Association of a Homozygous Nonsense Caveolin-1 Mutation with Berardinelli-Seip Congenital Lipodystrophy

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Context: Berardinelli-Seip congenital lipodystrophy (BSCL) is a rare recessive disease characterized by near absence of adipose tissue, resulting in severe dyslipidemia and insulin resistance. In most reported cases, BSCL is due to alterations in either seipin, of unknown function, or 1-acylglycerol-3-phosphate acyltransferase-β (AGPAT2), which catalyzes the formation of phosphatidic acid.

Objective: We sought to determine the genetic origin of the unexplained cases of BSCL. We thus sequenced *CAV1*, encoding caveolin-1, as a candidate gene involved in insulin signaling and lipid homeostasis. CAV1 is a key structural component of plasma membrane caveolae, and Cav1-deficient mice display progressive loss of adipose tissue and insulin resistance.

Design: We undertook phenotyping studies and molecular screening of *CAV1* in four patients with BSCL with no mutation in the genes encoding either seipin or AGPAT2.

Results: A homozygous nonsense mutation (p.Glu38X) was identified in *CAV1* in a patient with BSCL born from a consanguineous union. This mutation affects both the α- and β-CAV1 isoforms and ablates CAV1 expression in skin fibroblasts. Detailed magnetic resonance imaging of the proband confirmed near total absence of both sc and visceral adipose tissue, with only vestigial amounts in the dorsal sc regions. In keeping with the lack of adipose tissue, the proband was also severely insulin resistant and dyslipidemic. In addition, the proband had mild hypocalcemia likely due to vitamin D resistance.

Conclusions: These findings identify *CAV1* as a new BSCL-related gene and support a critical role for caveolins in human adipocyte function. **(*J Clin Endocrinol Metab* 93: 1129–1134, 2008)**

Berardinelli-Seip congenital lipodystrophy (BSCL) is a rare syndrome characterized by the absence of adipose tissue from birth or early infancy, resulting in severe dyslipidemia, insulin resistance, and muscular hypertrophy (1, 2). BSCL is a genetically heterogeneous disorder with autosomal recessive inheritance in which two genes have been implicated to date: *BSCL2*, which we identified on chromosome 11q13, encoding the protein seipin of unknown function (3), and *AGPAT2*, lo-

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Abbreviations: AGPAT2, 1-Acylglycerol-3-phosphate acyltransferase-β; BSCL, Berardinelli-Seip congenital lipodystrophy; CAV1, caveolin-1; MRI, magnetic resonance imaging; NR, normal range.

cated on chromosome 9q34 encoding the enzyme 1-acylglycerol-3-phosphate acyltransferase-β (4). This enzyme catalyzes the formation of phosphatidic acid, a key intermediate step in the synthesis of triglycerides and phospholipids. Most of the disease-causing mutations in *BSCL2* and *AGPAT2* are nonsense, splice, or frameshift mutations that are highly likely to lead to complete loss of gene function (3–8).

Although mutations in *BSCL2* or *AGPAT2* account for about 95% of reported cases (7), additional kindreds have been described in which the disease is not linked to either of these loci (2, 6, 7). To determine the etiology of BSCL in these remaining cases, we examined candidate genes involved in insulin signaling and adipocyte function. These studies included molecular screening of *CAV1* on chromosome 7q31, which encodes caveolin-1 (9). CAV1 is a highly conserved 22-kDa protein that is the key component of plasma membrane invaginations known as caveolae (10, 11). Loss of Cav1 expression in mice leads to progressive lipodystrophy and insulin resistance (12, 13). We now report the first case in humans of *CAV1* loss-of-function mutation, leading to generalized lipodystrophy and severe metabolic derangement, providing evidence for a critical role for CAV1 also in human adipocyte function.

Patients and Methods

Mutation screening

Genomic DNA was obtained from peripheral white blood cells by standard procedures. We obtained informed consent from individuals involved in the study and approval from local institutional ethics committees. *CAV1* exons and splice junctions were amplified by PCR using specific primers (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) before purification of PCR products on Sephadex columns and sequencing using Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA).

Magnetic resonance imaging (MRI)

Whole-body MRI was performed in the patient and in a Brazilian unaffected woman using a 1.5 Tesla system (GE Medical Systems, Milwaukee, WI, and Magneton, Siemens Medical Systems, Erlangen, Germany, respectively).

Cell studies

Primary fibroblast cultures established from skin biopsies were grown in DMEM/F12 containing 10% fetal calf serum (GIBCO, Invitrogen, Hercules, CA). Whole-cell lysates and detergent-resistant membrane fractions (14) were used for protein expression in Western blots with antibodies against caveolin-1, caveolin-2, flotillin-1, and flotillin-2 (BD Transduction Laboratory, Lexington, KY), insulin receptor β-subunit (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma-Aldrich, St. Louis, MO). Immunolocalization was performed after fixation (4% paraformaldehyde, 10 min) and permeabilization (0.5% Triton X-100, 10 min) followed by incubation with appropriate Alexa Fluor secondary antibodies. Fluorescence was visualized using a Zeiss (Oberkochen, Germany) laser-scanning confocal microscope (LSM 500).

Results

Identification of a loss-of-function mutation in CAV1

We performed molecular screening of *CAV1* in four patients with BSCL in whom mutations in *BSCL2* and *AGPAT2* had been excluded (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). A homozygous alteration in *CAV1* exon 2 was identified in one patient from a Brazilian consanguineous pedigree (Fig. 1). The nucleotide change c.112G→T leads to substitution of the glutamic acid residue at position 38 for a stop codon (p.Glu38X) (Fig. 2A). This nonsense mutation affects both the α- and the β-CAV1 isoforms (consisting, respectively, of residues 1–178 and 32–178) (Fig. 2B). The p.Glu38X mutation was found in the heterozygous state in the unaffected mother and two siblings (subjects III-4, IV-2, and IV-6). No DNA was available from the deceased father, who is inferred nevertheless also to have been heterozygous from haplotyping analysis (supplemental Fig. S2), or from an older sister (subject IV-3) who died at 17 yr old from fulminant hepatitis (Fig. 2C). The p.Glu38X mutation was absent from 740 control chromosomes including those from 94 Centre d'Etude du Polymorphisme Humain (CEPH) family founders and from 277 random Brazilian individuals. The sequence of *CAV1* was normal in three other patients diagnosed with BSCL without identified mutation in *AGPAT2* or *BSCL2*, suggesting that alterations in another gene are responsible for the disease in these cases. Furthermore, the sequence of *CAV1* was normal in 70 additional patients with BSCL with mutations in either *AGPAT2* (28 cases) or *BSCL2* (42

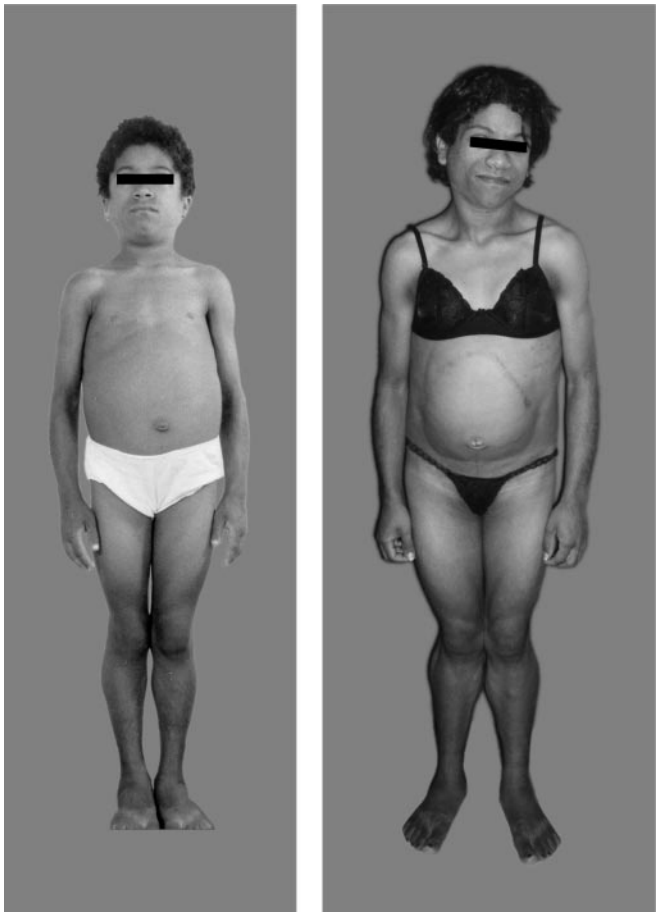

FIG. 1. Proband with BSCL at 8 yr 10 months old (*left*) and 20 yr old (*right*). Note the muscular appearance, acanthosis nigricans particularly on the neck and axillae, the protruding abdomen (liver and spleen), the thick curly hair, hirsutism, and prominent peripheral veins in the limbs.

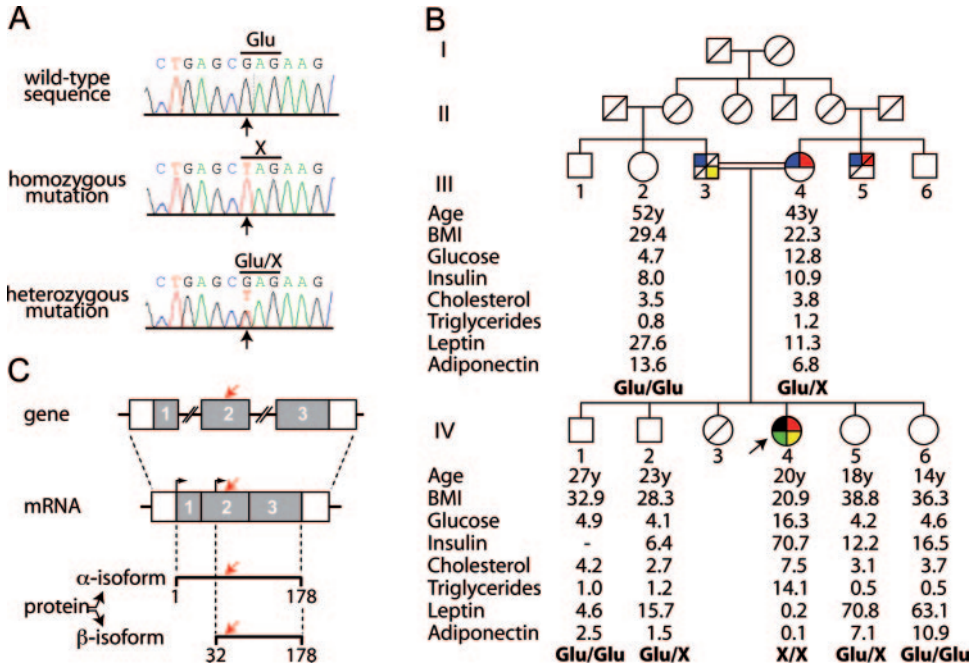

FIG. 2. *CAV1* mutation in the pedigree with BSCL. A, Chromatograms of *CAV1* exon 2 showing wild-type sequence with the normal nucleotide G (*arrow*) and mutant alleles with homozygous G→T mutation (*arrow*) in the homozygous state in the proband and in the heterozygous state in the mother. B, Schematic of wild-type *CAV1* gene, mRNA, and protein with α- and β-isoforms. The G→T substitution introduces a premature stop codon at position 38 (*red arrow*), truncating both isoforms in the N-terminal domain. C, Pedigree. The *black arrow* marks the proband. Individuals affected with lipodystrophy (*black*), hypertension (*blue*), hypertriglyceridemia (*green*), hypercholesterolemia (*yellow*), or type 2 diabetes (red) are indicated. Age, body mass index (BMI = weight in kilograms divided by height in square meters), fasting blood glucose (NR, 4–5.5 mmol/liter), plasma insulin (NR, 5–15 μU/ml), cholesterol (NR, <5.2 mmol/liter), triglycerides (NR, <1.7 mmol/liter), leptin (NR_{men}, 2.2–11.1 ng/ml; NR_{women}, 3.9–77.3 ng/ml), and adiponectin (NR, 1.2–19.9 μg/ml) are indicated. The homozygous Glu38X mutation is designated X/X, the heterozygous mutation Glu/X, and the wild-type Glu/Glu.

cases), which indicates that *CAV1* cannot be considered as a modifier gene, perturbed function of which would aggravate the phenotype of BSCL. Therefore, although the formal possibility of coinheritance of an unidentified causative mutation in this consanguineous pedigree remains, the *CAV1* p.Glu38X mutation is highly likely to be pathogenic with a recessive mode of inheritance of the BSCL phenotype.

Cellular significance of the CAV1 p.Glu38X mutation

As expected, skin fibroblasts from the proband expressed no detectable CAV1 protein on Western blot (Fig. 3A) or on immunofluorescent cell imaging (Fig. 3B). Furthermore, caveolin 2, another member of the caveolin family, is not detected, in agreement with what was observed in $Cav1^{-/-}$ mice (15). Despite these alterations, detergent-resistant membrane fractions can be isolated from the proband's fibroblasts containing flotillins but no caveolins (Fig. 3A).

Patient history

The proband carrying the homozygous *CAV1* p.Glu38X mutation was born at term with a birth weight of 2.9 kg. Facial lipoatrophy was reported at 3 months, but development was otherwise normal apart from recurrent episodes of pneumonia, chronic diarrhea, and poor growth. There was no intellectual impairment. Clinical evaluation at 8 yr revealed generalized lipoatrophy with muscular hypertrophy, organomegaly, and features of severe insulin resistance including acanthosis nigricans and hirsutism. Abdominal ultrasonography showed severe hepatosplenomegaly and hepatic steatosis but otherwise normal viscera. No bone cysts were seen radiologically. Diabetes mellitus was diagnosed at 13 yr, with poor glycemic control despite up to 3 U/kg·d sc insulin therapy. As in other generalized lipodystrophies, severe hypertriglyceridemia was observed, together with hypercholesterolemia and nearly undetectable plasma leptin and adiponectin (Fig. 2C).

At 9 yr old, hypocalcemia was observed [2.0 mmol/liter; normal range (NR), 2.22–2.67 mmol/liter], ultimately requiring treatment with oral calcitriol (0.5 μg/d). Investigation at 20 yr while taking this dose of calcitriol showed persisting low serum calcium (1.78 mmol/liter) and magnesium (0.57 mmol/liter; NR, 0.66–0.94 mmol/liter) but normal phosphate (0.94 mmol/liter; NR, 0.81–1.58 mmol/liter) and PTH toward the top of the normal range (50 ng/liter; NR, 11–62 ng/liter). Twenty-four-hour urinary excretion of calcium was 164 mg (NR, 100–250 mg). There was mild proteinuria but no acidosis. Although clouded by the clinical need to continue calcitriol treatment, these results collectively suggest a state of vitamin D resistance, with urinary calcium that is excessive in the face of significant hypocalcemia. There was no radiological evidence of vitamin D deficiency or resistance, although plain skeletal radiographs suggested osteopenia.

Height remained below the 2.5th centile from first evaluation at the age of 8 yr to the adult height of 1.46 m, in contrast to accelerated childhood growth with acromegaloid features commonly seen in lipodystrophy due to *AGPAT2* and *BSCL2* mutations. At 15 yr, surgical correction of functional megaesophagus of unknown cause was undertaken. At 20 yr, the proband had primary amenorrhea. Both blood pressure and echocardiogram were normal. There was no evidence of neoplasia, cardiomyopathy, or pulmonary disease.

Body fat distribution

The extent of lipodystrophy was studied when the proband was 19 yr-old using whole-body T1-weighted MRI (Fig. 4). Compared with an age-, sex-, and ethnic-matched control subject, both sc and visceral adipose tissue from the proband was nearly absent; only trace amounts of sc fat were detectable in the posterior neck and back regions, whereas it was absent in the cheeks and reduced in the temples. Visceral fat was also negligible in both intrathoracic and intraabdominal regions. In contrast, bone marrow fat, which is generally reduced or absent in BSCL linked to seipin or AGPAT2, was well preserved. So-called mechanical adipose depots were less severely affected, being reduced in the scalp but preserved in the retroorbital region. In the

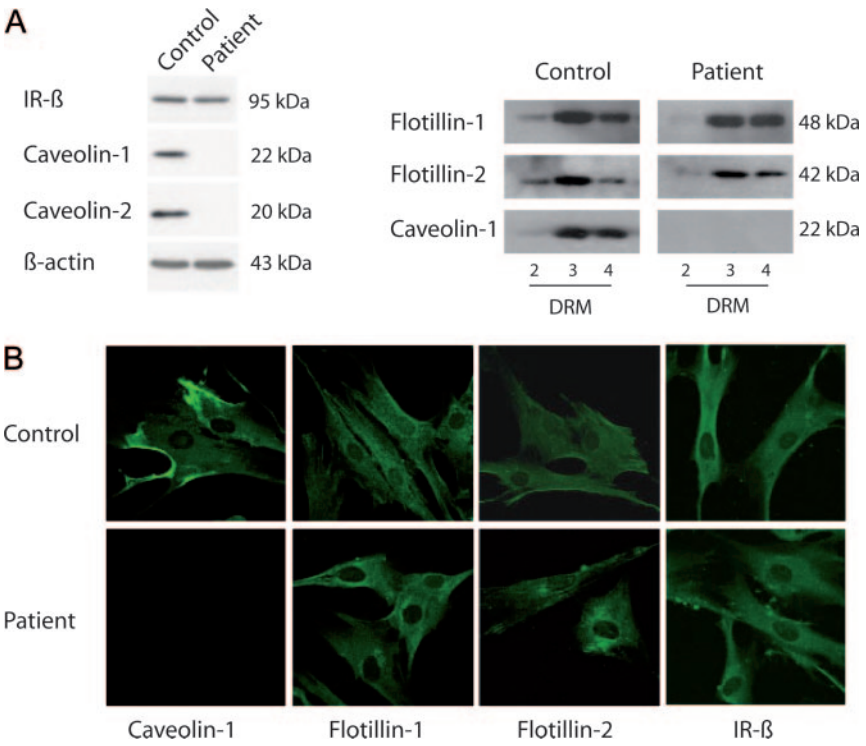

FIG. 3. Cell studies on skin fibroblasts derived from the patient and a control. Western blot analysis (A) on whole-cell lysates (*left*) and detergent-resistant membrane (DRM fractions 2–4) (*right*) and immunofluorescent cell imaging (B) confirm the absence of the CAV1 protein and its associated partner, CAV2. β-Actin and insulin receptor β-subunit (IRβ) were used as controls for protein loading or cell labeling. The absence of caveolins does not alter the subcellular distribution of flotillins or their presence in detergent-resistant membrane.

extremities, adipose tissue was preserved in the fingers and the plantar region with some loss of signal intensity.

Family history

The proband is the fourth child of first-cousin parents from Brazil. No other members of the kindred had lipodystrophy (Fig. 3), and subject IV-5, harboring the p.Glu38X mutation in the heterozygous state, was morbidly obese, suggesting that haploinsufficiency for *CAV1* does not lead to globally impaired accumulation of adipose tissue. The proband's father had hypercholesterolemia and hypertension, dying at 53 yr old from a myocardial infarction, and the mother had both hypertension and type 2 diabetes diagnosed in her 40s. However, the two known heterozygous siblings examined at 23 and 18 yr old (subjects IV-2 and IV-5) had normal metabolic parameters and blood pressure. The follow-up of this family will be required to determine whether *CAV1* haploinsufficiency cosegregates with these traits.

Discussion

Complete, or near complete, failure to accumulate adipose tissue from birth in the face of adequate nutrition is a rare disorder known as BSCL (1). It is due, in most cases, to loss-of-function mutations in the genes encoding either seipin or AGPAT2 and provides compelling evidence for the critical requirement of these genes for normal adipose tissue development (2–4, 6–8). We now report a case of BSCL due to homozygous disruption of CAV1 by the introduction of a premature stop codon very early in the coding sequence, affecting both the α- and the β-isoforms. This mutation is equivalent to a functionally null allele, as evidenced by the complete lack of detectable CAV1 protein in primary fibroblasts.

The phenotype of the patient with the homozygous *CAV1* p.Glu38X mutation fulfilled the criteria for the diagnosis of BSCL, with lipodystrophy, muscular hypertrophy, acanthosis nigricans, hyperandrogenism, hepatosplenomegaly, severe insulin resistance, and hypertriglyceridemia (1, 2). Unusual clinical features were, however, noticed, including the absence of accelerated linear growth in childhood with associated acromegaloid features and the presence of abnormal calcium homeostasis, although this has not been studied in detail in BSCL to date. Features most frequently linked to AGPAT2 deficiency, such as bone cysts, or to seipin deficiency, such as intellectual impairment, were not present (5, 8). BSCL linked to seipin usually appears as a more severe phenotype with a higher incidence of premature death and lipodystrophy of earlier onset with more extensive fat loss (5, 8, 16). Detailed imaging of the proband at 19 yr old confirmed near total absence of adipose tissue. In common with other patients with BSCL, both sc and visceral adipose tissues were nearly absent, with only vestigial amounts in the dorsal sc regions. In contrast, bone marrow fat, which is generally reduced or absent in BSCL, was well preserved. It has been suggested that AGPAT2 and seipin loss of function may be discriminated by examining so-called mechanical adipose depots serving mainly supportive or protective functions, for example in the retroorbital, palmoplantar and periarticular regions (16). These depots are preserved in AGPAT2 deficiency but are generally reported lost in seipin deficiency (6, 16). In the present case, such adipose depots were present but reduced, as shown by mild enophthalmos and reduction of MRI signal intensity in the extremities. Thus, the degree of lipodystrophy due to *CAV1* mutation may be intermediate between that linked to *AGPAT2* and that linked to *BSCL2*. However, more comprehensive genotype-phenotype correlation will be required to establish whether such detailed analysis of adipose tissue topography may permit clinical discrimination of these different genetic entities.

The severe metabolic derangements seen in BSCL, akin to those in other forms of lipodystrophy, are believed to be largely accounted for by loss of the ability to store dietary lipids, exacerbated by hyperphagia driven by low leptin levels, leading to harmful spillover of lipids to other insulin sensitive tissues (17, 18). The primary lack of adipose tissue may in principle be due to impaired triglyceride synthesis and/or storage, accelerated lipolysis, defective adipocyte differentiation, or destruction of the adipocytes. However, the mechanism of lipodystrophy in the context of either AGPAT2 or

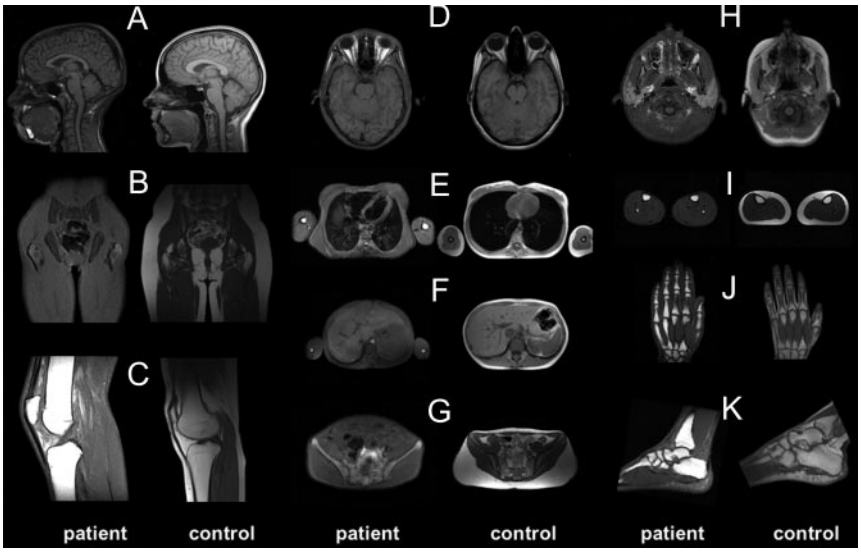

FIG. 4. MR images in the proband and an age-, sex-, and ethnically matched control subject. T1 weighted images from the proband are presented on the *left* and those from the control subject on the *right*: axial images of brain (D), neck (H), chest and arms (E), abdomen (F), pelvis (G), and legs (E–I); sagittal images of brain and upper neck (A) and knees (C) and coronal images of hips (B), left hand (J), and left foot (K). Subcutaneous fat is absent in the limbs and trunk, with only trace amounts in the posterior neck and back regions (A–C, E–G, and I). In the head, it is near absent in the scalp, reduced in the temples, but preserved in the retroorbital region (A, D, and H). In the extremities, adipose tissue is preserved in fingers (J) and plantar region (K), although it displays loss of brightness. Visceral fat is also negligible in the intrathoracic and intraabdominal regions (B and E–G). Bone marrow fat is preserved (C, J, and K). Muscular hypertrophy (C) is also evident.

seipin deficiency remains unclear, so the evidence for a critical role for CAV1 in humans presented here is of significant interest.

Caveolins are the main component of caveolae, which are present in many tissues, being most dense in adipocytes where they account for around 20% of the plasma membrane area (10, 11). Three caveolin isoforms have been described, but CAV3 is muscle specific, and although both CAV1 and CAV2 are expressed in adipocytes, only CAV1 has been shown to be both necessary and sufficient for caveolae formation (10, 12, 15). CAV1 is implicated as a negative regulator of cell growth, and hence as a putative tumor suppressor gene (19, 20), and as a key determinant of normal lipid homeostasis and insulin-regulated glucose uptake (21, 22). It is an important mediator of non-clathrin-dependent endocytosis (23), including lipid-regulated caveolar endocytosis to lipid droplets (24), which appears crucial for lipid handling during the regeneration of liver tissue (25). The marked hepatic steatosis in the patient described implies either that defects in lipid droplet formation in hepatocytes may be overcome by massively increased delivery of circulating lipid, in this case due to lipodystrophy, or that the mechanism of accumulation of intrahepatic lipid in the setting of lipodystrophy is different from that operating during the regeneration process.

Cav1$^{-/-}$ mice display progressive lipodystrophy, with ablation of the hypodermal fat layer and generalized reduction of white adipose tissue, and are protected against high-fat diet-induced obesity (10, 12, 13). They also exhibit elevated triglycerides and reduced leptin and adiponectin but only mild insulin resistance. Overt diabetes developed only in the context of prolonged high-fat feeding (21). However the phenotype in Cav1$^{-/-}$ mice is milder than seen in the human case described here. This is reminiscent of other mouse models of human severe insulin resistance, in which insulin resistance is reproduced only in the context of dietary or genetic manipulation to induce gross nutritional overload (26), and suggests that similar genetic perturbation of adipocyte function in humans and rodents may in general produce a much more severe phenotype in humans.

The clinically significant renal calcium wasting and low serum calcium despite therapy with activated vitamin D seen in the proband is reminiscent of the abnormal calcium homeostasis of Cav1$^{-/-}$ mice. Although these animals are not hypocalcemic, they have hypercalciuria due to impairment of renal calcium reabsorption, in association with urolithiasis in males but not in females (27). Immunohistochemical analysis of the kidney revealed mislocalization of the vitamin D-regulated plasma membrane calcium ATPase, an important calcium transporter that usually colocalizes with Cav1 in the epithelial cells of the distal convoluted tubule (27). These preliminary human findings suggest an essential role of CAV1 in regulating renal calcium handling and that loss of this function may contribute to renal damage.

At 20 yr old, the proband did not have other abnormalities reported in Cav1-null mice, in particular cardiomyopathy, neoplasia, and pulmonary fibrosis (10), although she did suffer recurrent episodes of pneumonia during infancy that subsequently recovered. Cav1$^{-/-}$ mice do not develop tumors spontaneously but exhibit higher tumorigenesis when exposed to carcinogens compared with their littermates (20). As a general growth signal inhibitor, CAV1 has been implicated in the pathogenesis of oncogenic cell transformation, tumorigenesis, and metastasis. However, its role in cancer may be complex because it has been suggested to act as a tumor suppressor or as a tumor promoter depending on the tumor type and/or the tumor stage (20). Interestingly, AGPAT2, loss of function of which also causes BSCL, was shown to be up-regulated in a wide variety of human tumor cells, especially in ovarian cancer cells (28), and so has been suggested as a potential prognostic, diagnostic, and therapeutic target in neoplasia (29).

The heterozygous carriers of the p.Glu38X mutation do not have lipodystrophy, but several phenotypes are seen within the pedigree. Obesity does not cosegregate with *CAV1* haploinsufficiency; however, cosegregation with type 2 diabetes, hypercholesterolemia, and hypertension (displayed by one or both parents but not by the children) cannot be ruled out, because these traits usually appear at an advanced age. It is noteworthy that *CAV1* has been proposed as a quantitative trait locus for blood pressure, glycemia, and triglyceridemia in hypertensive patients (30). The follow-up of this family and/or the identification of additional kindreds harboring loss-of-function mutations will be required to determine whether *CAV1* haploinsufficiency cosegregates with these phenotypes.

The discovery that CAV1 disruption leads to near total lipoatrophy firmly establishes the critical importance of caveolae in normal adipocyte function in humans, although the precise mechanism

of the loss of adipose tissue remains to be determined. The similar phenotypes of humans with loss of function of seipin, AGPAT2, or CAV1, three molecules with seemingly disparate cellular functions, is striking and may provide a genetic clue to functional connections between them and thus an insight into the *in vivo* physiology of adipose tissue. Lessons from these rare and extreme syndromes may ultimately yield important information of relevance to obesity and type 2 diabetes, which are among the greatest healthcare challenges in the world today.

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