Mutation in Pyrroline-5-Carboxylate Reductase 1 Gene in Families with Cutis Laxa Type 2

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Autosomal-recessive cutis laxa type 2 (ARCL2) is a multisystem disorder characterized by the appearance of premature aging, wrinkled and lax skin, joint laxity, and a general developmental delay. Cutis laxa includes a family of clinically overlapping conditions with confusing nomenclature, generally requiring molecular analyses for definitive diagnosis. Six genes are currently known to mutate to yield one of these related conditions. We ascertained a cohort of typical ARCL2 patients from a subpopulation isolate within eastern Canada. Homozygosity mapping with high-density SNP genotyping excluded all six known genes, and instead identified a single homozygous region near the telomere of chromosome 17, shared identically by state by all genotyped affected individuals from the families. A putative pathogenic variant was identified by direct DNA sequencing of genes within the region. The single nucleotide change leads to a missense mutation adjacent to a splice junction in the gene encoding pyrroline-5-carboxylate reductase 1 (*PYCR1*). Bioinformatic analysis predicted a pathogenic effect of the variant on splice donor site function. Skipping of the associated exon was confirmed in RNA from blood lymphocytes of affected homozygotes and heterozygous mutation carriers. Exon skipping leads to deletion of the reductase functional domain-coding region and an obligatory downstream frameshift. *PYCR1* plays a critical role in proline biosynthesis. Pathogenicity of the genetic variant in *PYCR1* is likely, given that a similar clinical phenotype has been documented for mutation carriers of another proline biosynthetic enzyme, pyrroline-5-carboxylate synthase. Our results support a significant role for proline in normal development.

Cutis laxa type 2 (ARCL2, [MIM 219200]) is an autosomal-recessive multisystem disorder with prominent connective-tissue features characterized by the appearance of premature aging, particularly wrinkled and lax skin with reduced elasticity. It falls in a family of related disorders including cutis laxa type 1 (MIM 219100), gerodermia osteodysplastica or Walt Disney dwarfism (MIM 231070), and wrinkly skin syndrome (MIM 278250) and is sometimes referred to as cutis laxa with growth and developmental delay, or as cutis laxa with joint laxity and retarded development. Definitive criteria for distinguishing all of these conditions unambiguously is lacking, involving a subjective element to clinical diagnosis. Thus, molecular diagnosis is proving increasingly useful in resolving these phenotypically similar disorders. Mutations have been identified in five genes in patients and families segregating these related clinical presentations, specifically Egf-containing fibulin-like extracellular matrix protein 2 (*EFEMP2*, alias fibulin 4 [MIM 604633]),[1,2] fibulin 5 (*FBLN5* [MIM 604580]),[3–6] ATPase, H^+ transporting, V0 subunit a2 (*ATP6V0A2* [MIM 611716]),[7] elastin (*ELN* [MIM 130160]),[8–11] and SCYL1 binding protein (*SCYL1BP1* [MIM 607983]).[12] Mutations have also been found in a sixth gene, aldehyde dehydrogenase 18 family, member A1 (*ALDH18A1*, alias pyrroline 5-carboxylate synthase [*PYCS*] [MIM 138250]), in patients described with a so-called neurocutaneous syndrome closely resembling cutis laxa type 2.[13–15] These six genes represent a variety of biochemical processes. The phenotypes also vary among the described patients, both clinically and biochemically, as to whether there is involvement of protein N- or O-glycosylation, found to be defective in association with the *ATP6V0A2* mutations, although this gene may not explain the symptoms of all such patients.[7,16–18] Notably, there are numerous literature reports of cases resembling this suite of phenotypes, either unexplained or untested for the known genes.[19–24]

In the course of regular clinical practice, we ascertained two Maritime Canadian pedigrees of French Acadian descent, one with four individuals diagnosed with ARCL2 and one with one such individual (Figures 1A and 1B). All of the patients displayed lax, wrinkled skin with reduced elasticity, lax joints, and mild craniofacial dysmorphic features (Figure 2). The loose skin was most prominent over the dorsum of the hands and feet. Craniofacial dysmorphism included microcephaly, broad and prominent forehead, prominent ears, blue sclerae, and sagging cheeks. The patients looked substantially older than their chronologic ages. Joint laxity was most prominent in the small joints of the hands and feet, and two patients had congenital hip dislocation. All patients had intrauterine growth retardation and at least some degree of postnatal growth deficiency. All five showed developmental delay. Two affected individuals had demonstrated agenesis of the corpus callosum, and one other had been found to

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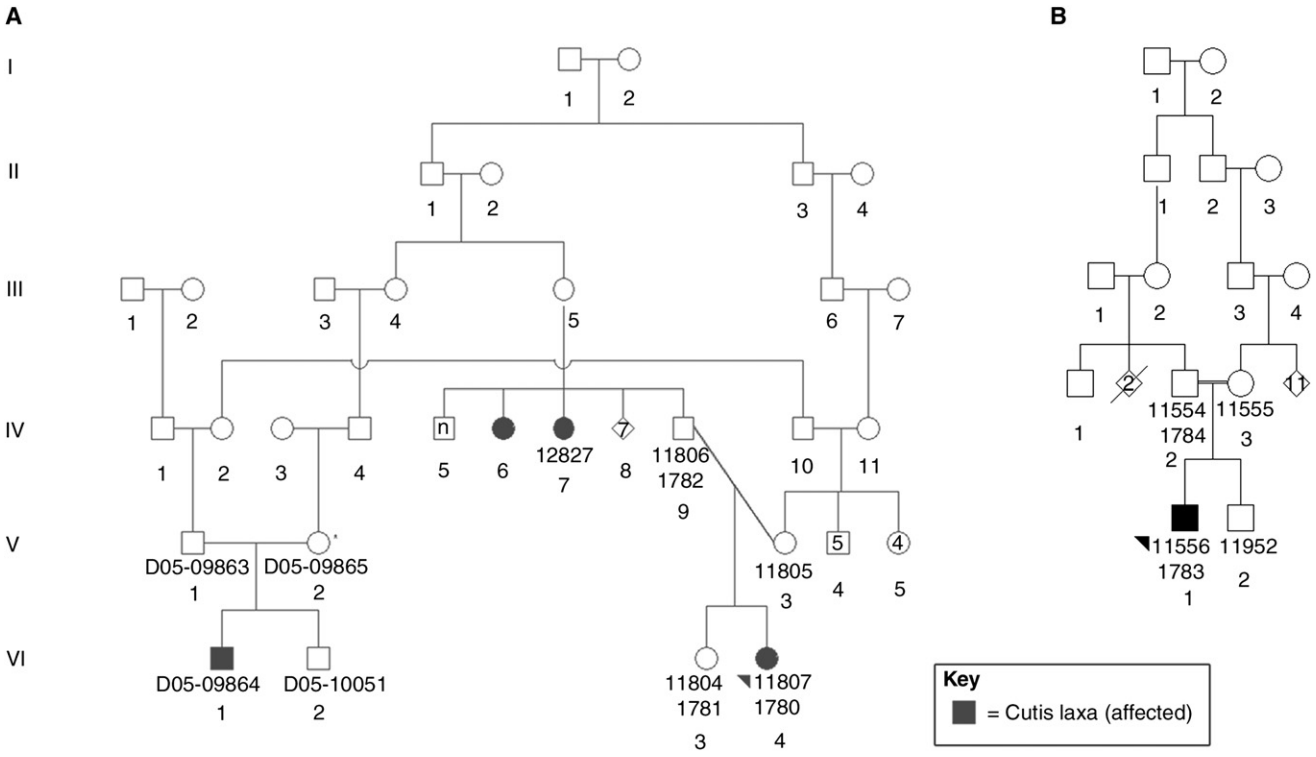

Figure 1. Maritime Families Segregating Cutis Laxa Type 2
Affected individuals VI:4 in the large family 1 and V:1 in the small family 2 (arrowheads) are the probands. Numbers below selected individuals are DNA and/or RNA sample numbers, as noted in text and figures. Clinically affected patients are indicated with filled black symbols.

have enlarged ventricles. There was no obvious metabolic pathology ascertained by routine blood and urine metabolite analysis. There was no corneal clouding or athetosis, as might be expected for de Barsy syndrome. There was no clinical evidence requiring X-ray or bone mineral density scan; hence, these procedures were not performed. Protein glycosylation and wound healing were normal. See Table 1 for detailed clinical findings. Approval for a research study was obtained from the Isaak Walton Killam (IWK) Hospital Research Ethics Board. All sampled family members or parents provided written informed consent to participate in the study. DNA was obtained from living patients' and relatives' blood samples via routine extraction methods. All procedures were in accordance with ethical and methodological standards for human experimentation.

Together, the clinical findings most resemble ARCL2 as described in the literature. In addition to sharing ethnic heritage, the two families reside near each other, consistent with the possibility of a founder effect. To test this, we genotyped a total of 13 DNA samples, including four of the five affected patients (sampled from both families) and nine unaffected relatives from the two pedigrees, using a high-density SNP genotyping array containing approximately 330,000 biallelic markers. Whole-genome SNP scanning was performed at McGill University and the Genome Quebec Centre for Innovation with the use of the Illumina HumanHap300v2_A panel. Homozygosity was assessed by computational inspection for long runs of consecutive homozygous SNPs identical by state in the four Maritime Canadian affected patients, followed by visual inspection of the longest runs for informativeness in unaffected family members.This analysis defined one chromosomal region of approximately 1.4 Mbp, near the tip of chromosome 17 (17q25.3), where all four genotyped affected patients from both families shared 134 consecutive SNPs homozygous with the same genotypic status (Figure 3, Table 2, Figure S1). The next largest region of sharing comprised only 40 consecutive SNPs and fell within the general distribution of miscellaneous short homozygous segments. Formal linkage analysis supported the chromosome 17 locus, although the two-point LOD scores were less than 2 for all hits in the genome (data not shown) as a result of the poorly informative pedigree structure. The region thus defined on chromosome 17 includes all or part of 50 RefSeq annotated genes.

For mutation detection, annotated coding exons were amplified from genomic patient DNA by PCR via standard methods and sequenced at McGill University and the Genome Quebec Centre for Innovation, or at Dalhousie University with Sanger fluorescent sequencing and capillary electrophoresis (Table S1). Sequence traces were analyzed with MutationSurveyor (Soft Genetics). One obvious gene candidate, nuclear prelamin A recognition factor (NARF), was screened first because of the association of lamin A with other progeroid genetic disorders. However, *NARF* did not contain any obvious causal mutations. In

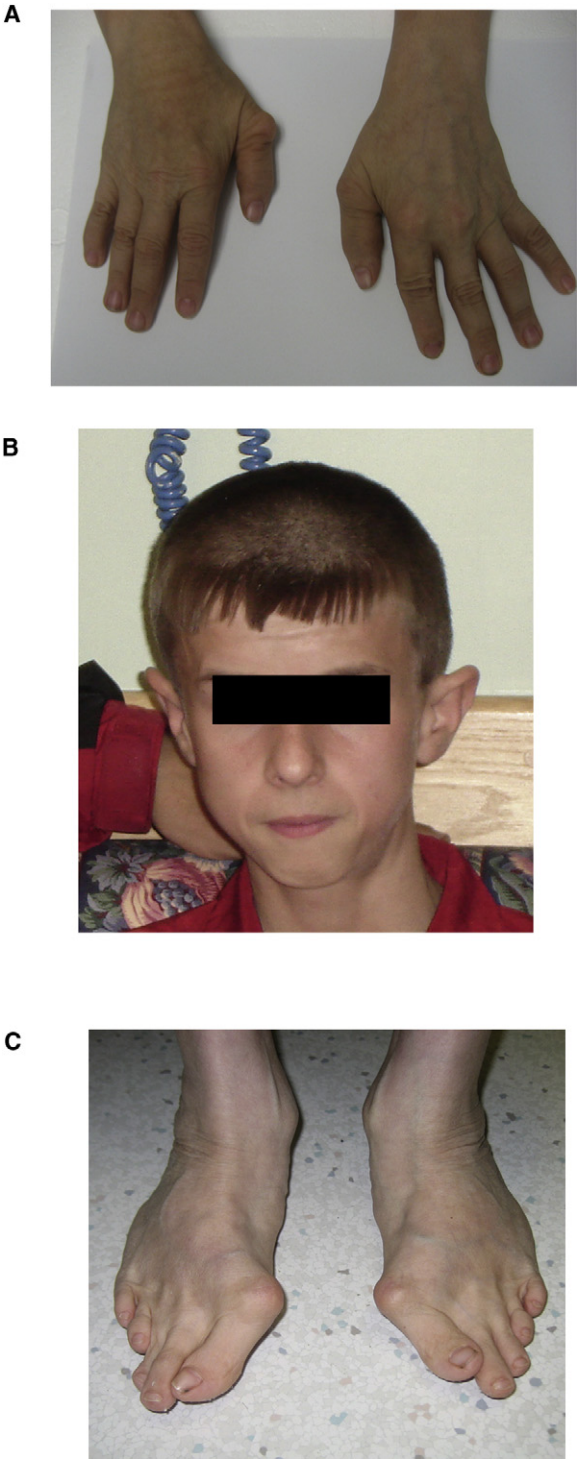

Figure 2. Clinical Presentations of Patients with Cutis Laxa Type 2
Shown are (A) hands (11156), (B) face (D05-09864), and (C) feet (11156) of case individuals aged 12, 11, and 12 years, respectively, at the time of photography. Note the appearance of premature aging, particularly wrinkled skin and joint laxity.

total, we sequenced most or all of the coding regions of 11 genes in the interval (*NARF* [MIM 705349], *HGS* [MIM 604375], *HEXDC*, *PYCR1* [MIM 179035], *RAB40B*, *ARHGDIA* [MIM 601925], *DUS1L*, *MRPL12* [MIM 602375], *NPLOC4*, *RAC3* [MIM 602050], and *TBCD* [MIM 604649]), until a potentially pathogenic variant, c.797G>A (p.Arg266Gln), was found in the gene encoding pyrroline-5-carboxylate reductase 1 (*PYCR1*). This was a single-nucleotide change, altering the last amino acid encoded by exon 6 of the gene, R266, and also potentially altering the splice donor site (Figure 4A). The mutation segregated in the family as expected on the basis of SNP genotyping: all four affected individuals were homozygous, all obligatory carriers were heterozygous, and some unaffected siblings were heterozygous for the mutation (data not shown) The mutation was not in dbSNP and was not found among 96 CEPH control DNAs. Among 142 Maritime population control samples, one sample was heterozygous for the mutation; there were no homozygotes. Our local control samples are known to include individuals of Acadian ethnicity, as in our ARCL2 patient families; therefore, it is not unlikely that a heterozygote for a recessively acting causal mutation might be observed among these controls. Our results are consistent with a founder effect of this mutation in the Acadian population; no other identified cases of ARCL2 from the region could be identified through intensive outreach among regional clinicians.

The potential pathogenic effect of the mutation was first studied bioinformatically. Residue R266 is completely conserved across vertebrate evolution (Figure S2). The missense mutation R266Q was predicted to have a deleterious effect on protein function by SIFT, PANTHER, and Align-GVGD, but was predicted as benign by PolyPhen (Table S2). The results suggest that the missense mutation R266Q might affect protein function. The potential impact of the mutation on splicing was also analyzed with NetGene2, SplicePort, and SplicePredictor. Genomic sequence NC_000017.9 was used as the input wild-type DNA sequence, and the DNA sequence with one base change, g.2767G>A, was used as the input mutant sequence. The exon 6 splice donor site was correctly predicted in the wild-type sequence by all three programs. The three methods also correctly identified other donor sites of the reference sequence. In the mutant sequence, however, the exon 6 donor site could no longer be identified by any of the three programs. This suggested that the mutation might also interfere with correct splicing of the primary *PCYR1* transcript. We tested this directly by examining RNA extracted from patients' blood lymphocytes.

We were able to amplify spliced products of *PYCR1* by nested RT-PCR from total RNA of blood lymphocytes (although not from salivary RNA). For sequencing of cDNA generated from lymphocyte RNA, buffy coats were prepared from fresh blood draws and approximately 5×10^5cells were extracted with the use of the QIAGEN RNeasy kit, in accordance with standard protocol for cells. Two microliters of total RNA was used as template for two rounds of nested RT-PCR with the Invitrogen M-MLV kit, including the use of Invitrogen RNaseOUT. An unrelated control sample generated the expected size product for mRNA, correctly splicing exons 5, 6, and 7 (Figures 4B and 4C).

Table 1. CL2 Clinical Details in Maritime Patients and the Literature

Clinical Feature	CL Type 2	WSS	GO	NC	11807	D05-09864	11556	12827	No DNA
General									
IUGR	+	+	+	+	+	+	+		
Failure to thrive	+	+	+	+	+	+	?		
Developmental delay	+	+	+	+	+	+	+	+	+
Feeding problems				reflux	+	reflux	-		+
DYSMORPHISM									
Microcephaly	+	+	+	+	+	+	+	?	+
Broad, prominent forehead		+			+	+	+	+	+
Long philtrum	+	-			+	-	-		
Midface hypoplasia		+			malar	+	-	+	
Epicanthal folds	+	+			-	-	-		
Downslanting palpebral fissures					+	-	+		
Large, bulbous nose		+			+		-		
Prognathism			+		-	+/−	-		
Retrognathia					-	-	-		
Large, protruding ears		+			+	+	+		
Hypertelorism	+	+/−			+	+	-		
Blue sclera	+/?	-			+	-	+	+	+
Sagging jowls	+	-	+		+	+	+	+	+
Large fontanelle	+	+/NM	-	-	+	+	+		
Pinched nose					+	+	+	+	
High palate					+	-	-		
Triangular face					+	+	-	+	+
Deep set eyes					-	+	+		
Musculoskeletal									
Lax joints	+	+	+	+	+	+	+	+	+
Hip dislocation	+	+	+	+	+	-	?	-	+
Winged scapulae	NM	+	-	-	-	-	-		
Spinal deformity		+			-	-	-		
Fractures					-	-	-		
Pectus excavatum	-	-	+		+	-	-		
Diminshed muscle mass		+		+	+	-	-		
Hypotonia	+	+	+	+	-	-	-		
Scoliosis					+	+	-		
Long digits					+	+	-		
Clasped thumb					+	+	+		
Cutaneous									
Wrinkly skin	+	+	+		+	+	+		
Prominent veins/translucent skin	+	+	+	+	+	+	+	+	+
Prominent palmar/plantar creases		+	+		-	+			
Lax skin with reduced elasticity			+	+	+	+	+	+	+

(*Continued on next page*)

Table 1. ***Continued***

Clinical Feature	CL Type 2	WSS	GO	NC	11807	D05-09864	11556	12827	No DNA
Radiology									
Osteoporosis			+		-				
Vertebral anomalies			+		-				
Wormian bones			+		-				+
Other									
Inguinal hernia	+	-/NM	+	1 of 4	-	-	+		
Umbilical hernia	+	-/NM	+		+ (?)	-	-		
Congenital heart disease		-/NM			-	-	-		+
Pulmonary emphysema					-	-	-		-
Renal defect	-	-/NM			-	-	-		
Hydrocephalus					-	+	-		+
Jaundice					+	-	-		+
CNS involvement					+	+	-		+
Agenesis of the corpus callosum					+	+	-		
Respiratory tract infection					?	+	-		?
Easy bruising					+	?	+		
Aortic root aneurysm					-	-	-		

Clinical features of Acadian ARCL2 patients (last 5 columns) versus literature review of other descriptions of cutis laxa type 2 with developmental delay, wrinkly skin syndrome (WSS), gerodermia osteodysplastica (GO), and neurocutaneous syndrome (NC).[13–15,21,22,44–46]

However, in two homozygous mutation-bearing individuals from our pedigree, a smaller size band was uniquely detected, which upon sequencing represented an aberrant splice product of exon 5 to exon 7, skipping exon 6 (Figures 4B and 4D, Figure S3). Heterozygous mutation carriers contained a mixture of RNA with normal and skipped products (Figures 4B and 4C). Skipping of exon 6 deletes 54 amino acids of the *PYCR1* protein, including the conserved reductase functional signature (Figure 5), and also generates an obligatory frameshift in the downstream exon(s), leading to premature termination of the open reading frame, and is reasonably presumed to be pathogenic. The nested RT-PCR protocol is qualitative, although signal strengths were comparable for the correctly spliced and skipped RNA-derived bands in the control and homozygous affected lymphocyte samples treated similarly. In the heterozygous carriers, the skipped RNA signal was weaker than that of the

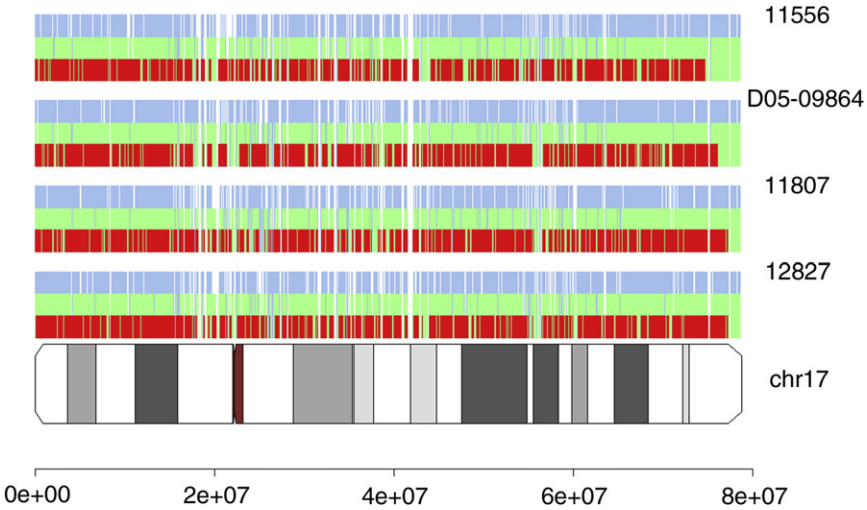

Figure 3. Homozygosity Mapping
Four ARCL2 mutation-affected individuals (family 1: IV:7, VI:1, VI:4; family 2: V:1) were genotyped as described in the text. They share a region of homozygosity close to the telomere of chromosome 17q (far right in figure). SNP genotypes (coded as AA, AB, or BB) for all of chromosome 17 are represented by vertical lines with different colors. AA: light blue, BB: light green, AB: red. Unaffected first-degree relatives were heterozygous for many SNPs within the shared homozygous region of the affected individuals at 17q25.3 (Figure S1).

Table 2. Homozygosity Analysis of CL2

nSNPs	Chr	BeginSNP	EndSNP	Begin	End	Size
134	17	rs9894429	rs6502043	77,207,216	78,634,366	1,427,151
40	1	rs4652869	rs12074848	35,151,521	36,357,664	1,206,144
33	20	rs6088813	rs7263536	33,438,595	34,110,871	672,277
29	2	rs1455653	rs11692344	198,186,565	198,504,108	317,544
29	4	rs6531772	rs6849339	33,432,805	34,032,949	600,145
28	7	rs4646450	rs6967487	99,104,254	99,440,127	335,874
28	10	rs2247247	rs715687	84,373,257	84,561,933	188,677
28	14	rs11626364	rs2109750	73,319,563	73,741,009	421,447
28	18	rs17240415	rs1431419	64,801,868	64,889,909	88,042
25	5	rs4267850	rs9293757	77,683,520	77,959,776	276,257

The ten longest runs of homozygous SNPs shared identically by state (IBS) in the four genotyped affected CL2 patients are shown in decreasing order of number of consistent contiguous SNPs. Headings indicate number of SNPs, chromosome, starting and ending SNPs (meaning first inconsistent heterozygous or non-IBS SNP at each end of each interval, yielding maximal interval extent), and beginning and ending nucleotides of each interval, according to human genome assembly hg36, based on first and last inconsistent SNPs. Interval size is given in megabase pairs (Mbp).

correctly spliced band in the same samples and gel lanes, suggesting some amount of nonsense-mediated decay or other destabilization of the incorrectly spliced product. However, the skipped product was readily detectable in both homozygous affected individuals and heterozygous carriers, so such an effect, if present, appears unrelated to the clinical penetrance of the mutation.

Our results strongly suggest that loss of *PYCR1* gene activity is responsible for the clinical ARCL2 phenotype in our patients. It is particularly noteworthy that this is the second enzyme of proline metabolism implicated in cutis laxa, the first being pyrroline-5 carboxylate synthase.[13–15] As noted above, mutations in *P5CS* have been reported with two types of clinical presentations. In the first report, patients exhibited developmental phenotypes similar to cutis laxa type 2, together with hyperammonemia. More recently, a different mutation in *P5CS* was reported in a family with a neurocutaneous syndrome also closely resembling cutis laxa type 2 but without any obvious metabolic abnormalities. If these are loss-of-function mutations, the result would presumably be a reduction in proline biosynthesis, due to reduction in cellular P5C pools. Similarly, loss of *PYCR1* activity in our patients would be expected to reduce proline biosynthesis. We measured urinary proline levels in our patients. Consistent with the hypothesis, though not definitive, none had any detectable proline in the urine, which places them at the low end of the normal urinary proline range of 0–67 umol/mmol creatinine. Blood proline levels were measured for the two male patients, D05-09864 and 11556 (see Figure 1), who had (nonfasting) levels of 116 and 282 μM, respectively. This placed them within the normal range of 70–440 μM. The *P5CS* patients have a much more severe neurologic phenotype than our patients: their degree of microcephaly is greater (smaller heads) and their retardation much more severe (nonambulatory), whereas our patients have only mild mental retardation. This could be due to differential functioning of the two genes, or to allele-specific effects. Ultimately, additional cases will be required for the determination of the full range of genotype-phenotype correlations of mutations in these two genes. In addition, there are several reports of cases sharing clinical features of moya-moya disease (MYMY2 [MIM 607151])and microcephalic osteodysplastic primordial dwarfism type II (MOPD2 [MIM 210720]).[25–27] MYMY2 is a neurological condition of the cerebral vasculature,[28] but MOPD2 shares some clinical features with ARCL2 and the neurocutaneous syndrome.[29,30] MOPD2 is associated with mutations in pericentrin 2 on chromosome 21 (MIM 605925).[31] However, MYMY2 maps near the telomeric region on chromosome 17q25, and a causative gene is yet to be identified.[32,33] Conceivably, the rare patients sharing clinical features of MYMY2 and MOPD2 could result from a chromosomal rearrangement on 17q25, including *PYCR1* plus an MYMY2 gene.

It is not immediately clear how reduction in proline biosynthesis could lead to many of the cutis laxa clinical manifestations. Proline is a major component of collagen, although the loose-skin phenotype appears more related to elastin. It is also unclear whether the observed circulating blood (normal) and urine (low) proline levels accurately reflect intracellular pools in the relevant tissues. However, proline plays another, essentially unrelated role in cellular metabolism, as part of an oxidative cycle involving NADH or NADPH. This cycle has been implicated in stress response in yeast and plants.[34–37] Moreover, *PYCR1* was originally identified as an induced proliferation gene called *PIG45*,[38,39] suggesting a possible role in cell growth regulation. There is another gene, *PYCR2*, on chromosome 1, which carries out the same reduction reaction as *PYCR1*, though possibly with a different cofactor. The differential roles of these two enzymes in organogenesis and cognitive

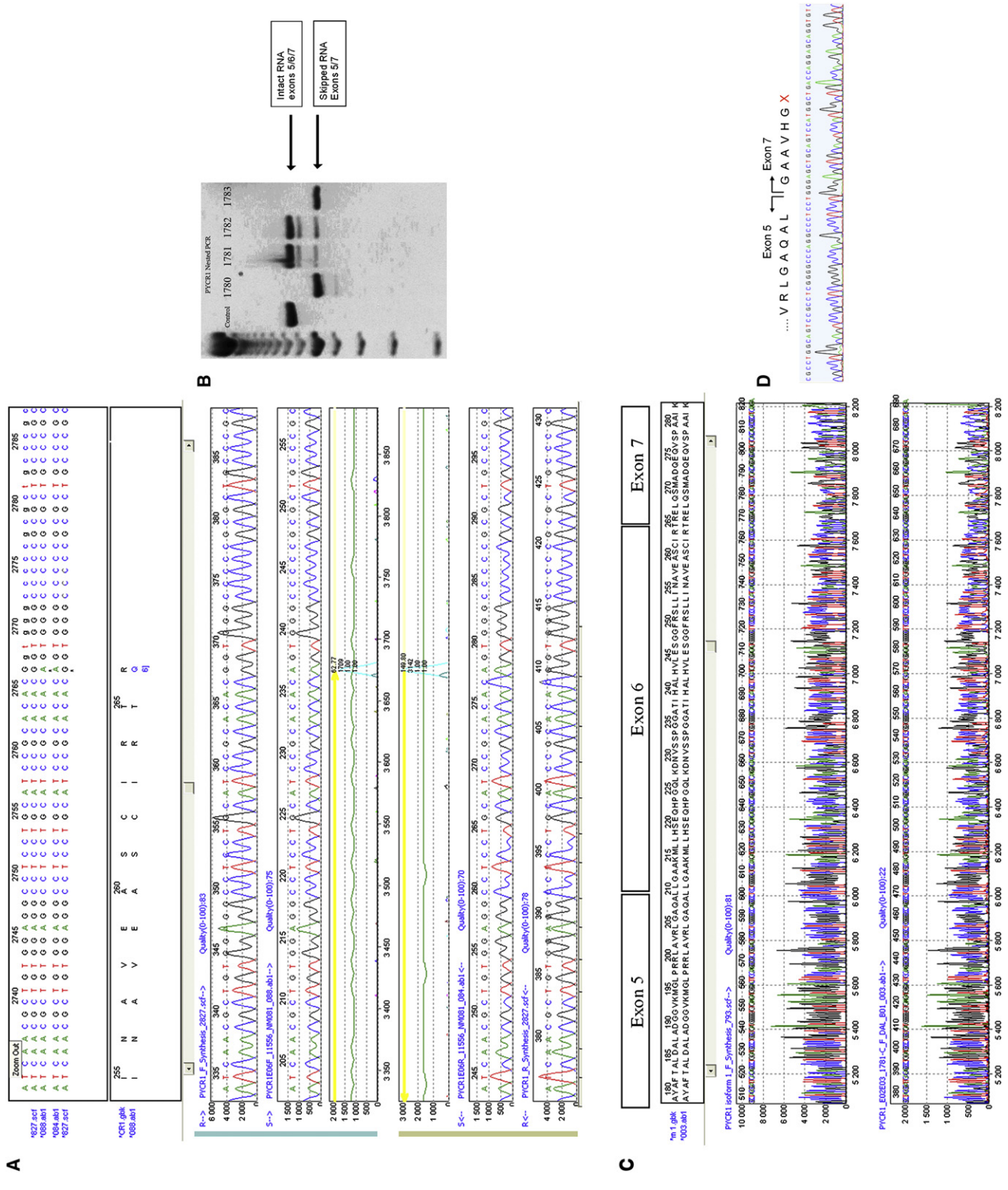

Figure 4. ***PYCR1*** **Mutations and In Vivo RNA Analysis**

(A) Genomic DNA sequence of affected patient 11556 (family 2: V:1), showing homozygous missense variant G-to-A at the last nucleotide of exon 6 of *PYCR1* (position 2766 of the reference sequence file generated for the gene by NCBI Mapviewer). Uppermost and lowermost traces are virtual chromatograms generated by the software from the consensus genome sequence. Inner chromatogram traces are forward and reverse sequences of patient PCR product. Central panels are forward and reverse mutation calls by Mutation Surveyor.

(B) Electrophoresis gel showing products of RT-PCR of RNA from blood with the use of exonic primers spanning PYCR1 exon 6. Lanes correspond, respectively, to RNA from an unrelated control, sample 1780 from affected homozygote no. 11807 (see Figure 1, family 1, VI:4), sample 1781 from unaffected heterozygote sibling no. 11804 (family 1, VI:3), sample 1782 from unaffected heterozygote parent

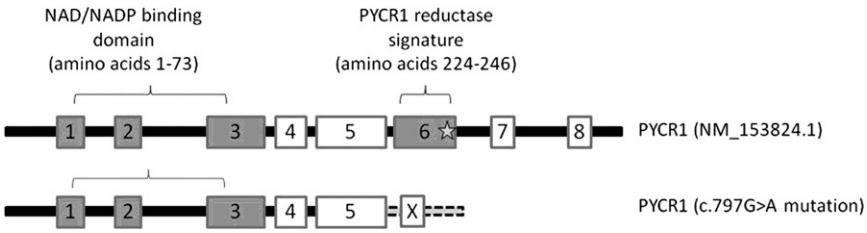

Figure 5. Protein Domain Structure of PYCR1 Indicating Effects of Deleting Exon *6* Plus Premature Truncation in Exon *7*

Note that this represents alternative transcript isoform 2. Isoform 1 retains the sequences between exons 7 and 8, leading to an alternative carboxy-terminus. Exclusion of exon 6 would similarly lead to a frameshift and premature protein truncation of isoform 1.

development remain to be understood.[40] Interestingly, a genetic hyperprolinemia syndrome has been suggested in schizophrenia, supporting a role of proline in normal brain development.[41] The genetic results suggest that proline metabolism may be an attractive target for novel therapeutics for skin conditions and, perhaps, wound healing.[42,43] Common genetic variants in proline-pathway genes could conceivably be involved in normal aging processes.

Supplemental Data

Supplemental Data include three figures and two tables and can be found with this article online at http://www.ajhg.org/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Align-GVGD, http://agvgd.iarc.fr/agvgd_input.php
MUSCLE, http://www.drive5.com/muscle/
NCBI, http://www.ncbi.nlm.nih.gov/
NetGene2, http://www.cbs.dtu.dk/services/NetGene2/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
PANTHER, http://www.pantherdb.org/tools/csnpScoreForm.jsp
PolyPhen, http://genetics.bwh.harvard.edu/pph/
SIFT, http://blocks.fhcrc.org/sift/SIFT.html
SplicePort, http://spliceport.cs.umd.edu/
SplicePredictor, http://deepc2.psi.iastate.edu/cgi-bin/sp.cgi
UCSC Genome Browser, http://genome.ucsc.edu/

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no. 11806 (family 1, IV:9), and sample 1783 from affected homozygote no. 11556 (family 2, V:1). Arrows indicate the major bands migrating at the size of correctly spliced (exons 5, 6, 7) or incorrectly spliced (exons 5, 7) PYCR1 mRNA.
(C) Sequence of cDNA from unaffected heterozygote 1781 (family 1: VI:3) intact RNA band (lower trace), aligned to virtual chromatogram of consensus cDNA sequence of exons 5–7 (upper trace).
(D) Sequence of cDNA from affected homozygote 1783 (family 2: V:1) skipped RNA band sequence, showing end of exon 5 spliced directly to beginning of exon 7, generating frameshift. See Figure S2 for alignment of skipped RT-PCR product to consensus cDNA sequence.

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