# TITLE: A Pathogenic Deletion in Forkhead Box L1 (*FOXL1*) Identifies the First Otosclerosis (*OTSC*) Gene

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# Materials and Methods

## Clinical Criteria and Evaluations

For linkage analyses (family-based study) we used conservative clinical criteria to assign affection status: affected were family members with otosclerosis confirmed by surgery; unaffected were blood relatives ≥ 60 years of age with normal (bilateral) hearing thresholds: all remaining members were considered "unknown status." All available medical charts and audiological reports were reviewed, and patients assessed, when possible, to update audiograms and confirm middle ear status. Classification of HL was based on the pure-tone threshold average of 0.5, 1.0 and 2.0 kHz as defined by the American Speech and Hearing Association. A difference of >10 dB HL between air and bone conduction sensitivity represented a significant conductive component associated with impaired sound transfer through the middle ear. In all cases, patients were managed with conventional hearing aids, corrective stapes surgery and/or implanted middle and inner ear prostheses.

### Discovery Cohort

We have an ongoing recruitment drive for otosclerosis families from otolaryngology clinics in Newfoundland and Labrador (NL). One of the largest is Caucasian of English extraction segregating autosomal dominant otosclerosis of varied clinical presentation among seven surgically-confirmed cases (**Table 1**). Onset of HL ranges from mid-teens to early twenties. The proband at 25 years had bilateral conductive HL that progressed by age 51 to mixed asymmetric loss (R severe; L moderate-severe) with air-bone gaps averaging 50 dB and profound loss bilaterally at 8000 Hz (**Figure 1A**, **Table 1**). Bilateral stapedectomies were clinically successful, with air-bone gaps resolved apart from slight residual conductive loss at 500 and 4000 Hz and functional hearing significantly restored at 52 years (**Figure 1A**). High frequency thresholds show minimal

improvement post-operatively, consistent with possible cochlear otosclerosis and/or presbycusis. The proband's parents (PIDs I-1, I-2, **Figure 2A**) both have HL and several siblings (PID II-9, II-11, II-14) had a similar clinical course and successful post-stapedectomy resolution of conductive loss in one or both ears (**Table 1**). Although sibling PID II-2 had a similar early progression of conductive loss, stapedectomy (R) at age 36 was unsuccessful, resulting in profound loss with no response to stimuli. Amplification with a conventional hearing aid was used until no longer effective for severe mixed loss (L) (**Table 1; Figure 1A**). PID II-2 had a middle ear implant at age 75 which was unsuccessful. Subsequent cochlear implantation at age 76 provided substantial functional improvement. Sibling PID II-3 had HL by mid-teens and wore hearing aids by age 18. No reliable responses to bone conduction stimuli were recorded on repeated tests from age 33-38, consistent with the medical report of profound SNHL bilaterally at age 38, and inadequate benefit from hearing aids (**Figure 1A; Table 1**). At 60 years of age, CT imaging confirmed “prominent” otosclerosis in PID II-3. Stapedectomy (R) was attempted without success. Cochlear implantation at age 61 significantly improved hearing function (self-report). In the next generation, PID III-2 experienced an onset of conductive HL (R) in mid-teens, and was followed by surgical exploration at age 17, which identified stapes fixation and a cholesteatoma which precluded stapedectomy. The report of a follow-up tympano-mastoidectomy to remove the cholesteatoma notes that the ossicular chain was left intact and re-mobilized; however, complete resolution of the air bone gap was not achieved (**Figure 1A,** PID III-2, age 18) and subsequent ossicular re-mobilization achieved only temporary improvement. By age 35, the conductive loss had advanced to moderately severe (R)

and moderate low frequency conductive loss (L) resulting in diagnosis of otosclerosis at age 35 (**Figure 1A**).

## Validation Cohort

For validation purposes, we used unrelated otosclerosis cases recruited from Canada (n=82), Finland (35) and Faroe Islands (n=20). A positve hit was identified in an Ontario case with profound mixed loss (R) and moderate low frequency SNHL (L) (**Figure 1B, Table 1**). Acoustic immittance testing revealed absent acoustic middle ear muscle reflexes despite normal middle ear compliance, a hallmark feature of otosclerosis. Hearing thresholds improved dramatically post-surgery, with near complete resolution of air-bone gaps, and noticeable improvement of bone conduction thresholds (**Figure 1B,** age 67) consistent with previous reports (Sperling et al. 2013; Vijayendra and Parikh 2011). Genealogical investigations yielded a multigenerational Caucasian family with autosomal dominant HL. This study was approved by institutional review boards at Memorial University (#1.186), Western University (#103679) and the Danish Research Ethical Committee (KF 01-234/02 and KF 01-108/03).

## Linkage to Published *OTSC* Loci and Susceptibility Genes

Genomic DNA was isolated from peripheral leukocytes according to a standard salting out procedure (Miller et al. 1988). Microsatellite markers were fluorescently labeled and amplified by PCR, run on ABI 3130xl or 3730 (Applied Biosystems) and analyzed with Gene Mapper v4.0. Pedigrees were drawn with Progeny (Progeny Genetics LLC) and haplotypes phased manually with the least number of recombination events. To test for linkage, seven family members (PID II-2, II-3, II-6, II-9, III-2, III-5, III-6; **Figure 2A**) were genotyped for markers spanning each *OTSC* disease interval and bracketing three

otosclerosis susceptibility genes (**Supplementary Table 1**). As well, a total of 17 relatives were genotyped with nine extra markers (*D16S518, D16S3049, D16S3098, D16S422, D16S2625, D16S520, D16S413, D16S3023, D16S3026*) mapping qter of the *OTSC4* disease interval. Two-point parametric linkage analyses (MLINK ver 5.1) were run for three markers per locus (assuming autosomal dominant inheritance, 99% penetrance and a gene frequency of 0.00). LOD scores were calculated at recombination fractions 0.000 to 0.5000. The proband was also sequenced for rare otosclerosis-associated variants in *SERPINF1* (Ziff et al. 2016).

## Sequencing, Variant Filtering and Validation

Positional candidate genes (UCSC Genome Browser and NCBI build 36.) were selected for sequencing based on function and/or gene expression. Oligonucleotide primers were designed (Primer 3) to amplify the longest isoform and to include all intron/exon boundaries and UTRs. Samples were Sanger sequenced on an ABI 3130xl/3730. For first pass variant filtering, we used three affected (PID II-3, II-9, III-2) and an unaffected spouse (PID II-4) (**Figure 2A)**. Variants either absent in affected samples or present in the unaffected spouse were removed from further study, as were variants with a MAF>2% using dbSNP and 1000 genomes. We used multiple *in silico* tools (SIFT, PolyPhen-2, PANTHER, Human Splicing Finder) and ClustalW for aa (amino acid) conservation, to evaluate the potential functional consequences of filtered variants.

For completeness, we next ran whole exome sequencing (WES) to screen all positional candidate genes. We selected five affected (PIDs II-2, II-6, II-9, II-11, II-14) and two senior (55, 60 yrs old) population controls with normal hearing thresholds for first pass variant filtering. WES was outsourced to the Genome Centre (McGill

University, QC, Canada) including library preparation (TrueSeq Prep Kit). Samples were run on the Illumina Hiseq 2000, generating 50-150 million 100 bp paired end reads. Reads >32 bp long were aligned to the 1000 genome reference using Burrows-Wheeler Aligner (BWA) and merged with Picard software (Broad Institute). Where multiple base mismatches and false positive variant calls were recorded, insertions and deletions were realigned using GATK software (DePristo et al. 2011; Van der Auwera et al. 2013). The percentage of aligned region coverage was detected using the Genome Centre's in-house database. The regions were identified as high coverage (>400), low coverage (<50), low mean mapq MQ (<20) and no data. Variants either absent in affected(s) or present in normal hearing control(s) were removed from further study. The remaining rare (MAF<1%) variants residing within linked regions (with a minimum of 20X coverage) were analysed with multiple *in silico* tools [samtools mpileup algorithm (Li et al. 2009), SnpSift (Cingolani et al. 2012), SnpEff (Cingolani et al. 2012), SIFT, PolyPhen-2 and PANTHER] including ClustalW to determine aa conservation. Variants of interest were validated by cascade sequencing in the NL family. Heterozygous variants co-segregating with otosclerosis in an autosomal dominant pattern were tested in the unrelated otosclerosis cases. Positive hits were genotyped and examined for potential allele sharing with the disease haplotype identified in the NL family.

## Computer Modelling of 2D Structure of Mutant Foxl1 C-Terminus

To initially assess the effect of the missing residues on FOXL1 structure, computer simulations were performed with the NAMD software (version 2.12) (Khajeh et al. 2020). The structure of the most C-terminal 69 residues of wildtype ($FOXL1_{CTERM}$) and the deletion mutant ($FOXL1_{MUT}$) were deduced by molecular dynamics simulations. Initial structures were generated in an extended state using the Protein in Atomistic details

coupled with Coarse-grained Environment (PACE) model (Han and Schulten 2012). The protein was solvated in a cubic simulation cell with a side length of 90 Å Langevin thermostat with a damping coefficient of 10 $ps^{-1}$ was employed to maintain temperature. All non-bonded interactions were shifted to zero between distances of 9 Å and 12 Å. The time-step for all simulations was set to 5 fs. This system was minimized, and an 8.5 ns equilibration molecular dynamics simulation was performed. Replica exchange molecular dynamics (REMD) (Zhou 2007) was then employed to sample the folded configurational space of these proteins. For both the mutant and wild-type proteins, 1 μs long simulations were performed with 32 replicas with temperatures ranging from 300 K to 500 K. The dominant structure in the 300 K replica was determined by clustering analysis based on the root mean squared distance (RMSD) of all protein atoms. Clustering analysis was performed in VMD 1.9.1 program using a 3 Å RMSD criteria.

## Experimental Measurements of 3D Structure of Mutant Foxl1 C-Terminus

To validate computer modelling of the 3D structure, $FOXL1_{MUT}$ and $FOXL1_{CTERM}$ were produced recombinantly in *E. coli,* purified by nickel affinity chromatography followed by size exclusion chromatography. Circular dichroism measurements were carried out on a Jasco J-810 spectropolarimeter (Jasco Inc.) in the far ultraviolet range (190-260 nm) with a 0.5 mm quartz cuvette at RT (average of 20 scans).

## Cell Culture and RNA Extractions

RNA was extracted from transformed B-cell lymphocytes from both affected and unaffected individuals (controls) using TRIzol-based methods (Thermo-fisher, Cat. #15596026). Osteoblast (hFOB 1.19) and HEK293A cell lines (ATCC) were maintained as adherent cells in Iscove's Modified Dulbecco's Medium (IMDM) F/12 (Life Technologies) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and

antibiotic-antimycotic (100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B as Fungizone®). hFOB 1.19 cells were maintained under the same culture conditions, but without phenol red, and G418 was added to a final concentration of 0.3 mg/ml. Lymphoblastoid cells were maintained in RPMI medium (Life Technologies). Total RNA was extracted using Trizol Reagent and treated with Ambion® TURBO™ DNase (Thermo Fisher). RNA was evaluated and quantified using a 2100 Bioanalyzer (Agilent) for samples with a RIN > 8.5 and cDNA synthesized with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). For mRNA expression, we used RT-PCR and *FOXL1* Taqman primers (L primer, CCTCCCTACAGCTACATCGC; R primer, TGTCGTGGTAGAAGGGGAA; hybrid probe, GGTCACGCTCAACGGCATCTA). *GAPDH* (Hs03929097_g1; Thermo Fisher) was used as an endogenous control (quantified by $\Delta\Delta$CT method and normalized using the Viia7$^{TM}$ system, Thermo Fisher).

## *FOXL1* Expression Constructs

To investigate the effect of the *FOXL1* 15-bp deletion on function, we transfected osteoblast cell line (hFOB1.19) with *FOXL1-WT* and *FOXL1-MUT* expression plasmids. We purchased two expression vector constructs (pReceiver-M02 and pReceiver-M29, cat. no. EX-E0843-M02, EX-E0843-M29-GFP) from GeneCopoeia containing wild type *FOXL1* (NM_005250) and generated two mutant constructs (*FOXL1* c.976_990del) by site directed mutagenesis (NOROCLONE biotech laboratories), using two empty plasmids as mock controls. Transfection conditions were optimized using Fugene HD (Roche) transfection reagent, diluting plasmid with Opti-MEM (Thermo Fisher) to 0.02µg /µl; Fugene HD in the ratio of 7:2 (Fugene HD in µl: plasmid DNA in µg) for 20 minutes

at RT and hFOB 1.19 cells were transfected with 100ul transfection mixture in a 6-well plate and incubated for 48 hrs at 34 C.

## Western Blot

To determine the effect of the *FOXL1* deletion on the quantity and location of Foxl1 protein, we used osteoblast cells transfected with the wildtype and mutant constructs and immunoblotting. *FOXL1* expression was determined using Anti-FOXL1 rabbit polyclonal IgG (ab83000, Abcam) (1µg/ml). Housekeeping proteins were detected with α tubulin (clone DM1A+DM1B, Abcam) (200 µg/ml) and anti-nuclear matrix protein p84 antibody (clone 5E10, Abcam) (1µg/ml). Horse Radish Peroxidase (HRP)-conjugated affiniPure $F(ab)_2$ fragment goat anti-mouse (GAM) IgG, Fc specific and HRP-conjugated affiniPure $F(ab)_2$ fragment goat anti-rabbit (GAR) IgG, and Fc specific antibodies (1:10,000 dilution) were obtained (Jackson Immunoresearch Labs Inc.). Protein lysates were obtained using the Nuclear Extract Kit (ActiveMotif) and protein concentration determined with the Bradford protein assay kit (BioRad). Samples were reduced using 0.2M mercaptoethanol, 10ug of protein per lane and subjected to 8% SDS PAGE. Following electrophoresis, proteins were transferred onto nitrocellulose and blocked with 5% milk powder in TBS-Tween (0.15M NaCl, 0.05 Tris pH 7.4, 0.05% Tween 20). Primary antibodies were incubated at 4$^{o}$C overnight and HRP-conjugated secondary antibodies were used to detect antibody binding. Signals were amplified using a chemiluminescence detection agent (Millipore). Immunoreactivity was visualized by scanning densitometry (Image Quant LAS 4000) and quantified (Image GE software) (GE Healthcare).

Supplementary: In-frame deletion in *FOXL1* causes otosclerosis

## Fluorescence Microscopy

Transfected HFOB1.19 cells were visualized with a Carl Zeiss AxioObserver A.1 microscope with standard fluorescence and brightfield/darkfield settings at X5 0,25 or X20 0,50 NA objectives. Images were captured using a Zeiss AxioCam MRM3 camera with Zeiss AxioVision 4.8 software. GFP-transfected cells were harvested by trypsin, followed by fixation in 1.0% paraformaldehyde (Sigma), and analysis of 5000–10,000 cells using a FACS Calibur flow cytometer (Becton-Dickinson).

## Luciferase Reporter Assay

To determine if the removal of 5 C-terminus residues in FOXL1 alters its ability to activate downstream transcription, we used a luciferase reporter assay. The reporter construct contained two copies of the *FOXL1* Consensus binding sequence [TATACATAAACAAGAA] (Pierrou et al. 1994) cloned into pGL3 (Promega) upstream of the *thymidine kinase* minimal promoter and the luciferase open reading frame (*Photinus*). 1.0 µg of constructs bearing the wildtype, or 15 base-pair mutant ORFs, or an empty expression vector containing no *FOXL1* sequence, were co-transfected into HEK293 cells with 250 ng of *FOXL1* luciferase reporter and 10 ng of constitutively active (*Renilla*) luciferase in 24- well dishes. Luciferase activity was measured using the dual Luciferase Assay kit (Promega) and six wells for each treatment, repeated three times. Ratios of *Photinus* and *Renilla* luciferase were calculated, and wildtype and mutant readings were compared to those from the empty expression vector (which was standardized to 1). Data are graphed as mean fold change +/- SD. A Fluoroskan Ascent (Labsystems) was used for all readings.

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**Supplemental Table 1.** Microsatellite markers genotyped spanning the disease intervals of 8 published OTSC loci and 3 otosclerosis associated genes.
* Markers mapping adjacent to published markers that were uninformative.

| Locus | Location | Disease Interval (Mb) | Microsatellite Markers (**Boundary Marker**) |
|---|---|---|---|
| *OTSC1* | 15q25-q26 | 14.5 | ***D15S127**, D15S652, D15S649, D15S1004, D15S157, **D15S657*** |
| *OTSC2* | 7q34-q36 | 16 | ***D7S495**, D7S684, D7S2202, D7S2513, D7S676, D7S1798, D7S2442, **D7S2426**, D7S1827* |
| *OTSC3* | 6p22.3-p21.3 | 17.4 | ***GAAT3A06**, D6S1660, D6S1545, D6S464, D6S273 D6S1568, D6S291, D6S1602, **D6S1680*** |
| *OTSC4* | 16q22.1q23.1 | 10 | ***D16S3107**, D16S3025, D16S3095, D16S752, D16S3106, D16S3139 D16S3018, D16S3115,**D16S3097*** |
| *OTSC5* | 3q22-p24 | 15.5 | ***D3S1292**, D3S3641, D3S1576, D3S3586, D3S3694, D3S1593, D3S3627, **D3S1744*** |
| *OTSC7* | 6q13-q16.1 | 13.47 | ****D6S467**, D6S280, D6S1596, D6S456, D6S1589, D6S460 D6S1652, D6S1595 D6S1613,***D6S450*** |
| *OTSC8* | 9p13.1-9q21.11 | 34.16 | ***D9S970,** D9S1844, D9S1862, D9S1879, D9S166**, D9S1799*** |
| *OTSC10* | 1q41-q44 | 26.1 | ***D1S2621**, D1S439, D1S2800, **D1S2811*** |
| *COL1A1* | 17q21.33 | - | ***D17S797**, D17S1795, D17S941, D17S809, **D17S788*** |
| *COL1A2* | 7q21.3 | - | ***D7S644**, D7S657, D7S2430, D7S821, **D7S651*** |
| *NOG* | 17q22 | - | ***D17S790**, D17S1607, D17S1606, ***D17S1161*** |

**Supplementary Table 2.** Two-point LOD scores between otosclerosis and microsatellite markers spanning eight *OTSC* loci, three otosclerosis-associated genes and a new *OTSC* locus qter of *OTSC4* (Extended *OTSC4*).

| Locus | Markers | Recombination fraction | | | | | |
|---|---|---|---|---|---|---|---|
| | | **0.000** | **0.100** | **0.200** | **0.300** | **0.400** | **0.500** |
| *OTSC1* | D15S127 | -4.096936 | -0.887153 | -0.387585 | -0.151425 | -0.035454 | 0.000000 |
| | D15S649 | -4.096936 | -0.264114 | -0.108875 | -0.048774 | -0.014267 | 0.000000 |
| | D15S657 | -0.300990 | -0.099513 | -0.029093 | -0.005594 | -0.000348 | 0.000000 |
| *OTSC2* | D7S495 | 0.301029 | 0.212668 | 0.130215 | 0.062729 | 0.016819 | 0.000000 |
| | D7S1798 | -4.096936 | -0.887153 | -0.387585 | -0.151425 | -0.035454 | 0.000000 |
| | D7S1827 | -13.494847 | -1.33082 | -0.581401 | -0.227145 | -0.053183 | 0.000000 |
| *OTSC3* | GAAT3A06 | -3.795907 | -0.887043 | -0.387574 | -0.151424 | -0.035454 | 0.000000 |
| | D6S273 | -3.795907 | -0.887043 | -0.387574 | -0.151424 | -0.035454 | 0.000000 |
| | D6S1680 | -3.795907 | -0.653891 | -0.226226 | -0.062029 | -0.007427 | 0.000000 |
| *OTSC4* | D16S3107 | -4.342935 | -0.766770 | -0.300033 | -0.086144 | 0.002510 | 0.000000 |
| | D16S3106 | -4.342935 | -0.636432 | -0.200119 | -0.038453 | 0.003295 | 0.000000 |
| | D16S3097 | -4.342935 | -0.598769 | -0.218146 | -0.063158 | 0.001094 | 0.000000 |
| Extended | D16S422 | 1.630082 | 1.269478 | 0.884142 | 0.488383 | 0.143531 | 0.000000 |
| *OTSC4* | D16S2625 | 1.630082 | 1.287609 | 0.908845 | 0.502244 | 0.140761 | 0.000000 |
| *(16q24)* | D16S520 | -0.176089 | -0.176583 | -0.153096 | -0.092488 | -0.028034 | 0.000000 |
| *OTSC5* | D3S3548 | 0.301025 | 0.210206 | 0.118188 | 0.044152 | 0.006429 | 0.000000 |
| | D3S1593 | -4.096936 | -0.755097 | -0.299465 | -0.104434 | -0.021215 | 0.000000 |
| | D3S1744 | -3.795907 | -0.653881 | -0.226224 | -0.062028 | -0.007427 | 0.000000 |
| *OTSC7* | D6S1619 | -3.795906 | -0.638142 | -0.218710 | -0.059726 | -0.007201 | 0.000000 |
| | D6S1595 | -4.096936 | -0.264114 | -0.108875 | -0.048774 | -0.014267 | 0.000000 |
| | D6S268 | -3.795906 | -0.638142 | -0.218710 | -0.059726 | -0.007201 | 0.000000 |
| *OTSC8* | D9S2148 | -4.045783 | -0.199700 | -0.047532 | -0.007852 | -0.000417 | 0.000000 |
| | D9S1862 | -13.494847 | -0.672523 | -0.254094 | -0.086982 | -0.018424 | 0.000000 |
| | D9S1799 | -0.300990 | -0.099513 | -0.029093 | -0.005594 | -0.000348 | 0.000000 |
| *OTSC10* | D1S2621 | -4.096936 | -0.264114 | -0.108875 | -0.048774 | -0.014267 | 0.000000 |
| | D1S2800 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| | D1S2811 | -4.096936 | -0.264114 | -0.108875 | -0.048774 | -0.014267 | 0.000000 |
| *COL1A1* | D17S797 | -0.300990 | -0.248075 | -0.151917 | -0.069146 | -0.017365 | 0.000000 |
| | D17S809 | -4.096936 | -0.443629 | -0.193798 | -0.075713 | -0.017727 | 0.000000 |
| | D17S788 | -4.096936 | -0.443629 | -0.193798 | -0.075713 | -0.017727 | 0.000000 |
| *COL1A2* | D7S644 | -3.795907 | -0.013984 | 0.073263 | 0.053196 | 0.016338 | 0.000000 |
| | D7S821 | -3.795902 | 0.025915 | 0.139021 | 0.118035 | 0.047724 | 0.000000 |
| | D7S651 | 0.602028 | 0.465356 | 0.318042 | 0.170249 | 0.049214 | 0.000000 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *NOG* | D17S790 | -4.698990 | -0.691749 | -0.345732 | -0.144865 | -0.035094 | 0.000000 |
| | D17S1606 | -4.096936 | -0.755097 | -0.299465 | -0.104434 | -0.021215 | 0.000000 |
| | D17S1161 | -4.096936 | -0.755097 | -0.299465 | -0.104434 | -0.021215 | 0.000000 |

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