Exome Sequencing Identifies *PDE4D* Mutations in Acrodysostosis

Hane Lee,[1,2] John M. Graham, Jr.,[3,9] David L. Rimoin,[1,3,4,9] Ralph S. Lachman,[5,9] Pavel Krejci,[9,10] Stuart W. Tompson,[8] Stanley F. Nelson,[1,2] Deborah Krakow,[1,6,7] and Daniel H. Cohn[7,8,*]

Acrodysostosis is a dominantly-inherited, multisystem disorder characterized by skeletal, endocrine, and neurological abnormalities. To identify the molecular basis of acrodysostosis, we performed exome sequencing on five genetically independent cases. Three different missense mutations in *PDE4D,* which encodes cyclic AMP (cAMP)-specific phosphodiesterase 4D, were found to be heterozygous in three of the cases. Two of the mutations were demonstrated to have occurred de novo, providing strong genetic evidence of causation. Two additional cases were heterozygous for de novo missense mutations in *PRKAR1A*, which encodes the cAMP-dependent regulatory subunit of protein kinase A and which has been recently reported to be the cause of a form of acrodysostosis resistant to multiple hormones. These findings demonstrate that acrodysostosis is genetically heterogeneous and underscore the exquisite sensitivity of many tissues to alterations in cAMP homeostasis.

Acrodysostosis (MIM 101800), also known as Arkless-Graham syndrome or Maroteaux-Malamut syndrome, is a pleiotropic disorder characterized by skeletal, endocrine, and neurological abnormalities.[1,2] Skeletal features include brachycephaly, midface hypoplasia with a small upturned nose, brachydactyly, and lumbar spinal stenosis. Endocrine abnormalities have been reported and include hypothyroidism and hypogonadism in males and irregular menses in females (summarized in Butler et al.). Developmental disability is a common finding but is variable in severity and can be associated with significant behavioral problems. Most cases are sporadic, and there is evidence of a paternal age effect,[3] suggesting that the phenotype might result from de novo point mutations. Perhaps as a result of the developmental disability and/or endocrine abnormalities, there are only a few examples of dominant transmission.[4,5] Recently, dominant mutations in *PRKAR1A* (MIM 188830), which encodes the cyclic AMP (cAMP)-dependent regulatory subunit of protein kinase A (PKA), were found in a subset of acrodysostosis cases resistant to multiple hormones.[6] The mutations resulted in reduced PKA activation by cAMP and led to a reduced hormone response in multiple tissues.

We studied five sporadic cases, four males and one female, who had clinical and radiographic phenotypes (Figure 1, Table 1) consistent with the diagnosis of acrodysostosis. A prior publication[7] contains additional clinical details on three of the five cases (International Skeletal Dysplasia Registry reference numbers R99-101A [case 1 in Graham et al.[7]], R99-514A [case 2], and 95-141A [case R1]). All studies were carried out with informed consent under a protocol approved by the institutional review board at Cedars-Sinai Medical Center. To determine the molecular basis of the phenotype in each case, we performed exome capture and sequence analysis. In three cases (R02-309A, R06-434A, and R95-141A), DNA from the unaffected parents was also available at the outset of the study, and we determined the exome sequences for the six parents to facilitate the identification of de novo mutations in these trios. High-molecular-weight genomic DNA was extracted from either blood or lymphoblastoid cell lines; the quality of the DNA samples was determined by a Qubit Fluorometer (Invitrogen) and a Bioanalyzer (Agilent). For each sample, we prepared the sequencing library with 3 μg of genomic DNA and used the Agilent SureSelect Target Enrichment System to construct an Illumina Paired-End Sequencing library (protocol version 2.0.1). The Agilent SureSelect Human All Exon 50Mb kit was used for the exome capture. Sequences for each sample (50 bp paired end) were determined on a single lane of an Illumina HiSeq2000 instrument, and a total of 82–123 million paired-end reads per sample were generated. We performed base-calling by using the real-time analysis (RTA) software provided by Illumina.

We aligned the sequence reads to human reference genome human_g1k_v37.fasta (downloaded from the Genome Analysis Toolkit [GATK] resource bundle in November 2010) by using Novoalign from the Novocraft Short Read Alignment Package; the adaptor-stripping and base-quality-calibration options were on. We used SAMtools version 0.1.15 to sort the aligned BAM files, and we removed potential PCR duplicates (rmdup) by

[1]Department of Human Genetics, University of California, Los Angeles, Los Angeles, CA 90095, USA; [2]Department of Pathology, University of California, Los Angeles, Los Angeles, CA 90095, USA; [3]Department of Pediatrics, University of California, Los Angeles, Los Angeles, CA 90095, USA; [4]Department of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA; [5]Department of Radiological Sciences, University of California, Los Angeles, Los Angeles, CA 90095, USA; [6]Department of Obstetrics and Gynecology, University of California, Los Angeles, Los Angeles, CA 90095, USA; [7]Department of Orthopedic Surgery, University of California, Los Angeles, Los Angeles, CA 90095, USA; [8]Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, Los Angeles, CA 90095, USA; [9]Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA; [10]Institute of Experimental Biology, Masaryk University and Department of Cytokinetics, Institute of Biophysics AS CR, 61265 Brno, Czech Republic
*Correspondence: dcohn@mcdb.ucla.edu
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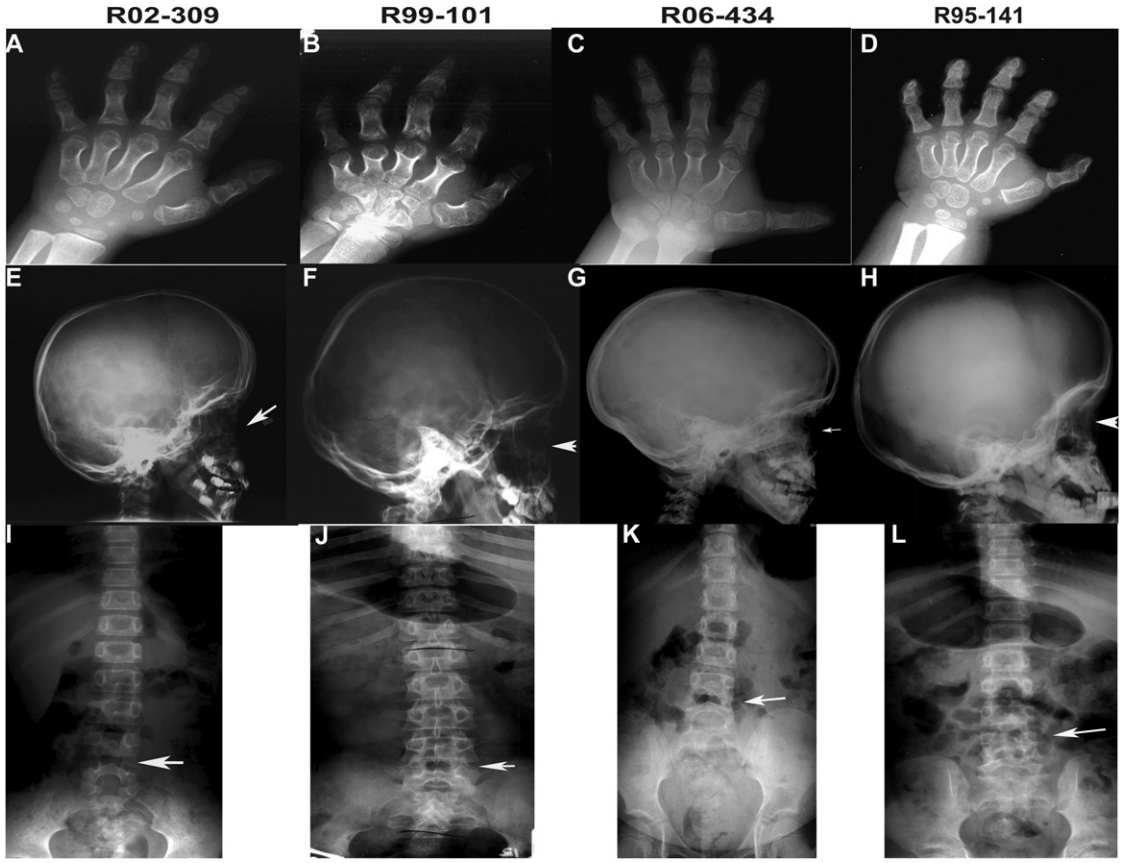

Figure 1. Radiographic Phenotype in Acrodysostosis Cases
For four of the cases, anteroposterior hand (A–D), lateral-skull (E–H), and lumbar-spine (I–L) radiographs are shown. Individuals R02-309 and R99-101 have mutations in *PRKAR1A*, and individuals R06-434 and R95-141 have mutations in *PDE4D*. Arrows on the lateral-skull films identify midface hypoplasia. Arrows on the lumbar-spine films indicate absence of normal interpedicular widening in the lumbar vertebrae; the absence of such widening predisposes the affected individuals to spinal stenosis. The case numbers are indicated across the top.

using Picard. On average, 88.2% of the reads were uniquely aligned to the reference genome. The PCR duplication rate varied between 5.1% and 8.2%, and there was an average estimated library size of 704 million unique fragments. The on-target rate, or capture specificity, varied from 60% to 63.8%. The mean coverage across the captured regions was 97×, and approximately 92% of the targeted bases were covered by ≥ 10 reads for each exome.

We performed local realignment for each sample by using the GATK "IndelRealigner" tool, and we recalibrated base qualities by using the GATK "TableRecalibration" tool according to GATK's recommendation (Best Practice Variant Detection with the GATK version 2). Variants were simultaneously called with the GATK "Unified Genotyper" tool for all 11 samples (the five cases and six unaffected parents). Small indels were called with the "-glm DINDEL" option. The dbSNP132 file downloaded from the GATK resource bundle was used so that the known SNP positions were annotated in the output VCF (variant call format) file. Only the variants found within the protein coding regions of the captured exons were reported with the –L option. The interval file that we used is available upon request. Using the GATK "VariantFiltrationWalker" tool, we hard filtered both the SNPs and INDELs to remove low-quality variants. As suggested by GATK, we used the following parameters for standard filtration: (1) the clusterWindowSize was 10, (2) mapping quality of zero was >40, (3) quality by depth was <5.0, and (4) strand bias was >-0.10.

We annotated the "PASS"-ed variants that were not found at dbSNP132 positions by using SeattleSeqAnnotation version 6.16 (SNPs and INDELs were annotated separately). Both NCBI (National Center for Biotechnology Information) full genes and CCDS (consensus coding sequence) 2010 gene models were used for the annotation. Variants present in the 1,000 Genomes Database (March 2010 release) or dbSNP131 as well as those resulting in synonymous coding changes or found outside the coding region were removed from further analysis.

The annotated variants were first examined in the trios and were further filtered under a rare dominant model. Because acrodysostosis is dominantly inherited and was sporadic in the cases studied, we prioritized the variants to examine the de novo variants. We identified potential de novo variants by selecting the heterozygous variants found only in the case but not in the parents, and we

Table 1. Clinical Findings in the Five Cases of Acrodysostosis

	R06-434A	R95-141A	R99-514	R02-309A	R99-101A
Sex	female	male	male	male	male
Locus	*PDE4D*	*PDE4D*	*PDE4D*	*PRKAR1A*	*PRKAR1A*
Skeletal abnormalities					
Short stature	no	mild	mild	mild	mild
Small hands	yes	yes	yes	yes	yes
Midface hypoplasia	yes	yes	yes	yes	yes
Lumbar stenosis	unknown	yes	yes	yes	yes
Neurological abnormality					
Developmental disability	no	significant	mild	mild	mild
Endocrine abnormalities					
Hypothyroidism	no	no	congenital	no	congenital
Hypogonadism	unknown	cryptorchidism	no	no	unilateral undescended testis
Hearing loss	no	no	no	no	moderate mixed

manually inspected the raw reads of these variants to verify that each was absent from the parental sequences.

Individual R06-434A had two de novo variants, and both were of good quality (Table 2). Individual R95-141A also had two potential de novo variants, but one variant was found in a poor coverage region, and there was insufficient coverage in the parental samples for this variant to be reliably called. Two de novo variants (c.682C>G [p.Gln228Glu] in R06-434A and c.1769A>C [p.Glu590Ala] in R95-141A) found in these first two individuals were located in the same gene, *PDE4D* (RefSeq accession number NM_001104631.1; MIM 600129), and an additional *PDE4D* variant (c. 2018G>A [p.Gly673Asp]) was identified in a third individual, R99-514. All *PDE4D* variants were confirmed by Sanger-sequence analysis of PCR-amplified fragments, and the unaffected parents were shown to not carry the changes identified in their offspring. In the third individual (R99-514), the *PDE4D* variant was not found in DNA from the mother, and the father could not be studied because he is deceased. These data provide strong genetic evidence that the *PDE4D* mutations are causative.

Individual R02-309A had three potential de novo variants. However, one variant showed evidence that the same nonreference allele was present in one of the parents even though it was not called as a variant, leaving two potential de novo variants in this individual (Table 2). Both variants were confirmed by Sanger-sequence analysis of PCR-amplified fragments containing the changes. One of the de novo variants (c.1004G>C [p.Arg335Pro]) was located in *PRKAR1A* (RefSeq accession number NM_002734.3), the gene previously associated with

Table 2. De Novo Variants Identified by Exome Sequencing in the Five Cases of Acrodysostosis

Individual	Chromosome	Genomic Position	Reference Sequence	Variant Sequence	Locus	cDNA Position	Protein Change	De Novo?	Polyphen-2 Prediction	SIFT Prediction
R06-434A	5	58,489,328	G/G	G/C	*PDE4D*	c.682C>G	p.Gln228Glu	yes	probably damaging	damaging
R06-434A	7	148,963,588	C/C	C/T	*ZNF783*	c.187C>T	p.Arg63Cys	yes	–	damaging
R95-141A	5	58,272,238	T/T	T/G	*PDE4D*	c.1769A>C	p.Glu590Ala	yes	probably damaging	damaging
R99-514	5	58,270,903	C/C	C/T	*PDE4D*	c.2018G>A	p.Gly673Asp	not in mother	probably damaging	damaging
R02-309A	17	66,526,448	G/G	G/C	*PRKAR1A*	c.1004G>C	p.Arg335Pro	yes	probably damaging	damaging
R02-309A	2	175,264,813	T/T	T/C	*SCRN3*	c.302T>C	p.Leu108Ser	yes	probably damaging	tolerated
R99-101	17	66,526,424	T/T	T/C	*PRKAR1A*	c.980T>C	p.Ile327Thr	yes	probably damaging	damaging

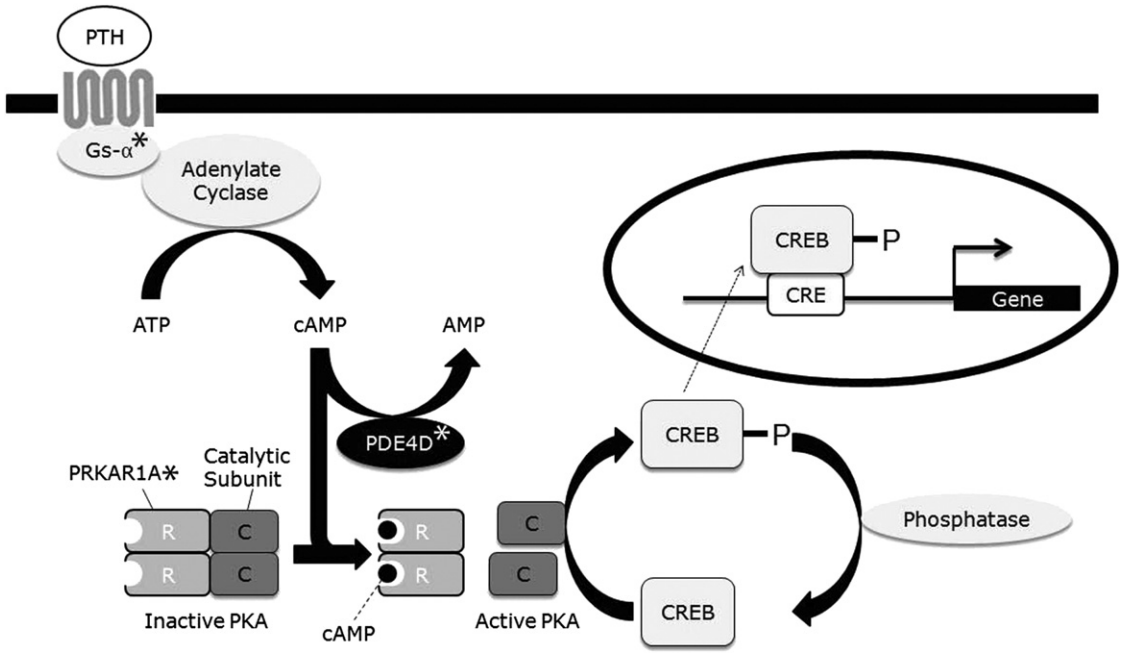

Figure 2. cAMP Signaling Cascade
Ligand binding (represented in this example by PTH, but other ligands and receptors can stimulate cAMP synthesis), activates Gs-α and stimulates cAMP synthesis by adenylate cyclase. The binding of cAMP by PRKAR1A, the cAMP-dependent regulatory subunit, leads to the dissociation and activation of PKA and the subsequent phosphorylation of cAMP response element binding (CREB), nuclear translocation, and expression of downstream genes. PDE4D phosphodiesterase activity modulates cAMP levels. Mutations (indicated by asterisks) in the genes encoding these three components of the pathway result in a spectrum of clinically related disorders— acrodysostosis for mutations in *PDE4D* or *PRKAR1A* or Albright hereditary osteodystrophy for mutations in *GNAS*, the gene encoding Gs-α.

acrodysostosis with hormone resistance.[6] Individual R99-101 was also found to have a variant (c.980T>C [p.Ile327Thr]) in *PRKAR1A*, and subsequent Sanger-sequence analysis of a PCR-amplified fragment confirmed the mutation and demonstrated its absence from DNA derived from the parents; this analysis indicated that the variant resulted from a de novo event. Therefore, acrodysostosis in these latter two individuals appears to have resulted from *PRKAR1A* mutations.

All five missense variants (three in *PDE4D* and two in *PRKAR1A*) were predicted to be damaging by PolyPhen-2 (Polymorphism Phenotyping version 2) and/or SIFT, two commonly used tools that predict the functional consequences of amino acid changes on the basis of sequence homology and the physical properties of the amino acids. None of these variants were observed in an internal exome dataset of 48 individuals affected by different medical conditions, in a group of 250 published exome datasets,[8,9] or among the 5,379 exomes available from the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project Exome Variant Server (ESP5400).

The findings described here thus demonstrate that acrodysostosis can result from missense mutations in *PDE4D*, the gene encoding cAMP-dependent phosphodiesterase 4D. *PDE4D* encodes at least five isoforms that differ at their amino-terminal ends as a result of alternate transcription start sites or alternative splicing.[10] The encoded proteins range in size from 508 to 810 amino acids, and the three longer isoforms contain two highly evolutionarily conserved upstream regions (UCR1 and UCR2) and the large catalytic domain. The two shorter isoforms lack the amino-terminal UCR1 domain, which regulates catalytic activity along with UCR2.[11] The p.Gln228Glu substitution alters a conserved residue in the UCR1 region, indicating that disruption of the longer isoforms alone is enough to cause a phenotypic effect in the target tissues and result in acrodysostosis. The p.Glu590Ala and p.Gly673Asp substitutions alter conserved catalytic-domain amino acids, indicating that these residues are essential for normal PDE4D activity.

The results of this study also confirm that mutations in *PRKAR1A*, which encodes the cyclic AMP-dependent regulatory subunit of PKA, can also lead to acrodysostosis.[6] The two substitutions, p.Arg335Pro and p.Ile327Thr, found in *PRKAR1A* were different than the recurrent mutation (p.R368*) previously reported,[6] but all three mutations were in exon 11, which encodes part of the highly conserved cAMP-binding domain B. Binding of cAMP by PRKAR1A is required for the release and activation of PKA (Figure 2), which then phosphorylates and activates CREB; this process then leads to the expression of downstream targets. This suggests that these mutations could cause reduced cAMP binding and result in reduced PKA activation and, consequently, reduced downstream signaling. This mechanism would distinguish the acrodysostosis mutations from the *PRKAR1A* mutations that cause Carney

complex (the mutations that cause Carney complex primarily lead to reduced PRKAR1A synthesis, lack of regulatory control of PKA activation, and derepression of CREB-mediated targets).[12]

The clinical and radiographic phenotypes (summarized in Table 1) facilitated comparing the acrodysostosis cases with the typical symptoms associated with either *PDE4D* or *PRKAR1A* mutations. Mild short stature with small hands was present in all of the cases, including those with *PRKAR1A* mutations previously described,[6] regardless of the locus involved. Similarly, stenosis of the lumbar spine and midface hypoplasia with a small nose were consistent findings both clinically and radiographically (Figure 1). However, endocrine abnormalities were variable; hypothyroidism was documented in just two of the individuals, R99-514 (who had a *PDE4D* mutation) and R99-101 (who had a *PRKAR1A* mutation). Hypothyroidism persisted in individual R99-101 but spontaneously resolved in individual R99-514 when he reached three years of age. However, firm conclusions cannot be made from these observations because the number of cases studied thus far is too small. One of the four male individuals with a *PDE4D* mutation (R95-141A) had cryptorchidism. One of the *PRKAR1A* individuals described here, R99-101, exhibited a unilateral undescended testis, and both of the males previously described[6] had cryptorchidism, indicating that hypogonadism can be found in cases with defects in either gene. From a neurological viewpoint, four of the five individuals studied had some degree of developmental disability, and one individual (R95-141A) displayed significant behavioral problems. Thus, it is difficult to distinguish acrodysostosis cases with *PDE4D* mutations from those with *PRKAR1A* mutations by clinical observation only.

The acrodysostosis phenotype is similar to that of *Pde4d*-knockout mice.[13] As in humans with acrodysostosis, *Pde4d*-null mice exhibit reduced growth and midface hypoplasia. Females with acrodysostosis have been reported to have irregular menses, and knockout mice have reduced fertility associated with decreased ovulation and oocyte degeneration. These observations suggest that the human mutations lead to reduced PDE4D activity. Because the heterozygous knockout mice were phenotypically normal and had essentially normal phosphodiesterase activity,[13] it appears that haploinsufficiency for PDE4D activity has no phenotypic consequence. Because PDE4D is a dimer, the data suggest the possibility that the missense alleles identified in the acrodysostosis cases might cause the phenotype via a dominant-negative effect on the protein.

Albright hereditary osteodystrophy (MIM 103580) shares phenotypic features, including short stature, brachydactyly, hormone resistance, and varying degrees of developmental disability, with acrodysostosis and results from mutations in *GNAS*[14] (MIM 139320), the gene encoding the adenylate cyclase activating protein Gs-α. Gs-α, PDE4D, and PRKAR1A are all components of the cAMP signaling pathway (Figure 2). The disruption of *PRKAR1A* and *GNAS* causes downregulation of the cAMP signaling cascade in response to an external signal, such as parathyroid hormone (PTH). Although decreased PDE4D activity might be predicted to increase cAMP levels, it has been suggested[13] that inactivation of PDE4D-mediated negative feedback would cause a permanent desensitization state of the cAMP signaling pathway; this desensitization would paradoxically lead to a significant reduction in the cAMP response. Consequently, the phenotypic effects resulting from *PDE4D* mutations would be similar to those resulting from *PRKAR1A* and *GNAS* defects.

PDE4D is orthologous to *Drosophila dunce*, which has been shown to play a role in learning and memory in flies.[15] Flies deficient in dunce have reduced cAMP phosphodiesterase activity,[16] a reduction which results in defects in both associative and nonassociative memory.[17] Although increased branching of terminal neuronal processes has been observed in *dunce* larvae (implicating abnormal brain morphology as an element of the phenotype[18]), alterations that occur in the biochemical process of memory as a result of altered cAMP levels in the mushroom body of the *Drosophila* brain appear to be the predominant effect of dunce mutations.[19] Because most acrodysostosis cases exhibit significant developmental disabilities, the data presented here raise the possibility that PDE4D deficiency disrupts a highly evolutionarily conserved neurological pathway.

Thus, a variety of genetic defects that alter cAMP metabolism produce disorders with a related constellation of findings, which include short stature with brachydactyly, endocrine abnormalities, and developmental disability. However, the precise role of PDE4D in the skeleton, particularly in growth-plate cartilage, is not well understood. Loss of cAMP activity as a result of a chondrocyte-specific knockout of Gs-α revealed severe growth-plate abnormalities, accelerated hypertrophic chondrocyte differentiation with ectopic cartilage formation, and increased parathyroid hormone-related peptide expression in periarticular chondrocytes.[20] Individuals with acrodysostosis have been reported to exhibit accelerated bone maturation as well as ectopic bone formation,[21,22] supporting the hypothesis that a component of the cartilage phenotype might be reduced activity of the cAMP signaling cascade. It remains to be determined whether modulation of cAMP levels could ameliorate the phenotypic consequences of mutations in the pathway in any meaningful way, especially in the primary target tissues of the skeleton, brain, and endocrine organs. Understanding the complexity of cAMP regulation among the affected tissues would be an important step in achieving this goal.

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Web Resources

The URLs for data presented herein are as follows:

Exome Variant Server, http://evs.gs.washington.edu/EVS/
Genome Analysis Toolkit, ftp://gsapubftp-anonymous@ftp.broadinstitute.org
Novocraft Short Read Alignment Package, http://www.novocraft.com
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
Picard, http://picard.sourceforge.net/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/bgi.shtml
SAMtools, http://samtools.sourceforge.net/
SeattleSeqAnnotation, http://snp.gs.washington.edu/SeattleSeqAnnotation131/
SIFT, http://sift.jcvi.org/

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