CBP/p300-Interacting Transactivator, with Glu/Asp-Rich C-Terminal Domain, 2, and Pre-B-Cell Leukemia Transcription Factor 1 in Human Adrenal Development and Disease

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Context: Disorders of adrenal development result in significant morbidity and mortality. However, the molecular basis of human adrenal development, and many forms of disease, is still poorly understood.

Objectives: We evaluated the role of two new candidate genes, CBP/p300-interacting transactivator, with Glu/Asp-rich C-terminal domain, 2 (*CITED2*), and pre-B-cell leukemia transcription factor 1 (*PBX1*), in human adrenal development and disease.

Design: *CITED2* and *PBX1* expression in early human fetal adrenal development was assessed using RT-PCR and *in situ* hybridization. The regulation of *CITED2* and *PBX1* by steroidogenic factor-1 (SF-1) and dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome, gene-1 (DAX1) was evaluated in NCI-H295R human adrenocortical tumor cells by studying promoter regulation. Finally, mutational analysis of *CITED2* and *PBX1* was performed in patients with primary adrenal disorders.

Results: *CITED2* and *PBX1* are expressed in the human fetal adrenal gland during early development. Both genes are activated by SF-1 in a dose-dependent manner in NCI-H295R cells, and, surprisingly, PBX1 is synergistically activated by SF-1 and DAX1. Mutational analysis failed to reveal significant coding sequence changes in individuals with primary adrenal disorders.

Conclusions: *CITED2* and *PBX1* are likely to be important mediators of adrenal development and function in humans, but mutations in these genes are not common causes of adrenal failure in patients in whom a molecular diagnosis remains unknown. The positive interaction between DAX1 and SF-1 in regulating *PBX1* may be an important mechanism in this process. **(*J Clin Endocrinol Metab* 94: 678–683, 2009)**

Adrenal failure can be difficult to diagnose in children and is associated with significant mortality and morbidity. Although 21-hydroxylase deficiency and autoimmune Addison disease remain the most likely diagnoses in early infancy and childhood, respectively, a range of metabolic, infectious, infiltrative/destructive, and developmental etiologies can result in a spectrum of adrenal disorders presenting throughout the pediatric and adolescent years (1). Because some of these conditions

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2008-1064 Received May 16, 2008. Accepted October 28, 2008.
First Published Online November 4, 2008

Abbreviations: AHC, Adrenal hypoplasia congenita; *CITED2*, CBP/p300-interacting transactivator, with Glu/Asp-rich C-terminal domain, 2; DAX1, dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome, gene-1; *PBX1*, pre-B-cell leukemia transcription factor 1; SF-1, steroidogenic factor-1; WT, wild type.

have different natural histories, potential associated features, and varying modes of inheritance, making a correct diagnosis and undertaking appropriate treatment and counseling are essential.

The past decade has seen steady progress in our understanding of the molecular basis of adrenal disease, especially in the area of adrenal development and regulation (2–4). Several single gene disorders causing "adrenal hypoplasia" have now been reported (2). For example, secondary adrenal hypoplasia can result from pituitary dysfunction or isolated ACTH deficiency (*e.g. HESX1*, *SOX3*, *LHX3*, *LHX4*, and *TPIT*). ACTH resistance ("familial glucocorticoid deficiency") can result from defects in ACTH signaling pathways and related mechanisms (*e.g. MC2R*, *MRAP*, and *AAAS*), and primary adrenal hypoplasia most frequently occurs as an X-linked condition due to deletions or mutations in the orphan nuclear receptor dosage-sensitive sex reversal, adrenal hypoplasia congenita (AHC), critical region on the X chromosome, gene-1 (DAX1) (*NR0B1*), although rare cases due to defects in other factors (*e.g.* steroidogenic factor-1 [SF-1, *NR5A1*]) have been reported (2, 5–8). At present, a molecular diagnosis can be reached in approximately 50% of infants or children presenting with adrenal hypoplasia or resistance, and an increasing number of syndromic associations and "non-classic" variants of adrenal disorders are being described (4, 5, 7, 9–13). However, although substantial progress has been made, a significant proportion of cases of syndromic and nonsyndromic adrenal hypoplasia and related disorders currently remain unexplained.

Two candidate genes emerging as potential causes of primary adrenal hypoplasia from work in mice are the transcriptional regulators CBP/p300-interacting transactivator, with Glu/Asp-rich C-terminal domain, 2 (*CITED2*) (Mendelian Inheritance in Man 602937), and pre-B-cell leukemia transcription factor 1 (*PBX1*) (Mendelian Inheritance in Man 176310). Targeted disruption of *Cited2* in mice results in adrenal agenesis, neurological defects, and cardiac malformations (14), whereas *Pbx1*-deficient mice have severe adrenal hypoplasia together with pancreatic dysfunction, impaired gonadal development, and skeletal abnormalities (15). Although both knockout models are embryonic lethal, *Cited2* haploinsufficient animals have markedly impaired adrenal development when crossed with either *Sf1*+/− or *Wt1*+/− strains (16), and *Pbx1* haploinsufficient animals are viable, and have smaller adrenal glands with impaired adrenocortical growth and steroidogenesis (17).

To date, the role of *CITED2* and *PBX1* in humans is poorly understood, but they are candidate genes for unexplained cases of adrenal hypoplasia with or without associated features. Here, we demonstrate the expression of these genes in human fetal adrenal development, their regulation by SF-1 and DAX1, and mutational analysis of *CITED2* and *PBX1* in a cohort of patients with primary adrenal disorders.

Materials and Methods

RT-PCR

Human fetal adrenal tissue from 7 and 10 wk gestation was provided by the Medical Research Council/Wellcome Trust funded Human Developmental Biology Resource with Research Ethics Committee approval and informed consent. RNA was extracted using the TRIzol method (Invitrogen Corp., Paisley, UK), and RT-PCR was performed according to the manufacturer's protocol (35 cycles, Access Quick RT-PCR System; Promega, Southampton, UK; detailed conditions are available on request). Primers for *CITED2* were located within exon 2 (forward, 5′-CAGGAAGGTCCCCTCTATGTG-3′, and reverse, 5′-GCGCCGTAGTGTATGTGCTC-3′), and for *PBX1* within exon 4 (forward, 5′-GTTCCCGATTTCTGGATGC-3′) and within exon 6 (reverse, 5′-CATGGGCTGACACATTGGTA-3′). Glyceraldehyde-3-phosphate dehydrogenase was used as a positive control.

In situ hybridization

In situ hybridization analysis of *CITED2* and *PBX1* in early human adrenal tissue was performed with ethical approval through the In House Gene Expression Service of the Human Developmental Biology Resource. Human embryos/fetuses at selected stages were dissected and fixed in 4% paraformaldehyde, then dehydrated and embedded in paraffin wax. Sections of 7 μm were cut using a standard microtome and mounted on Superfrost Plus slides (BDH, Poole, UK). *In situ* hybridization was performed essentially as described by Wilkinson (18) using digoxigenin 11 incorporated riboprobes generated from a pOTB7 vector containing the 1903-bp full cDNA sequence of *CITED2* and a pCR4-TOPO vector containing the 1388-bp full cDNA sequence of *PBX1* (both plasmids obtained from the Mammalian Gene Collection/National Institutes of Health, Integrated Molecular Analysis of Gnomes and their Expression identification nos. 3640855 and 8069084, respectively). For antibody detection, slides were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (diluted 1:1000, containing 2% fetal calf serum). Expression patterns were visualized using the nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt system (Roche, Welwyn Garden City, UK). Sections were mounted in VectaMount (Vector Laboratories, Burlingame, CA) and analyzed using the Axioplan2 imaging system (Zeiss, Jena, Germany). Sense probes for *CITED2* and *PBX1* were tested on adjacent sections, and showed no staining above background levels.

Reporter and expression vector construction

Based on previously published mouse data (14, 17) and analysis for putative SF-1 binding sites (MatInspector, www.genomatix.de) (19), a 3.3-kb upstream region of the *CITED2* promoter and a 910-bp upstream region of the *PBX1* promoter were PCR amplified and cloned into a pGL4.10[luc2] luciferase reporter vector (Promega). Expression vectors (pCMX) containing SF-1 [wild-type (WT) and mutant G35E] and DAX1 (WT and mutants R267P, A300P, I439S) cDNAs have been described previously (10, 20–22).

Transient gene expression assays

Transient gene expression assays were performed in 96-well plates (Techno Plastic Products, Trasadingen, Switzerland) using a NCI-H295R human adrenocortical tumor cell line, Lipofectamine 2000 (Invitrogen), and a dual-luciferase reporter assay system (Promega) with cotransfection of pRLSV40 Renilla luciferase (Promega) as a marker of transfection efficiency.

To analyze the effects of SF-1 on *CITED2* and *PBX1* regulation, increasing doses of pCMXWT or mutant SF-1 expression vectors (10, 20, 50, and 100 ng/well) were cotransfected with either pGL4.10-CITED2-luc or pGL4.10-PBX1-luc reporters (100 ng/well). Activation of the promoters by SF-1 and DAX1 was studied using 100 ng pCMXWTSF1 together with increasing doses of pCMXWTDAX1 (2, 5, 10, 20, and 50 ng/well). The synergistic effects of DAX1 were evaluated further using DAX1 mutants associated with severe (R267P, A300P) or milder (I439S) phenotypes (50 ng/well) (10, 22).

In all studies, cells were lysed 48 h after transfection and luciferase assays performed using a FLUOstar Optima fluorescence microplate reader (BMG Labtech, Aylesbury, UK). All data were standardized for

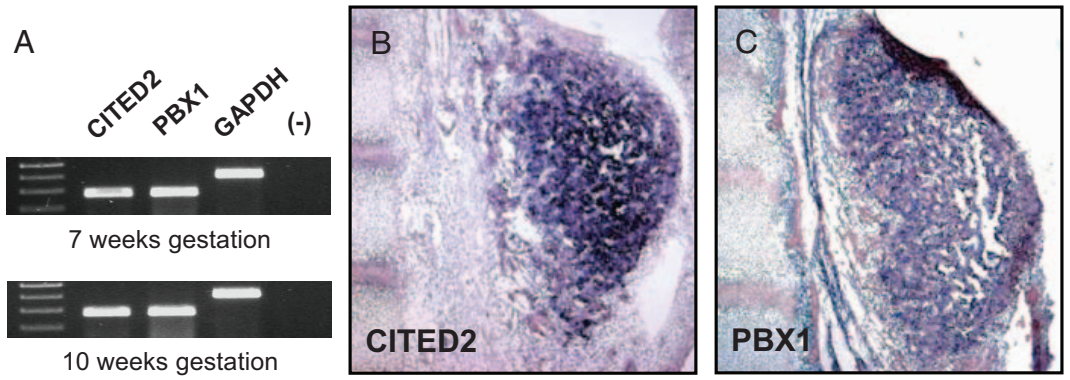

FIG. 1. *CITED2* and *PBX1* expression in human fetal adrenal. A, RT-PCR of *CITED2* and *PBX1* at 7 and 10 wk gestation. (−), water control. B and C, *In situ* hybridization of *CITED2* and *PBX1* in human fetal adrenal tissue at Carnegie Stage 20 (7 wk gestation). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Renilla coexpression. Results are shown as the mean ± SEM of at least three independent experiments, each performed in triplicate.

Patient cohort

Direct sequencing of *CITED2* and *PBX1* was undertaken in a diverse cohort of patients with primary adrenal failure, with or without associated features. In many cases, mutations in several other relevant candidate genes [*DAX1* (*NR0B1*), *SF1* (*NR5A1*), *MC2R*, *AAAS*, *CYP11A1*, *STAR*, and *ACD*] had been excluded (supplemental Table 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org), and common steroidogenic defects (21-hydroxylase deficiency), autoimmune disorders, and metabolic disorders (*e.g.* X-linked adrenoleukodystrophy) were not detected. In a cohort from one center, 36 patients (31 males, five females) were analyzed for mutations in *CITED2* and *PBX1*. Additional features present in several patients with more complex phenotypes included gonadal, cardiac, and renal abnormalities, and intrauterine growth restriction or Intrauterine growth retardation, Metaphyseal dysplasia, AHC, and Genital anomalies syndrome features (9) (supplemental Table 1A). In another cohort, *PBX1* was analyzed in 20 patients with a predominant ACTH-resistance phenotype (supplemental Table 1B). Finally, *CITED2* was sequenced in a group of 15 patients with a predominant adrenal hypoplasia phenotype (supplemental Table 1C). The Human Random Control-1 DNA Panel (British Caucasian) (Human Random Control-1 DNA Panel, European Collection of Cell Cultures, UK) was used as control genomic DNA for the analysis of previously unreported polymorphisms.

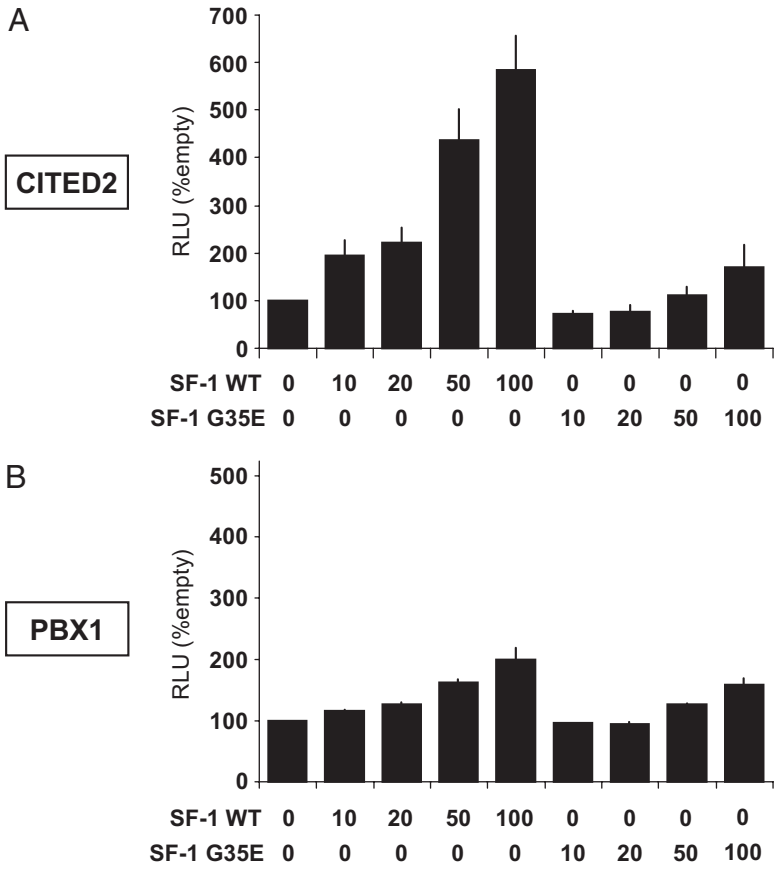

FIG. 2. Activation of *CITED2* (3.3 kb) (A) and *PBX1* (910 bp) (B) promoters by WT or mutant (G35E) SF-1 (0–100 ng/well) in NCI-H295R cells. Data shown as mean ± SEM of three experiments each performed in triplicate. RLU, Relative light units.

Mutational analysis

After institutional board approval and with informed consent, genomic DNA was extracted from peripheral blood lymphocytes, and the entire coding regions of *CITED2* (exon 2, three primer pairs) and *PBX1* (exons 1–9, nine primer pairs) were amplified by PCR (specific conditions and primer sequences available on request). PCR products were purified by gel extraction (QIAGEN, Crawley, UK) or by using exonuclease I (New England Biolabs, Ipswich, MA)/shrimp alkaline phosphatase (USB, Columbus, OH) and then subjected to direct sequencing using dye terminator sequencing kits (dRhodamine/BigDyev1.1; PE Applied Biosystems Inc., Foster City, CA) in an automated capillary based sequencer (MegaBACE1000; Amersham Biosciences Inc., Piscataway, NJ). Sequencher version 4.1 (Genecodes Corp., Ann Arbor, MI) was used to analyze the data.

Results

CITED2 and *PBX1* expression

Analysis of *CITED2* and *PBX1* by RT-PCR showed abundant expression of these genes in RNA derived from 7 and 10-wk human fetal adrenal glands (Fig. 1A). This expression was confirmed by *in situ* hybridization on human fetal adrenal tissue at Carnegie Stage 20 (7 wk gestation) (Fig. 1, B and C).

Transcriptional regulation of *CITED2* and *PBX1*

Several putative SF-1 binding sites were identified within the proximal promoter regions of *CITED2*

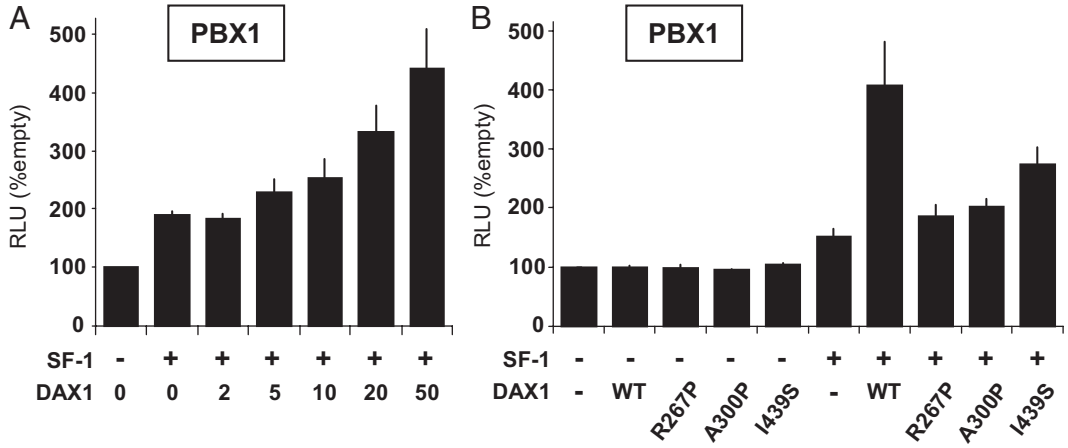

FIG. 3. A, Cotransfection of increasing doses of WT DAX1 (0–50 ng/well) together with WT SF-1 (100 ng/well) shows a dose-dependent activation of the *PBX1* promoter. B, By comparing the effects of WT DAX1 alone (50 ng/well, lane 2), WT SF-1 alone (100 ng/well, lane 6), and WT DAX1 (50 ng/well) and WT SF-1 (100 ng/well) together (lane 7), synergistic activation of the *PBX1* promoter is seen. This synergistic activation is attenuated when naturally occurring DAX1 mutants associated with severe (R267P, A300P) or mild (I439S) forms of X-linked adrenal hypoplasia are studied (all 50 ng/well). Data represent the mean ± SEM of triplicate experiments (unpaired *t* tests: WT >R267P, A300P; $P < 0.01$; I439S >R267P, A300P; $P < 0.05$). RLU, Relative light units.

(3.3 kb) and *PBX1* (910 bp), as has been reported previously in the mouse (12). Cotransfection of SF-1 with reporter constructs containing these regions showed dose-dependent activation of the *CITED2* promoter by SF-1 (up to 6-fold) (Fig. 2A), whereas the *PBX1* promoter showed 2-fold activation (Fig. 2B).

Coexpression of the nuclear receptor DAX1 with SF-1 had only limited effects on regulation of the *CITED2* promoter (data not shown). In contrast, synergistic activation was observed when SF-1 and DAX1 were coexpressed with the *PBX1* promoter (4-fold higher than empty vector, 2.7- fold greater than SF-1 alone) (Fig. 3). This increased activation was lost when DAX1 mutants associated with severe X-linked adrenal hypoplasia (R267P, A300P) were cotransfected instead of WT cDNA, and partially reduced when the I439S mutant associated with a milder, late-onset form of X-linked adrenal hypoplasia was studied (Fig. 3B).

Mutational analysis of *CITED2* and *PBX1*

Mutational analysis of *CITED2* (n = 51) and *PBX1* (n = 56) in individuals with primary adrenal failure failed to reveal significant coding sequence changes. Two polymorphisms were found in *CITED2*. The previously reported c.21C>A transversion (refSNP rs1131400) was found in 12% of the alleles (consistent with control data). The previously unreported c.*47G>C transversion was present in 2.0% of patient alleles and 2.2% of 186 control alleles. Two polymorphisms were detected in *PBX1*. The previously described c.61G>A transversion (refSNP rs2275558) was found as a heterozygous change in 26% of patient alleles. The novel c.191 + 37_40delTTTT (intron 1–2) change was present in 17% of patient alleles and in 13% of 128 control alleles.

Discussion

Although significant progress has been made in our identification of several genetic causes of primary adrenal hypoplasia and ACTH resistance syndromes, the underlying etiology remains unknown in a substantial proportion of cases. Recently, the description of adrenal phenotypes associated with deletion of *Cited2* or *Pbx1* in the mouse has provided potential candidate genes for analysis in patients with disorders of adrenal development and function.

Here, we show that *CITED2* and *PBX1* are both expressed during the early stages of human fetal adrenal development at a time when the gland is undergoing significant morphological and functional differentiation (3, 23) and concordant with expression of SF-1 (24).

Because SF-1 is an important regulator of many target genes involved in adrenal development and function, we hypothesized that SF-1-dependent regulation of *CITED2* and *PBX1* might occur in humans. Recent studies have shown that Sf-1 can regulate *Pbx1* expression in mice (17), and that Pbx1 and Cited2 may in turn mediate *Sf1*(*Nr5a1*) expression in *in vitro* and *in vivo* systems (16, 25). By focusing on a human adrenal cell line, we have shown that SF-1 can strongly activate the human *CITED2* promoter. Furthermore, although SF-1 appears to be a relatively weak activator of the minimal promoter of human *PBX1*, synergistic activation of this promoter by SF-1 and DAX1 was observed in adrenal cells. Although mutations in both DAX1 and SF-1 can result in variable degrees of adrenal insufficiency, it remains enigmatic how these two transcription factors interact during adrenal development and function because most studies have shown that DAX1 can act as a repressor of SF-1-mediated transactivation (22, 26–28). However, a recent report by Verrijn Stuart *et al.* (29) has shown activation of the *CYP11B1* promoter by SF-1 and DAX1 in an adrenal cell line, and both underexpression and overexpression of *Dax1*(*Nr0b1*) have had a detrimental effect on testis development (30–34). Together with the synergistic activation of the *PBX1* promoter shown in this report, our findings suggest that DAX1 may also have an activating role during certain stages of development, on specific promoters, or together with cell-specific transcriptional complexes. This mechanism may contribute to the impaired definitive zone de-

velopment in patients with X-linked AHC because the synergy of DAX1 was attenuated when naturally occurring severe and partial DAX1 mutants were studied.

Given the phenotype of *Cited2* and *Pbx1* knockout and haploinsufficient mice, together with the human expression and transcriptional data obtained, mutational analysis of *CITED2* and *PBX1* was undertaken in a cohort of patients with adrenal hypoplasia or adrenal failure phenotypes. A heterogeneous cohort was deliberately chosen because it was possible that associated features could be present (*e.g.* cardiac defects with *CITED2* and skeletal abnormalities with *PBX1*) (15, 35), or that a variable spectrum of milder phenotypes could be seen if severe loss-of-function was not compatible with survival. Although several reported and novel polymorphisms were found, no nonsynonymous mutations in *CITED2* and *PBX1* were discovered. It is possible that haploinsufficiency or copy number variations of these genes could cause a phenotype in humans, which would not have been detected in our analysis. However, because biallelic variants (heterozygous single nucleotide polymorphisms) were detected in most of the patients studied, haploinsufficiency of *CITED2* and/or *PBX1* does not appear to be frequent. Therefore, *CITED2* and *PBX1* are likely to be important mediators of adrenal development and function in humans, but mutations in these genes are unlikely to be a common cause of adrenal hypoplasia or adrenal failure in those patients in whom the etiology remains unknown.

Acknowledgments

We thank Larry Jameson, Masafumi Ito, and Ron Evans for plasmids and reagents. We also thank the many pediatricians and physicians who have referred patients. Human embryonic material was provided by the Medical Research Council/Wellcome Trust-funded Human Developmental Biology Resource, Institute of Child Health.

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B.F.-d.-S. holds a scholarship from Capes/Brazil (4798066). D.M. is supported by the Biological Network of Rare Diseases, French Health Ministry. A.H. is supported by a grant of the Deutsche Forschungsgemeinschaft, Germany (HU895/3-5). F.B. is supported by a grant of the Landesstiftung Baden-Wurttemberg (P-LS-ASN/5). The Human Developmental Biology Resource receives funding from The Wellcome Trust and Medical Research Council. J.C.A. holds a Wellcome Trust Senior Research Fellowship in Clinical Science (079666). Part of this work was undertaken at Great Ormond Street Hospital/University College London Institute of Child Health which received a proportion of funding from the Department of Health's National Institute for Health Research Biomedical Research Centres funding scheme.

Disclosure Statement: The authors have nothing to disclose.

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