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SHORT REPORT

Biallelic mutations in *FLNB* cause a skeletal dysplasia with 46,XY gonadal dysgenesis by activating β-catenin

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Filamin B (FLNB) functions as a switch that can affect chrondrocyte development and endochondral bone formation through a series of signaling molecules and transcription factors that also affect Sertoli cell development. Here, we report a subject with a novel skeletal dysplasia and co-existing 46,XY gonadal dysgenesis and biallelic mutations in *FLNB*. Whole exome sequencing was performed to identify mutations. Quantitative polymerase chain reaction (qPCR) and flow variant assays were performed to quantify RNA, proteins and phosphorylated proteins. The TOPFLASH reporter was performed to quantify β-catenin activity. Mutations were identified in the *FLNB* gene (*FLNB*:p.F964L, *FLNB*:p.A1577V). These mutations increased binding of FLNB protein to the MAP3K1 and RAC1 signal transduction complex and activated β-catenin and had different effects on phosphorylation of MAP kinase pathway intermediates and SOX9 expression. Direct activation of β-catenin through the FLNB-MAP3K1-RAC1 complex by *FLNB* mutations is a novel mechanism for causing 46,XY gonadal dysgenesis. The mechanism of action varies from those reported previously for loss of function mutations in *SOX9* and gain-of-function mutations in *MAP3K1*.

KEYWORDS

disorder of sex development, Filamin B, functional assays, skeletal dysplasia

1 | INTRODUCTION

Filamin B (FLNB) is 1 of 3 members of the filamin family of cytoskeletal proteins that induce actin polymerization and interact with signal transduction molecules.[1,2] FLNB functions as a switch that can control cartilage development and endochondral bone formation. Both biallelic nonsense and monoallelic missense mutations in *FLNB* have been shown to be associated with distinct skeletal dysplasias.[3–7] Biallelic nonsense mutations cause the autosomal recessive spondylocarpotarsal (SCT) syndrome of short stature and vertebral, carpal and tarsal fusions. Missense mutations cause the autosomal dominant, perinatal lethal boomerang dysplasia (BD) and atelosteogenesis I and III phenotypes (AOI, AOIII). Missense mutations also cause Larsen syndrome, a disorder of multiple joint dislocations, craniofacial abnormalities and accessory carpal bones. None of these conditions has been associated with 46,XY disorders of sex development (DSDs).

Developing chondrocytes and Sertoli cells share a repertoire of signaling molecules that influence the expression of transcription factors that determine cell fates.[8–12] Mutations in some of these signaling molecules or their structural anchors could result in co-existing DSDs and skeletal dysplasias. Here, we show that each of the biallelic FLNB mutations observed in a subject with a skeletal dysplasia and 46,XY gonadal dysgenesis increases the activity of β-catenin and has other molecular phenotypic effects.

2 | METHODS

2.1 | Case report

The subject was born small for gestational age at 37 weeks (birth weight 2.04 kg, length 17.5 in., both <3 percentile). Her family history was notable for her father having tracheomalacia at birth, congenital emphysema, intellectual disability and a conduct disorder. Her mother had mild scoliosis. The subject had dolichocephaly, low-set ears,

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midface hypoplasia, cleft palate with micrognathia and kyphoscoliosis. Scoliosis survey showed kypholevoscoliosis of the thoracic spines and irregularities of the ribs with a crowded appearance of the vertebral bodies (Figure 1). External genitalia were normal female. An abdominal ultrasound showed a normal uterus with an endometrial stripe and small bilateral gonads. Hand films showed fifth finger clinodactyly. Karyotype was 46,XY. Array CGH did not reveal pathogenic duplications or deletions. Whole exome sequencing showed biallelic variants in *FLNB* (NM_001457.3) c.2890T>C p.F964L and c.4730C>T p.A1577V. The mother was found to have the c.2890T>C p.F964L variant and the father was found to have the c.4730C>T p.A1577V variant. The p.F964L variant was reported once in the EXAC database, whereas the p.A1577V variant was not reported (http://exac.broadinstitute.org/).

2.2 | Functional analysis of mutations

To determine the pathogenicity, wild type and variant cDNAs were cloned into a cDNA construct in an expression vector, transfected into NT2/D1 cells and TOPFLASH β-catenin activity and quantitative analyses of RNA, proteins, and protein-protein interactions were performed.[9,13] The effects of *FLNB* variants were compared to those of the p.L189P mutation in the *MAP3K1* gene that causes 46,XY DSD, because *MAP3K1* mutations expressed in NT2/D1 cells switched the

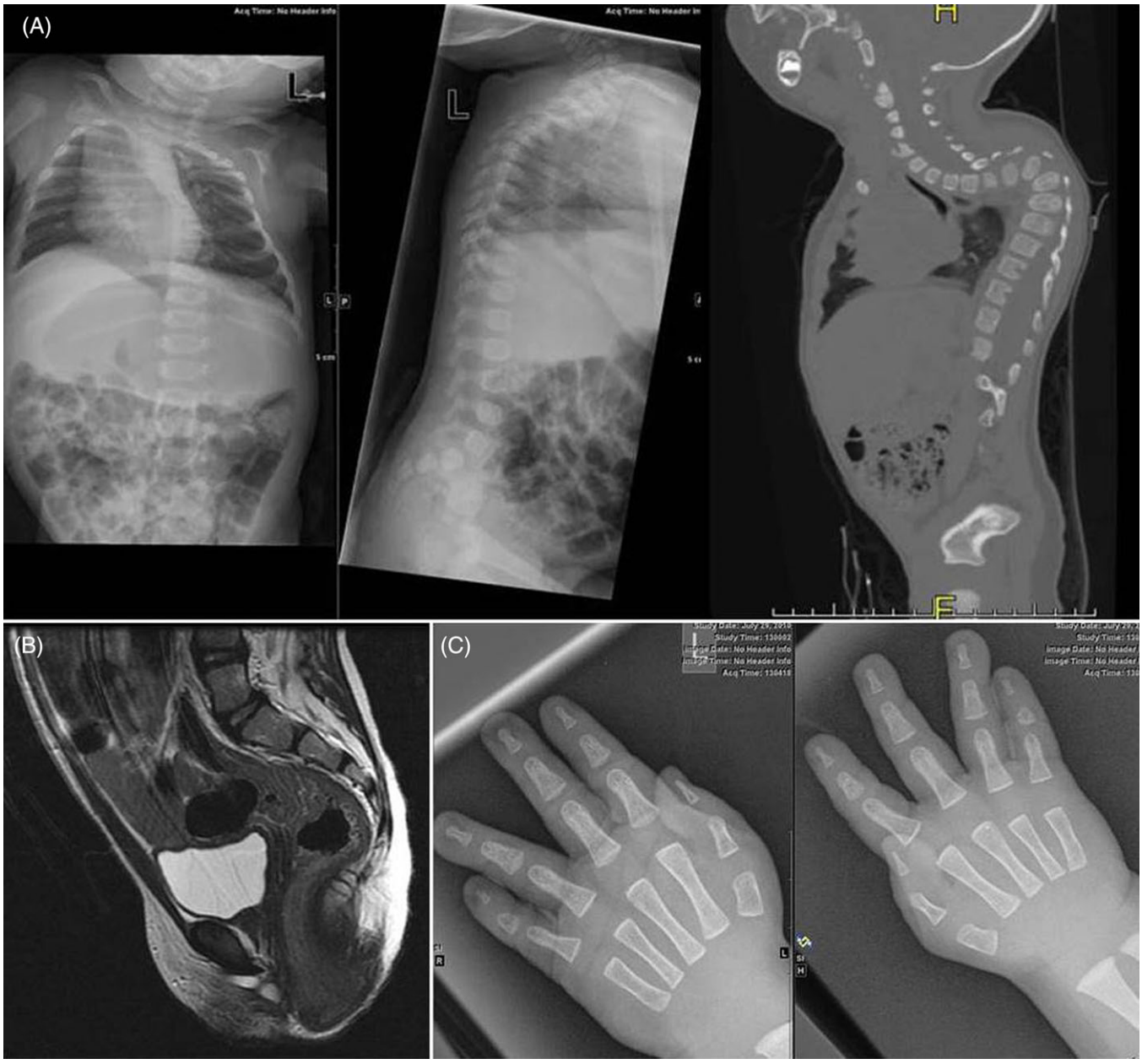

FIGURE 1 Imaging studies demonstrating skeletal dysplasia and gonadal dysgenesis. (A) Frontal and lateral view of the entire spine show marked kyphoscoliosis with multiple segmentation anomalies most pronounced in the lower cervical and upper thoracic spine, sagittal reconstructions of the CT show marked kyphoscoliosis. (B) Sagittal T2-weighted MR images of the pelvis shows a rudimentary vagina posterior and inferior to the bladder without any defined uterus superior. (C) Images of both hands show mild bilateral clinodactyly. [Color figure can be viewed at wileyonlinelibrary.com]

balance of gene expression and the patterns of protein phosphorylation observed in the testis-determining pathway to those observed in the ovarian-determining pathway.[9] This study was approved by the Albert Einstein College of Medicine Institutional Review Board (#2011-620).

3 | RESULTS

Transfection with both variant *FLNB* cDNA constructs (*FLNB*:p.F964L and *FLNB*:p.A1577V) increased β-catenin activity relative to transfection with the wild type construct at 24 hours ($P = 2 \times 10^{-4}$ and .01) and at 48 hours (P = .001 and .04; Figure 2). In addition, the increase observed at 24 hours for *FLNB*:p.F964L was significantly higher than that observed for *FLNB*:p.A1577V (P = .002), suggesting a differential effect of these 2 mutations. Co-transfection with both variant *FLNB* cDNA constructs increased β-catenin activity relative to transfection with the wild type construct at 24 and 48 hours (P = .001 and .03; Figure S1 in Appendix S1, Supporting information). These effects differed from those produced by wild type and mutant *MAP3K1*. Transfection with the wild type *MAP3K1* construct had no effect of β-catenin luciferase activity, whereas the mutant *MAP3K1* p.L189P construct showed an increase in β-catenin activity at 48 hours (P = .001), but not 24 hours.

Transfection with wild type *FLNB* cDNA increased expression of SOX9 relative to transfection with empty vector at 24 hours (P = .01) and at 48 hours (P = .03). The 2 *FLNB* mutant cDNAs showed different effects. Relative to the FLNB wild type construct, the *FLNB*:p.F964L mutant construct showed no effect on SOX9 at 24 hours and a significant reduction at 48 hours (P = .02). The *FLNB*:p.A1577V was significantly higher at 24 hours (P = .01) but had no effect at 48 hours (Figure 2). Co-transfection with both variant *FLNB* cDNA constructs decreased SOX9 expression activity relative to transfection with the wild type construct at 48 hours (P = .01; Figure S1 in Appendix S1). Transfection with the *MAP3K1* wild type construct increased SOX9 expression at both these time points (P = .01 at 24 hours and .01 at 48 hours) and transfection with the mutant *MAP3K1*:p.L189P reduced expression of SOX9 relative to wild type at both of these time points (P = .02 for 24 hours and .002 at 48 hours).

FLNB is part of a signal transduction complex that activates β-catenin and includes the MAP3K1 and RAC1 proteins.[14] Mutations in FLNB could augment activation of β-catenin by increasing binding of RAC1 and MAP3K1. Relative to wild type, transfection with the *FLNB*:p.F964L and *FLNB*:p.A1577V mutant constructs increased binding of FLNB to RAC1 ($P = 1 \times 10^{-5}$ and 2×10^{-9}; Figure 3), FLNB to MAP3K1 ($P = 5 \times 10^{-5}$ and 3×10^{-4}) and RAC1 to MAP3K1 (P = .001 and 4×10^{-4}; Figure 3). Relative to wild type, *MAP3K1*:p.

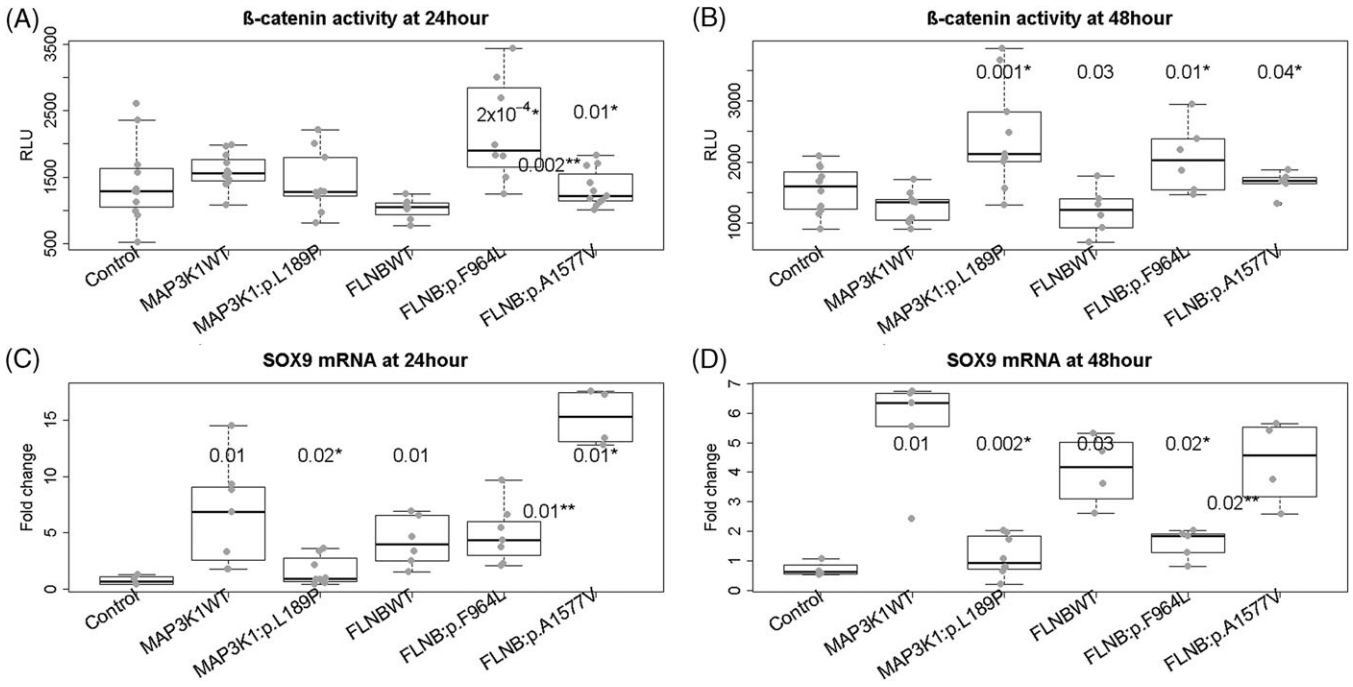

FIGURE 2 (A,B) Box plots demonstrating quantification of normalized TOPFLASH activity using Cignal TCF/LEF luciferase reporters and compared in a pairwise fashion. Co-transfections of wild type and mutant plasmids for *FLNB* and *MAP3K1* (*FLNB*:p.964L, *FLNB*:p.A1577V and *MAP3K1*:p.L189P) with Cignal TCF/LEF luciferase reporters. Control is sham transfection with vector. The TOPFLASH activity was measured at 24 and 48 hours. The standardized results were compared to control NT2/D1 cells transfected with empty plasmids by the Mann-Whitney test. (C,D) Box plots demonstrating quantification of SOX9 mRNA by Quantitative polymerase chain reaction (qPCR) and compared in a pairwise fashion. Transfection of wild type and mutant plasmids for *FLNB* and *MAP3K1* (*FLNB*:p.964L, *FLNB*:p.A1577V and *MAP3K1*:p.L189P). Control is sham transfection with empty vector. SOX9 mRNA was measured using the Taqman qPCR gene expression assay at 24 and 48 hours. For these experiments, the results were normalized to the housekeeping gene, GAPDH, standardized, and then compared to control NT2/D1 cells transfected with empty plasmids by the Mann-Whitney test. No asterisk represents wild type to control comparison. * represents mutant to wild type comparison. ** represents pairwise comparison of *FLNB*:p.F964L and *FLNB*:p.A1577V

L189P cDNA increased binding FLNB to MAP3K1 ($P = 2 \times 10^{-4}$), but decreased binding of FLNB to RAC1 ($P = 2 \times 10^{-4}$) and RAC1 to MAP3K1 ($P = .005$).

The expression of SOX9 and the activity of β-catenin can be affected by the phosphorylation of p38 and ERK1/2, targets downstream of MAP3K1. The *MAP3K1* p.L189P mutant construct increased p38 and ERK1/2 phosphorylation at 24 and 48 hours, relative to wild type (Figure S2 in Appendix S1).[9] Different effects were observed for phosphorylation of p38 and ERK1/2 in response to transfection with the mutant *FLNB* constructs. *FLNB*:p.F964L had no effect on the phosphorylation of either p38 or ERK1/2 at both time points (Figure S1 in Appendix S1). *FLNB*:p.A1577V had no effect at 24 hours and increased ERK1/2 phosphorylation at 48 hours, compared to wild type ($P = .07$). This mutant construct decreased p38 phosphorylation at 24 hours ($P = .003$), but had no effect at 48 hours.

4 | DISCUSSION

To date, combined skeletal dysplasia and DSD phenotypes have been observed only in subjects with loss of function mutations in *SOX9*.[15,16] Thus, this study extends the range of genes, which when mutated, can produce the dual phenotype of skeletal dysplasia and 46,XY DSD. The subject's compound heterozygous mutations had a joint effect in causing her phenotype because neither of her parents had a severe skeletal dysplasia and her father did not have a DSD. (Her mother would not be affected due to her gender.) The nature of skeletal dysplasia was distinct from those previously reported to be caused by *FLNB* mutations, as the subject's disorder was neither lethal nor associated with joint dislocations.[3,7] The FLNB protein forms dimers and, thus, only variant homodimers or heterodimers would form in the patient's cells.

The mechanism of action of the mutant variant *FLNB* alleles is distinct from the loss of function mutations in *SOX9* that cause campomelic dysplasia. The FLNB protein tethers RAC1 and MAP3K1, molecules that have also been shown to modulate the activities of SOX9 and β-catenin.[14] The *FLNB* mutations increase binding of RAC1 and MAP3K1 to FLNB protein and activation of β-catenin. The 2 FLNB mutations also had differential effects. The *FLNB*:p.F964L mutation did not have any effects on ERK1/2 and p38 phosphorylation, whereas the FLNB:p.A1577V increased phosphorylation of ERK1/2 slightly at 48 hours and decreased phosphorylation of p38 at 24 hours. The increased phosphorylation of ERK1/2 may have contributed to the increased activity of β-catenin, whereas the decreased p38 phosphorylation at 24 hours could account for the increased expression of SOX9 observed with this mutant construct, as noted, phosphorylated p38 is a transcriptional inhibitor of SOX9.[12,17] The mechanism of action of *FLNB* mutations is also distinct from mutations in *MAP3K1* that are associated with decreased SOX9 expression and increased β-catenin activity. Those gain of function mutations increase phosphorylation of p38, ERK1/2 and GSK3β, stabilizing β-catenin and decreasing transcription of SOX9.

This study has several implications. Subjects with skeletal dysplasias or 46,XY gonadal dysgenesis should be assessed for dual phenotypes, as similar mechanisms are affecting gonadal and bone development. Variants in newly identified candidate sex determining genes should be tested for their effects on the balance of SOX9 and

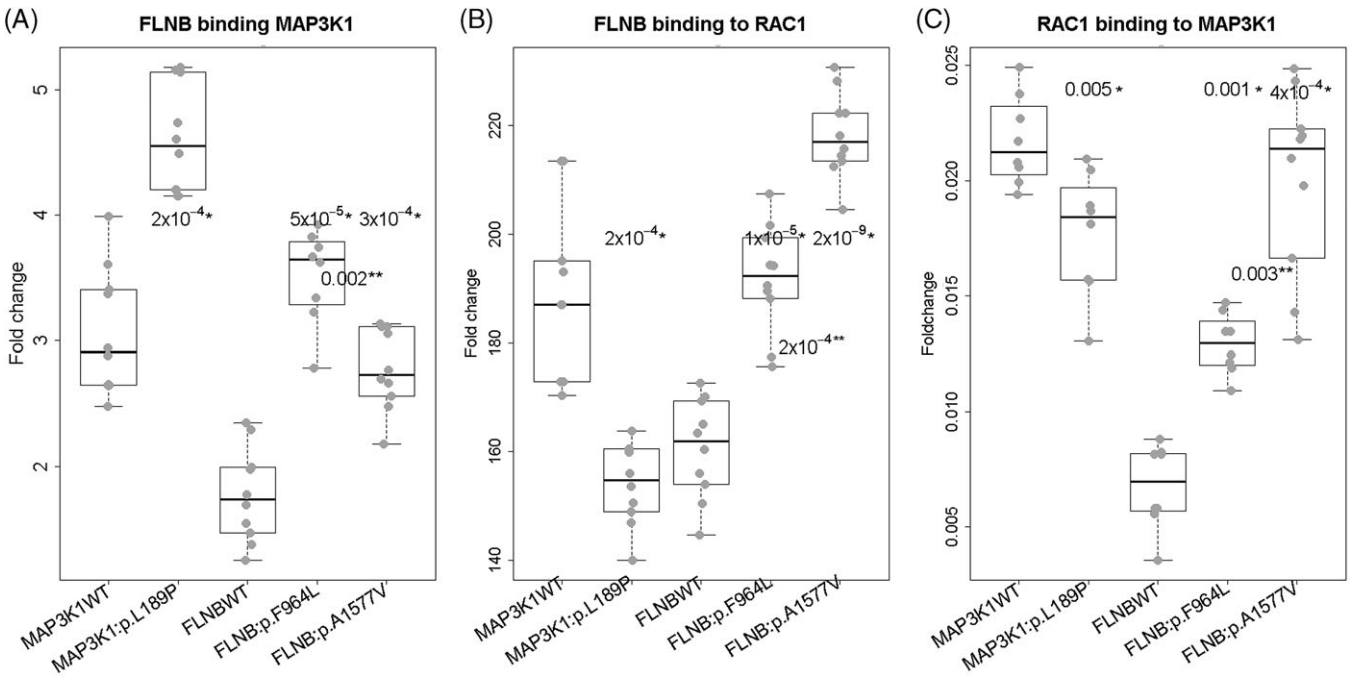

FIGURE 3 (A) Box plots demonstrating that *FLNB* and *MAP3K1* mutations increase FLNB binding to MAP3K1 relative to wild type, as judged by co-immunoprecipitation assays. (B) *FLNB*.p.964L and *FLNB*.p.A1577V increased binding of FLNB to RAC1, relative to wild type. (C) *FLNB*.p.964L and *FLNB*.p.A1577V increase binding of RAC1 to MAP3K1 relative to wild type, whereas *MAP3K1*.p.L189P decreases binding of RAC1 to MAP3K1. The signal quantification is normalized to the bait target, either MAP3K1 or RAC1, standardized and compared in a pairwise fashion. No asterisk represents wild type to control. * represents mutant to wild type. ** represents comparison of FLNB:p.F964L and FLNB:p.A1577V

β-catenin activities and their intermediate regulators for pathogenicity. Study of other molecular targets in these pathways should be applied to subjects with FLNB skeletal dysplasias to account for the considerable phenotypic heterogeneity.

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Conflict of interest

Nothing to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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