Supplementary information

Nucleotide-sugar transporter SLC35D1 is critical to chondroitin sulfate synthesis in cartilage and skeletal development in mouse and human

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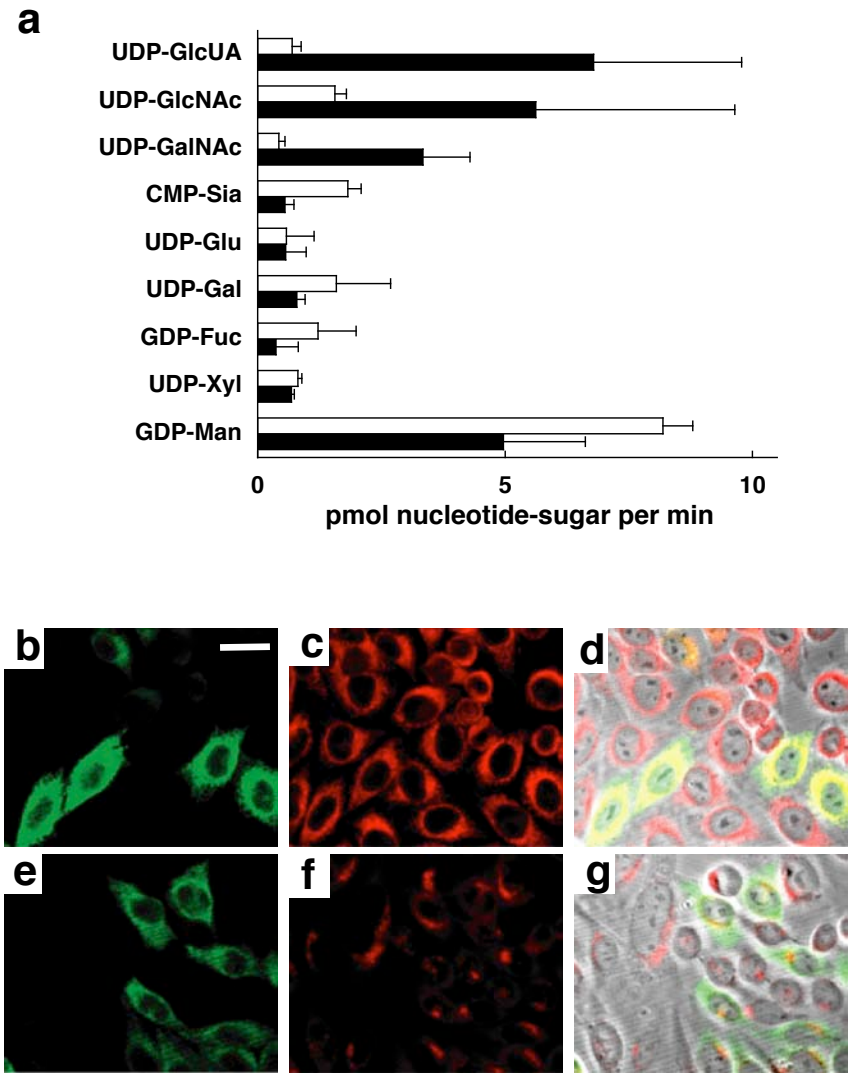
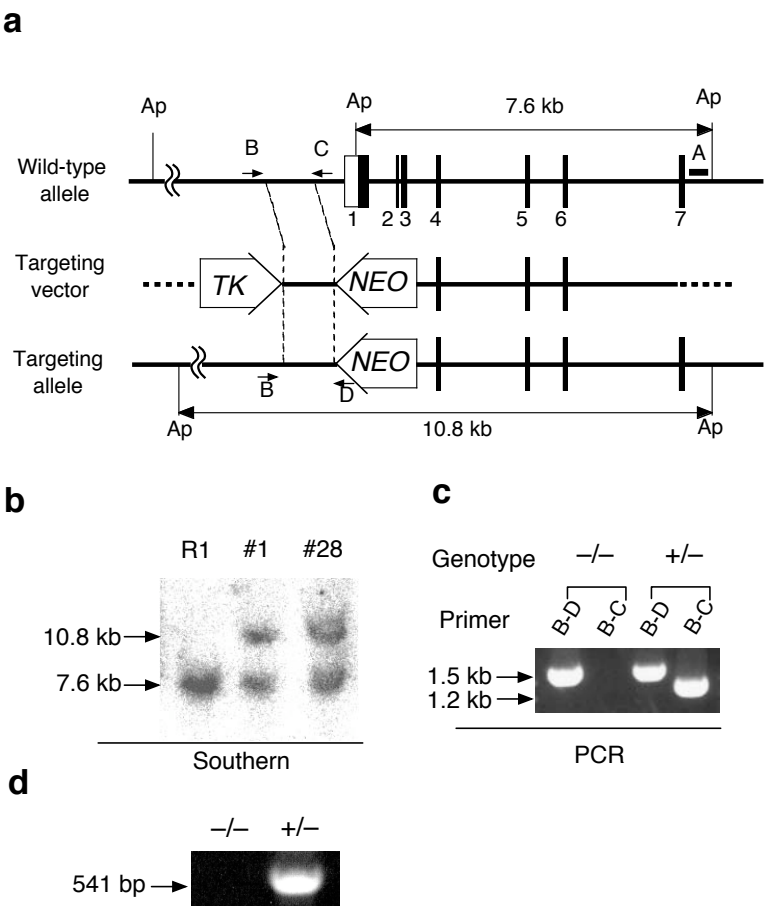


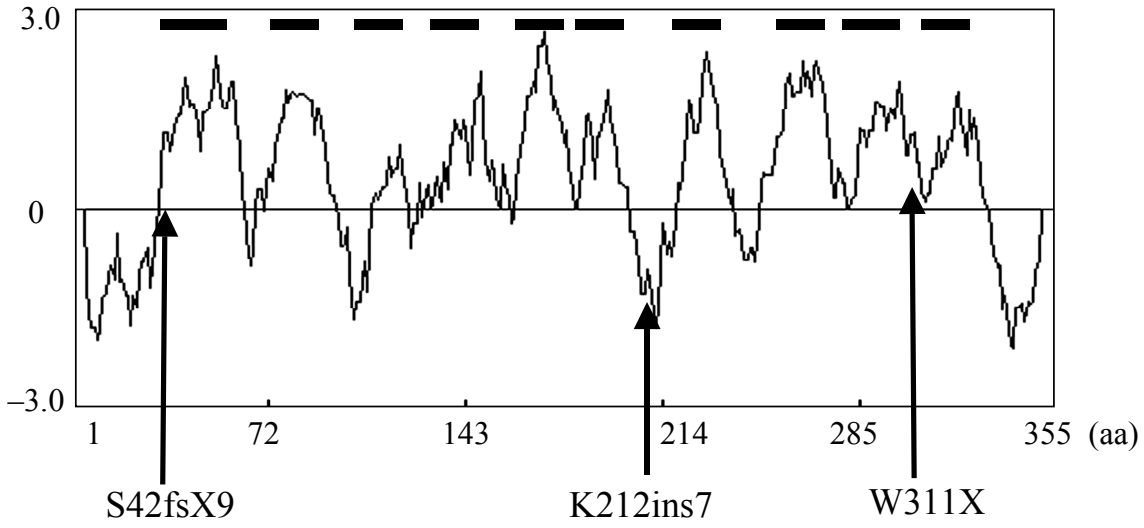
Supplementary Figure 1

(**a**) Nucleotide-sugar transporter activity of mouse Slc35d1 protein. Microsomes were prepared from yeast cells transfected with empty or mouse Slc35d1 expression vector. The incorporation of nucleotide-sugars for 1 min at 30 °C per mg protein of microsomes is illustrated for Slc35d1 expressing (black column) and non-expressing (white column) microsomes. Each value represents the mean±s.d. of duplicate experiments. Slc35d1-expressing microsomes showed greater incorporation of UDP-GlcUA, UDP-GlcNAc and UDP-GalNAc relative to controls. (**b**–**g**) Subcellular localization of mouse Slc35d1. CHO-K1 cells were transfected with pMKIT-neo-mSlc35d1nHA to express mouse Slc35d1 tagged with HA-epitope at the N-terminus. After 48 hours, indirect immunofluorescence in transfected cells was observed using anti-HA rat mAb (**b**, **e**), rabbit anti-ERp57 (ER marker) serum (**c**) and rabbit anti-α-mannosidase II (Golgi marker) serum (**f**). (**d**), merge of (**b**) and (**c**); (**g**), merge of (**e**) and (**f**). Note that Slc35d1 was located in the ER (**c**). Scale bar, 20 μm.



Supplementary Figure 2

Generation of *Slc35d1*-deficient mouse. (**a**) Targeting strategy. Partial genomic organization of the *Slc35d1* gene (top), targeting vector (middle), and targeted allele (bottom). Exons are denoted by rectangles, and coding regions are filled. The exon number is indicated under the rectangle. The targeting vector was constructed by replacing of the genomic fragment containing exons 1–3 of *Slc35d1* with the PGK-neo cassette (*NEO*) and attachment of the HSV-tk cassette (*TK*) to its flank for the negative selection marker. Ap, *Apa*I. (**b**) Genomic Southern analysis of recombinant ES clones. Genomic DNAs of ES clones were digested with *Apa*I, and analyzed by Southern hybridization, using genomic fragment A in **a**. The representative results of the original ES cells (R1) and two clones of homologous recombinant (#1 and #28) are shown. (**c**) PCR analyses of the targeted mouse. Genotype was determined by PCR using the specific primers shown in **a** for wild type (B and C), and for targeted alleles (B and D). (**d**) RT-PCR analysis for *Slc35d1* in lung RNA from an 18.5 dpc embryos. Homozygous mice did not express a 541-bp band derived from *Slc35d1* mRNA.



Supplementary Figure 3

Hydrophobicity analysis of human SLC35D1 protein and positions of the mutations identified in Schneckenbecken dysplasia. The plot was based on a calculation using the hydrophobicity values of Kyte and Doolittle [Kyte and Doolittle 1982]. The x-axis represents the amino acid position within the SLC35D1 protein sequence, and the y-axis represents the hydrophobicity values of the amino acid analyzed. SLC35D1 is predicted to have 10 transmembrane domains (thick horizontal bars). S42fsX9 is the mutation in Subject 1, K212ins7X (K212insLSSNLKLX) and W311X are mutations found in Subject 2.

Reference

Kyte, J., & Doolittle, R.F. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132 (1982).

Supplementary table 1 Quantitative analysis of the glycosaminoglycans (GAG) in the long bones of *Slc35d1*- deficient mouse.

	unsaturated disaccharide composition (%)*[1]	
	Slc35d1	
	–/–	+/+
Chondroitin sulfate (CS)		
ΔDi-0S	18.4	16.9
ΔDi-4S	77.6	79.3
ΔDi-6S	2.7	3.4
ΔDi-UA2S	0.0	0.0
ΔDi-diSE	0.5	0.2
ΔDi-diSB	0.5	0.1
ΔDi-diSD	0.3	0.1
ΔDi-triS	0.0	0.0
Total (μg/mg)*[2]	100 (5.0)	100 (35.4)
Heparan sulfate (HS)		
ΔUA-GlcNAc	64.4	67.8
ΔUA-GlcNS	18.7	17.3
ΔUA-GlcNAc6S	2.2	1.4
ΔUA2S-GlcNAc	0.6	0.5
ΔUA-GlcNS6S	3.8	2.7
ΔUA2S-GlcNS	7.0	6.7
ΔUA2S-GlcNAc6S	0.0	0.0
ΔUA2S-GlcNS6S	3.3	3.6
Total (μg/mg)*[2]	100 (0.26)	100 (0.31)

*[1] GAG extracts of long bones from embryos at 18.5 dpc were analyzed.
*[2] Specific amounts of CS and HS in the tissues (μg/mg) were deduced from the composition of unsaturated disaccharides.

Supplementary Table 2 Summary of the subjects that were screened for the *SLC35D1* mutation

Sample No.	Registry	ID	Information
SBD1	ISDR	R84-089	18-19 week fetus. Two affected siblings in the family. Firm radiographic diagnosis.
SBD2	ISDR	R93-208	Subject 2. Firm radiographic diagnosis. No histology.
SBD3	ISDR	R94-087A	Not typical, but some similarities.
SBD4	ISDR	R02-170	33 week fetus. Long bones better formed than most subjects.
SBD5	ESDN	1262	Subject 1. Firm radiographic and histology diagnosis.
SBD6	JSDC	820177	Not typical, similar to spondylo-metaphyseal dysplasia, Sedaghatian type.

ISDR: International Skeletal Dysplasia Registry, ESDN: European Skeletal Dysplasia Network,
JSDC: Japanese Skeletal Dysplasia Consortium.

Supplementary case report

Subject 1.

ESDN (European Skeletal Dysplasia Network) 1262 [Nikkels et al. 2001]. A male fetus was the second child of consanguineous parents of Mediterranean origin. On ultrasound at 20 weeks of gestation, he was seen with severe hydrops, extremely short extremities, and a small thorax. Termination of pregnancy was performed at 22 weeks. Radiographs showed typical findings for Schneckenbecken dysplasia, including small ilia with snail-like appearance, platyspondylia with oval vertebral bodies and general shortness of long bones with dumbbell-like appearance **(Fig. Aa,b**, the next page). The chondroosseous morphology also is typical for Schneckenbecken dysplasia. Hypercellular cartilage with scare extra-cellular matrices and absence of columnar alignment of proliferating chondrocytes **(Fig. Ac**). Chondrocytes in the proliferating zone are large with round, centrally located nuclei (**Fig. Ad**).

Reference

Nikkels, P.G. et al. Schneckenbecken dysplasia, radiology, and histology. Pediatr. Radiol. 31, 27-30 (2001).

Subject 2.

ISDR (International Skeletal Dysplasia Registry), R93-208. The female infant was the product of a 36-week uncomplicated pregnancy from unrelated parents. At delivery, she was hydropic and noted to have a narrow chest and a protuberant abdomen with a large umbilical hernia. The face was small and flat with a hypoplastic midface and a small nose. The limbs were very short in all segments with bilateral clubfeet. The infant died of respiratory insufficiency in the immediate neonatal period. Radiographs showed features typical of Schneckenbecken dysplasia. The ilia was short with a snail-like appearance and a flattened acetabular roof. The long bones were short and the metaphyses were abnormally wide. The spine was abnormal, with inter-pedicular narrowing, particularly in the lumbar region.

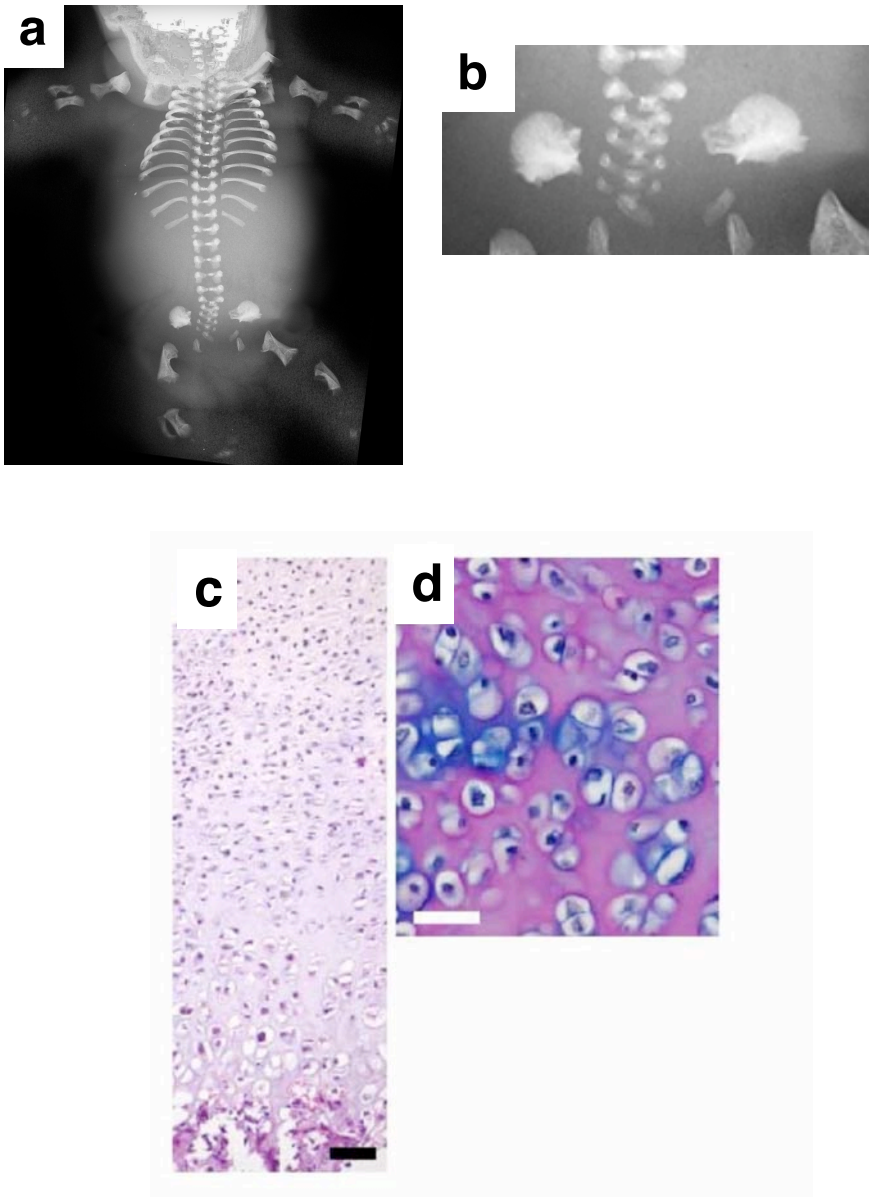


Figure A

(**a**,**b**) Radiographs of Subject 1. (**a**) Anterroposterior radiograph. Extremely short long bones with dumbbell-like appearance. (**b**) The snail-like appearance of the ilia due to medial bone projection from the inner iliac margin. (**c**,**d**) Cartilage pathology of Subject 1. (**c**) Lower magnification (scale bar, 100 μm), HE stain. (**d**) Higher magnification (scale bar, 25 μm), PAS-alcian blue stain.