

## Hypomorphic Mutations in TONSL Cause SPONASTRIME Dysplasia

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# 1 **Hypomorphic Mutations in *TONSL* Cause SPONASTRIME Dysplasia**

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49 **Abstract**

50 SPONASTRIME dysplasia is a rare recessive skeletal dysplasia characterized by short  
51 stature, facial dysmorphism, and aberrant radiographic findings of the spine and long bone  
52 metaphysis. No causative genetic alterations for SPONASTRIME dysplasia have yet been  
53 determined. Using whole exome sequencing, we identified biallelic *TONSL* mutations in 10 of 13  
54 individuals with SPONASTRIME dysplasia. *TONSL* is a multi-domain scaffold protein that interacts  
55 with DNA replication and repair factors, and plays critical roles in resistance to replication stress and  
56 maintenance of genome integrity. We show here that cellular defects in dermal fibroblasts from  
57 individuals are complemented by expressing wild-type *TONSL*. In addition, *in vitro* cell-based assays  
58 and *in silico* analyses of *TONSL* structure support the pathogenicity of those *TONSL* variants.  
59 Intriguingly, a knock-in *Tonsl* mouse model leads to embryonic lethality, implying the physiological  
60 importance of *TONSL*. Overall, these findings indicate that genetic variants resulting in reduced  
61 function of *TONSL* cause SPONASTRIME dysplasia and highlight the importance of the *TONSL* in  
62 embryonic development and postnatal growth.

63  
64 **Introduction**

65 SPONASTRIME dysplasia (MIM: 271510), the denotation of which originate from  
66 SPONDylar and NASal alterations with STRIated METaphyses,<sup>1</sup> is a very rare but distinct entity that  
67 can be categorized as a spondyloepimetaphyseal dysplasia, transmitted as an autosomal recessive trait.  
68 To date, only 15 individuals with this disorder have been reported<sup>1-10</sup> based on the diagnostic criteria  
69 of a unique combination of clinical and radiological findings, as suggested by Langer et al.<sup>5</sup> The  
70 major clinical features are mild to moderate short-limb type dwarfism, relatively large head with a  
71 prominent forehead, as well as epicanthic folds observed in infancy or early childhood. However, the  
72 clinical criteria are nonspecific and radiological features must be present for a diagnosis. Diagnostic  
73 criteria based on the radiological features focus on the changes in the lumbar vertebrae and  
74 metaphyseal changes in the long bones. Metaphyseal irregularities and striations, as proposed by the  
75 disease's name, are also important diagnostic criteria, but are not seen as consistently as the lumbar

76 vertebral changes.<sup>5</sup> To date, causative genetic mutations for SPONASTRIME dysplasia have not been  
77 determined, and thus a genetic test for this disease is not yet clinically available.

78 Tonsoku-like DNA repair protein (TONSL) is a multi-domain scaffold protein that interacts  
79 with DNA replication and repair factors including anti-silencing function 1 (ASF1), minichromosome  
80 maintenance complex component helicases (MCM helicases), H3 histone, H4 histone, and MMS22-  
81 like protein (MMS22L).<sup>11-15</sup> TONSL consists of 1,378 amino acids with annotated domains such as  
82 eight tetratricopeptide repeats (TPR), three Ankyrin (ANK) repeats, an ubiquitin-like domain (UBL),  
83 and seven leucine-rich repeats (LRR).<sup>13</sup> It was reported that the TONSL-MMS22L heterodimer plays  
84 a key role in homologous recombination required for repairing spontaneous replication-associated  
85 DNA lesions. At the cellular level, depleting TONSL causes pronounced defects in the rate of cell  
86 proliferation and enhances the sensitivity to camptothecin (CPT), a topoisomerase 1 inhibitor that  
87 induces DNA breakage at replication forks.<sup>12,13</sup> Specifically, TONSL-MMS22L is recruited to the  
88 sites of stalled replication forks during normal S phase by replication protein A (RPA1, RPA2 and  
89 RPA3) bound to single-strand DNA (ssDNA), and promotes RAD51 loading for strand invasion.<sup>16</sup>  
90 Therefore, it was demonstrated that small interfering RNA (siRNA)-mediated TONSL knockdown  
91 leads to loss of damage-induced RAD51 foci formation in cells treated with genotoxic agents.<sup>11,12,16</sup>

92 In this study, in order to identify the causative genetic alteration for SPONASTRIME  
93 dysplasia, we recruited 13 individuals, including a previously reported case,<sup>10</sup> from four different  
94 ethnicities, who satisfied the diagnostic criteria of the disease. By performing whole exome  
95 sequencing and Sanger sequencing, we identified autosomal recessive hypomorphic and loss-of-  
96 function (LoF) mutations in the *TONSL* (MIM: 604546) gene of individuals with SPONASTRIME  
97 dysplasia. In further studies, using the dermal fibroblasts from individuals, *in vitro* cell-based assays,  
98 *in silico* structure simulation, and *in vivo* knock-in mouse model, we demonstrated the pathogenicity of  
99 *TONSL* variants, suggesting that defects in replication-associated DNA damage repair and the  
100 resultant inefficient cell proliferation due to *TONSL* mutations might be the underlying pathogenic  
101 mechanism for SPONASTRIME dysplasia.

102

103 **Material and Methods**

104 **Subjects.** Written informed consent was obtained from the individuals or their parents. The  
105 Institutional Review Boards of the Seoul National University Hospital, Seoul, Republic of Korea and  
106 Samsung Medical Center, Seoul, Republic of Korea approved the studies.

107 **Whole exome sequencing (WES) and whole genome sequencing (WGS).** To identify genomic  
108 variants that cause SPONASTRIME, we performed WES. Additionally, WGS was conducted in cases  
109 where only a single pathogenic *TONSL* allele was identified by WES (P01-1 and P01-2). Basic  
110 statistics of the WES data are summarized in Table S2. Based on the inheritance pattern of the  
111 individuals, we hypothesized that the disease follows an autosomal recessive fashion. Thus, we  
112 eliminated variants that did not satisfy the following criteria: (1) Variants showing allele frequency <  
113 1% in ESP6500 and 1000 Genome Project; (2) Variants not found in our in-house database; (3)  
114 Protein altering variants; and (4) High quality of reads (read number > 20, QS > 30, or minor allele  
115 frequency > 20%). The resulting list of variants is displayed in Table S3. For the structural variants  
116 from WGS data, Manta (0.20.2) was used with default settings and Control-FREEC (6.4) for  
117 identifying copy number variants. Window size was set as 10,000 and read counts were normalized  
118 based on GC-content bias for Control-FREEC. CNV type was classified based on genome ploidy  
119 value 2, where values below 2 denoted loss and values above 2 denoted gain.

120 **Amino acid conservation and base-level functionality analyses.** To evaluate functionality of nine  
121 missense variants in *TONSL*, orthologous sequences from 61 mammalian species were downloaded  
122 from the UCSC browser and aligned with the human *TONSL*. The CADD and GERP scores across  
123 *TONSL* coding sequences were downloaded from dbNSFP.<sup>17</sup>

124 **Long-range PCR.** Long-range PCR (LR-PCR) was conducted to analyze the exon 23 deletion of  
125 *TONSL* in P01-1, P01-2 and the mother using the following primers: *TONSL*-exon22-F: 5'-  
126 GAAGAGACTGCCAAGCCAAG-3' and *TONSL*-exon24-R: 5'-TACCATTCTGTGGCCCTTC-3'.

127 **Sanger sequencing.** Sequencing of *TONSL* candidate variants found from WES or WGS analysis  
128 were conducted using standard PCR and Sanger sequencing methods (primer sequences available  
129 upon request). Sequence data were aligned to the reference sequence Sequencher software (Gene

130 Codes Corporation, Ann Arbor, MI, USA).

131 **Reverse transcription-PCR and cloning.** To investigate the splicing changes caused by the splicing  
132 site and deep intronic mutations in P11, reverse transcription-PCR and cloning of the amplicon was  
133 performed. The mRNA was harvested from the circulating leukocytes of proband and parents using  
134 QIAamp RNA Blood Mini Kit (Qiagen, Germany). The cDNA were transcribed using Transcriptor  
135 First Strand cDNA Synthesis Kit and then PCR amplification was carried out using the primers  
136 TONSL4F 5'-TATGACCACTGCCAGTCGAG-3' and TONSL11R 5'-  
137 TGAGCTCCCGTAGTCTGGTT-3', which encompass both paternal and maternal mutations. After  
138 PCR based cloning using an All in One™ PCR Cloning Kit (Biofact, Korea), 30 colonies were picked  
139 for PCR and sequencing analyses using the same primers.

140 **Cell culture, cell immortalization, mutagenesis and TONSL cell line establishment.** Dermal  
141 fibroblasts from individuals were grown in high glucose and no glutamine DMEM (Gibco, 10313)  
142 supplemented with 15% fetal bovine serum (Gibco), glutamine (Gibco, 35050-061), MEM non-  
143 essential amino acid (Gibco, 11140-050), penicillin and streptomycin (Gibco, 15140-122) and grown  
144 in 5% CO<sub>2</sub> and 3% O<sub>2</sub> at 37°C. BJ foreskin fibroblasts used as a normal control and were obtained  
145 from ATCC. HeLa, U2OS and 293T cells were grown in high glucose DMEM (Gibco, 11965)  
146 supplemented with 10% fetal bovine serum, penicillin and streptomycin (Gibco, 15140-122) and  
147 grown in 5% CO<sub>2</sub> at 37°C. Dermal fibroblasts from individuals were transformed by human papilloma  
148 virus E6 and E7 protein and immortalized by catalytic subunit of human telomerase (hTERT) through  
149 retroviral transduction. HPV16 E6E7 genes (a gift from Howley Lab, Harvard Medical School,  
150 Boston, Massachusetts, USA) were subcloned into pMSCVneo (Clontech) and used to transform the  
151 dermal fibroblasts. pWZL-hTERT was used to immortalize the dermal fibroblasts from individuals.  
152 The gene coding wild-type TONSL was amplified from a cDNA library prepared from U2OS cells by  
153 polymerase chain reaction (PCR) with the following primers TONSL-F (5'-  
154 GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAGCCTGGAGCGCGAGC-3') and  
155 TONSL-R (5'-  
156 GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGAGGCGCCGAAAGAAGAGC-3'). The

157 PCR product was cloned into a Gateway BP vector, pDONR223, using BP clonase. The pDONR223  
158 clone was sequenced and then recombined into pHAGE vectors using LR clonase (Thermo Fisher  
159 Scientific). Using the pDONR223-TONSL template, we generated the point mutation plasmids used  
160 in this study using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Genomics). The  
161 primers used to generate TONSL mutations are listed in Table S5.

162 **Fiber analysis.** For replication fork stalling, P03 cells were pulse-labelled with 25  $\mu\text{M}$  5-chloro-20-  
163 deoxyuridine (CldU; Sigma-Aldrich C6891), washed with PBS, pulse-labelled with 250  $\mu\text{M}$  5-iodo-  
164 20-deoxyuridine (IdU; Sigma-Aldrich I7125), and harvested. Cells were washed and resuspended to  
165  $5 \times 10^5$  cells/ml in PBS. The cells were lysed in spreading buffer (200 mM Tris-HCl, pH 7.5, 50 mM  
166 EDTA, 0.5% SDS) on glass slides. DNA fibers were spread by gravity, then fixed with  
167 methanol:acetic acid (3:1), denatured with 2.5M HCl. CldU was detected using rat anti-BrdU  
168 antibody (clone BU1/75, ICR1; Abcam, ab6326; 1:750) and IdU with mouse anti-BrdU antibody  
169 (clone B44; BD Biosciences, 347583; 1:750). Slides were fixed using 4% paraformaldehyde, then  
170 stained with Alexa Fluor 594 or 488 conjugated secondary antibodies (Life Technologies). Images  
171 were acquired using a Nikon Eclipse Ni microscope with 60 $\times$  oil-immersion objectives and NIS-  
172 Elements software (Nikon Instruments). ImageJ software (US National Institutes of Health; NIH) was  
173 used to analyze replication fork structures (>1000 fork structures) and CldU/IdU track lengths (>300  
174 ongoing forks). For CPT treatment (DNA damage repair) fiber analysis, cells were treated with 100  
175  $\mu\text{M}$  CldU for 30 minutes, then 250  $\mu\text{M}$  5-iodo-20-deoxyuridine (IdU; Sigma-Aldrich I7125) for 30  
176 minutes with 2.5  $\mu\text{M}$ /50 nM CPT or DMSO. DNA plug was prepared by  $\sim 2\text{-}4.5 \times 10^5$  cell/plug using  
177 by low-melting agarose (Bio-Rad 161-3112), followed by lysis with 20 mg/ml Proteinase K (Roche  
178 03115828001) for two days at 50 $^\circ\text{C}$ . To stretch the DNA fibers, 22 mm $\times$ 22 mm silanized coverslips  
179 (Genomic Vision) were dipped into the DNA solution for 13 minutes and pulled out a constant speed  
180 (300  $\mu\text{M}/\text{s}$ ) using a Molecular Combing System (Genomic Vision MCS-001). The coverslips were  
181 baked for 4 hours at 60 $^\circ\text{C}$  and incubated with acid for denaturation. CldU- and IdU-labeled tracts were  
182 detected by incubating for 2 hours at room temperature (RT) with rat anti-BrdU antibody (dilution  
183 1:100 detects BrdU and CldU; Abcam 6326) and mouse anti-BrdU antibody (1:10, detects BrdU and



184 IdU; Becton Dickinson347580). Slides were fixed in 4% paraformaldehyde/PBS and incubated for 1  
185 hour at RT with Alexa Fluor 488-conjugated goat anti-rat antibody (dilution 1:100, A21208;  
186 Molecular Probes/Thermo Fisher) or Alexa Fluor 568-conjugated goat anti-mouse antibody (dilution  
187 1:100, A21124; Molecular Probes/Thermo Fisher). Finally, they were mounted with ProLong Gold  
188 Antifade Reagent (Molecular Probes) and stored at -20 °C. DNA fibers were observed with Carl Zeiss.  
189 Axio Observer 7 & ApoTome 2 (Motorized Fluorescence Microscope with Grid Projection) 63  
190 objective. For each experiment, a total of 200 DNA fibers were analyzed, where the number of DNA  
191 fibers was measured with ImageJ.

192 **siRNA transfection, cell proliferation and CPT sensitivity assay.** The siRNA targeting *TONSL*  
193 coding regions are listed in Table S6. Here,  $3 \times 10^5$  cells were plated in 6-well plates and transfected  
194 siRNA in reverse transfection manner. Cells were transfected a second time after 24 hours. Then 48  
195 hours after the second transfection, 4,000 cells were plated in 4 wells (12 well plates, SPL) for cell  
196 counting, and 1,000 cells were plated in 4 wells (96 well plates, Corning #3603) for Hoechst staining.  
197 The cells in the 12-well plates were counted using Z1 Coulter Particle Counter (Beckman Coulter)  
198 and the nuclei were counted using Cytation 3 (BioTek) for Hoechst stained cells in the 96-well plates.  
199 Then, 1,000 cells were plated in 96 well plates in 4 wells and were treated with increasing  
200 concentrations of CPT 24 hours after plating. The cells were counted 5 days after treatment. Cells  
201 were then stained with Hoechst and counted using Gen 5 (BioTek). For cell lines from individuals,  
202 40,000 cells were plated in 6-well plates in triplicates, and after 24 hours, increasing concentration of  
203 CPT was treated. Four days after drug treatment, cells were passed 1:4 to 6 well plates and counted 4  
204 days after using Z1 Coulter Particle Counter (Beckman Coulter).<sup>18</sup>

205 **Immunoblot.** Cell lysate was prepared by boiling cells in 95 °C for 5 minutes in 2X SDS sample  
206 buffer (RBC). For resolution of the proteins, 7.5% precast gels (Bio-Rad) were used, and  
207 immunoblotting was performed with anti-HA (Biolegend; MMS-101R, Lot B224726), anti-*TONSL*  
208 (Bethyl: A303-843A, Lot #1), anti-GAPDH (Santa Cruz: SC-25778, Lot K0615), anti-phosphorylated  
209 CHEK1 (Cell Signaling: 2348P, Lot #11), anti-phosphorylated CHEK2 (Cell Signaling: 2661P, Lot  
210 #11) and anti- $\alpha$ -Tubulin (Abfrontier: LF-PA0146, Lot MJL01-02) antibodies to detect each protein.

211 **Immunofluorescence (BrdU/Rad51).** For immunofluorescence,  $3 \times 10^5$  cells were plated in 6-well  
212 plates (SPL) with cover glass. For HeLa and U2OS cells, siRNA was treated in reverse and forward  
213 transfection manner and plated 48 hours after the second transfection. After 24 hours, cells were either  
214 treated with CPT (500  $\mu$ M) overnight or BrdU (20  $\mu$ g/mL) for 4 hours, then fixed with 3.7%  
215 formaldehyde in PBS. For BrdU incorporation, the Invitrogen protocol was followed. Cells were  
216 washed in PBS and fixed in 3.7% formaldehyde in PBS for 15 minutes. After washing cells in PBS,  
217 cells were permeabilized in 0.1% Triton X-100 buffer for 20 minutes, then in 1N HCl for 10 minutes  
218 on ice. Cells were then incubated in 2N HCl for 10 minutes in room temperature (RT), then in  
219 phosphate/citric acid buffer for 10 minutes. Cells were washed in permeabilization buffer twice, then  
220 incubated with Alexa Fluor® 488 conjugated anti-BrdU primary antibody overnight at RT. For all  
221 others, cells were fixed in 3.7% formaldehyde for 15 minutes, then permeabilized with 0.1% Triton X-  
222 100 for 20 minutes with PBS washing in between. After washing with PBS twice, the cells were  
223 blocked with PBG (0.2% [w/v] cold fish gelatin, 0.5% BSA in PBS) for 1 hour at room temperature.  
224 Then, the cells were incubated with 1:7000 of anti-Rad51 antibody (Abcam, Rb:ab133534, Lot #:  
225 GR219215-36), and 1:1000 anti- $\gamma$ -H2A.X antibody (Cell Signaling, #9718S, Lot #13) in PBG  
226 overnight at 4°C. The following day, cells were washed three times with PBG and incubated with  
227 fluorescent-conjugated secondary antibody for 30 minutes. Secondary antibodies were purchased  
228 from Abcam (Ms Alexa Fluor® 594: ab150112; Rb Alexa Fluor® 594: ab150064; Ms Alexa Fluor®  
229 488: ab150109 and Rb Alexa Fluor® 488: ab150061). After washing with PBG three times, the  
230 coverslips were embedded in Vectashield (Vector Laboratories) supplemented with DAPI. Nikon A1  
231 confocal microscope was used equipped with a CFI-Apochromat 60X NA-1.4 oil objective, the A1-  
232 DUG GaAsP multi detector unit, and the NIS-Element C-ER software. For the BrdU assay, the ratio  
233 was calculated by dividing the number of BrdU incorporated cells by the number of total cells  
234 counted.

235 **Animals and ethics statement.** All mice were purchased from Taconic Biosciences (Dae Han Biolink  
236 Co., Ltd., Chungbuk, Republic of Korea) and housed at the specific pathogen-free (SPF) facility of  
237 the Yonsei Laboratory Animal Research Center. Animal experimental procedures were conducted in

238 accordance with the Korean Food and Drug Administration (KFDA) guidelines, reviewed, and  
239 approved by the Institutional Animal Care and Use Committees (IACUC) at Yonsei University  
240 (Permit Number: 201506-322-02).

241 **Preparation of CRISPR/Cas9 mRNA and donor DNA.** Cas9 mRNA was synthesized using a  
242 mMESSAGE mMACHINE® T7 Ultra kit (Ambion) and diluted to working concentration in  
243 endonuclease-free injection buffer (0.25 mM EDTA, 10 mM Tris, pH7.4) immediately before  
244 microinjection. A plasmid encoding the *S. pyogenes* Cas9 (SpCas9) protein<sup>19</sup> was obtained from  
245 ToolGen, Inc. (Seoul, Republic of Korea). The crRNAs were designed by searching for “NGG” or  
246 “CCN (the reverse complement sequence of NGG)” sequences near the point mutation target sites.

247 The crRNA sequences used in this study were: crRNA1 5'-GGTCCAGCCCCCTCCCATCC-3',  
248 crRNA2 5'-GAACCCGGATGGGAGGGGGC-3', crRNA3 5'-CCGGGTTTCGAGTTCAAATTC-3',  
249 and crRNA4 5'-CCTGAATTTGAACTCGAACC-3'. The 106-bp synthesized single stranded  
250 oligonucleotide (ssDNA) used as donor DNA was p.Arg924Trp ssDNA 5'-

251 TAGAAACTTCTGTCTTCTGACTGTCCCCTCCCTCTGTCTTTCCTGCTAGCTTCTGGTCCAGC  
252 CTCCTCCTATCTGGGTTTCGGGTTTCAGATTCAGGATAACCTTTTCCTCATCCCCGTTCCC-3'.

253 The tracrRNA, crRNAs targeting the *Tonsl* gene, and ssDNA donor for homology-directed repair were  
254 obtained from Integrated DNA Technologies (IDT).

255 **Microinjection.** Microinjection of one-cell embryos was performed as previously described.<sup>20</sup> In  
256 brief, 3–4 week old C57BL/6 female mice were superovulated by intra-peritoneal (i.p.) injections of 5  
257 IU pregnant mare serum gonadotropin (PMSG, Sigma) and 5 IU human chorionic gonadotropin  
258 (hCG, Sigma) at 48 hour intervals. The fertilized eggs were then collected from the superovulated  
259 females crossed with stud males, and were microinjected with a mixture of Cas9 mRNA (50 or 100  
260 ng/μL), four crRNAs (10, 25, or 50 ng/μL each), tracrRNA (tracrRNA mixed with crRNA at 1:1 molar  
261 ratio), and donor ssDNA (200 ng/μL). Microinjections were performed in the cytoplasm of one-cell  
262 embryos using a piezo-driven manipulator (Prime Tech), followed by embryo transfer into the  
263 oviducts of pseudo-pregnant ICR mice to produce a living mouse.

264 **Founder screening and genotyping PCR.** To screen founders carrying the p.Arg924Trp mutation in

265 the *Tonsl* gene, PAGE-PCR assay was performed as previously described<sup>21</sup> using genomic DNA  
266 obtained from newborns that were produced from the microinjected embryos. In brief, the genomic  
267 regions spanning the crRNA target site were amplified by PCR. After simply denaturing and  
268 annealing the PCR products, the resulting products were analyzed by acrylamide gel electrophoresis.  
269 Then, the candidates were cloned in T-Blunt PCR Cloning Vector (SolGent Co., Ltd., Republic of  
270 Korea), and were validated by direct sequencing analysis (Cosmobiotech Co., Ltd., Republic of  
271 Korea). The primer sequences used in PAGE-PCR were: F 5'-TGAATGCAGAGCCTGCAGAGA-3'  
272 and R 5'-TCTAGGGAGCAGAGTGCCAAG-3'. For genotyping PCR, DNA was extracted from tails  
273 or yolk sacs. Primers F, 5'-AAGCAGTCTTCAGCATGGGACT-3' and WT R, 5'-  
274 AACTCGAACCCGGATG-3' were used to identify the *Tonsl* WT allele. Primers F and KI R 5'-  
275 ACCCAGATAGGAGGAG-3' were used to identify the *Tonsl* p.Arg924Trp allele. The primer WT R  
276 is annealed specifically to the sequence of the *Tonsl* WT allele, whereas KI R to the sequence of the  
277 *Tonsl* p.Arg924Trp allele.

278 **Embryo collection.** Gestation was dated by the detection of a vaginal plug (as E0.5) or following *in-*  
279 *vitro* fertilization (IVF) and embryo transfer. Embryos were either fixed in 10% neutral buffered  
280 formalin (Sigma-Aldrich) or snap-frozen in liquid nitrogen. Stereomicroscope images were generated  
281 from specimens of fixed embryos.

282

283

## 284 **Results**

### 285 ***Diagnosis and clinical information of individuals with SPONASTRIME dysplasia***

286 We recruited 13 individuals, including a previously reported case,<sup>10</sup> satisfying the diagnostic  
287 criteria of the disease from four different ethnicities – Korean, Indian, Finnish and Brazilian (African  
288 black and non-Latin European). Their clinical features are presented on Table 1 and case reports (see  
289 Supplemental note). The median of height standard deviation scores (SDS) of all the individuals of  
290 this study was -4.9 (ranging from -0.9 to -10.0). They were short with height SDS down to -10, but a  
291 mildly affected individual (P03) had a height SDS of only -0.9. All the individuals recruited shared

292 similar facial dysmorphism (Figure 1A). Other clinical findings shared by more than one individual  
293 included short dental root, airway narrowing, cataracts and joint laxity. Radiographic features were  
294 characterized by distinct changes in vertebrae and metaphyses of the long bones. It is worth noting  
295 that the radiographic features changed and became more conspicuous with age (Figure 1B and Table  
296 S1).

### 297 ***Identification of TONSL mutations in individuals with SPONASTRIME dysplasia***

298 To identify the pathogenic mutation causing the disease, whole exome sequencing (WES) of  
299 individuals and available family members was conducted in eight individuals from seven families  
300 (five Koreans, one Brazilian, and one Finnish; P01-1 to P07) (Tables S2 and S3). Written informed  
301 consent was obtained from each individual or their parents, and the study was approved by the  
302 Institutional Review Board. Based on the hypothesis that this disease is inherited in an autosomal  
303 recessive manner, we retrieved rare variants (<1% frequency in the public database) that were  
304 specifically harbored by the probands in homozygous or compound heterozygous status from the eight  
305 individuals. Among the individual-specific variants that followed a recessive pattern, variants in  
306 *TONSL* were present in four individuals (Table 2). The remaining four individuals harbored single  
307 variants in the gene and required further analysis to verify whether they also fit into the recessive  
308 model. For example, subsequent whole genome sequencing analysis of the sibling probands (P01-1  
309 and P01-2) identified an exon 23 deletion in the maternal allele, which was validated by polymerase  
310 chain reaction (PCR). Next, an additional cohort from India was recruited and subjected to singleton  
311 WES (P08 to P10), which led to the discovery of biallelic variants in *TONSL*. Finally, we screened  
312 two additional individuals (one Korean and one Indian) for such *TONSL* variants by Sanger  
313 sequencing and identified biallelic mutations in one (P11) and a single mutation in the other (P12).  
314 P11 has a splicing site variant and a deep intronic variant whose subsequent changes in mRNA were  
315 confirmed by reverse transcription-PCR. Only one mutation was identified in the *TONSL* gene in the  
316 three probands, P05, P06 and P12 (Table 2). Because the parents harboring the same mutation were  
317 unaffected, it is highly likely that the individuals had undetermined variants in the remaining allele of  
318 *TONSL*, such as a cryptic structural variation or a noncoding variation. All the *TONSL* mutations

319 identified were confirmed by Sanger sequencing (Figure S1A-B). No significant variation was found  
320 in other skeletal dysplasia-related genes in affected individuals. Ten out of thirteen individuals  
321 (76.9 %) carried one LoF allele and one missense allele (Table 2). Except for nonsense mutations,  
322 which may lead to nonsense-mediated mRNA decay, *TONSL* missense mutations were found in  
323 various locations throughout the protein-coding region (Figure 2A), mostly within functionally  
324 annotated domains (8 out of 9). The nine missense variants displayed complete or near-complete  
325 evolutionary conservation across vertebrate species (Figure 2B) and were found in low frequency in  
326 the healthy population (5/9 not found in the ExAC database, all nine  $<1.0 \times 10^{-4}$ ). To predict the  
327 functionality of the missense variants, the CADD and GERP scores were compared with the rest of  
328 the amino acid residues of the protein and displayed significant differences (Figure 2C and Figure  
329 S2A-D). *TONSL* is tolerant to LoF variants (ExAC pLI = 0.00), but no individual in this study carried  
330 LoF variants in both alleles, implying a critical but minimal requirement of *TONSL* function for  
331 survival.

332

### 333 *Complementation of defects in dermal fibroblasts from individuals by expression of wild-type*

#### 334 *TONSL*

335 In an attempt to validate that *TONSL* variants are causal for the SPONASTRIME phenotype,  
336 we established primary dermal fibroblasts from two individuals, P03 and P04. Immunoblot analysis  
337 showed a pronounced decrease in *TONSL* levels compared to those in normal human fibroblasts, BJ  
338 cells (Figure 3A). siRNA treatment abrogated endogenous *TONSL* protein levels (Figure 3A and  
339 Figure S5A). To further validate the causative nature of *TONSL* variants, we transduced an empty  
340 vector (EV) and HA-tagged wild-type (WT) *TONSL* into P03 and P04 cells (Figure 3B) and  
341 performed functional complementation assays. Expressing WT *TONSL* successfully rescued the  
342 enhanced sensitivity to CPT (Figure 3C) and DNA damage-induced RAD51 foci formation in both  
343 cell lines (Figure 3D and E). As controls for the CPT sensitivity assay, we used BJ cells, and the  
344 FANCP(also known as SLX4) deficient dermal fibroblasts, the FANCP cells, derived from an  
345 individual with Fanconi anemia. The FANCP cells were found to be sensitive to CPT, which was

346 rescued by introducing wild-type SLX4 protein through lentiviral transduction.<sup>18,22</sup> CPT sensitivity  
347 assay of P03 and P04 cells were designed similar to that of the previous FANCP cell assay by using  
348 EV and wild-type TONSL complemented cells. In addition to the normal control BJ cells, EV as well  
349 as wild-type SLX4 FANCP cells were used for comparing CPT sensitivity. In addition, to confirm the  
350 impaired DNA replication caused by TONSL variants found in individuals, BrdU incorporation assay  
351 was performed and quantified by immunofluorescence. As expected, the BrdU incorporation ratios in  
352 P03/EV and P04/EV cells were less than that in BJ cells, whereas the complemented cells showed  
353 increased BrdU incorporation (Figure 3F). In a study by Duro et al., it was reported that although  
354 *TONSL* knock-down cells are sensitive to CPT, they are not sensitive to hydroxyurea (HU) compared  
355 to control cells.<sup>6</sup> In order to test if the fibroblasts from individuals with mutant *TONSL* display similar  
356 characteristics, we treated P03 and P04 cells with HU. Consistent with previous reports, both P03 and  
357 P04 cells were not sensitive to HU (Figure S3). As mentioned earlier, TONSL is involved in  
358 homologous recombination, and its impairment results in decreased cell proliferation and increased  
359 sensitivity to CPT.<sup>12, 13</sup> Because homologous recombination is an important part of DNA damage  
360 repair and thus DNA replication, it is possible that TONSL variants may increase the occurrence of  
361 stalled replication fork due to DNA damage repair defects. In order to evaluate the impairment of  
362 replication restart and DNA damage repair of the mutant *TONSL*, we performed fiber analysis with  
363 fibroblast from P03 and normal control cell (Figure S4). We used nucleotide analogs CldU and IdU to  
364 track the first label origin and the second label origin newly synthesized DNA. Ongoing forks have  
365 both CldU and IdU tracks, whereas stalled forks can be monitored by CldU-only labeled tracks. The  
366 percentage of each tracks from the total of all CldU incorporated tracks was then calculated to  
367 compare the changes in ongoing or stalled replication forks. Compared to normal human fibroblast,  
368 the percentage of ongoing replication fork was slightly decreased in P03 cells, and the number of  
369 stalled forks was significantly increased in P03 cells compared to normal cells (Figure S4). Next, we  
370 performed fiber analysis for CPT sensitivity using P03 and P04 cells with EV and WT-TONSL and  
371 BJ cells as controls. When treated with 50 nM of CPT, P03/EV and P04/EV cells showed decreases in  
372 replication length compared to BJ cells, which were rescued by WT-TONSL (Figure 3G). Taken

373 together, these data display strong evidence that the TONSL variants found in individuals impair  
374 DNA replication and repair capacity, all of which were rescued by WT-TONSL, demonstrating the  
375 pathogenic nature of *TONSL* mutations in the disease phenotype.

376

### 377 ***Validation of pathogenic TONSL mutations in in vitro cell-based assay***

378 To systematically evaluate the pathogenicity of *TONSL* variants found in other individuals  
379 whose primary fibroblasts were not available, we established an *in vitro* cell-based assay using HeLa  
380 cell lines. We focused on missense *TONSL* mutations since nonsense mutations may be subject to  
381 nonsense-mediated mRNA decay. First, we tested if we could recapitulate the results of previous  
382 reports showing that TONSL depletion leads to enhanced sensitivity to CPT.<sup>11,12</sup> In order to  
383 accomplish this, we established an assay system using HeLa cell line, where mutant *TONSL* is  
384 expressed but the endogenous wild-type is selectively knocked down. The strategy was to establish  
385 individual HeLa cell lines that stably express each mutant *TONSL* by lentiviral transduction, then  
386 treating cells with siRNA targeting *TONSL* 3'-untranslated region (UTR), which selectively knocks  
387 down only the endogenous *TONSL* mRNA. If successful, the HA-tagged *TONSL* is expressed, and any  
388 cellular defects due to *TONSL* mutation can be observed without the interference of the endogenous  
389 *TONSL*. WT-TONSL and EV cell lines were established as positive and negative controls. To first test  
390 the siRNA's, HeLa cells were treated with siRNA targeting the coding region and the 3' UTR. As  
391 shown in Figure S5A-B, depleting TONSL by targeting the coding or the 3' UTR region in HeLa cells  
392 resulted in hypersensitivity to CPT. Then, HeLa cells stably expressing the individual EV, HA-tagged  
393 *WT-TONSL* and the HA-tagged *TONSL* missense mutant were successfully established (Figure S5C).  
394 The siRNA targeting the *TONSL* 3' UTR selectively knocked down endogenous *TONSL*, but not HA-  
395 tagged WT or mutant *TONSL* (Figure 4A). After confirming the selective knockdown of endogenous  
396 *TONSL* and decreased TONSL protein levels, EV, WT-TONSL and TONSL variant cells were treated  
397 with siRNA targeting *TONSL* 3' UTR, and were then grown for 5 days to monitor cell proliferation.  
398 Overall, a noticeable proliferation defect was observed in TONSL variant HeLa cells compared to  
399 WT-TONSL cells (Figure 4B). Of these, proliferation of cells with P02\_p.Gly973Arg, P01-1;P01-



400 2\_p.Arg934Trp, P05\_p.Asp364His, and P08\_p.Ser174Asn variants were significantly inhibited to a  
401 similar level as that of siTONSL-treated HeLa-EV cells (Figure 4B). This result shows that all  
402 examined TONSL variants directly impact cell proliferation, but to varying degrees. At the same time,  
403 we evaluated phosphorylated Checkpoint (CHEK1) and Checkpoint kinase 2 (CHEK2) after  
404 endogenous TONSL depletion in the individual TONSL variant HeLa cells. We found that the check-  
405 point is activated in cells with proliferation defects (Figure 4C), suggesting that the functional  
406 impairment of TONSL leads to genome instability, which results in cell cycle arrest and inhibition of  
407 cell division. To further investigate the role of each TONSL variants in repairing replication-  
408 associated DNA damage, a CPT sensitivity assay was performed. Consistent with the proliferation  
409 results, the CPT sensitivity of the TONSL variant cells was comparable to that of siTONSL-treated  
410 HeLa-EV cells, which lack TONSL (Figure 4D). In addition, we modeled the wild-type and TONSL  
411 variants based on the existing available structure to determine the basis of aberrant function of the  
412 TONSL variants (Figure S6). p.Glu539Lys (P03) and p.Arg558Gln (P04) altered the polar interactions  
413 between amino acids. Exon 23 deletion from individuals P01-1 and P01-2 changed the curvature of  
414 LRR domain significantly, and the p.664delLeu (P09) altered the direction of the C-terminal of the  
415 helix (Figures S6A-G). These changes deformed the overall structures, which may influence binding  
416 affinity between variant TONSL and its binding partners (Figure S6). From the studies using *in vitro*  
417 cell-based assay and *in silico* structural analyses, we conclude that the identified TONSL variants  
418 compromise TONSL functional activity, which potentially leads to human diseases.

419

#### 420 ***Embryonic lethality of Tonsl knock-in mouse model***

421 To better understand the pathophysiology and clinical correlations, we generated a  
422 CRISPR/Cas9-mediated Tonsl knock-in (KI) mouse carrying a p.Arg924Trp substitution in the *Tonsl*  
423 gene, akin to its human counterpart TONSL p.Arg934Trp variant found in individuals P01-1 and P01-  
424 2 (Figure 5A-C and S7A). Heterozygous *Tonsl*<sup>+/Arg924Trp</sup> (HT) mice were fertile and showed normal  
425 gross morphology. Intriguingly, no offspring littermates (n = 38) carrying the biallelic p.Arg924Trp  
426 variant were obtained from heterozygous intercrosses, while their WT and HT littermates were born

427 in a 1:2.8 ratio (Figure 5D), suggesting that the homozygotes were not viable. For development  
428 analysis, we collected embryos from embryonic days 10.5 to 12.5 (E10.5–E12.5) and observed a  
429 1:1.4:0.6 ratio of WT:HT:KI embryos (Table S4). However, all homozygous *Tonsl*<sup>Arg924Trp/Arg924Trp</sup>  
430 embryos showed early embryonic lethality with fetal growth restriction at around stage E10.5 (Figure  
431 5E and S7B-D). The yolk sacs of homozygous *Tonsl*<sup>Arg924Trp/Arg924Trp</sup> embryos lacked visible blood  
432 vessels and were smaller than others (Figure S7E). Consistent with *in vitro* cell-based assays of the  
433 human TONSL p.Arg934Trp variant, these results suggest that the *Tonsl* p.Arg924Trp variant is not  
434 functional, and that the lack of this functional protein is likely the major reason for embryonic  
435 lethality. Taken together, these findings demonstrated the physiological importance of functional  
436 *Tonsl* protein in embryonic development and the pathogenic nature of the TONSL p.Arg934Trp  
437 variant.

438

## 439 **Discussion**

440 In this study, we report the identification of *TONSL* mutations causative for 13 individuals  
441 with SPONASTRIME dysplasia. We demonstrate that the endogenous TONSL protein level of  
442 dermal fibroblast cells from individuals presented with SPONASTRIME dysplasia is significantly  
443 lower than that of wild-type control cells. Furthermore, we found that those cells from individuals  
444 exhibit enhanced sensitivity to CPT, reduced DNA damage-induced RAD51 foci formation, and  
445 impaired replication capacity, all of which are successfully rescued by WT-TONSL through lentiviral  
446 transduction. Given the importance of TONSL function in replication fork stability during normal S  
447 phase,<sup>23</sup> it is reasonable to speculate that pathogenic *TONSL* variants result in impaired cell  
448 proliferation, which is critical for embryonic development and postnatal growth.<sup>24</sup> Similarly,  
449 mutations in various genes important for preventing replication-associated DNA lesions have been  
450 identified in individuals with growth retardation.<sup>22,24,25</sup>

451 We found increased phosphorylation of CHEK1 and CHEK2 (Figure 4C) in cells with  
452 TONSL variant in the absence of endogenous TONSL or siRNA-mediated TONSL depletion even  
453 without treatment of genotoxic agents. CHEK1 is mainly phosphorylated by Ataxia-telangiectasia and

454 Rad3 related (ATR), and deleterious mutations in the *ATR* (MIM:601216) gene which results in  
455 Seckel syndrome (MIM: 210600), characterized by microcephaly, dwarfism, progeria, and mental  
456 retardation.<sup>25,26</sup> ATR responds to a variety of DNA damage, and together with ATR interacting  
457 protein (ATRIP), recognizes RPA bound to single stranded DNA, which is often the result of a stalled  
458 replication fork.<sup>27</sup> We observed that mutation in *TONSL* did not affect RPA foci formation in response  
459 to CPT-induced DNA damage in cells from SPONASTRIME dysplasia individuals (unpublished  
460 data), implying that TONSL functions downstream of ATR activation. Therefore, it is possible to  
461 speculate that alterations in the ATR, which function upstream of TONSL, may lead to broader  
462 defects, whereas loss of TONSL function may cause milder disease phenotypes such as  
463 SPONASTRIME dysplasia. It remains unknown why alterations in *TONSL* result specifically in  
464 SPONASTRIME dysplasia, and understanding the genotype to phenotype correlation requires further  
465 study.

466 Our knock-in mouse model clearly demonstrated that even a single point mutation,  
467 p.Arg924Trp, corresponding to p.Arg934Trp identified in human SPONASTRIME dysplasia, causes  
468 embryonic lethality in mouse. Although this finding in the mouse model does not directly translate to  
469 the pathogenicity of human disease, it provides evidence supporting that the TONSL p.Arg934Trp  
470 variant might be causative of SPONASTRIME dysplasia, and highlights the physiological importance  
471 of TONSL. Homozygous p.Arg924Trp variant in mice *Tonl* has severe developmental impact, where  
472 fetal growth retardation was observed approximately at E10.5, resulting in embryonic lethality.  
473 However, the heterozygotes surviving to adulthood show no apparent defects. Consistent with the *in*  
474 *vitro* cell-based assay (Figure 4B-D), these results suggest that *Tonl* proteins carrying the  
475 p.Arg924Trp variant are not functional, and that the lack of this functional protein is considered to be  
476 the main reason for embryonic lethality of *Tonl* KO mouse (Brendan Lee, personal communications)  
477 and even our KI models. However, unlike in mouse, the human TONSL p.Arg934Trp variant is found  
478 in a subgroup of individuals with SPONASTRIME dysplasia. The discrepancy between human and  
479 mouse phenotypes might be due to the difference in transcript isoforms found in humans and mice.  
480 According to the GENCODE basic project,<sup>28</sup> two transcripts are predicted to be translated in humans,

481 whereas mouse only have one transcript. Among the two transcripts in humans, the shorter isoform  
482 lacks the domain containing the Arg934 residue as well as the UBL and LRR domains. Whether the  
483 shorter isoform has any function in humans has yet to be elucidated, but the possibility that it provides  
484 partial function for survival cannot be excluded. More research is needed to understand the TONSL  
485 variants, since the presence of another TONSL isoform could have prevented lethality in humans.  
486 Although further study is required to understand how the decreased function of TONSL leads to the  
487 specific phenotype of SPONASTRIME dysplasia, we believe that our findings increase our  
488 understanding of the pathogenesis of this disease.

489

#### 490 **Description of Supplemental Information**

491 Supplemental Information includes clinical case reports, seven figures, six tables, supplemental  
492 methods and references.

493

#### 494 **Declaration of Interests**

495 The authors declare no competing interests.

496

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514

#### 515 **Accession Numbers**

516 The raw genome data can be downloaded at <http://biodata.kr/> (Submission ID: 1711075636).

517

#### 518 **Web Resources**

519 Online Mendelian Inheritance in Man: <http://www.omim.org>

520 UCSC browser: <http://genome.ucsc.edu>

521 Protein data bank: <https://www.rcsb.org/>

522 ExAC browser: <http://exac.broadinstitute.org/>

523

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609 **Figure Titles and Legends**

610

611 **Figure 1. Characteristic facial appearance and radiographic findings of individuals with**  
612 **SPONASTRIME dysplasia.** (A) Facial photos of individuals from three different ethnicities. They  
613 share midfacial hypoplasia, depressed nasal root, short and upturned nose, prognathism, and a relatively  
614 large head size with a prominent forehead. P05 at age 5 years, P06 at age 5 years, and P08 at age 13  
615 years. (B) Lateral spine view of P02 at age 3 years and 8 months (3.8) showing taller anterior vertebral  
616 body and convex anterior endplates, and that of P01-2 at age 13 years showing biconcavity of the  
617 endplates. Hip and knee anteroposterior views of P02 at age 3.8 years showing metaphyseal irregularity  
618 and vertical striation and small, dysplastic epiphyses, and those of P01-2 showing residual avascular  
619 necrosis of the left femoral head, and mixed dense striations and lucent area at the metaphysis of the  
620 knee.

621

622 **Figure 2. *TONSL* mutations identified in individuals with SPONASTRIME dysplasia.** (A)  
623 Pathogenic variants in *TONSL* found from SPONASTRIME individuals are displayed on *TONSL*  
624 protein with known functional domains. Variants in red were analyzed for variant functionality. (B)  
625 Evolutionary conservation of the nine missense variants found in SPONASTRIME individuals. (C)  
626 As parameters of missense variant ("Pathogenic") functionality of individuals, CADD and GERP  
627 values were plotted along with residues that overlapped with the ExAC database ("ExAC") and  
628 residues that were not polymorphic ("Other residues").

629

630 **Figure 3. Complementation of dermal fibroblasts from individuals P03 and P04 with the *TONSL***  
631 **cDNA.** (A) *TONSL* protein levels and corresponding siRNA-mediated *TONSL* depletion of cells  
632 from individuals with SPONASTRIME dysplasia. BJ cells were used as a normal control. The basal  
633 *TONSL* protein level in cells derived from individuals was lower than that in BJ cells, and all were

634 successfully depleted by siRNA treatment. C: siControl, T: siTONSL. **(B)** Stable protein level of wild-  
635 type TONSL through lentiviral transduction in fibroblasts derived from both P03 and P04. EV and  
636 WT denote the empty vector and wild-type, respectively. The asterisk indicates the cross-reacting  
637 band. **(C)** Cells from individuals were sensitive to CPT, which were rescued to a level comparable to  
638 that of BJ cells by transducing WT-TONSL. The indicated cells in triplicate were exposed to different  
639 concentration of CPT ranging 0-32 nM. After 5 days, the number of the cells were counted using a  
640 coulter counter, and the total number of cells at each concentration was divided by the number of  
641 untreated cells. FANCP/EV, a Fanconi anemia-derived cell line lacking *Slx4*, and the genetically  
642 isogenic SLX4 complemented cells, FANCP/WT-SLX4 were used as controls. The error bars  
643 represent the standard deviation (SD) of three replicates. **(D)** Representative images of CPT-induced  
644 DNA damage Rad51 and  $\gamma$ H2A.X foci. P03/EV, P03/WT-TONSL, P04/EV and P04/WT-TONSL cells  
645 were treated with CPT (50 nM) overnight, followed by fixing and staining with anti-RAD51 and anti-  
646  $\gamma$ H2A.X antibodies. Impaired DNA damage induced RAD51 foci formation of those cells from  
647 individuals was recovered to that of normal cells by transducing WT-TONSL. **(E)** Statistical analysis  
648 of Rad51 foci. Percentage was calculated by counting nuclei with  $n \geq 10$  Rad51 foci divided by the  
649 total nuclei for non-treated cells, or divided by  $\gamma$ H2A.X positive nuclei for CPT treated cells. The  
650 error bars represent the SD. **(F)** BrdU incorporation into DNA was reduced in P03/EV and P04/EV  
651 cells, which were rescued to a normal level by transducing WT-TONSL. The BrdU incorporation ratio  
652 was calculated by dividing the number of BrdU incorporated cells by the number of total cells  
653 counted. The error bars represent the SD of three replicates. **(G)** DNA fiber analysis of CPT-treated  
654 SPONASTRIME individual-derived cell lines. BJ cells were used as control. Schematic of the  
655 experiment is shown on the top and the representative DNA fiber is shown on bottom. The red line  
656 indicates median value; \*\*:  $p = 0.003$ ; \*\*\*:  $p < 0.001$ .

657

658 **Figure 4. *In vitro* cell-based assay of TONSL variants shows defects in cell proliferation and**  
659 **enhanced sensitivity to camptothecin.** (A) Protein level of recombinant wild-type or individual

660 TONSL variant in HeLa cells upon endogenous TONSL depletion using siRNA against the *TONSL* 3'  
661 UTR. The TONSL antibody is able to detect both endogenous and HA-tagged TONSL, whereas the  
662 HA antibody is not able to detect endogenous TONSL. The asterisk indicates the cross-reacting band.  
663 **(B)** Cell proliferation assay showing varied proliferation rate of cells expressing mutant *TONSL*. EV  
664 cells were treated with either non-targeting siCtrl or siTONSL targeting the 3' UTR. The rest of the  
665 cells were treated with siTONSL targeting the 3' UTR. The cell proliferation rate was normalized to  
666 the cell number on day 1. Error bar represents SD of three replicates. siCtrl: siControl. **(C)** TONSL  
667 variants in the absence of endogenous TONSL lead to checkpoint activation. Whole cell extracts of  
668 HeLa cells expressing mutant *TONSL* were treated with siRNA against 3' UTR and analyzed by  
669 immunoblot with DNA damage response factors, phosphorylated CHEK1 (pCHEK1) or  
670 phosphorylated CHEK2 (pCHEK2). **(D)** TONSL variant cell lines were treated with 3' UTR siRNA as  
671 in b, then treated with increasing concentrations of CPT. Cells were stained with Hoechst and nuclei  
672 were counted 5 days after CPT treatment. Cell survival was normalized to that in vehicle-treated cells.  
673 The error bars represent SD from three replicates.

674

675 **Figure 5. Generation and analysis of homozygous *Tonsl* p.Arg924Trp knock-in mouse using**  
676 **CRISPR-Cas9.** **(A)** A schematic diagram showing the mouse *Tonsl* locus and the enlarged sequences  
677 of the exon 18 of the *Tonsl* gene, along with the sequences of the *Tonsl*<sup>Arg924Trp</sup> allele. Blue letters in  
678 the *Tonsl*<sup>WT</sup> allele indicate a proto-spacer adjacent motif (PAM) sequence. Red letters indicate the  
679 substitution (C to T) target nucleotide in the *Tonsl*<sup>WT</sup> and the *Tonsl*<sup>Arg924Trp</sup> alleles. The amino acid  
680 sequences from the *Tonsl*<sup>WT</sup> and the *Tonsl*<sup>Arg924Trp</sup> alleles are shown at the top of the nucleotide  
681 sequences. The substituted nucleotides for synonymous and target mutations are shown at the bottom  
682 of the *Tonsl*<sup>Arg924Trp</sup> allele sequences by black and red asterisks (\*), respectively. Forward (F) and  
683 Reverse (R) PCR primers for genotyping are indicated. **(B)** Genotyping PCR for the *Tonsl*<sup>WT</sup> and the  
684 *Tonsl*<sup>Arg924Trp</sup> alleles. The upper and bottom panels show the PCR products that were amplified from  
685 the *Tonsl*<sup>WT</sup> (115 bp) and the *Tonsl*<sup>Arg924Trp</sup> (108 bp) alleles, respectively. **(C)** Chromatogram

686 displaying the sequence of the  $Tonsl^{WT}$  and the  $Tonsl^{Arg924Trp}$  locus. **(D)** Genotype distribution of  
687 offspring from heterozygous intercrosses. **(E)** Gross morphology of whole embryos at stage E11.5.  
688  $Tonsl^{+/+}$  and  $Tonsl^{+/Arg924Trp}$  mouse embryos show normal development, whereas  $Tonsl^{Arg924Trp/Arg924Trp}$   
689 embryo exhibits a growth retardation with the abnormal development of eyes and limbs.

**Table 1.** Clinical features of individuals in this series

ID	P01-1 <sup>a</sup>	P01-2 <sup>a</sup>	P02	P03	P04	P05	P06	P07 <sup>b</sup>	P08	P09	P10	P11	P12
<b>Ethnicity</b>	Korean	Korean	Korean	Korean	Brazilian (African black and non-Latin European)	Korean	Finnish	Korean	Indian	Indian	Indian	Korean	Indian
<b>Age of initial presentation (years. months)</b>	8.5	6	0.2	13	2.2	2.7	At birth	36	13	14	11	3.5	1.9
<b>Gender</b>	F	M	M	M	M	F	F	M	M	F	F	F	F
<b>Consanguinity</b>	No	No	No	No	Yes	No	No	No	No	No	No	No	No
<b>Initial presentation</b>	short stature	short stature	short stature	radiographic abnormality of knee	short stature	short stature	short stature	neck and shoulder pain	short stature	short stature	short stature and limb deformity	short stature	short stature
<b>Height (cm) (SDS)</b>	104.1 (-3.6)	93.4 (-4.7)	47.9 (-4.9)	145.2 (-0.9)	76 (-4.0)	74 (-4.5)	40 (-5.6)	136 (-6.2)	120 (-4)	119 (-7)	93 (-10)	73.6 (-5.6)	53 (-10)
<b>Age of latest measurement (years)</b>	17.5	15	5.7		13	6	12.6	37	13	14	11	4.2	2
<b>Ht cm (SDS) (latest)</b>	124.4 (-6.8)	143.5 (-4.8)	82.6 (-6.0)		122 (-4.6)	98.8 (-3.4)	85.8 (-10.4)	136 (-6.2)	120 (-4)	119 (-7)	93 (-10)	80.6 (-5.8)	
<b>Facial dysmorphism</b>	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
<b>Cognitive impairment</b>	(-)	(-)	(-)	(-)	borderline	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
<b>Other findings</b>	short dental roots	short dental roots	hypothyroidism; nystagmus; hypospadias; airway narrowing <sup>c</sup>		recurrent pneumonia in the 1 <sup>st</sup> year; short dental roots	hypothyroidism; recurrent pneumonia until age 5 years; precocious puberty	cataracts; cerebrovascular aneurysm rupture; weak voice	Arnold-Chiari malformation; cataracts			joint laxity	joint laxity; hip subluxation; airway narrowing <sup>c</sup>	

<sup>a</sup> These two individuals are siblings

<sup>b</sup> Previously reported (Jeong et al.<sup>10</sup>)

<sup>c</sup> Airway narrowing was composed of glottic narrowing and diffuse tracheal narrowing.

M: male, F: female, SDS: standard deviation score

**Table 2.** *TONSL* mutations identified in 13 individuals and their predicted protein changes

<b>ID</b>	<b>Mutation 1</b>	<b>Mutation2</b>	<b>Genetic study</b>		
<b>P01-1</b> <sup>a</sup>	exon 23 deletion <sup>b</sup>	c.2800C>T	p.Arg934Trp	quad WES	
<b>P01-2</b> <sup>a</sup>	exon 23 deletion <sup>b</sup>	c.2800C>T	p.Arg934Trp	quad WES	
<b>P02</b>	c.1531C>T	p.Gln511*	c.2917G>A	p.Gly973Arg	trio WES
<b>P03</b>	c.735C>G/ c.737delT <sup>c</sup>	p.Cys245Trpfs*20	c.1615G>A	p.Glu539Lys	trio WES
<b>P04</b>	c.1673G>A	p.Arg558Gln	c.1673G>A	p.Arg558Gln	trio WES
<b>P05</b>	c.1090G>C	p.Asp364His	Not identified	Not identified	quad WES
<b>P06</b>	c.578+1G>T	Not identified	Not identified	Not identified	trio WES
<b>P07</b> <sup>g</sup>	c.2907C>A	p.Tyr969*	c.3862G>C	p.Glu1288Gln	singleton WES
<b>P08</b>	c.1471_1472delTC	p.Ser491Argfs*65	c.521G>A	p.Ser174Asn	singleton WES
<b>P09</b>	c.1459G>A / c.1989_1991del <sup>d</sup>	p.Glu487Lys / p.664delLeu	c.125G>A	p.Arg42His	singleton WES
<b>P10</b>	c.295delT	p.Ser99fs*59	c.1459G>A	p.Glu487Lys	singleton WES
<b>P11</b>	c.578+1G>A	p.Thr151Argfs*42 <sup>e</sup>	c.1291-11_1291-14delCCTC	p.Arg431Profs*6/ p.431_441del <sup>f</sup>	Sanger
<b>P12</b>	c.1459G>A	p.Glu487Lys	Not identified	Not identified	Sanger

<sup>a</sup> These two individuals are siblings

<sup>b</sup> g.145657122\_145658684del

<sup>c</sup> Paternal allele harbors a substitution followed by deletion of one nucleotide.

<sup>d</sup> Paternal allele harbors a substitution and an in-frame deletion.

<sup>e</sup> RT-PCR revealed mRNA sequence change, predicting a truncated protein or nonsense-mediated mRNA decay

<sup>f</sup> RT-PCR and cloning its products revealed wild-type (14/25) and two different mutant mRNAs formed by retention of a part of intron 10 (5/25) and by skipping of a part of exon 11 (6/25), which predicted these two different polypeptides.

<sup>g</sup> All the individuals with two or three mutations were confirmed to be biallelic by testing the parents, except P07, whose parents had already passed away, by testing the siblings.

WES: whole exome sequencing