

A novel *PHF6* mutation results in enhanced exon skipping and mild Börjeson-Forssman-Lehmann syndrome

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BFLS is a rare X linked recessive disorder characterised by moderate to severe mental retardation, obesity with gynaecomastia, hypogonadism, and large prominent ears.¹ However, a clear diagnosis is often difficult. BFLS may be confused with other obesity related mental retardation syndromes as there can be intrafamilial and interfamilial phenotypic variability and women may also be affected, possibly because of skewed X inactivation. Following the identification of the *PHF6* gene (Xq26–27) as the cause of BFLS, Turner *et al* showed that the phenotype may be milder and broader than originally appreciated.²

Mutations in the *PHF6* gene have been identified in 11 families and they are localised throughout the gene, suggesting a loss of function mechanism.^{3–5} Primary sequence analysis has suggested that *PHF6* encodes a transcriptional regulator based on the presence of two PHD zinc finger domains, a motif common to chromatin remodelling proteins including the ATRX protein. Nonetheless, little is known of the function of the *PHF6* protein or how different mutations give rise to the phenotypic variability observed in patients. Clearly, the identification of additional mutations will enhance our understanding of *PHF6* function and provide insight into the clinical spectrum of this disorder. Here we describe mutational analysis in two Canadian families, one with classic features of BFLS and another with milder phenotypic features.

METHODS

Case reports

The affected individual from family 1 (fig 1A) was diagnosed at age 19 because of a classical phenotype that was consistent with that described in the most comprehensive clinical review of BFLS patients.⁶ He is severely intellectually handicapped with psychiatric challenges that include self-injurious behaviour. He has characteristic facial features, a stooped kyphoscoliotic posture, central obesity, marked gynaecomastia, hypogonadism, incomplete virilisation, and anomalies of the hands and feet. In contrast, the two affected half brothers in family 2 (fig 1C) have a less severe phenotype, both physically and intellectually. Patient III-1 had several of the diagnostic features of BFLS including large prominent ears, truncal obesity, and gynaecomastia. However, he had only mild to moderate intellectual handicap and his digital findings were less striking than those of the proband in family 1. His half brother (III-2) had global developmental delay and some characteristic facial findings, yet otherwise displayed few of the typical physical features of BFLS, the exception being very large and prominent ears. Similarly, their mother and maternal grandmother displayed mild physical features of BFLS, most notably the large ears (in both) and tapered fingers (in the mother). Intellectually, both were able to function independently but had significant academic difficulties. A more detailed clinical description will be presented elsewhere (Graham GE, *et al*, in preparation).

Key points

- Börjeson-Forssman-Lehmann syndrome (BFLS) is caused by mutations in the *PHF6* gene located in Xq26. Classic BFLS patients present with moderate to severe mental retardation, epilepsy, obesity, gynaecomastia, hypogonadism, typical hands, and minor facial anomalies which include large ears and excessive facial fat.
- Mutational analysis for two Canadian families are presented—one family with classical phenotypic features of the disease and a second with a less severe phenotype including mild developmental delay.
- The affected individual in family 1 with classical features of BFLS had a missense mutation (Arg257Gly) in exon 8. The affected individuals in family 2 with milder BFLS features had an A to G substitution at position –8 in intron 2, creating a cryptic acceptor splice site. RT-PCR analysis demonstrated two spliced products: a larger transcript (+7 bp) in which the novel acceptor splice site was used, and increased levels of a novel spliceform (also found in normal individuals) in which exon 3 was skipped (*PHF6*Δexon3).
- The *PHF6*Δexon3 transcript maintains the ORF and is properly localised to the nucleolus. The novel *PHF6*Δexon3 protein may retain partial function resulting in the milder BFLS phenotype in this family.

Written consent was obtained from all individuals in the study and mutation testing was in accordance with the Ottawa Hospital research ethics board approved protocol 1998577-01H.

Mutational analysis

DNA isolated from peripheral blood was used to amplify each exon (and the corresponding flanking intron sequence for analysis of splice junctions) of the *PHF6* gene according to the previously published protocol.³ Polymerase chain reaction (PCR) products were run on a 1% agarose gel and purified with a Qiaquick gel extraction kit (Qiagen, Valencia, California, USA). Purified PCR products were directly sequenced by the Ottawa Genomics Innovation Centre. DNA sequence was assembled and analysed for mutations using the Sequencher program (Gene Code Corporation). Subsequent to the identification of the mutation in intron 3, primers were designed to amplify exons 2–4 as follows: BFLSrtF (5'-CTACAAGACAGCGCAAATG-3') and BFLSrtR (5'-GCAATGCACAGTGGTAGTG-3'). RNA (10 µg) isolated from EBV transformed lymphoblasts was reverse transcribed using random primers and Superscript II as previously

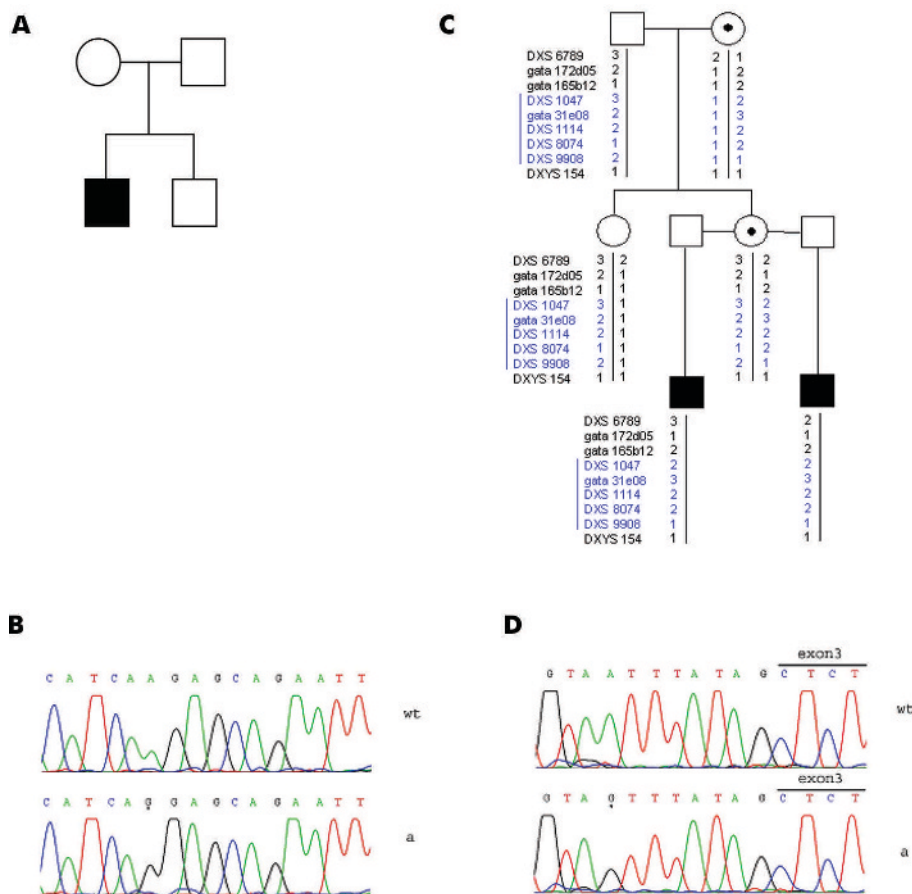


Figure 1 Mutational analysis in two Canadian families with Börjeson-Forsman-Lehmann syndrome (BFLS). (A) The affected individual from family 1 is a 27 year old man with classical findings of BFLS. (B) Sequence chromatograms of a normal (wt) or affected family member (a), demonstrating an A to G transition (marked with a dot) at position 769 which results in an Arg257Gly missense mutation in this patient. (C) The pedigree of family 2 complete with the corresponding haplotype analysis. The markers in the Xq26-27 region are shown in blue. (D) Sequence chromatograms of a normal (wt) or affected family member (a) demonstrating an A to G transition (position indicated by dot) in intron 2 which creates a novel cryptic acceptor splice site.

described.⁷ PCR amplification was undertaken for 35 cycles using the following conditions: 30 seconds at 94°C, 40 seconds at 56°C, and 30 seconds at 72°C. Samples amplified in the absence of reverse transcriptase served as controls. Southern blot analysis was undertaken using standard conditions for oligonucleotide hybridisation⁸ with the oligonucleotide BFLS_{exon2-4} (5'-GCATGATGTG-3').

Isolation of polysomes

EBV transformed lymphoblast cell lines established from patients and normal individuals were expanded (1×10^7) before treatment with 0.1 mg/ml cyclohexamide for three minutes at 37°C. Cells were harvested, washed twice with ice cold phosphate buffered saline containing cyclohexamide, and resuspended in 1 ml of lysis buffer (1% Triton-X, 0.3 M NaCl, 15 mM MgCl₂, 15 mM Tris-HCl (pH 7.4), 0.1 mg/ml cyclohexamide, and 100 units of RNase inhibitors (Amersham)) at 4°C. The nuclei were removed by centrifugation at $1000 \times g$ for five minutes at 4°C. The supernatants were subjected to an additional spin at $10\,000 \times g$ for five minutes at 4°C to remove any residual cellular debris and generate the cytoplasmic fraction. The cytoplasmic lysate was then layered onto a 10 ml continuous sucrose gradient (10–50% sucrose; 15 mM MgCl₂, 15 mM Tris-HCl (pH 7.4), 0.3 M NaCl). After a 90 minute centrifugation at 39 000 rpm at 4°C in an SW41-Ti rotor (Beckman), the absorbance across the gradient was read at 254 nm.

Fractions were collected every 30 seconds and snap frozen in liquid nitrogen. RNA was recovered from individual or pooled fractions by phenol-chloroform extraction and ethanol precipitation, and finally purified with an RNeasy kit (Qiagen). Each RNA sample was reverse transcribed and amplified using primers specific for exons 2–4 of the *PHF6* gene or actin, as described above.

Immunofluorescence and transient transfection

The following primers were designed to amplify and directionally clone the coding sequence of the *PHF6* gene: *PHF6*Fwd-2 (5'-GCTAAGCTTATGTCAAGCTCAG-3'), and *PHF6*Rev (5'-GCTGAATTCCTAGTTTCCATTA-3'). Purified PCR products were digested with EcoRI and HindIII, cloned into the pEGFP-C3 expression plasmid (BD Biosciences Clontech, Mississauga, Ontario, Canada), and sequenced for confirmation. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in 5% CO₂ at 37°C. Cells were transiently transfected with the GFP-*PHF6* constructs using Lipofectamine 2000 (Life Technologies Inc) as previously described.⁹ Cells were processed after 24 hours by fixing in cold ethanol/methanol (3:1) and counterstaining with 4',6-diamidino-2-phenylindole (DAPI). Co-localisation to nucleoli was examined using a mouse anti-human nucleoli monoclonal antibody (Chemicon, Temecula, California, USA; MAB1277). Anti-mouse Alexa fluor 594 conjugated secondary antibodies

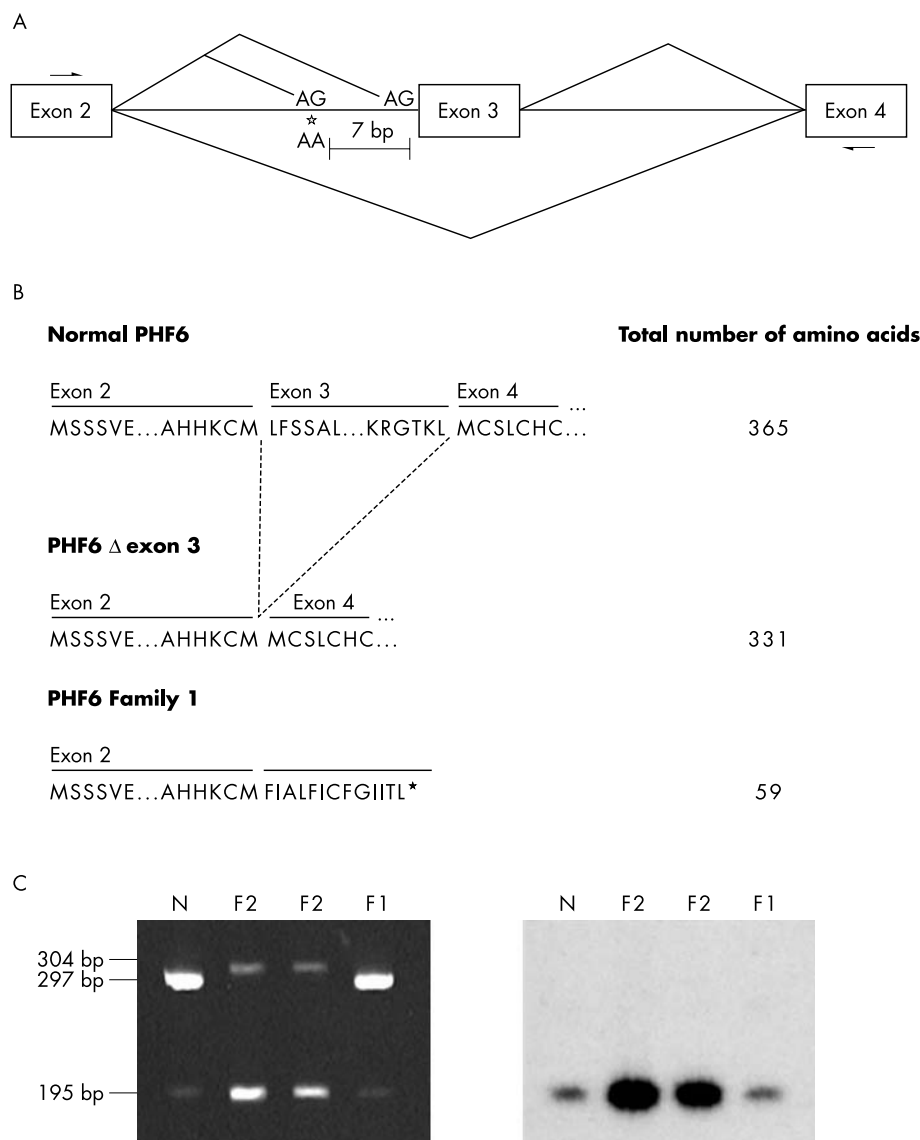


Figure 2 A novel *PHF6* spliceform lacking exon 3. (A) Schematic diagram showing the exon structure surrounding the cryptic acceptor splice site identified in family 2 and the corresponding splicing possibilities amplified by RT-PCR using primers in exons 2 and 4 (depicted by arrows). (B) The excision of exon 3 maintains the reading frame generating a protein 34 amino acids shorter in length. Use of the cryptic splice site results in a frameshift and early truncation of the *PHF6* protein. (C, left) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis using primers located in exons 2 and 4 shows a high level of *PHF6* Δ exon 3 transcript in the affected brothers from family 2 (F2) compared with normal individuals (N), or the affected individual from family 1 (F1). (C, right) Southern hybridisation using an oligonucleotide comprising the last five nucleotides of exon 2 and the first five of exon 4 confirmed the absence of exon 3 in the lower band.

were obtained from Molecular Probes (Eugene, Oregon, USA). Fluorescent images were captured using a Zeiss Axioplan 2 microscope outfitted with an AxioCam camera and AxioVision software.

RESULTS

Mutation analysis

Two Canadian families with a diagnosis of BFLS were examined for mutations in the *PHF6* gene. The proband of family 1 (fig 1A) presented with the classical hallmarks of BFLS and he was found to have an A to G change at nucleotide 769 within exon 8 (fig 1B) which results in the substitution of glycine for an arginine residue (Arg257Gly). This mutation has previously been described in another BFLS family³ and lies within an uncharacterised domain of the *PHF6* protein.

The second family (fig 1C) was diagnosed with BFLS despite a milder phenotype than is typically described and apparent in the proband of family 1. As family 2 was suspected to have a mild form of BFLS we first examined 16 markers spanning the X chromosome. Both affected boys, their mother (II-3), and their maternal grandmother (I-2) shared a common haplotype for markers in the Xq26–27 region (DXS1047, gata31e08, DXS1114, DXS8074, and DXS9908; fig 1B). We proceeded to screen the *PHF6* gene, identifying an A to G change within intron 2 that was located eight nucleotides upstream of the acceptor splice site (fig 1D). The nucleotide change creates a cryptic 3' splice site (AG) which was also present in the proband's affected brother, his mother, and his maternal grandmother (data not shown). It was not present in unaffected members of the family or in DNA isolated from over 100 control individuals, suggesting

that this alteration does not represent a rare polymorphism but is probably a disease causing mutation (data not shown). Use of the cryptic acceptor splice site would predict the inclusion of seven nucleotides, thereby creating a frame shift and truncation of the protein soon afterwards (fig 2B). We hypothesised that the mild phenotype associated with this mutation resulted from the use of both acceptor splice sites within intron 2 such that a significant amount of normally spliced product would be produced (fig 2A).

Altered splicing of the *PHF6* gene

To assess the functional significance of the splicing defect, lymphocyte RNA was isolated from affected individuals from both families and from a normal individual for RT-PCR analysis across exons 2–4 (fig 2A). In the patient samples with the cryptic splice site alteration, we expected to observe bands of 297 base pairs (bp) and 304 bp that corresponded to the normally spliced product and the mutant product containing the additional seven nucleotides, respectively. However, we observed only a small amount of the mutant product and a prominent fragment of 195 nucleotides (fig 2C). Sequencing of the larger band confirmed that it was the mutant band containing the additional 7 bp of intronic sequence.

The size of the smaller band was consistent with a fragment lacking exon 3 and it was also present, albeit at very low amounts, in normal control samples and in the patient with a *PHF6* mutation in the coding region (fig 2C). We confirmed that exon 3 was skipped in the patient sample by sequencing the smaller fragment. The low abundance of the Δ exon3 transcript in normal samples made sequencing somewhat difficult, so for confirmation we designed an oligonucleotide that contained the last five nucleotides of exon 2 and the first five nucleotides of exon 4 for hybridisation to the PCR products. As shown in fig 2C, we confirmed that normal individuals consistently produce a small amount of the shorter product; it thus represents a novel spliceform of the *PHF6* gene (referred to herein as *PHF6* Δ exon3). Alternate splicing of the *PHF6* gene was previously documented but the spliceform reported retained intron 10 sequence.³ Exclusion of exon 3 maintains the ORF, creating a putative protein that is shortened by 34 amino acids, although it would retain all of the known functional motifs, including the two PHD fingers and the multiple nuclear and nucleolar localisation signals (fig 2B).

Assessment of the *PHF6* protein lacking exon 3

The *PHF6* protein is a putative transcriptional regulator that localises to the nucleolus when fused to GFP.³ The elucidation of the precise function of *PHF6* requires the generation of valuable reagents such as antibodies. Nonetheless, to determine if the *PHF6* Δ exon3 protein was translated and could moderate the BFLS phenotype we examined whether the RNA was associated with functional ribosomes. Lymphocytes from the patient and normal individuals were fractionated on a sucrose gradient to isolate the polysome fraction (fig 3A). RNA was isolated from five pooled fractions corresponding to 40 S (fraction 1) and 60 S (fraction 2) ribosomal particles, 80 S monoribosomes (fractions 3 and 4), and the polysome fractions (fraction 5). RT-PCR analysis showed that both actin and the *PHF6* mRNA are associated with polysomes (fig 3B). Moreover, the *PHF6* Δ exon3 transcript is also associated with polysomes, suggesting that *PHF6* Δ exon3 represents a functional transcript destined for translation.

As another measure of whether the *PHF6* Δ exon3 protein can compensate for loss of the full length protein we examined whether its nucleolar localisation was maintained in HeLa cells transfected with a GFP-*PHF6* Δ exon3 expression

construct. We observed strict nuclear expression of the fusion protein that was concentrated in the nucleolus, as confirmed by co-localisation with an anti-nucleoli antibody for both the wild type and *PHF6* Δ exon3 proteins (fig 4). Similarly, the Arg257Gly mutation in family 1 did not affect subnuclear localisation. The truncated protein of 59 amino acids created by the use of the cryptic splice site and the inclusion of seven additional nucleotides gave a weak, non-nuclear signal when fused to GFP (data not shown). Taken together, these studies imply that the *PHF6* Δ exon3 protein is translated and probably functionally compensates for loss of the *PHF6* protein.

DISCUSSION

Here we present two Canadian families with features of Börjeson-Forsman-Lehmann syndrome and mutations in the *PHF6* gene. One patient presented with classical features

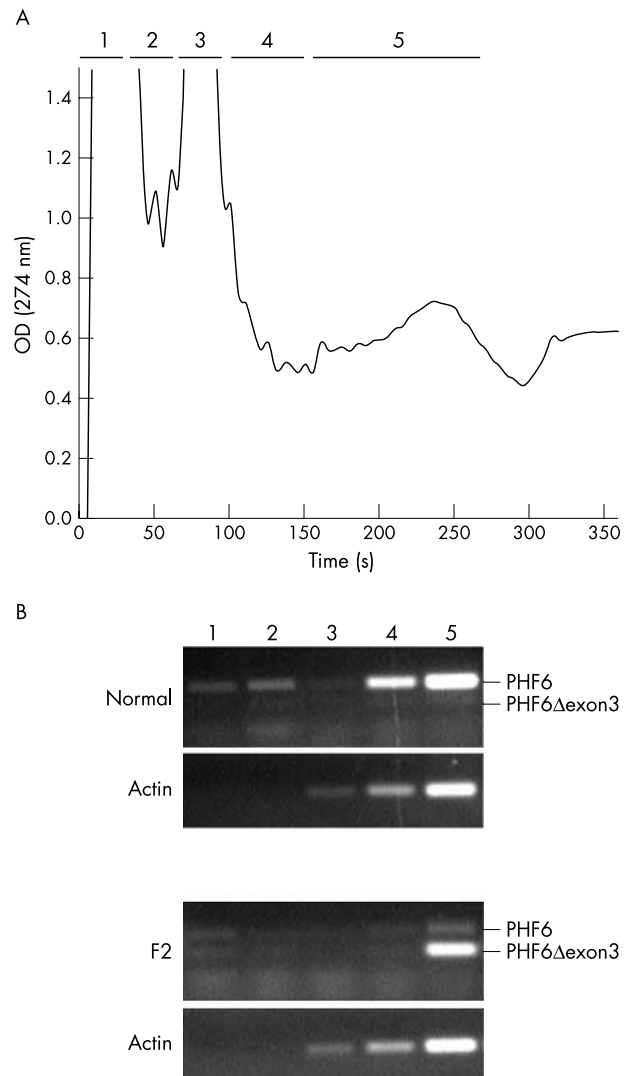


Figure 3 *PHF6* Δ exon3 transcripts are associated with polysomes. (A) Lymphoblasts isolated from patients or normal individuals were used to isolate polysomes. The graph depicts a typical isolation and the fractions used for RNA analysis. Typically, the ribosomal 40 S and 60 S particles are collected in fractions 1 and 3, while the 80 S monoribosomes and polysomes correspond to fractions 4 and 5. (B) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that the *PHF6* transcript with or without exon 3 is associated with polysomes, as is actin mRNA.

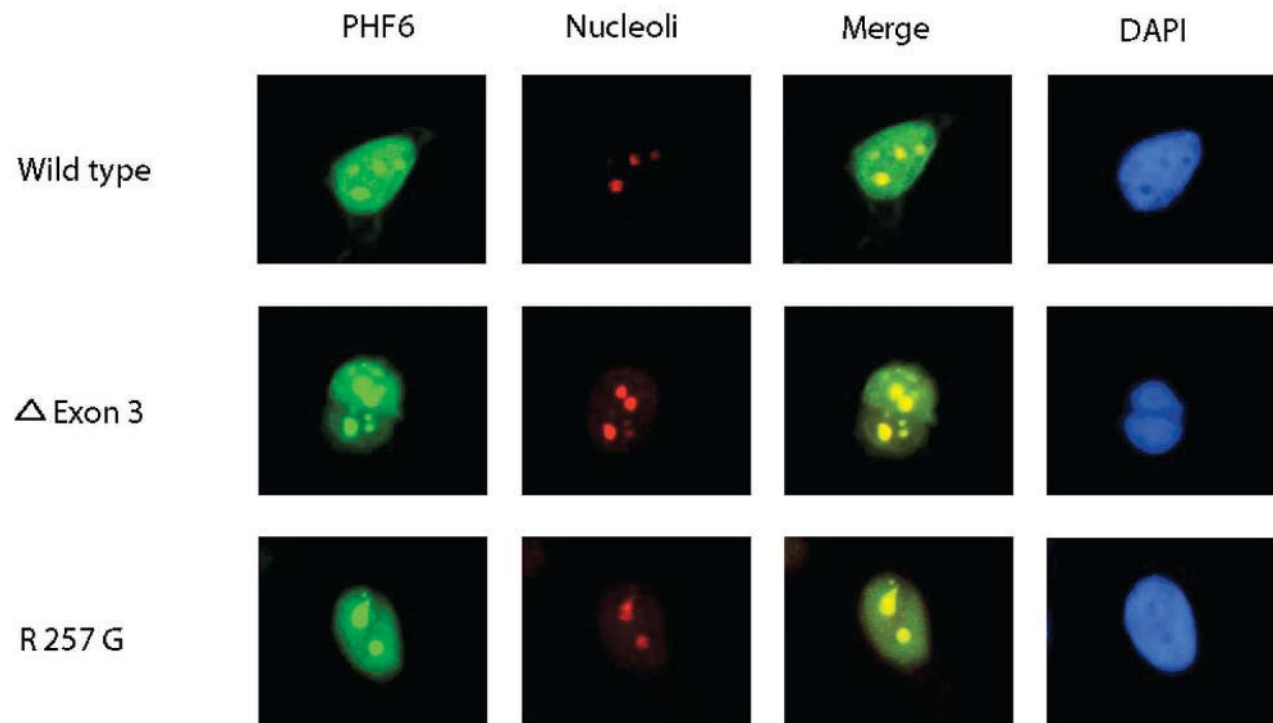


Figure 4 The GFP-*PHF6*Δexon3 fusion protein localises to the nucleolus. Previous studies showed that *PHF6* encodes a nucleolar protein. GFP fusion proteins comprising either *PHF6* wild type, *PHF6*Δexon3, or the R257G mutations were transfected into HeLa cells and co-localisation to the nucleolus was demonstrated using an antibody against nucleoli, suggesting that the shorter *PHF6*Δexon3 protein is correctly localised.

of BFLS (family 1) and we identified an A→G nucleotide change that resulted in an Arg257Gly missense mutation within *PHF6*. This mutation was identical to that previously described in an Australian patient.³ The other mutation we identified was a novel splicing mutation in intron 2 that resulted from an A→G nucleotide transition creating a cryptic 3' splice site eight nucleotides from the canonical site. To date, the two mutations in our Canadian families increase the number of unique mutations in the *PHF6* gene to 10, identified from 13 families. The mutations are mainly missense⁶; additionally, two truncations, a single amino acid deletion, and a splicing defect (this report) have also been identified. The *PHF6* protein contains two conserved PHD domains suggesting a role in transcription but is otherwise a protein of unknown function. However, unlike the ATRX syndrome, in which 60% of the mutations fall within the PHD domain,¹⁰ only one mutation has been identified within these conserved motifs of the *PHF6* gene. The broad range of mutations are localised throughout the gene and thus a "loss of function" model is suggested as the disease mechanism. However, the discovery of the precise mechanism whereby different mutations alter *PHF6* protein function will require additional studies.

The *PHF6* gene is alternatively spliced with two splice variants, *PHF6a* and *PHF6b*, that differ in size by the inclusion of intron 10 in the *PHF6b* isoform.³ Here we identified a novel spliceform that lacked exon 3 (*PHF6*Δexon3). The *PHF6*Δexon3 transcript was present in both normal and patient samples, yet semiquantitative RT-PCR analysis across exons 2–4 suggests that it is normally of relatively low abundance. Nonetheless, skipped exons have been shown to serve many purposes, for example to provide tissue specificity, or to alter cellular location or function.^{11–13} In this regard, exclusion of exon 3 maintains the ORF and reduces the protein product by the 34 amino acids preceding

the first PHD domain. Future studies will be aimed at identifying the functional significance of exon 3 to *PHF6* protein function.

Interestingly, transcript analysis from family 2 showed that the normal 3' splice site was not used; only the cryptic site, generating either a fragment increased in size by seven nucleotides, or the *PHF6*Δexon3 transcript. A failure to detect any wild type mRNA in the patient samples suggests various possibilities. The cryptic splice site may be dominant over the normal site, or the mutation may alter a splice enhancer to suppress splicing of exon 3. Mutations located outside of traditional splice sites have been associated with exon skipping and human disease.^{14–15} Indeed, such mutations have led to the identification of exonic and intronic splicing enhancers and their corresponding sequences.^{16–17} Although it is possible that splicing enhancers have become more active in the presence of the mutation to induce exon skipping, the simplest explanation is that the prevalence of the *PHF6*Δexon3 transcript arises because primary use of the cryptic splice site leads to a transcript that undergoes nonsense mediated decay.¹⁸

Regardless, the presence of the *PHF6*Δexon3 transcript suggests that it may account for the milder phenotype observed in these patients, and the exciting possibility that exon 3 contains a domain critical to some aspects of *PHF6* function. In support of this idea we showed that the *PHF6*Δexon3 transcript is most probably translated through its association with polysomes. Furthermore, *PHF6*Δexon3-GFP fusion proteins were localised to nucleoli, suggesting that the shorter *PHF6* polypeptide is correctly targeted within the cell. Taken together, this suggests that the *PHF6*Δexon3 translated protein probably maintains many of the properties of the full length *PHF6* protein and as such would functionally compensate for the loss of *PHF6* in the affected individuals of family 2.

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Conflicts of interest: none declared

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