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# 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 determined. Using whole exome sequencing, we identified biallelic TONSL mutations in 10 of 13 53 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 individuals are complemented by expressing wild-type TONSL. In addition, in vitro cell-based assays 57 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 function of TONSL cause SPONASTRIME dysplasia and highlight the importance of the TONSL in 61 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. To date, only 15 individuals with this disorder have been reported<sup>1-10</sup> based on the diagnostic criteria 68 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

vertebral changes.<sup>5</sup> To date, causative genetic mutations for SPONASTRIME dysplasia have not been
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 MMS22like protein (MMS22L).<sup>11-15</sup> TONSL consists of 1,378 amino acids with annotated domains such as 81 eight tetratricopeptide repeats (TPR), three Ankyrin (ANK) repeats, an ubiquitin-like domain (UBL), 82 and seven leucine-rich repeats (LRR).<sup>13</sup> It was reported that the TONSL-MMS22L heterodimer plays 83 a key role in homologous recombination required for repairing spontaneous replication-associated 84 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 induces DNA breakage at replication forks.<sup>12,13</sup> Specifically, TONSL-MMS22L is recruited to the 87 sites of stalled replication forks during normal S phase by replication protein A (RPA1, RPA2 and 88 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 leads to loss of damage-induced RAD51 foci formation in cells treated with genotoxic agents.<sup>11,12,16</sup> 91 In this study, in order to identify the causative genetic alteration for SPONASTRIME 92 dysplasia, we recruited 13 individuals, including a previously reported case,<sup>10</sup> from four different 93 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 struture 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.

#### 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
- 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
- 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
- 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'-TACCATTTCTGTGGCCCTTC-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<sup>™</sup> 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)

supplemented with 15% fetal bovine serum (Gibco), glutamine (Gibco, 35050-061), MEM non-

essential amino acid (Gibco, 11140-050), penicillin and streptomycin (Gibco, 15140-122) and grown

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)

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

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 GGGGACAAGTTTGTACAAAAAGCAGGCTTAATGAGCCTGGAGCGCGAGC-3') and

155 TONSL-R (5'-

156 GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGAGGCGCCGAAAGAAGAGC-3'). The

PCR product was cloned into a Gateway BP vector, pDONR223, using BP clonase. The pDONR223 157 158 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  $\mu$ M 5-chloro-20-163 deoxyuridine (CldU: Sigma-Aldrich C6891), washed with PBS, pulse-labelled with 250 µM 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 antibody (clone BU1/75, ICR1; Abcam, ab6326; 1:750) and IdU with mouse anti-BrdU antibody 168 169 (clone B44; BD Biosciences, 347583; 1:750). Slides were fixed using 4% paraformaldehyde, then 170 stained with Alexa Fluor 594or 488 conjugated secondary antibodies (Life Technologies). Images 171 were acquired using a Nikon Eclipse Ni microscope with 60× 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 μM CldU for 30 minutes, then 250 μM 5-iodo-20-deoxyuridine (IdU; Sigma-Aldrich I7125) for 30 minutes with 2.5  $\mu$ M/50 nM CPT or DMSO. DNA plug was prepared by ~2-4.5×10<sup>5</sup> cell/plug using 176 177 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°C. To stretch the DNA fibers, 22 mm×22 mm silanized coverslips 179 (Genomic Vision) were dipped into the DNA solution for 13 minutes and pulled out a constant speed 180 (300 µM/s) using a Molecular Combing System (Genomic Vision MCS-001). The coverslips were 181 baked for 4 hours at 60  $^{\circ}$ C and incubated with acid for denaturation. CldU- and IdU-labeled tracts were detected by incubating for 2 hours at room temperature (RT) with rat anti-BrdU antibody (dilution 182 1:100 detects BrdU and CldU; Abcam 6326) and mouse anti-BrdU antibody (1:10, detects BrdU and 183

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 1:100, A21124; Molecular Probes/Thermo Fisher). Finally, they were mounted with ProLong Gold 187 188 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* 

coding regions are listed in Table S6. Here,  $3 \times 10^5$  cells were plated in 6-well plates and transfected

hours after the second transfection, 4,000 cells were plated in 4 wells (12 well plates, SPL) for cell

siRNA in reverse transfection manner. Cells were transfected a second time after 24 hours. Then 48

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)

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,

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>

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196

205 Immunoblot. Cell lysate was prepared by boiling cells in 95 °C for 5 minutes in 2X SDS sample

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-α-Tubulin (Abfrontier: LF-PA0146, Lot MJL01-02) antibodies to detect each protein.

**Immunofluorescence (BrdU/Rad51).** For immunofluorescence, 3×10<sup>5</sup> cells were plated in 6-well 211 212 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 treated with CPT (500 µM) overnight or BrdU (20 µg/mL) for 4 hours, then fixed with 3.7% 214 215 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 blocked with PBG (0.2% [w/v] cold fish gelatin, 0.5% BSA in PBS) for 1 hour at room temperature. 223 224 Then, the cells were incubated with 1:7000 of anti-Rad51 antibody (Abcam, Rb:ab133534, Lot #: 225 GR219215-36), and 1:1000 anti-y-H2A.X antibody (Cell Signaling, #9718S, Lot #13) in PBG overnight at 4°C. The following day, cells were washed three times with PBG and incubated with 226 227 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.

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

accordance with the Korean Food and Drug Administration (KFDA) guidelines, reviewed, and
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

- 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'-GGTCCAGCCCCTCCCATCC-3',

248 crRNA2 5'-GAACCCGGATGGGAGGGGGC-3', crRNA3 5'-CCGGGTTCGAGTTCAAATTC-3',

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 CTCCTCCTATCTGGGTTCGGGTTCAGATTCAGGATAACCTTTTCCTCATCCCCGTTCCC-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
- 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
- 120 ng/µL), four crRNAs (10, 25, or 50 ng/µL each), tracrRNA (tracrRNA mixed with crRNA at 1:1 molar
- 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.
- Founder screening and genotyping PCR. To screen founders carrying the p.Arg924Trp mutation in

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

regions spanning the crRNA target site were amplified by PCR. After simply denaturing and

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

is annealed specifically to the sequence of the Tonsl WT allele, whereas KI R to the sequence of the

277 Tonsl p.Arg924Trp allele.

from specimens of fixed embryos.

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

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284 Results
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#### 285 Diagnosis and clinical information of individuals with SPONASTRIME dysplasia

We recruited 13 individuals, including a previously reported case,<sup>10</sup> satisfying the diagnostic criteria of the disease from four different ethnicities – Korean, Indian, Finnish and Brazilian (African black and non-Latin European). Their clinical features are presented on Table 1 and case reports (see Supplemental note). The median of height standard deviation scores (SDS) of all the individuals of this study was -4.9 (ranging from -0.9 to -10.0). They were short with height SDS down to -10, but a mildly affected individual (P03) had a height SDS of only -0.9. All the individuals recruited shared similar facial dysmorphism (Figure 1A). Other clinical findings shared by more than one individual
included short dental root, airway narrowing, cataracts and joint laxity. Radiographic features were
characterized by distinct changes in vertebrae and metaphyses of the long bones. It is worth noting
that the radiographic features changed and became more conspicuous with age (Figure 1B and Table
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 unaffected, it is highly likely that the individuals had undetermined variants in the remaining allele of 317 TONSL, such as a cryptic structural variation or a noncoding variation. All the TONSL mutations 318

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 the healthy population (5/9 not found in the ExAC database, all nine  $<1.0 \times 10^{-4}$ ). To predict the 326 327 functionality of the missense variants, the CADD and GERP scores were compared with the rest of the amino acid residues of the protein and displayed significant differences (Figure 2C and Figure 328 S2A-D). TONSL is tolerant to LoF variants (ExAC pLI = 0.00), but no individual in this study carried 329 330 LoF variants in both alleles, implying a critical but minimal requirement of TONSL function for 331 survival.

332

# Complementation of defects in dermal fibroblasts from individuals by expression of wild-type 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 vector (EV) and HA-tagged wild-type (WT) TONSL into P03 and P04 cells (Figure 3B) and 340 341 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 cell lines (Figure 3D and E). As controls for the CPT sensitivity assay, we used BJ cells, and the 343 FANCP(also known as SLX4) deficient dermal fibroblasts, the FANCP cells, derived from an 344 individual with Fanconi anemia. The FANCP cells were found to be sensitive to CPT, which was 345

rescued by introducing wild-type SLX4 protein through lentiviral transduction.<sup>18,22</sup> CPT sensitivity 346 347 assay of P03 and P04 cells were designed similar to that of the previous FANCP cell assay by using EV and wild-type TONSL complemented cells. In addition to the normal control BJ cells, EV as well 348 as wild-type SLX4 FANCP cells were used for comparing CPT sensitivity. In addition, to confirm the 349 350 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 homologous recombination, and its impairment results in decreased cell proliferation and increased 358 359 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 BJ cells as controls. When treated with 50 nM of CPT, P03/EV and P04/EV cells showed decreases in 371 replication length compared to BJ cells, which were rescued by WT-TONSL (Figure 3G). Taken 372

together, these data display strong evidence that the TONSL variants found in individuals impair
DNA replication and repair capacity, all of which were rescued by WT-TONSL, demonstrating the
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 treating cells with siRNA targeting TONSL 3'-untranslated region (UTR), which selectively knocks 386 387 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. Overall, a noticeable proliferation defect was observed in TONSL variant HeLa cells compared to 398 WT-TONSL cells (Figure 4B). Of these, proliferation of cells with P02\_p.Gly973Arg, P01-1;P01-399

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, we evaluated phosphorylated Checkpoint (CHEK1) and Checkpoint kinase 2 (CHEK2) after 403 endogenous TONSL depletion in the individual TONSL variant HeLa cells. We found that the check-404 405 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 variants based on the existing available structure to determine the basis of aberrant function of the 411 412 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 LRR domain significantly, and the p.664delLeu (P09) altered the direction of the C-terminal of the 414 415 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

To better understand the pathophysiology and clinical correlations, we generated a CRISPR/Cas9-mediated Tonsl knock-in (KI) mouse carrying a p.Arg924Trp substitution in the *Tonsl* gene, akin to its human counterpart TONSL p.Arg934Trp variant found in individuals P01-1 and P01-2 (Figure 5A-C and S7A). Heterozygous Tonsl<sup>+/Arg924Trp</sup> (HT) mice were fertile and showed normal gross morphology. Intriguingly, no offspring littermates (n = 38) carrying the biallelic p.Arg924Trp 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 1:1.4:0.6 ratio of WT:HT:KI embryos (Table S4). However, all homozygous Tonsl<sup>Arg924Trp/Arg924Trp</sup> 429 430 embryos showed early embryonic lethality with fetal growth restriction at around stage E10.5 (Figure 5E and S7B-D). The yolk sacs of homozygous *Tonsl*<sup>Arg924Trp/Arg924Trp</sup> embryos lacked visible blood 431 432 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 lethality. Taken together, these findings demonstrated the physiological importance of functional 435 436 Tonsl protein in embryonic development and the pathogenic nature of the TONSL p.Arg934Trp variant. 437

438

#### 439 Discussion

In this study, we report the identification of TONSL mutations causative for 13 individuals 440 441 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 exhibit enhanced sensitivity to CPT, reduced DNA damage-induced RAD51 foci formation, and 444 445 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 phase,<sup>23</sup> it is reasonable to speculate that pathogenic *TONSL* variants result in impaired cell 447 proliferation, which is critical for embryonic development and postnatal growth.<sup>24</sup> Similarly, 448 449 mutations in various genes important for preventing replication-associated DNA lesions have been identified in individuals with growth retardation.<sup>22,24,25</sup> 450 451 We found increased phosphorylation of CHEK1 and CHEK2 (Figure 4C) in cells with TONSL variant in the absence of endogenous TONSL or siRNA-mediated TONSL depletion even 452

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 retardation.<sup>25,26</sup> ATR responds to a variety of DNA damage, and together with ATR interacting 456 protein (ATRIP), recognizes RPA bound to single stranded DNA, which is often the result of a stalled 457 replication fork.<sup>27</sup> We observed that mutation in *TONSL* did not affect RPA foci formation in response 458 459 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 SPONASTRIME dysplasia. It remains unknown why alterations in TONSL result specifically in 463 464 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 embryonic lethality in mouse. Although this finding in the mouse model does not directly translate to 468 469 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 Tonsl 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 Tonsl 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 Tonsl 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 mouse phenotypes might be due to the difference in transcript isoforms found in humans and mice. 479 According to the GENCODE basic project,<sup>28</sup> two transcripts are predicted to be translated in humans, 480

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
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493	
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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						
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#### 609 Figure Titles and Legends

610

611 Figure 1. Characteristic facial appearance and radiographic findings of individuals with 612 **SPONASTSRIME dysplasia.** (A) Facial photos of individuals from three different ethnicities. They share midfacial hypoplasia, depressed nasal root, short and upturned nose, prognathism, and a relatively 613 614 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 PO2 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 endplates. Hip and knee anteroposterior views of P02 at age 3.8 years showing metaphyseal irregularity 617 and vertical striation and small, dysplastic epiphyses, and those of P01-2 showing residual avascular 618 619 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)

Pathogenic variants in TONSL found from SPONASTRIME individuals are displayed on TONSL
protein with known functional domains. Variants in red were analyzed for variant functionality. (B)
Evolutionary conservation of the nine missense variants found in SPONASTRIME individuals. (C)
As parameters of missense variant ("Pathogenic") functionality of individuals, CADD and GERP
values were plotted along with residues that overlapped with the ExAC database ("ExAC") and
residues that were not polymophic ("Other residues").

629

Figure 3. Complementation of dermal fibroblasts from individuals P03 and P04 with the *TONSL*cDNA. (A) TONSL protein levels and corresponding siRNA-mediated *TONSL* depletion of cells
from individuals with SPONASTRIME dysplasia. BJ cells were used as a normal control. The basal
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 WT denote the empty vector and wild-type, respectively. The asterisk indicates the cross-reacting 636 band. (C) Cells from individuals were sensitive to CPT, which were rescued to a level comparable to 637 638 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 represent the standard deviation (SD) of three replicates. (D) Representative images of CPT-induced 643 DNA damage Rad51 and yH2A.X foci. P03/EV, P03/WT-TONSL, P04/EV and P04/WT-TONSL cells 644 were treated with CPT (50 nM) overnight, followed by fixing and staining with anti-RAD51 and anti-645 646  $\gamma$ 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  $n \ge 10$  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 indicates median value; \*\*: *p* = 0.003; \*\*\*: *p* < 0.001. 656

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

TONSL variant in HeLa cells upon endogenous TONSL depletion using siRNA against the TONSL 3' 660 661 UTR. The TONSL antibody is able to detect both endogenous and HA-tagged TONSL, whereas the HA antibody is not able to detect endogenous TONSL. The asterisk indicates the cross-reacting band. 662 (B) Cell proliferation assay showing varied proliferation rate of cells expressing mutant TONSL. EV 663 cells were treated with either non-targeting siCtrl or siTONSL targeting the 3' UTR. The rest of the 664 cells were treated with siTONSL targeting the 3' UTR. The cell proliferation rate was normalized to 665 the cell number on day 1. Error bar represents SD of three replicates. siCtrl: siControl. (C) TONSL 666 667 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 were counted 5 days after CPT treatment. Cell survival was normalized to that in vehicle-treated cells. 672 The error bars represent SD from three replicates. 673

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

- 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.
- $\label{eq:construction} 688 \qquad Tonsl^{+/+} \ and \ Tonsl^{+/Arg924Trp} \ mouse \ embryos \ show \ normal \ development, \ whereas \ Tonsl^{Arg924Trp/Arg924Trp}$
- 689 embryo exhibits a growth retardation with the abnormal development of eyes and limbs.

 Table 1. Clinical features of individuals in this series

ID	<b>P01-1</b> <sup>a</sup>	<b>P01-2</b> <sup>a</sup>	P02	P03	P04	P05	P06	<b>P07</b> <sup>b</sup>	P08	P09	P10	P11	P12
Ethnicity	Korean	Korean	Korean	Korean	Brazilian (African black and non-Latin European)	Korean	Finnish	Korean	Indian	Indian	Indian	Korean	Indian
Age of initial presentation (years. months)	8.5	6	0.2	13	2.2	2.7	At birth	36	13	14	11	3.5	1.9
Gender	F	М	М	М	Μ	F	F	М	М	F	F	F	F
Consanguinity	No	No	No	No	Yes	No	No	No	No	No	No	No	No
Initial presentation	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
Height (cm) (SDS)	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)
Age of latest measurement (years)	17.5	15	5.7		13	6	12.6	37	13	14	11	4.2	2
Ht cm (SDS) (latest)	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)	
Facial dysmorphism	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Cognitive impairment	(-)	(-)	(-)	(-)	borderline	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Other findings	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	hypothyroi dism; recurrent pneumonia until age 5 years; precocious puberty	cataracts; cerebro- vascular 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

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

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

<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

 $^{\rm f}$  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