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A New Subtype of Multiple Synostoses Syndrome Is Caused by a Mutation in *GDF6* That Decreases Its Sensitivity to Noggin and Enhances Its Potency as a BMP Signal

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Abstract

Growth and differentiation factors (GDFs) are secreted signaling molecules within the BMP family that have critical roles in joint morphogenesis during skeletal development in mice and humans. Using genetic data obtained from a six-generation Chinese family, we identified a missense variant in *GDF6* (NP_001001557.1; p.Y444N) that fully segregates with a novel autosomal dominant synostoses (*SYNS*) phenotype, which we designate as SYNS4. Affected individuals display bilateral wrist and ankle deformities at birth and progressive conductive deafness after age 40 years. We find that the Y444N variant affects a highly conserved residue of GDF6 in a region critical for binding of GDF6 to its receptor(s) and to the BMP antagonist NOG,

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Disclosures

All authors state that they have no conflicts of interest.

Additional Supporting Information may be found in the online version of this article.

and show that this mutant GDF6 is a more potent stimulator of the canonical BMP signaling pathway compared with wild-type GDF6. Further, we determine that the enhanced BMP activity exhibited by mutant GDF6 is attributable to resistance to NOG-mediated antagonism. Collectively, our findings indicate that increased BMP signaling owing to a GDF6 gain-of-function mutation is responsible for loss of joint formation and profound functional impairment in patients with SYNS4. More broadly, our study highlights the delicate balance of BMP signaling required for proper joint morphogenesis and reinforces the critical role of BMP signaling in skeletal development.

Keywords

GDF6; NOG; MULTIPLE SYNOSTOSES SYNDROME; BMP/TGF- β SIGNALING

Introduction

BMP signaling plays diverse regulatory roles in the skeleton. Mice lacking the ability to respond to canonical BMP signaling or those with hyperactive BMP signaling fail to undergo normal endochondral ossification, consistent with the idea that BMP signaling must be tightly regulated to avoid deleterious effects during skeletal development.⁽¹⁻⁶⁾ Within the BMP family, *growth and differentiation factor (GDF) 5* and *GDF6* are known to have critical roles in joint formation, the process by which skeletal elements separate and develop the articulations that allow for ambulation,^(7,8) consistent with the observation that BMP signaling imbalances can lead to abnormal joint morphogenesis.⁽⁹⁾ As would be predicted from the skeletal anomalies found in mice lacking *Gdf5*, human mutations in *GDF5* result in joint anomalies; proximal symphalangism (SYM) and multiple synostoses syndromes (SYNS) are caused by increased GDF5 activity,⁽¹⁰⁻¹³⁾ whereas brachydactyly (BDA and BDC) results from reduced GDF5 activity.⁽¹⁴⁻¹⁸⁾ Although *Gdf6* mutations in mice specifically affect joint morphogenesis, to date, human loss-of-function *GDF6* mutations underlie Klippel-Feil syndrome (KFS; MIM 118100), Leber congenital amaurosis-17 (LCA17, MIM 615360), and isolated microphthalmia (MCOP4, MIM 613094), diseases whose skeletal involvement is not specific to joint formation.⁽¹⁹⁻²¹⁾ Intriguingly, patients with Leri's pleonosteosis (LP), an autosomal-dominant rheumatic condition characterized by flexion contractures of the interphalangeal joints, limited motion of multiple joints, and short, broad metacarpal, metatarsal, and phalanges, were found to carry a micro-duplication at 8q22.1 involving *GDF6*,⁽²²⁾ suggesting that a gain in GDF6 activity altering the amount of BMP signaling might have specific deleterious effects on joint morphogenesis.

Here, using genetic data obtained from a six-generation Chinese family, we identify *GDF6* as a locus for a novel subtype of SYNS, which we name SYNS4. All affected individuals had joint fusions of variable degrees and also exhibited progressive conductive deafness after the age of 40 years. By analyzing the signaling activity of the mutant GDF6 protein linked to SYNS4, we show that this *GDF6* mutation results in loss of GDF6 inhibition by the BMP signaling antagonist NOG. The increased chondrogenesis that occurs from enhanced BMP signaling at sites of *GDF6* expression appears to be responsible for joint fusions at birth and loss of hearing in adults with this mutation.

Materials and Methods

Exome sequencing

The ethics committee of the Shanghai Children's Medical Center approved the study, and informed consent was obtained from all participants. DNA was extracted from patients' peripheral blood and sheared with a Covaris S2 Ultrasonicator (Covaris, Woburn, MA, USA) to create fragments of 150 to 200 bp. Sequencing library preparation and target capture were performed using the SureSelect^{XT} Library Prep Kit and the SureSelect^{XT} Human All Exon v4 Capture Library (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Clusters were generated by isothermal bridge amplification with an Illumina cBot station, and sequencing was performed using the Illumina HiSeq 2000 System (Illumina, San Diego, CA, USA), with 100 base-pair read-lengths. Four samples were pooled into a single flow cell lane.

Analysis of sequencing data

Base calling and quality assessment were performed using Illumina Sequence Control Software (SCS) with Real Time Analysis (RTA). Alignment of sequence reads to the reference human genome (Human 37.3, SNP135) was performed using NextGENe software (SoftGenetics, State College, PA, USA). Mean read coverage was ~150×, with >95% of targets covered at >20×. All single nucleotide variants (SNVs) and indels were saved in VCF format and uploaded to Ingenuity Variant Analysis (Ingenuity Systems, Redwood City, CA, USA) for filtering and interpretation. Variants reported in public databases (ie, 1000 Genomes Project and NHLBI Exome Sequencing Project) were used as a frequency filter.

Mutation verification

Candidate SNVs were confirmed by Sanger sequencing. Primers were designed using Primer3 (v.4.0.0) online software.⁽²³⁾ Regions of candidate SNVs were amplified using KAPA2G Robust PCR Kit (Kapa Biosystems, Wilmington, MA, USA). PCR products were purified with ExoSAP-IT Kit (GE Healthcare, Aurora, OH, USA) and sequencing reactions were prepared using the BigDye Direct Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA). The final products were purified via agarose gel and recovered with the QIAquick Gel Extraction Kit (Qiagen GMBH, Hilden, Germany). Capillary electrophoresis sequencing was performed using an ABI Prism 3730XL Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequence data were analyzed with Mutation Surveyor DNA Variant Analysis Software (SoftGenetics).

Plasmids and cloning

GDF6 cDNA was amplified from wild-type and Y444N mutant *GDF6* Myc-DDK-tagged vectors (Origene, Rockville, MD, USA) and subcloned into pcDEF3 to generate tag-free vectors.⁽²⁴⁾ Wild-type *GDF5* vector was obtained from the Dana-Farber/Harvard Cancer center DNA Resource Core. Plasmids encoding wild-type human *BMP2* and *ALK2*, as well as wild-type mouse *Alk3* and *Alk6* were obtained elsewhere and have been described previously.^(24,25) *GDF5* Y490N and *BMP2* Y385N mutant vectors were generated via PCR using Prime Star HS DNA polymerase (TaKaRa, Shiga, Japan), with wild-type *BMP2* or

GDF5 vectors serving as a template. The IDWT4F-luciferase reporter plasmid, in which firefly luciferase is driven by 4 copies of a BMP-responsive element (BRE) in the human *ID1* gene, was described previously.⁽²⁶⁾ All final constructs were confirmed by sequencing.

Recombinant protein expression

GDF6 was transiently transfected into HEK 293T cells. Serum-free conditioned media was collected 24 hours post-transfection, concentrated on Amicon Ultra-15 filter units (Merck KGaA, Darmstadt, Germany), separated on 12% SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with GDF6 antibodies (Abcam, Cambridge, MA, USA). The concentration of GDF6 in the conditioned media was interpolated from a standard curve generated with rhGDF6 (Genetics Institute, Cambridge, MA, USA). Analysis was performed using ImageJ software.⁽²⁷⁾ Each experiment was repeated 3 times, with representative results shown in Supplemental Fig. S1.

Generation of C2C12-ALK6 cells

ALK6 expression plasmid was transfected into C2C12 cells using Lipofectamine2000 (Life Technologies) according to the manufacturer's instruction. One day after transfection, fresh medium was added containing 800 µg/mL of G418 (TEKnova, Hollister, CA, USA). Cells were cultured in G418-containing medium for 3 weeks. *ALK6* expression was confirmed by RT-qPCR.

Alkaline phosphatase (ALP) assays

C2C12-ALK6 cells were treated with GDF6, GDF5, or BMP2, either alone or in combination with NOG (R&D Systems, Minneapolis, MN, USA) for 72 hours. Cells were then fixed with an acetone-ethanol mixture (50:50, v/v) and incubated with a substrate solution composed of 0.1 M diethanolamine, 1 mM MgCl₂, and 1 mg/mL p-nitrophenyl phosphate. The reaction was terminated by adding 3 M NaOH, and absorbance values were measured at 405 nm.⁽²⁸⁾ For each condition, 3 replicates were performed in parallel.

Luciferase assays

BMP signaling was monitored using IDWT4F-luciferase reporter plasmid. Briefly, C2C12-ALK6 cells were plated in 96-well plates at a density of 1×10^4 cells/well 1 day before the assay. The cells were co-transfected with IDWT4F-luciferase and pRL-SV40 Renilla luciferase (Promega, Madison, WI, USA). One day after transfection, cells were treated with different doses of WT or Y444N GDF6 for 24 hours. Luciferase activity was measured and normalized to renilla luciferase with the Dual-Glo Luciferase assay system (Promega). Biological and technical triplicates were performed.

Micromass cultures

The mouse limb bud cell line, MLB13MYC clone 14, was used for micromass culture.⁽²⁹⁾ Briefly, cells were plated at a density of 2×10^5 cells/7.5 µL drop and maintained in DMEM with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. After 1 day in culture, the medium was replaced with chondrogenic medium (DMEM supplemented with 1% FBS, 50 µg/mL ascorbic acid, 0.1 µM dexamethasone, 40 µg/mL L-proline, 1 mM sodium

pyruvate, ITS+ Premix Universal Culture Supplement [Corning Inc., Corning, NY, USA], 100 IU/mL penicillin, and 100 µg/mL streptomycin) with or without 50 ng/mL *GDF6*. Culture medium was replaced every 2 to 3 days. One week after stimulation, the micromass cultures were fixed and stained with Alcian blue overnight. Quantification of Alcian blue dye was determined at 620 nm after extraction with 6M guanidine-HCl.⁽¹⁶⁾ For each condition, 3 replicates were performed in parallel.

Statistical analysis

Comparisons were made using two-tailed Student's *t* test. Results are shown as mean $\pm$ SD ($n = 3$). Values of $p < 0.05$ were considered significant.

Results

Identification of a novel *GDF6* mutation as a cause of SYNS

A six-generation Chinese pedigree with autosomal dominant SYNS was identified (Fig. 1). Forty-four affected individuals (24 male and 20 female) and 29 unaffected individuals from generations III to VI were studied. These individuals are listed along with a summary of their clinical features in Supplemental Table S1. Affliction status for most individuals was determined based on physical examination. All affected individuals had congenital bilateral foot deformities (examples shown in Fig. 2A) with diminished ankle flexion, resulting in debilitating pain and difficulty with ambulation. Computed tomography (CT) 3D bone reconstructions revealed that the joints between nearly all the tarsal bones were completely absent (Fig. 2B). The calcaneocuboid joint was partially fused (Fig. 2B, arrows), whereas only the intercuneiform joints between the medial cuneiform, intermediate cuneiform, and navicular bones remained completely unfused (Fig. 2B, black arrowheads). All the tarsometatarsal joints were fused, except for the joint between the cuboid and 5th metatarsal bones (Fig. 2B, white arrowheads). The intermetatarsal joints between metatarsal bones 2 to 4 were also fused. The first metatarsophalangeal joint was fused in 1 affected individual (Fig. 2B, gray arrowhead), whereas the distal interphalangeal joints were fused in 2 of the affected individuals (Fig. 2B, asterisks).

The hands of affected individuals appeared normal; however, radiographic examination revealed variable carpal bone fusion. In 1 patient, the lunate bone remained isolated, with carpometacarpal fusion involving the 2nd and 3rd metacarpals (Fig. 2C, upper panel). In another patient, the trapezoid, capitate, and caphoid bones were fused on the radial side, leaving the trapezium isolated, whereas the hamate bone, lunate bone, and triquetrum bone were fused on the ulnar side, leaving the pisiform bone isolated (Fig. 2C, middle panel).

Fusions in the fingers, elbows, knees, or spine were not observed in affected individuals; however, they exhibited progressive conductive deafness after the age of 40. Computed tomography (CT) scans of the skull showed no obvious abnormalities nor were any ocular abnormalities observed. Stature, facial features, and intelligence were all normal. Collectively, these findings were consistent with the diagnosis of SYNS.

To identify the causal gene for this autosomal-dominant disorder, we first searched for coding variants in known SYNS genes (ie, *NOG*, *GDF5*, *FGF9*) by Sanger sequencing (data

not shown). After failing to find any, we performed whole exome sequencing for 2 affected individuals (V-9 and V-22, who are related as third cousins). Fourteen candidate SNVs were identified after a filtering cascade (Supplemental Table S2). Among these, the variant (c. 1330T>A/p.Y444N) in *GDF6* was recognized as the most likely causal gene because of its functional relevance and its homology to *GDF5*, the disease gene in SYNS2. We then evaluated the segregation of this variant by Sanger sequencing (Supplemental Fig. S2) in 33 family members, including 18 affected individuals and 15 unaffected individuals, and observed complete co-segregation of the mutation with SYNS in the pedigree (LOD score >7 for affected individuals only; LOD score >11 for all genotyped individuals). None of the other variants we identified via whole-exome sequencing co-segregated with the phenotype; moreover, the *GDF6* mutation was absent from 105 Chinese controls and all public databases. Taken together, these results implicate *GDF6* as a new causal gene for SYNS. We designate the SYNS described in this study as SYNS4 to distinguish it from SYNS caused by mutations in *NOG*, *GDF5*, *FGF9* (known as SYNS1, SYNS2, and SYNS3, respectively). A comparison of the clinical features accompanying these four SYNS subtypes can be found in Table 1.

GDF6 Y444N protein exhibits increased activity because of NOG resistance

The Y444 residue of GDF6 affected in individuals with SYNS4 is conserved in all vertebrate GDF6 orthologues (Fig. 3A). It is also conserved among other GDFs and BMPs (Fig. 3B). Although GDF6 has not been crystallized, it is highly homologous to GDF5 at the amino acid level.⁽³⁰⁾ Using the GDF5 protein structure as a reference, we localized the Y444 residue (equivalent to Y490 of GDF5) to a TGF β domain predicted to interface with type 1 BMP receptors and NOG (Fig. 3C, D).⁽¹²⁾

To examine the effect of the missense mutation on GDF6 activity, we first tested its ability to stimulate alkaline phosphatase (ALP) activity in C2C12 cells engineered to overexpress *Activating Receptor-Like Kinase 6* (*ALK6*). This system provides a sensitive readout of GDF activity (Supplemental Fig. S3).⁽¹²⁾ Accordingly, wild-type (WT) GDF6 was able to increase ALP activity in a dose-dependent fashion (Fig. 4A). Strikingly, Y444N GDF6 induced higher ALP activity than WT GDF6 in ALK6-overexpressing cells at nearly every dose tested (Fig. 4A), suggesting that Y444N represents a gain-of-function mutation. We further found that Y444N GDF6 was able to stimulate activity of a Smad1/5/8 reporter (IDWT4F-luciferase) in C2C12-ALK6 cells more potently compared with WT GDF6 (Fig. 4B), consistent with enhanced BMP signaling. Finally, Y444N GDF6 displayed increased chondrogenic activity compared with WT GDF6 in micromass cultures of immortalized limb bud cells (Fig. 4C). Collectively, these results demonstrate that the Y444N mutation in GDF6 is hypermorphic in nature.

The Y444N mutation affects a residue located within the NOG-GDF6 binding interface, suggesting that the gain of function may reflect an altered interaction with this potent BMP antagonist. Accordingly, we found that addition of NOG inhibited WT GDF6-induced ALP activity in a dose-dependent manner, whereas it had no such effect on GDF6 Y444N (Fig. 5A). Interestingly, the homologous mutation in GDF5 (Y490N) conferred similar resistance to NOG (Fig. 5B), whereas the homologous mutation in BMP2 (Y385N) did not (Fig. 5C),

suggesting that this highly conserved residue specifically mediates binding between NOG and GDFs. Based on our in vitro results, we infer that the Y444N mutation renders GDF6 insensitive to NOG, resulting in exaggerated BMP signaling and chondrogenic activity at the sites where GDF6 and NOG are co-expressed. The joint fusions observed in patients with SYNS4 likely reflect such site-specific misregulation of BMP-induced chondrogenesis.

Discussion

GDFs are secreted signaling molecules within the BMP family that have critical roles in joint morphogenesis during embryonic development.^(7,8) Alterations in GDF activity underlie a growing list of human skeletal disorders, and prominent among these are syndromes brought about by the interruption of the normal segmentation process that creates individual skeletal elements. Although the mechanistic links between genotype and phenotype are not completely clear, mutations in *GDF5* have been observed to selectively increase or decrease BMP signaling. The former are associated with symphalangism (SYM1B, MIM #615298) and synostoses (SYNS2; MIM #610017),⁽¹⁰⁻¹³⁾ whereas the latter are associated with various subtypes of brachydactyly (BDA1C, MIM #615072; BDA2, MIM #112600; BDC, MIM #113100).⁽¹⁴⁻¹⁸⁾ Based on our molecular analysis, the GDF6 Y444N mutation belongs to the former category, with increased BMP signaling resulting from reduced sensitivity to NOG.

A role for *GDF6* in joint morphogenesis has been established in mice. *GDF6* knockout mice display anomalies in both wrist and ankle joints and also exhibit reduced size and altered shapes of middle-ear bones.⁽⁷⁾ In humans, *GDF6* loss-of-function mutations result in Klippel-Feil syndrome (KFS; MIM 118100), Leber congenital amaurosis-17 (LCA17, MIM 615360), and isolated microphthalmia (MCOP4, MIM 613094).⁽¹⁹⁻²¹⁾ Joint defects accompanying these mutations affect the spine more commonly than hands or feet, especially in patients with KFS. On the other hand, patients carrying a microduplication of a genomic segment (8q22.1) encompassing the *GDF6* locus exhibit hand and foot abnormalities, including flexion contractures of the interphalangeal joints and limited motion of multiple joints.⁽²²⁾ The hands and feet of these patients are also short and broad, a feature not shared by SYNS4 patients. Nevertheless, this further supports the idea that gain-of-function mutations in *GDF6* cause defects in joint formation at these particular sites.

In molecular assays, the mutant GDF6 protein in this study is nearly indistinguishable from mutant versions of *GDF5* resulting from substitutions (E491K and N445K, N445T) characterized by others.^(12,14) These mutant proteins all exhibit NOG resistance with a corresponding increase in BMP signaling. Although patients harboring these mutations share several symptoms, including wrist and ankle fusions, they also exhibit important differences. For example, patients with the *GDF5* N445K, N445T mutation have fusions affecting the elbows, fingers, and toes, whereas patients with the *GDF5* E491K or *GDF6* Y444N mutations have fusions affecting the fingers or toes, respectively, while sparing the elbows.^(12,13) The distinctive clinical features accompanying the *GDF6* mutation compared with those accompanying the *GDF5* mutations could reflect different patterns and/or levels of expression of each protein, mediated by the unique genomic loci in which each gene is situated. Localization studies in mice support the idea that both genes are expressed in

developing joints; however, *Gdf6* expression is restricted to a subset of joints including the wrists and ankles, as well as joints of the middle ear.^(7,31) Interestingly, its appearance in the ear joints occurs subsequent to their segmentation, suggesting a later role in their development that is consistent with the late-onset hearing loss found in SYNS4 patients. It is also worth noting that individuals carrying *GDF5* or *GDF6* mutations are heterozygous for their respective alleles. Because GDFs are dimeric molecules, afflicted individuals have the potential to form twice as many distinct dimer combinations as individuals possessing two wild-type copies of *GDF5* and *GDF6*. This, in turn, might permit gradations in signaling that could explain the variable phenotypes occurring with these mutations.

To date, 3 of the 4 SYNS identified are the result of increased BMP signaling (SYNS1, SYNS2, and here SYNS4), whereas SYNS3 is caused by a loss-of-function mutation in *FGF9*.⁽³²⁾ During organogenesis, the FGF and BMP pathways often have opposing effects, such as those found in the developing limb bud,⁽³³⁾ and it has been reported that FGF signaling may attenuate BMP signaling in specific contexts.^(34,35) An intriguing possibility that might unify the molecular mechanisms underlying SYNS is that reduced FGF9 signaling found in SYNS3 creates a gain of function in BMP activity, consistent with that observed in SYNS1, 2, and 4. Further study of SYNS3 would be needed to determine the validity of this idea.

The resistance of the mutant GDF6 protein to NOG-mediated antagonism is consistent with the location of the Y444 residue within a region predicted to interface with *NOG*. Presumably, the single amino acid substitution destabilizes the interaction between the two molecules, although this remains to be formally demonstrated. The conservation of Y444 by nearly every BMP family member further hints at the important role played by this residue. Interestingly, an analogous substitution in GDF5 (Y490N) but not BMP2 (Y385N) was able to confer NOG resistance, suggesting a unique role for this residue in mediating interactions between NOG and GDFs.

In conclusion, we identified *GDF6* as a locus for a novel subtype of SYNS that we designate as SYNS4. In vitro data indicate that this mutation reduces GDF6 inhibition by NOG. This, in turn, likely leads to increased chondrogenic activity because of enhanced BMP signaling at sites of *GDF6* expression, including a subset of synovial joints present in the wrists, ankles, and within the inner ear. Our findings highlight the dynamic role that BMP signaling plays in skeletal development, and more specifically indicate that the balance between GDFs and their antagonist NOG is critical for joint morphogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A novel multiple synostoses syndrome displays an autosomal-dominant pattern of inheritance in a six-generation Chinese family. Of 76 offspring in this family, 46 (60.5%) are affected (shaded symbols). Twenty-one affected offspring are females (circles) and 25 are males (squares). Symbols with strikethroughs indicate deceased individuals. Roman numerals indicate generation number. Asterisks (*) indicate individuals subjected to whole-exome sequencing. Individuals shown in red were genotyped for the GDF6 variant.

Fig. 2.

Affected individuals exhibit varying degrees of foot deformity accompanied by multiple joint fusions in the hands and feet. (A) Feet from 4 affected individuals are shown. (B) Reconstructed 3D computed tomography images of feet from 3 affected individuals show complete fusion of nearly all tarsal bones (t = talus; n = navicular; ca = calcaneus; cb = cuboid; c = cuneiform). The calcaneocuboid joint was partially fused (arrows), whereas only the intercuneiform joints between the medial cuneiform, intermediate cuneiform, and navicular bones remained completely unfused (black arrowheads). All tarsometatarsal joints were fused, except for the joint between the cuboid and 5th metatarsal bones (white arrowhead). Fusions involving the metatarsophalangeal joint (gray arrowhead), as well as the interphalangeal joints (asterisks), were found in some individuals. (C) Hand X-ray images of 2 affected individuals (upper and middle panels) show extensive carpal bone fusions. An unaffected individual is shown for comparison (lower panel). A fusion involving the carpometacarpal joint is highlighted (arrow), and unfused carpal bones are indicated (td = trapezoid; tm = trapezium; s = scaphoid; l = lunate; c = capitate; h = hamate; t = triquetrum; p = pisiform).

Fig. 3.

The SYNS-associated mutation in GDF6 affects a highly conserved residue predicted to mediate NOG binding. (A) A multi-species comparison of the GDF6 amino acid sequence showing conservation of the Y444 amino acid residue among vertebrate GDF6 orthologues. (B) Comparison with other GDFs and BMPs reveals the GDF6 Y444 residue is conserved among homologs as well. (C) The Y444N missense mutation occurs in the TGF β (mature) domain of GDF6. (D) Homology model of the NOG-GDF6 complex. GDF6 and NOG dimers are shown in cyan and green, respectively, with the GDF6 Y444 residue shown in

red. An enlarged image highlighting the proximity of Y444 to the NOG clip is shown in the lower panel. This model was generated using PyMOL open-source software,⁽³⁶⁾ with the NOG-BMP7 complex structure serving as template (Protein Data Bank entry 1M4U).⁽³⁷⁾

Fig. 4.

The GDF6 Y444N mutation creates a more potent ligand than WT GDF6. (A) WT GDF6 dose-dependently stimulates ALP activity in C2C12-ALK6 cells (open circles), whereas GDF6 Y444N stimulates even higher levels of ALP activity at equivalent doses (closed circles). (B) GDF6 Y444N stimulates activity of a BMP-responsive luciferase reporter in C2C12-ALK6 cells more potently than WT GDF6. (C) GDF6 Y444N stimulates chondrogenesis more potently than WT GDF6 in micromass cultures of the immortalized mouse limb bud cell line, MYLB13MYC (clone 14). Values are expressed as mean $\pm$ SD (n

= 3). Double (**) and triple asterisks (***) indicate p values less than 0.005 and 0.001, respectively.

Fig. 5.

The GDF6 Y444N mutant protein is resistant to NOG. (A–C) ALP activity induced by GDF6, GDF5, or BMP2 in C2C12-ALK6 cells was inhibited dose-dependently by NOG. The Y444N mutation abolished this effect on GDF6 (A), as did the analogous mutation in GDF5 (B). In contrast, the analogous mutation in BMP2 had no effect on NOG-mediated antagonism (C). Values are expressed as mean $\pm$ SD ($n = 3$).

Table 1

Characteristic Features of the 4 SYNS Subtypes

	SYNS1	SYNS2	SYNS3	SYNS4
Hand	Carpal fusions, symphalangism, hypoplasia or aplasia of various digital phalanges and corresponding nails	Carpal fusions, symphalangism, brachydactyly	Carpal fusions, symphalangism	Carpal fusions
Feet	Tarsal fusions, shortened toes	Tarsal fusions, symphalangism, shortened toes or hypoplastic toenails	Tarsal fusions, involving first metatarsal, cuneiform, and navicular	Tarsal fusions, symphalangism
Elbow	Humeroradial synostoses, bilateral elbow joint dysplasia	Humeroradial synostoses	Humeroradial synostoses, semidislocation or cubital valgus	Normal
Spine	Cervical vertebral fusions	Vertebral fusions	Lumbar vertebral fusions	Normal
Hearing	Progressive conductive deafness in early childhood	Normal	Normal	Progressive conductive deafness after age 40 years
Other	Characteristic facial features, including long, arrow face, broad, tubular nose with lack of alar flare, short philtrum, and thin upper vermillion	Broad hemicylindrical nose	n/a	n/a