites of pMSCV(PB)puro, to generate a fusion protein with a 107aa C-terminal addition that included a Myc-DDK tag and produce pMSCV(PB)-Itm2a. The Itm2a cDNA was also amplified and cloned into the EcoR1 and BamHI sites of pIRES(PB)-EGFP with a separate 69aa C-terminal addition also including a Myc-DDK tag to produced pCMV(PB)Itm2a. Itm2a shRNAs were purchased from origene (TF501127 – shRNA sequences in Table 1) in the pRFP-C-RS vector and piggyBaC-terminal repeats were cloned into each shRNA plasmid and a scramble control (TR30015) plasmid as described above. Six dual constructs were generated as follows; the U6 promoter and shItm2a/shControl (TR30015 scramble) were amplified from the original pRFP-C-RS backbone and inserted into the AflII site of pIRES(PB)-EGFP empty vector and the described previously pCMV(PB)-Flag-LMNA-WT and pCMV(PB)-Flag-LMNA-R482W to produce dual LMNA overexpression/ITM2A knockdown constructs and the relevant controls. The constructs were named as follows; pCMV(PB)-Flag-LMNA-WT.shItm2a, pCMV(PB)-Flag-LMNA-WT.shControl, pCMV(PB)-Flag-LMNA-R482W.shItm2a, pCMV(PB)-Flag-LMNA-R482W.shControl or empty vector control pCMV(PB).shItm2a and pCMV(PB).shControl. Itm2a promoter fragments (2 kb and 0.5 kb) were amplified from mouse genomic DNA and inserted into the EcoRI and HindIII sites of pGluc(PB)basic. The PB transposon pCyL50 construct and mPB mouse codon optimized piggyBac transposase used in all stable transfections were obtained from the Wellcome Trust Sanger Institute.[65,66] All amplified PBTR, genes and promoter segments were verified by sequencing. Primer sequences are listed in Table 1.

Table 1. primers used to amplify piggyBac transposon terminal repeats 5′PBTR and 3′PBTR, for cloning into pIRES2-EGFP, pMSCVpuro, pRFP-C-RS, pGlucBasic.

Primer name	Gibson primer sequence
5′PBTR.pIRES2-EGFP5′	cgccatgcattagttatGGTACCTCGCGCGACTTG
5′PBTR.pIRES2-EGFP3′	cccgtaattgattactatGCTAGCCAACAAGCTCGTC
3′PBTR.pIRES2-EGFP5′	cgccatgcattagttatTTAATTAACGAGAGCATAATATTG ATAT
3′PBTR.pIRES2-EGFP3′	cccgtaattgattactatGAGCTCGGTATTCACGAC
5′PBTR.pMSCVpuro5′	tactgagagtgcaccaGGTACCTCGCGCGACTTG
5′PBTR.pMSCVpuro3′	ggtatttcacaccgcaGCTAGCCAACAAGCTCGTC
3′PBTR.pMSCVpuro5′	gataacgcaggaaagaaTTAATTAACGAGAGCATAATAT TGATATC
3′PBTR.pMSCVpuro3′	gctggccttttgctcaGAGCTCGGTATTCACGAC
5′PBTR.pRFPCRS5′	ccggccggatcggtgGGTACCTCGCGCGACTTG
5′PBTR.pRFPCRS3′	gtctttccactggggGCTAGCCAACAAGCTCGTC
3′PBTR.pRFPCRS5′	cactggccaattggttTTAATTAACGAGAGCATAATATTG ATAT
3′PBTR.pRFPCRS3′	cgcggctacaattgttGAGCTCGGTATTCACGAC
5′PBTR.pGlucbasic5′	agtgccacctgacgtGGTACCTCGCGCGACTTG
5′PBTR.pGlucbasic3′	ccgatccgtcgacgtGCTAGCCAACAAGCTCGTC
3′PBTR.pGlucbasic5′	gtatcttatcatgtctgtaTTAATTAACGAGAGCATAATATT GATAT
3′PBTR.pGlucbasic3′	ctagaggtcgacggtaGAGCTCGGTATTCACGAC
U6.shRNA.F	caaactcatcaatgtatcAATTCCCCAGTGGAAAGAC
U6.shRNA.R	cgcttacaatttacgccCTGACACACATTCCACAG
pGluc.ltm2a.2kb.F	gatcgggagatcttggTACTTTTCTGAATAATACAATGTG GACTTC
pGluc.ltm2a.1.5kb.F	gatcgggagatcttggACATCCTGCTTCTAAGGTCC
pGluc.ltm2a.1kb.F	gatcgggagatcttggACACCAGCATCTGGTTATATTG
pGluc.ltm2a.0.5kb.F	gatcgggagatcttggGTTGCAGAACTCAGAAACC
pGluc.ltm2aprom.R	ccgagctcggtaccaGGTGAATCTTCGGGCTGC
pIRES2-EGFP.LMNA.F	ctaccggactcagatcATCGAATTAATACGACTCATTATAG
pIRES2-EGFP.LMNA.R	taccgtcgactgcagCATGATGCTGCAGTTCTG
pMSCVpuro.ltm2a.F	gccggaattagatctcAATTCGTCGACTGGATCC
pMSCVpuro.ltm2a.R	tcccctacccggtagTAAACCTTATCGTCGTCATC
shItm2a.1	CGTGCCATTGACAAATGCTGGAAGATTAG
shItm2a.2	TGTTGGTGGAGCCTGCATTTACAAGTACT
shItm2a.3	GGCGGCAATTATTCACGACTTTGAGAAGG
shItm2a.4	GATGTAGAGGCGCTCGTCAGTCGCACTGT

Immunoblotting

Cell lysates were prepared as follows: 3T3-L1,3T3 NIH or MEF cells were washed with PBS and scraped into lysis buffer (2% SDS, 62.5 mM Tris-HCL; pH 6.8, 10% glycerol, 0.01% bromophenol blue, 41.6 mM DTT). Lysates were sonicated for approx. 10 seconds and centrifuged at 16,000 x *g* for 20 mins at 4°C. For SDS-PAGE, lysates were boiled for 5 minutes and cooled on ice before being separated on 8–12% polyacrylamide gels. Samples were transferred to nitrocellulose membranes (Amersham Protran 0.2 μm NC) and protein transfer was confirmed by Ponceau staining. Membranes were blocked for 1 hr at room temperature and immunoblotted with primary antibodies at 4°C overnight, as recommended by antibody manufacturers. Membranes were then incubated with HRP-conjugated secondary antibody (1:2000 dilution, 5% milk – Amersham ECL IgG, HRP-linked whole ab, GE Healthcare Life Sciences), for 1 hr at room temperature and visualized with SuperSignal West Pico/Femto Chemiluminescent Substrate. Primary antibodies: ectopic lamin A overexpression was detected with monoclonal ANTI-FLAG antibody (Clone M2 – F1804, Sigma Aldrich) and endogenous lamin A was detected with a lamin A/C polyclonal Rabbit antibody (#2032 Cell Signaling). Itm2a overexpression was detected with The

DYKDDDDK tag Antibody (mAB, Mouse, A00187 – GenScript) and an ITM2A Rabbit Polyclonal Antibody (14407–1-AP, Proteintech). Endogenous PPARγ was detected with a PPARγ Rabbit mAB (81D8, #2443 Cell Signaling). β-actin and α-tubulin were detected with the mouse Monoclonal Anti-β-Actin (AC-15) antibody (A5441, Sigma Aldrich) and the mouse Monoclonal Ant-α-tubulin (B-5–1–2) antibody (T6074, Sigma Aldrich), respectively.

Real-time qPCR

Total RNA was isolated from 3T3-L1 and MEF cell biologic triplicates using Trizol reagent, and 1–2 μg of RNA was reverse transcribed to cDNA with the Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instructions. Quantitative qPCR was performed using IDT PrimeTime qPCR probe-based assays, and HOT FIREPol Probe qPCR Mix Plus (ROX) (Solis Biodyne) on the AB7300 Real-Time PCR cycler. All IDT Probe/primer assays were validated with a custom IDT gblock titration. Expression of each gene was normalized to NoNo expression and relative mRNA expression was calculated using the 2(-Delta Delta C(T)) method. IDT PrimeTime qPCR probe-based predesigned assay IDs are listed in Table 2.

Luciferase reporter assay

3T3-L1 cells were stably transfected (as described above) and seeded in triplicate for each differentiation treatment (MDI/DI/D) in 12 well plates. Cells were grown to confluence, and induced to differentiate as described above. Small aliquots of media were taken at 24 hr intervals post media changes and stored at – 20°C until assayed for luciferase activity. Samples (10 μl) were assayed for *Gaussia* secreted luciferase activity with 1.43 μM Coelenterazine (NanoLight Technology) substrate in PBS on the Veritas Microplate Luminometer (Turner Biosystems) as described previously.[67] The luciferase activity directed by each ITM2A promoter fragment was normalized to that of the pGluc(PB)basic empty vector construct to account for the weak promoter activity of the piggyBac transposable arms in the vector backbone.[66] Adipogenesis was assessed at day 8 of differentiation when cells were fixed and stained with Oil Red O as described above.

Table 2. Assay IDs of IDT PrimeTime qPCR probe-based predesigned assays used in quantitative qPCR analysis.

Gene	Assay ID
NoNo	Mm.PT.58.16299938
LMNA	Hs.PT.58.39267032
Itm2a	Mm.PT.58.11424567
CEBPα	Mm.PT.58.30061639.g
PPARγ	Mm.PT.56a.31161924

Statistical analysis

Student's *t*-tests (2-tailed, assuming equal variance) were used to calculate statistical significance, indicated as follows: $^{*} = P < 0.05$;$^{**} = P < 0.01$; $^{***} = P < 0.001$.

Abbreviations

ASC	Adipocyte Stem Cells
CREB	cAMP response element-binding protein
CEBPα	CCAAT/enhancer binding protein α
cAMP	Cyclic adenosine monophosphate
FXR1P	fragile X-related protein 1
FPLD2	Dunnigan-type familial partial lipodystrophy
ITM2A	Integral membrane protein 2
IBMX	3-Isobutyl-1-methylxanthine
MSCs	Mesenchymal Stem Cells
MEFs	Mouse Embryonic Fibroblasts
MCE	mitotic clonal expansion
PPARγ	peroxisome proliferator-activated receptor γ
PB	piggyBac
qPCR	Quantitative real-time PCR
SREBP1	sterol response element binding protein 1
SPPL2	signal peptide peptidase-like 2
TR	terminal repeats
WT	wild type

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The authors declare no conflict of interest.

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