Deregulation of Fragile X-related protein 1 by the lipodystrophic lamin A p.R482W mutation elicits a myogenic gene expression program in preadipocytes

Anja R. Oldenburg¹, Erwan Delbarre¹, Bernd Thiede², Corinne Vigouroux³,⁴,⁵,⁶ and Philippe Collas¹,*

¹Stem Cell Epigenetics Laboratory, Institute of Basic Medical Sciences and Norwegian Center for Stem Cell Research, Faculty of Medicine, University of Oslo, PO Box 1112, Blindern, Oslo 0317, Norway, ²The Biotechnology Centre of Oslo, University of Oslo, PO Box 1125, Blindern, Oslo 0317, Norway, ³INSERM, UMR S938, Centre de Recherches Saint-Antoine, Paris F-75012, France, ⁴UPMC Université Paris 06, UMR S938, Paris F-75005, France, ⁵ICAN, Institute of Cardiometabolism and Nutrition, Paris, France and ⁶AP-HP, Hôpital Tenon, Service de Biochimie et Hormonologie, Paris F-75020, France

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The nuclear lamina is implicated in the regulation of various nuclear functions. Several laminopathy-causing mutations in the LMNA gene, notably the p.R482W substitution linked to familial partial lipodystrophy type 2 (FPLD2), are clustered in the immunoglobulin fold of lamin A. We report a functional association between lamin A and fragile X-related protein 1 (FXR1P), a protein of the fragile X-related family involved in fragile X syndrome. Searching for proteins differentially interacting with the immunoglobulin fold of wild-type and R482W mutant lamin A, we identify FXR1P as a novel component of the lamin A protein network. The p.R482W mutation abrogates interaction of FXR1P with lamin A. Fibroblasts from FPLD2 patients display elevated levels of FXR1P and delocalized FXR1P. In human adipocyte progenitors, deregulation of lamin A expression leads to FXR1P up-regulation, impairment of adipogenic differentiation and induction of myogenin expression. FXR1P overexpression also stimulates a myogenic gene expression program in these cells. Our results demonstrate a cross-talk between proteins hitherto implicated in two distinct mesodermal pathologies. We propose a model where the FPLD2 lamin A p.R482W mutation elicits, through up-regulation of FXR1P, a remodeling of an adipogenic differentiation program into a myogenic program.

INTRODUCTION

The nuclear envelope (NE) separates the nuclear genome from the cytoplasm and regulates many nuclear functions. Interfacing the inner nuclear membrane and chromatin is the nuclear lamina, a meshwork of intermediate filament proteins called lamins (1). Lamins are grouped into A-type lamins (lamins A and C, referred to as ‘lamin A’ from here on), splice variants of the LMNA gene, and B-type lamins encoded by the LMNB1 and LMNB2 genes. Whereas B-type lamins are ubiquitously expressed, A-type lamins are developmentally regulated and expressed in most lineage progenitors and differentiated cells. Lamins interact with the cytoskeleton, chromatin, transcription factors and signaling molecules (1–3), and play a role in genome organization (4). They emerge, therefore, as pleiotropic modulators of cellular functions.

Mutations in nuclear lamins lead to alterations in nuclear architecture (5), signal transduction and gene regulation (3,6), and cause disease. At least 15 disorders, commonly called laminopathies, are linked to over 300 mutations throughout the LMNA gene (3,7). The C-terminal immunoglobulin (Ig) fold of lamin A is a hotspot for mutations linked to myodystrophies and partial lipodystrophies (8,9). Among them, the Arg453 to Trp (p.R453W) mutation causes autosomal-dominant Emery–Dreifuss muscular...
dystrophy (OMIM#181350) (10). Another mutation in the Ig fold substitutes Arg482 also with a Trp (p.R482W) and is linked to Dunnigan-type familial partial lipodystrophy type 2 (FPLD2, OMIM#151660) (11,12). FPLD2 primarily affects adipose tissue, resulting in partial lipoatrophy mainly in the limbs, and metabolic disorders leading to insulin resistance (13). FPLD2 patients also show muscle hypertrophy in the lipoatrophic areas, although this muscle mass presents abnormalities (14–17). Whether a connection exists between the lipoatrophy and myo-hypertrophy symptoms in FPLD2 is unknown. The p.R482W substitution affects the charge of the Ig fold and its interaction with DNA (18) and proteins (19), suggesting that integrity of the Ig fold is essential for proper lamin function.

The fragile X-related (FXR) family of proteins contains the fragile X mental retardation protein (FMRP) and two autosomal paralogs of FMRP, FXR1P and FXR2P, encoded by the FXR1 and FXR2 genes (20). FXR1P is an RNA-binding protein that associates with DICER, microRNAs (miRNAs) and the miRNA machinery to either down-regulate (21) or stimulate (22) translation of its target mRNAs. FXR1P exists in several isoforms (23), including some involved in muscle development (24,25). Fxr1 knock-out mice show muscle wasting (24), and in Xenopus and zebrafish fxr1 down-regulation disrupts somite development (26) and causes muscle abnormalities and cardiomyopathy (27). In humans, deregulated FXR1P expression in muscle occurs in patients with facio-scapulo humeral dystrophy (OMIM#158900), a prevalent myodystrophy in adults and children (28). FXR1P has thus up to now been implicated in muscle development.

Interestingly, fragile X-associated tremor/ataxia syndrome (FXTAS; OMIM#300623), a neurodegenerative disorder affecting carriers of CGG repeats of the FMR1 gene, is associated with neuronal nuclear inclusions containing FMR1 mRNA (29) and lamin A (30). Overexpression of an FMR1 5‴UTR with CGG repeats also results in lamin A inclusions coinciding with FMR1 mRNA, suggesting that FXTAS pathology may be associated with a deregulation of lamin A function (31). There is, however, to our knowledge no link between lamin A and FXR proteins.

We report here the first evidence of an interaction between lamin A and FXR1P, which is deregulated by the lamin A p.R482W mutation. Lamin A down-regulation or the p.R482W substitution leads to FXR1P up-regulation and displacement from the NE. Cells from FPLD2 patients also show up-regulated FXR1P. In human adipocyte progenitors, elevated FXR1P deregulates adipogenesis and promotes a myogenic gene expression program, suggesting a switch from an adipogenic to myogenic induction pathway. Our results provide insights on a cross-talk between proteins hitherto implicated in distinct pathologies affecting fat and muscle tissues.

RESULTS
Lamin A peptide arrays spanning the Ig fold identify FXR1P as a lamin A/C-interacting protein
To gain functional insight into the lamin A p.R482W mutation causing FPLD2, we searched for proteins that differentially interact with the Ig fold of wild-type and mutant lamin A. We used an in vitro peptide mapping approach (Supplementary Material, Fig. S1A), where peptides spanning most of the Ig fold were immobilized on a membrane and incubated with a cell extract. Peptide-bound proteins were identified by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by mass spectrometry or western blotting, or by far-western blotting.

We synthesized 20 amino acid overlapping wild-type and mutant lamin A peptides spanning residues A434–A500 of the Ig fold (Supplementary Material, Fig. S1B). Among these peptides, ‘lamin A-R482 peptides’ spanned residues S463–A500, while ‘lamin A-W482 peptides’ spanned the same residues but contained a W in position 482. A peptide from Ezrin, a T-cell plasma membrane protein (32), was used as a control for unspecific binding (Supplementary Material, Fig. S1C). Immunoblots of these peptides were first used to capture interacting proteins from an extract of Jurkat cells. Fractions retained on lamin A-R482 (471RQNGDPLLTYRFPPKFTLK490) and lamin A-W482 (471RQNGDDLLTYWFPPKFTLK490) peptides were eluted and resolved by SDS–PAGE. These two peptides were used because in our preliminary analysis, eluates from each peptide in the library (Supplementary Material, Fig. S1B) revealed most differential Coomassie blue staining with these (data not shown). A search for proteins differentially retained by these peptides revealed a notably ~80 kDa band in the lamin A-R482-bound fraction (Supplementary Material, Fig. S1D). Proteins in this band were identified by liquid chromatography coupled to mass spectrometry. We reproducibly identified nine proteins, none of which have been reported as lamin A partners in interaction databases, such as APID (bioinfo.dep.usal.es/ apid/index.htm) FunCoup (funcoup.sbc.su.se/) and PINA (cgb garvan.unsw.edu.au/pina/). Among these proteins was FXR1P, a member of the fragile X-related protein family.

We next sought to substantiate the detection of FXR1P in a complex interacting with lamin A peptides in an adipogenic context. Human primary adipose stem cells (ASCs) isolated from liposuction material from non-obese donors (33) express FXR1P as a specific 80 kDa protein (Fig. 1A). FXR1P expression was confirmed by knock-down with an shRNA to the FMR1 mRNA (Fig. 1A), while we note that the 50 kDa product is likely not specific (34). An overlay with anti-FXR1P antibodies of ASC cellular fractions retained on lamin A-R482 and lamin A-W482 peptides shows that FRX1P is enriched in fractions retained by the lamin A-R482 peptides, particularly with R482 in the mid-position (Fig. 1B). FXR1P detection is weaker with lamin A peptides bearing an R → W mutation at position 482 (lamin A–W482 peptides; Fig. 1B). Identification of FXR1P was confirmed by elution, SDS–PAGE and immunoblotting of fractions bound to the peptides (Fig. 1B and C). FXR1P was not detected on peptides spanning residues 434–471 of the Ig fold (lamin A-R453 peptides; Supplementary Material, Fig. S1E). These results indicate that FXR1P is enriched in a complex interacting in vitro with peptides spanning R482 in the Ig fold. This association is weakened, though it does occur, by the lamin A p.R482W mutation occurring in FPLD2.

Deletion of the Ig fold, or the lamin A p.R482W mutation, impairs interaction with FXR1P in primary adipocyte progenitors
Interaction of FXR1P with lamin A in ASCs was shown by co-immunoprecipitation of both proteins using anti-lamin A/C or anti-FXR1P antibodies (Fig. 2A). Moreover, a GFP-Trap
pull-down of green fluorescent protein (GFP)-coupled lamin A (GFP-lamin A) transiently expressed in ASCs co-precipitates endogenous FXR1P (Fig. 2B; GFP-LaA). This interaction is, however, impaired in a pull-down of GFP-lamin A (R482W), indicating that the R482W substitution affects the interaction of lamin A with FXR1P (Fig. 2B and C). In addition, a GFP-Trap pull-down of lamin A truncation lacking the Ig fold (GFP-lamin A[D428–547], also referred to as GFP-lamin A[Ig-fold]), reveals essentially no interaction with FXR1P (Fig. 2B and C), showing the necessity of the Ig fold for this association. To rule out the possibility that co-precipitation of FXR1P with lamin A reflects protein aggregation in insoluble complexes, we transiently expressed in LMNA knock-down ASCs GFP-tagged C-terminal lamin A(389–664) peptides that lack the rod domain and are unable to multimerize. These peptides do not anchor in the NE and remain 0.1% Triton X-100-soluble in the nucleoplasm (not shown). GFP-Trap pull-down of GFP-lamin A(389–664) co-precipitates ~5-fold more FXR1P than GFP-lamin A(389–664)(R482W) (Fig. 2D and E). Therefore, FXR1P is able to interact with lamin A, and the R482 residue within the Ig fold is critical for this association. These results suggest a link between a lipidostrophic laminopathy and FXR1P, a protein hitherto known to be involved in muscle development and implicated in myopathies (24,28).

**Lamin A recruits a fraction of FXR1P to the NE**

Identification of FXR1P in a complex with lamin A predicts that at least a fraction of FXR1P would co-localize with lamin A at the NE. Accordingly, we detect in ASCs FXR1P at the nuclear periphery in addition to the cytoplasm (Fig. 3A). Moreover, extraction of ASCs with 0.004% digitonin after fixation, which permeabilizes the plasma membrane but not nuclear membranes, prevents immunodetection of FXR1P (and as expected lamin A) at the nuclear periphery, but not in the cytoplasm (Fig. 3B). This is consistent with FXR1P localization on the nucleoplasmic side of the NE where lamin A resides. These results indicate that a fraction of FXR1P is enriched with lamin A at the NE, consistent with the ability of both proteins to co-immunoprecipitate.

We next determined whether FXR1P localization at the nuclear periphery depends on lamin A expression. Lamin A knock-down with an shRNA to LMNA (Supplementary Material, Fig. S2) abolishes detection of FXR1P at the nuclear periphery (Fig. 3C). Conversely, expression of GFP-lamin A maintains perinuclear FXR1P staining (Fig. 3D). In addition, expression of GFP-lamin A(R482W) or GFP-lamin A[Ig fold] abolishes the perinuclear localization of FXR1P (Fig. 3D), indicating displacement from the NE. Thus, perinuclear localization of FXR1P depends on lamin A expression, requires the Ig fold of lamin A and is abolished by the R482W substitution. This suggests that lamin A anchors a fraction of FXR1P on the nucleoplasmic face of the NE.

**FXR1P is up-regulated and redistributed in fibroblasts from patients with FPLD2**

Weakening of the interaction between lamin A and FXR1P by the R482W mutation raises the possibility of interplay between these proteins in cells from FPLD2 patients. Whereas normal fibroblasts display low or barely detectable levels of FXR1P, FXR1P is strongly up-regulated in fibroblasts from three unrelated FPLD2 patients with the lamin A p.R482W mutation (Fig. 4A and B). Knock-down of the FXR1 mRNA in FPLD2 patient fibroblasts abolishes FXR1P detection.
Confirming that the up-regulated protein is the product of the FXR1 gene, this up-regulation does not occur at the transcriptional level as we detect similar FXR1 mRNA levels in control and FPLD2 patient cells (Supplementary Material, Fig. S3A). In addition, FXR1P is markedly redistributed in FPLD2 fibroblasts, with enrichment in the cytoplasm and impoverishment in the nucleus compared with controls (Fig. 4D). We conclude that FXR1P is up-regulated post-transcriptionally in FPLD2 patient fibroblasts with the lamin A p.R482W mutation.

Alteration of lamin A level and composition post-transcriptionally up-regulates FXR1P

Up-regulation of FXR1P in FPLD2 patient fibroblasts raises the question of whether this may be mimicked by manipulating lamin A level or composition in ASCs. Lamin A down-regulation results in elevated FXR1P (Fig. 5A and B), as in normal fibroblasts (not shown). Expression of GFP-lamin A (R482W) also increases the FXR1P level compared with GFP alone or GFP-lamin A (despite up-regulation of FXR1P by the latter; Fig. 5A and B). As noted earlier in FPLD2 fibroblasts, these elevations are post-transcriptional as they do not occur at the FXR1 mRNA level (Supplementary Material, Fig. S3B). These results are consistent with a functional association between lamin A and FXR1P, the impact of which is deregulated by altered lamin A level or composition in the lamina. Thus, lamin A is not only involved in localizing FXR1P at the NE but also in regulating FXR1P level.

FXR1P up-regulation phenocopies the adipogenic block of ASCs deficient in lamin A

FPLD2 patients present a partial lipodystrophy phenotype, including subcutaneous lipoatrophy in the limbs (11,35). ASCs used in our study originate from the thighs and buttock, that is, from anatomical regions presenting lipoatrophy in FPLD2. Thus, we evaluated the impact of deregulating lamin A on the adipogenic potential of ASCs and whether any effect could be mimicked by altering FXR1P level.

Human ASCs efficiently differentiate into adipocytes in vitro as judged by Oil Red-O lipid staining (Fig. 6A–C) and by induction of adipogenic genes (CEBPA, PPARG, SREBP1, FABP4; Fig. 6D). In contrast, lamin A knock-down impairs adipogenic differentiation (Fig. 6A–D). Some differentiation can occur in the LMNA shRNA cell population; however, tracking expression of the LMNA shRNA by co-expression of TurboRFP shows an adipogenic block in all LMNA shRNA-expressing cells.
In contrast to GFP alone, stable expression of GFP-lamin A(R482W) also affects differentiation (Fig. 6A–D). Overexpression of wild-type GFP-lamin A has a more moderate impact on adipogenesis (Fig. 6A–C), although early gene expression is affected (Fig. 6D). Thus, lamin A expression is required for adipogenic differentiation of human ASCs. Given the effect of down-regulating lamin A on differentiation, we also established that knocking-down lamin A in ASCs did not cause cell death (not shown), nor cell cycle arrest (which would be sufficient to inhibit adipogenesis (36)). In fact, expression of LMNA shRNA in ASCs results in an average of 1.4-fold increase in the cell number compared with controls after 9 days under proliferative conditions (not shown). This is consistent with the proliferative ability of Lmna−/− fibroblasts (37,38) and with the observation that lamin A knock-down in neuroblastoma cells elicits a more aggressive phenotype inconsistent with growth arrest (39). Our findings are also consistent with adipogenic differentiation defects following deregulation of lamin A expression in mesenchymal stem cells (40) and in 3T3-L1 preadipocytes expressing wild-type and R482W mutant lamin A (37). They differ, however, from data in Lmna−/− embryonic fibroblasts which can form adipocytes (37). This suggests that the role of lamin A in adipocyte differentiation may depend on species or on the adipogenic commitment of the cells examined. Of note, mice lacking A-type lamins are lean but do not develop lipodystrophy or the insulin resistance seen in FPLD2 patients (41).

Since FXR1P is up-regulated in cells from FPLD2 patients, and this effect is mimicked by expression of GFP-lamin A(R482W) and by knock-down of lamin A, we assessed the impact of overexpressing FXR1P on the adipogenic differentiation capacity of ASCs. Remarkably, FXR1 overexpression inhibits differentiation, both at the gene expression and phenotypic levels (Fig. 6A and B). Thus, overexpression of FXR1P replicates the adipogenic failure elicited by lamin A down-regulation. This suggests that lamin A and FXR1P are functionally linked in the regulation of adipogenic gene induction in adult human adipocyte progenitors.

FXR1P up-regulation stimulates a myogenic transcription program in ASCs

ASCs are programmed for adipogenic differentiation, and we have earlier shown that they display no ability to differentiate into myocytes (42). Interestingly, a clinical feature increasingly
recognized among FPLD2 patients is muscle hypertrophy in the lipoatrophic areas (11,35,43,44). The mesodermal origin of the affected tissues (fat and muscle), the up-regulation of FXR1P in FPLD2 fibroblasts and the role of FXR1P in muscle differentiation (24) suggest that muscle hypertrophy in FPLD2 patients may be related to the lipoatrophy phenotype, and that this relationship may implicate a deregulation of FXR1P expression.

To address this possibility, we assessed how FXR1P overexpression in ASCs affected myogenic gene expression. In undifferentiated, non-stimulated ASCs (cultured in proliferating medium), FXR1P-GFP overexpression induces the myogenic regulator genes MYF5, MYOD1 and MYOG (Fig. 7A). Expression of GFP alone has no marked effect (Fig. 7A). In quiescent muscle satellite cells, MYF5 protein is weakly expressed but its co-expression with MYOD is required for MYOG activation (45). In addition, while PAX7 is not activated in FXR1P-overexpressing ASCs, its paralog PAX3 is induced (Fig. 7A). PAX7 protein is expressed in muscle satellite cells and together with PAX3 is an upstream regulator of MYOD expression; however, it is not strictly necessary for MYOD or MYOG induction (45). These results suggest that elevated FXR1P levels in ASCs can stimulate a myogenic gene expression profile in these cells.

We next determined whether FXR1P elevation in ASCs caused by the lamin A p.R482W mutation would elicit expression of myogenin, an essential marker of myogenesis. Indeed, ASCs stably expressing GFP-lamin A(R482W) also express myogenin (Fig. 7B and C). GFP-lamin A also elicits some myogenin expression, albeit to a lesser extent, while GFP alone has no effect (Fig. 7B and C). Myogenin induction, however, does not occur after lamin A knock-down (Fig. 7B and C), in line with the myodystrophic phenotype of Lmna−/− mice (38). These results suggest that induction of FXR1P overexpression by the lamin A p.R482W mutation stimulates a myogenic differentiation program in adipocyte progenitor cells.

**DISCUSSION**

Our findings link lamin A p.R482W causing FPLD2, to FXR1P, a protein of the fragile X-related family essential for muscle development. Expression of lamin A p.R482W causes FXR1P up-regulation in adipocyte progenitors and similarly, fibroblasts from FPLD2 patients harbor abnormally high FXR1P levels. Disruption of the lamin A–FXR1P interaction by the R482W mutation, and displacement of FXR1P from the nucleus in FPLD2 cells, could be due to pre-lamin A expression in these cells (46,47) and steric inhibition of mature lamin A-FXR1P interaction at the NE. However, our peptide binding data show that the R482W substitution is sufficient to impair this interaction in vitro. Structure determination and mutation analysis show that the p.R482W mutation leads to a positive charge reduction in the Ig fold (9), which could perturb interaction with partners. FXR1P is predicted to be negatively charged at pH ≥6.5 (http://www.scripps.edu/~cdputnam/protcalc.html), consistent with a weaker interaction with a lamin A mutant harboring a reduced positive charge in the Ig fold.

Recurrent clinical signs may provide a pathophysiological context for deregulation of FXR1P expression in FPLD2. FPLD2 patients display muscle hypertrophy particularly in the limbs (14–17), which could be linked to abnormal FXR1P levels. FPLD2 hypertrophic muscles show functional and histological abnormalities (15,16), including adipose infiltration in FPLD2. Only high-molecular weight FXR1P isoforms (≥84 kDa) are up-regulated in muscle (28), as we detect in differentiated myoblasts. These isoforms are not detected in cells from FPLD2 patients (this paper), so abnormal muscle mass may speculatively be associated with the lack of up-regulation of the proper FXR1P isoform(s). Our results are nevertheless consistent with a model where LMNA mutations causing FPLD2 are associated with deregulated FXR1P expression with implications for muscle development and homeostasis. Alternatively, cross-talk between lamin A and ERK signaling on one hand (48), and between ERK and p21-activated kinase 1 (PAK1) which phosphorylates FXR1P (49), on the other hand, raises the possibility that FXR1P is not properly phosphorylated in FPLD2. This would affect FXR1P activity (49). Reports of lamin A mutations leading to abnormalities in signaling pathways mediated through protein kinases in muscle (50–52) lend support to a deregulation of the FXR1P phosphorylation pathway.

**Deregulation of lamin A expression post-transcriptionally up-regulates FXR1P**

Up-regulation of FXR1P by altering the lamin A level or composition without affecting FXR1P mRNA levels is consistent with a post-transcriptional role of lamin A in FXR1P expression.
Translation of FXR1P is regulated by miRNAs (53–55), thus lamin A may be involved in regulating miRNA genes which control FXR1P translation. Chromatin associated with the nuclear lamina contains many genes that are mainly inactive (4). Lamin–genome interactions can be influenced by lamin A mutations (56,57), which may cause de-repression of target miRNA-encoding genes. The R482W mutation lowers the affinity of the Ig fold for DNA and nucleosomes in vitro (18), and in cells may affect its association with loci including miRNA genes. Supporting this view, muscles from patients with lamin A-linked muscular dystrophy show deregulation of several miRNAs (58). Our preliminary data also show differential expression of several miRNAs in FPLD2 versus normal fibroblasts (A.O.R, Eivind Lund, P.C., unpublished), some of which are predicted targets for FXR1. It will be important to determine which miRNAs are deregulated by lamin A mutations, and how these miRNAs affect mesodermal differentiation pathways.

An intact lamina is essential for the regulation of FXR1P level, as FXR1P is intriguingly up-regulated by altering lamina composition in three different ways (lamin A down-regulation or overexpression, or expression of mutant lamin A). Alteration in the lamina would affect nuclear import of FXR1P, possibly by modifying nuclear pore constitution (59), deregulating its
cytoplasmic level. FXR1P normally shuttles between the cytoplasm and the nucleus (60), and lamin A recruits FXR1P to the NE, a process abolished by the p.R482W mutation. Thus, the lamin A mutation may impair nuclear retention of FXR1P leading to its cytoplasmic accumulation. The granular distribution of FXR1P in the cytoplasm is reminiscent of localization in RNA granules, aggregates of mRNAs, ribosomes and RNA-binding proteins (61), and FXR1P overexpression enhances its co-localization with ribosomes and mRNAs (61). As these complexes are sites of translation (34), cytoplasmic accumulation of FXR1P may impact translation regulation.

Up-regulation of FXR1P inhibits an adipogenic differentiation program

In the cytoplasm, altered FXR1P stoichiometry within the DICER complex may deregulate miRNA pathways resulting in unscheduled translation or absence of down-regulation of target mRNAs. Similarly to the recruitment of mRNAs into RNA granules by FXR1P in neurons (61), excess FXR1P in ASCs may sequester mRNAs of adipogenic regulators expressed in these cells, e.g. CEBPA, CEBPB or CEBPG (33), inhibiting their translation and activation of their target genes on differentiation; for instance, the CEBPG mRNA has eight binding sites for FXR1P (62), making it a potential FXR1P target. This scenario is consistent with the adipogenic failure of ASCs containing excess FXR1P. Changes in the lamina may also affect adipocyte differentiation through impaired organization of the cytoskeletal–nuclear lamina axis (63). Failure to differentiate could also be due to deregulation of transcription factors by the mutated lamin (37). Instances of gene regulation by sequestration of transcription factors by the lamina exist, such as SMADs and the adipogenic factor SREBP1 (614). Affinity of lamin A for SREBP1 is reduced in cells from FPLD2 patients with the R482W mutation, and mistargeting of SREBP1 has been proposed as a mechanism of impaired adipogenesis in lamin A-linked lipodystrophies (65).

FXR1P up-regulation promotes a myogenic gene induction program in adipose progenitors

The increased muscle mass detected in lipodystrophic regions in FPLD2 patients (14–17) raises the hypothesis of a transition between an adipogenic homeostatic program and a myogenic differentiation program. This does not occur after down-regulating lamin A despite the up-regulation of FXR1P, consistent with the myodystrophic phenotype of Lmna-null mice (38). Thus, an intact lamina is necessary for muscle differentiation and homeostasis. The interaction between lamin A and FXR1P, and the role of lamins in muscle differentiation may be mediated through FXR1P, which is required for this process (24–28). It may, however, also be mediated through protein kinase signaling pathways cross-talking with lamin A and regulating myogenesis (48,50,52). In undifferentiated ASCs, overexpression of FXR1P elicits a myogenic gene expression profile and expression of lamin A p.R482W promotes myogenin expression. FXR1P can repress or activate translation of specific mRNA targets in a cell context-dependent manner (22). One may speculate that FXR1P functions as a switch controlling adipogenic and myogenic differentiation downstream of lamin A. Interestingly, lipofibroblasts of mesodermal origin exist in several organs (66–68), and their adipogenic phenotype can be altered to that of myofibroblasts under pathological conditions (66,67). Additionally, selection of adipogenic or myogenic differentiation pathways by embryonic fibroblasts has been shown to involve tension-induced/inhibited proteins (TIPS) 1.
and 3, which respectively promote myogenic and adipogenic lineages (69). Molecular switches such as TIPs controlling mesenchymal cell fate decision between myogenic and adipogenic lineages may provide a molecular basis to explore mechanisms underlying muscle hypertrophy in FPLD2 patients.

MATERIALS AND METHODS

ASCs
ASCs were isolated from liposuction material from thighs and buttock of non-obese non-diabetic women aged 30–44, after approval by the Regional Committee for Research Ethics for South Norway (REK S-06387a). ASCs were cultured in DMEM/F12/10% fetal calf serum as described (33). ASCs at passages 8–10 pooled from three donors were used.

Primary skin fibroblasts
All fibroblast donors gave their informed consent for these studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hoˆpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. All fibroblast donors gave their informed consent for these studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France. Normal control studies, which were approved by the institutional review board of Hôpital Saint Antoine, Paris, France.

Subcellular fractionation
Fraction of fibroblasts into nuclear and cytoplasmic fractions was done as described (70).

Vectors
A GFP-tagged open-reading frame clone of human FXR1, transcript variant1, was purchased from Origene. PCR products of the C-termini (residues 389–664) of lamin A and lamin A(R482W) were cloned using KpnI and EcoRI sites into the EGFP vector (Clontech). GFP-lamin A(Δ428–547) was constructed by site-directed mutagenesis (Stratagene). Full-length GFP-lamin A, GFP-lamin A(R482W) and GFP control cDNAs were subcloned (NcoI/NotI sites) into a ptoCMV-vector (kindly provided by Dr. Fahri Saatcioglu, University of Oslo). GFP alone was used as a control. Lentiviral vectors for overexpression of GFP and GFP-lamin A fusion constructs were generated from a pGIPZ vector (Open Biosystems, Thermo Fisher Scientific) by replacing the shRNA-coding sequence with the relevant GFP-lamin A sequences into Xbal/NotI sites. Lentiviruses were produced in 293 T cells. ASCs were transduced and selected with 1 μg/ml puromycin for 8 days to select shRNA-expressing cells. shRNA expression was induced with 1 μg/ml doxycycline together with 0.1 μg/ml puromycin for 6–25 days. For FXR1 silencing, lentiviruses containing an shRNA to FXR1 (sc-35423-V) and a non-silencing control (sc-36870-V) were purchased from Santa Cruz Biotechnology. ASCs and fibroblasts were transduced and selected with 1 μg/ml puromycin for 8 days. Cells expressing the shRNA were maintained in 0.1 μg/ml puromycin.

Adipogenic differentiation
ASCs were induced to differentiate into adipocytes for 6 or 21 days as indicated using 1-methyl-3-isobutyl xanthine, dexamethasone, insulin and indomethacin, and lipids were stained on day 21 with Oli red-O as described (33).

Immunofluorescence
Immunolabeling was as described (71), unless indicated otherwise using antibodies to lamin A/C (1:100; Santa Cruz sc-7292) or FXR1P (5 μg/ml; Abcam 519790). DNA was stained with DAPI.

Immunoblotting
Proteins were resolved by 10% SDS–PAGE, transferred onto Immobilon-FL membranes (Millipore) and blocked with Odyssey blocking buffer (LI-COR). Membranes were incubated with antibodies to lamin A/C (1:1000; Santa-Cruz sc-7292; or a rabbit antibody; 1:1000 (19)), lamin B1 (1:100; Santa Cruz sc-6216), FXR1P (5 μg/ml; Abcam 56386), myogenin (1:100; Abcam 1835) and a non-silencing control (sc-36870-V) was purchased from Origene. PCR products of the C-termini (residues 389–664) of lamin A and lamin A(R482W) were cloned using KpnI and EcoRI sites into the EGFP vector (Clontech). GFP-lamin A(Δ428–547) was constructed by site-directed mutagenesis (Stratagene). Full-length GFP-lamin A, GFP-lamin A(R482W) and GFP control cDNAs were subcloned (NcoI/NotI sites) into a ptoCMV-vector (kindly provided by Dr. Fahri Saatcioglu, University of Oslo). GFP alone was used as a control. Lentiviral vectors for overexpression of GFP and GFP-lamin A fusion constructs were generated from a pGIPZ vector (Open Biosystems, Thermo Fisher Scientific) by replacing the shRNA-coding sequence with the relevant GFP-lamin A sequences into Xbal/NotI sites. Lentiviruses were produced in 293 T cells. ASCs were transduced and selected with 1 μg/ml puromycin for 8 days to select shRNA-expressing cells. shRNA expression was induced with 1 μg/ml doxycycline together with 0.1 μg/ml puromycin for 6–25 days. For FXR1 silencing, lentiviruses containing an shRNA to FXR1 (sc-35423-V) and a non-silencing control (sc-36870-V) were purchased from Santa Cruz Biotechnology. ASCs and fibroblasts were transduced and selected with 1 μg/ml puromycin for 8 days. Cells expressing the shRNA were maintained in 0.1 μg/ml puromycin.

Transient transfections were performed with a Nucleofector (Lonza) in batches of ~4 × 10^5 cells mixed with 2 μg DNA. After electroporation, cells were cultured for 24–48 h on coverslips in 24-well plates for immunofluorescence or in flasks GFP-Trap® pull-down.

LMNA and FXR1 silencing by shRNA
The shRNA targeting the 5′ UTR of the LMNA mRNA was as follows, with the underlined sequence specific for LMNA: 5′tcgttgcagctgaagctgctcgtcttgaagcagcagtagtagagagctggagtctcggctctgctgag3′. The sequence was cloned into BamHI/Mlu sites into the pGIPZ vector mentioned above. Lentiviruses were produced, and ASCs transduced and cultured as above for 8 days to select shRNA-expressing cells. shRNA expression was induced with 1 μg/ml doxycycline together with 0.1 μg/ml puromycin for 6–25 days. For FXR1 silencing, lentiviruses containing an shRNA to FXR1 (sc-35423-V) and a non-silencing control (sc-36870-V) were purchased from Santa Cruz Biotechnology. ASCs and fibroblasts were transduced and selected with 1 μg/ml puromycin for 8 days. Cells expressing the shRNA were maintained in 0.1 μg/ml puromycin.

Adipogenic differentiation
ASCs were induced to differentiate into adipocytes for 6 or 21 days as indicated using 1-methyl-3-isobutyl xanthine, dexamethasone, insulin and indomethacin, and lipids were stained on day 21 with Oli red-O as described (33).

Immunofluorescence
Immunolabeling was as described (71), unless indicated otherwise using antibodies to lamin A/C (1:100; Santa Cruz sc-7292) or FXR1P (5 μg/ml; Abcam 519790). DNA was stained with DAPI.

Immunoblotting
Proteins were resolved by 10% SDS–PAGE, transferred onto Immobilon-FL membranes (Millipore) and blocked with Odyssey blocking buffer (LI-COR). Membranes were incubated with antibodies to lamin A/C (1:1000; Santa Cruz sc-7292; or a rabbit antibody; 1:1000 (19)), lamin B1 (1:1000; Santa Cruz sc-6216), FXR1P (5 μg/ml; Abcam 56386), myogenin (1:100; Abcam 1835) and γ-tubulin (1:10000; Sigma T5326). Proteins were visualized using IRDye-800- or IRD IRDye-680-coupled secondary antibodies.

Immunoprecipitation
For immunoprecipitation, cells were lysed in 20 mM Hepes/HCl pH 8.2, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, a protease inhibitor mix (Roche) and 1% Triton X-100 for 25 min followed by sonication with a probe sonicator (B. Braun Biotech; 35% power, 0.4 s intervals for 1 min 10 s.). Lysates were cleared by centrifugation (15 min, 15000g, 4°C) and by pre-incubation with Protein A/G PLUS-Agarose beads (Santa Cruz) for 90 min at 4°C. Lysates were then incubated for 90 min at 4°C with antibodies to lamin A/C (Santa Cruz sc-7292), FXR1P (Abcam 56386) or lamin B1 (Santa Cruz
GFP-Trap® pull-down

GFP-Trap® pull-down was done in extracts of ASCs. Cells at 80% confluence were harvested 48 h after transfection, suspended in cell lysis buffer (20 mM Hepes/HCl pH 8.2, 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1 mM PMSF and protease inhibitor mix), sedimented by centrifugation (10 min, 800 g, 4°C) and lyzed by incubation in cell lysis buffer containing 0.1% Triton X-100 for 45 min followed by sonication as described above. The lysate was recovered after centrifugation (2 × 100 g, 4°C) and incubated for 90 min at 4°C with GFP-Trap® beads (Chromotek) pre-equilibrated in cell lysis buffer. After incubation, beads were washed three times in lysis buffer and pulled-down proteins dissolved in 2 × Laemmli buffer for SDS–PAGE.

Peptide arrays

Peptides were synthesized with Fmoc (N-(9-fluorenyl)methoxy carbonyl) protection chemistry onto cellulose membranes using a Multipep automated peptide synthesizer (Intavis Bioanalytical Instruments) as described (72).

Solid phase pull-down from overlaid nuclear/cell extracts

For peptide overlay experiments, whole cell extracts of ASCs were prepared as described above for GFP-Trap® pull-down. The extract was aliquoted, snap-frozen and stored at −80°C. Extracts of Jurkat-TAG cells were similarly prepared. For membrane overlays, extracts from 10⁶ cells were incubated overnight at 4°C on membranes harboring lamin A peptides in quadruplicates. Membranes were washed twice 10 min in lysis buffer (see GFP-Trap® pull-down) and twice in high-salt lysis buffer (lysis buffer/1 M NaCl). Peptide-bound proteins were eluted by cutting the peptide spots and boiling them in 1× Laemmli buffer for SDS–PAGE.

Protein identification by LC-MS/MS

The Coomassie G-250 stained SDS–PAGE gel band selected (see Results) was in-gel digested using 0.1 μg sequencing grade trypsin (Promega, Madison, WI, USA) in 20 μl of 50 mM ammonium bicarbonate, pH 7.8 and purified by C₁₈ ZipTips (Millipore). Peptides were analyzed by an ESI-ion trap/Orbitrap (LTQ Orbitrap XL, Thermo Scientific) mass spectrometer coupled to a nano-LC system (73). Data were acquired using Xcalibur v2.5.5 and processed using ProteoWizard v3.0.331. Proteins were identified using an in-house version of the protein identification software Mascot v2.3 (73) and mass spectra were searched against the Swiss-Prot database (Human; 20 411 sequences).

Reverse transcription-polymerase chain reaction

Reverse transcription-PCR was done from 1μg total RNA (Qiagen RNeasy) using the Iscript cDNA synthesis kit (BioRad). Relative mRNA levels were determined by quantitative PCR with IQ SYBR® Green using GAPDH as a reference and the ΔΔCt method. PCR conditions were 95°C for 3 min and 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Primers are listed in Supplementary Material, Table S1.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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