TUFT1, a novel candidate gene for metatarsophalangeal osteoarthritis, plays a role in chondrogenesis on a calcium-related pathway

Eeva Sliz, Mari Taipale, Maiju Welling, Sini Skarp, Viivi Alaraudanjoki, Jaakko Ignatius, Lloyd Ruddock, Ritva Nissi, Minna Männikko.

Abstract

Osteoarthritis (OA) is the most common degenerative joint disorder and genetic factors have been shown to have a significant role in its etiology. The first metatarsophalangeal joint (MTP I) is highly susceptible to development of OA due to repetitive mechanical stress during walking. We used whole exome sequencing to study genetic defect(s) predisposing to familial early-onset bilateral MTP I OA inherited in an autosomal dominant manner. A non-synonymous single nucleotide variant rs41310883 (c.524C>T, p.Thr175Met) in TUFT1 gene was found to co-segregate perfectly with MTP I OA. The role of TUFT1 and the relevance of the identified variant in pathogenesis of MTP I OA were further assessed using functional in vitro analyses. The variant reduced TUFT1 mRNA and tuftelin protein expression in HEK293 cells. ATDC5 cells overexpressing wild type (wt) or mutant TUFT1 were cultured in calcifying conditions and chondrogenic differentiation was found to be inhibited in both cell populations, as indicated by decreased marker gene expression when compared with the empty vector control cells. Also, the formation of cartilage nodules was diminished in both TUFT1 overexpressing ATDC5 cell populations. At the end of the culturing period the calcium content of the extracellular matrix was significantly increased in cells overexpressing mutant TUFT1 compared to cells overexpressing wt TUFT1 and control cells, while the proteoglycan content was reduced. These data imply that overexpression of TUFT1 in ATDC5 inhibits chondrogenic differentiation, and the identified variant may contribute to the pathogenesis of OA by increasing calcification and reducing amount of proteoglycans in the articular cartilage extracellular matrix thus making cartilage susceptible for degeneration and osteophyte formation.
Introduction

Osteoarthritis (OA) is one of the most common musculoskeletal disorders worldwide and its prevalence is predicted to increase in the future [1]. OA is a disease of the whole joint [2] and the main pathologic changes are progressive loss of articular cartilage, joint space narrowing, osteophyte formation, subchondral bone sclerosis, and cyst formation [3]. These lead to pain and loss of joint function in OA patients [4]. During the development of OA chondrocytes start to proliferate and express matrix-degrading enzymes leading to matrix remodeling involving hypertrophic maturation of chondrocytes and calcification of cartilage [5]. In general, OA is considered as a complex trait caused by interplay between genetic and environmental factors [6]. In twin studies the influence of genetic components has been predicted to be as high as 39–65% depending on joint site [7]. Although OA generally lacks a clear Mendelian pattern of inheritance, rare familial early-onset forms with autosomal dominant inheritance have been described [8–11].

Foot OA is poorly studied in comparison to hip or knee OA, although the first metatarsophalangeal (MTP I) joint is often affected by OA [12]. The prevalence of radiographic MTP I OA has been estimated to be from 6.3% to 39% in middle-aged and older adults [13]. Foot OA shares many risk factors with other forms of OA, like age and obesity. Also, mechanical stress, trauma and inflammation are often associated with foot OA [12]. Individuals with symptomatic MTP I OA experience localized pain and stiffness during movement and therefore have difficulties in various physical tasks such as walking [14].

Nissi et al. (2011) reported a family with early-onset bilateral foot arthritis limited to the MTP I joint [15]. The family history strongly suggests an autosomal dominant inheritance. In order to identify the genetic defect(s) predisposing to this disorder, we performed whole exome sequencing on the aforementioned family and identified a variant in \textit{TUFT1} co-segregating with the disease. \textit{TUFT1}, encoding tuftelin protein, is previously known to be involved in enamel mineralization [16] and is recognized as a candidate gene for dental caries [17], but is suggested to have a universal or multifunctional role due to its expression in multiple cell and tissue types [18–20]. In cartilage, \textit{TUFT1} expression is strongest in the deeper, mineralizing zones [21]. Interestingly, \textit{TUFT1} expression has been shown to be regulated \textit{in vitro} by hypoxia-inducible factor 1-alpha and hedgehog pathways, both essential for cartilage and bone formation [22,23]. However, the precise function of tuftelin is not fully known. We show that \textit{TUFT1} participates in regulation of chondrocyte differentiation and that the identified variant gives rise to extracellular matrix (ECM) modifications observed in association with OA. We propose that \textit{TUFT1} is a novel candidate gene for foot OA and that the identified variant is likely to be involved in the pathogenesis of foot OA in the studied family.

Results

Whole exome sequencing

Three affected individuals from a Finnish family with MTP I OA (Fig 1) and two unrelated individuals (controls) were analyzed using whole exome sequencing. Summary of the exome sequencing and variant annotation is shown in Table 1. The three patients shared in total 48 860 single nucleotide variants (SNVs) and 4 670 insertions and deletions (indels). Following the variant filtering steps, altogether 33 SNVs and five indels (S1 Table) were selected for validation by Sanger sequencing in all nine family members whose DNA was available for the study (four affected, five unaffected, Fig 1). The variant rs41310883 (c.524C>T) in \textit{TUFT1} was found heterozygous in all four patients, but was not detected in healthy family members, being
the only variant perfectly co-segregating with the MTP I OA in the family. The variant introduces a threonine to methionine substitution at position 175 (p.Thr175Met, NP_064512).

Detection of Copy Number Variations (CNVs)

CNVs calls were generated from exome data to screen possible structural genome variations such as deletions and duplications. Altogether 160 CNVs were called in chromosomes 1–22. Of these, 142 were deletions and 18 were amplifications. The three affected family members had a mutual CNV region on chromosome 17 where two patients (II-4 and III-1) showed a heterozygous deletion and one patient (III-2) had an amplification (data not shown). Annotation to the Database of Genomic Variants showed that this region has been previously reported to be a common CNV region [24].

The effect of c.524C>T on mRNA and protein expression

The function of the identified variant was studied in vitro in human embryonic kidney (HEK293) cells. Cells transfected with the mutant TUFT1 construct (HEK293-mutTUFT1) generated less (P < 0.001) TUFT1 mRNA than cells transfected with the wt TUFT1 construct (HEK293-wtTUFT1, Fig 2A). Consistent with this, the variant attenuated tuftelin protein expression (Fig 2B and S1 Fig).

Table 1. Summary statistics for exome sequence data analysis steps.

|                                | SNV count | Indel count |
|--------------------------------|-----------|-------------|
| Autosomal variants shared by the three affected family members | 48 860    | 4 670       |
| Shared by the affected family members, not found in the controls | 5 794     | 525         |
| Novel and rare variants (MAF≤0.01 or unknown) | 739       | 366         |
| Exonic or splicing            | 143       | 12          |
| Harmful in silico prediction  | 33        | 5           |
| Co-segregates with MTP I OA*  | 1         | 0           |

SNV, single nucleotide variant; indel, insertions and deletions, *validation by Sanger sequencing in nine family members.

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The role of TUFT1 overexpression in chondrocyte differentiation

To study the functional role of tuftelin in chondrogenesis and mineralization, ATDC5 cells stably overexpressing wt or mutant tuftelin (ATDC5-wtTUFT1 or ATDC5-mutTUFT1, respectively) and empty vector controls (ATDC5-ctrl) were grown in calcifying conditions in the presence of insulin, ascorbic acid and beta-glycerophosphate (βGP) for 15 days. Expression of three marker genes for chondrogenic differentiation, sex determining region Y-box 9 (Sox9), type II collagen (Col2a1), aggrecan (Agc1), and four marker genes for chondrocyte hypertrophy, runt-related transcription factor 2 (Runx2), type X collagen (Col10a1), matrix metalloproteinase 13 (Mmp13), and alkaline phosphatase (Alpl) were studied at three time points using real-time quantitative polymerase chain reaction (qPCR).

Overexpression of both wt and mutant TUFT1 significantly influenced the expression of Col2a1 and Agc1 (P = 2.95x10^{-15} and P = 1.86x10^{-12}, respectively, Fig 3A, S2 Table), but did not affect the expression of Sox9 (P = 0.461, Fig 3A, S2 Table): in ATDC5-mutTUFT1 cells Col2a1 expression differed from control cells on all the measurement days whereas in ATDC5-wtTUFT1 cells the difference reached statistical significance on days 8 and 15 (S3 Table). Additionally, Col2a1 expression was significantly lower in ATDC5-mutTUFT1 cells in comparison to ATDC5-wtTUFT1 cells on days 12 and 15 (P = 0.008 and P = 0.045, respectively, Fig 3A, S3 Table). Agc1 expression in both ATDC5-mutTUFT1 and ATDC5-wtTUFT1 cells deviated from the expression seen in control cells, while there was no statistically significant difference between the mutant and wt cells (S3 Table).

No statistically significant differences were observed in the Runx2 expression on any of the days (Fig 3B, S3 Table). Col10a1 expression showed a similar trend in ATDC5-ctrl and ATDC5-wtTUFT1 cells. On day 15 expression of Col10a1 in the ATDC5-mutTUFT1 cells was significantly lower when compared to the ATDC5-wtTUFT1 cells (P = 0.007, Fig 3B, S3 Table). The expression of Mmp13 was significantly lower in both ATDC5-wtTUFT1 and ATDC5-mutTUFT1 cells in comparison to the control cells on day 15 (P = 2.09x10^{-5} and P = 0.011, Fig 3B, S3 Table). The expression of Alpl in ATDC5-mutTUFT1 cells was significantly higher in control cells already on day eight (P = 0.005, Fig 3B, S3 Table), whereas on day
The Alpl expression in ATDC5-mutTUFT1 cells was significantly lower than in control cells (P = 2.12x10^-4, Fig 3B, S3 Table).

Expression of TUFT1 was assessed at three time points (Fig 3C). Comparable with the study done in HEK293 cells, TUFT1 expression was lower in the ATDC5-mutTUFT1 cells than in the ATDC5-wtTUFT1 cells at all time points. All three cell populations showed an increasing trend of TUFT1 expression during the 15-day period. Results of gene expression studies in ATDC5 cells are presented in Fig 3, and the test statistics are summarized in S2 and S3 Tables.

**Contribution of TUFT1 overexpression to extracellular matrix mineralization and nodule formation**

Calcium and proteoglycan content in the extracellular matrix (ECM) of the ATDC5 cells were studied at three time points along the 15-day culture period using Alizarin red and Alcian blue stainings, respectively. In general, the contents of both calcium (Fig 4A) and proteoglycans (Fig 4B) in the ECM of both ATDC5-mutTUFT1 and ATDC5-wtTUFT1 cells deviated from what was seen in control cells at the three time points. The most evident deviation was the significant increase in the calcium content in the ECM of ATDC5-mutTUFT1 cells on day 15.
On the contrary on day 15 the ATDC5-mutTUFT1 cells showed reduced amount of proteoglycans in the ECM when compared with the control and ATDC5-wtTUFT1 cells (Fig 4B).

During the differentiation experiment, control cells begin to form cartilaginous nodules and ECM mineralization centres, seen as concentrated black deposits in Fig 5A. Nodule formation in ATDC5-wtTUFT1 cells was impaired when compared with control cells, and no mineralization centres were observed (Fig 5B). Nodule formation was also impaired in the ATDC5-mutTUFT1 cells and mineralization occurred disorderly throughout the cell culture (Fig 5C) compatible with the observed increase in the ECM calcium (Fig 4A).

Discussion

In the present study we identified a rare nonsynonymous variant (rs41310883, c.524C>T, p.Thr175Met) in TUFT1 that co-segregates with MTP I OA in a Finnish family. TUFT1 encodes tuftelin protein which plays a role in enamel mineralization [16,17], but is also suggested to have a more universal function due to its wide expression in multiple cell and tissue types [18–20]. Our in vitro experiments showed that the c.524C>T variant decreased both TUFT1 mRNA and tuftelin protein expression in TUFT1 overexpressing HEK293 cells, and
altered the calcium and proteoglycan content in the ECM of ATDC5 cells. Interestingly, over-expression of both wt and mutant TUFT1 in ATDC5 cells altered chondrogenic differentiation, as indicated by atypical expression of differentiation marker genes and diminished formation of cartilage nodules. Our findings suggest that TUFT1 plays a role in chondrocyte differentiation and cartilage mineralization apparently on a calcium-related pathway, and the identified variant likely contributes to the disease phenotype in the studied family by giving rise to cartilage ECM modifications often seen in association with OA.

The identified c.524C>T variant was found to alter the amount of calcium and proteoglycans in the ECM of ATDC5 cells over a 15-day culture period suggesting that the mutation alters cell functions involved in the assembly of ECM. Particularly the highly increased extracellular calcium on day 15 was the distinguishable feature that seemed to be due to the c.524C>T variant rather than due to TUFT1 overexpression. The variant introduces an amino acid residue that is larger in size and more hydrophobic than the wt residue likely disrupting proper folding of the protein [25]. The mutation also resides in one of the two coiled-coil domains in tuftelin which may also contribute to altered function of tuftelin, as coiled-coil domains are important for proper protein folding and known to be crucial in multiple biological functions [26]. Furthermore, the mutation locates on one of tuftelin’s ten evolutionarily conserved phosphorylation sites that are predicted to function in chelation of calcium [27].

The observed effect of the mutation could be due to tuftelin’s altered affinity to calcium or due to improper folding disturbing protein-protein interactions or due to both of these. Increased calcification and degraded proteoglycans are distinctive features of osteoarthritic cartilage [5,28], and therefore it seems likely that the identified mutation is involved in the pathogenesis of the MTP I OA in the studied family.

Seemingly TUFT1 overexpression alone is sufficient to interfere with chondrogenesis, as we discovered that in addition to ATDC5-mutTUFT1 cells chondrogenic differentiation was diminished also in ATDC5-wtTUFT1 cells. Both of the cell populations showed a decreasing trend of chondrogenic differentiation marker genes Col2a1 and Agc1 during the 15-day culture period while in the control cells the expression strengthened over time, as expected. In addition, in the beginning of chondrogenesis mesenchymal precursor cells should start to condensate to form nodules [29], whereas we observed that the nodule formation was diminished in both of the TUFT1 overexpressing ATDC5 cell populations. This advocates that proper timing and/or quantity of TUFT1 expression is crucial for the differentiation to proceed appropriately.

Similar findings were obtained in a study done on mice, which indicated that tuftelin overexpression disturbed the growth of carbonated calcium hydroxyapatite crystallites in developing enamel and the phenotype was most evident in mice with the greatest tuftelin expression [30].

Tuftelin appears to function at the very beginning of the differentiation process: we observed that the differentiation marker gene expression as well as the ECM mineralization in both ATDC5-mutTUFT1 and ATDC5-wtTUFT1 cells deviates from what is seen in the control cells already on day eight. A highly similar phenotype with impaired marker gene expression and diminished nodule formation during differentiation has previously been described in mouse chondrocytes with inactivated Sox9 [31,32]. However, we did not detect significant difference in the expression of Sox9 between the ATDC5-mutTUFT1, ATDC5-wtTUFT1 and ATDC5-ctrl cells suggesting that the described phenotype arises downstream from Sox9 expression. We hypothesize that TUFT1 overexpression inhibits differentiation signaling downstream from Sox9 by altering cells’ calcium metabolism. This could further interfere the normal function of calcium-dependent adhesion molecule N-cadherin which is expressed in the early chondrogenesis following Sox9 expression, but prior to expression of Col2a1 and Agc1 [33]. During chondrogenesis N-cadherin plays a role in cell aggregation [34,35] an event which has been proposed to be a crucial step in the early phase of the differentiation cascade,
influencing also COL2A1 expression in vitro in chick chondrocytes [36]. Assessing the effect of the identified mutation in relation to the function of N-cadherin could be an interesting topic for further studies.

This study has some limitations. Whole exome sequencing allows us to detect only the coding and surrounding region variants, while non-coding variations are missed. Regulatory regions locating in non-coding intronic and intergenic regions have been associated with OA (11) as well as with many other complex diseases [37–39]. However, it has been estimated that approximately 85% of mutations with large phenotypic effects are located on protein coding regions, and thus Mendelian traits result most often from exonic or splice-site mutations [40]. Another limitation is that only one family was available to the study. Unfortunately, population samples available from the same geographical area as the studied family, such as the Northern Finland Birth Cohorts (NFBC, http://www.oulu.fi/nfbc/), do not provide a suitable phenotype (radiographically determined arthritis of the first metatarsophalangeal joint) to perform a population level replication, nor does the chip-based genotype data from NFBC allow replication of rare variants, as they are excluded from the data for technical reasons. Disease symptoms limited to the MTP I joint may partially be explained by joint loading or site specific methylation profiles [41,42]. An animal model could inform more on tuftelin’s function during development of MTP I OA, but would be challenging to implement due the specific characteristics of human foot anatomy and impact of physical loading and environmental factors on the disease phenotype. A genome editing method, such as CRISPR-Cas9, would need to be applied to generate a mouse model with the specific TUFT1 mutation, as TUFT1 knockout mice do not display deformities in joints, bones or enamel [43]. A genome editing method could be also applied to generate an in vitro model that would help to better distinguish the effect of the identified mutation from TUFT1 overexpression.

Results of the present study implicate that TUFT1 plays a role in chondrocyte differentiation and cartilage mineralization apparently on a calcium-related pathway, and thus can be considered as a novel susceptibility gene for MTP I OA. Our findings also advocate that investigation of rare variants and familial forms of complex phenotypes can provide valuable information about pathogenic mechanisms behind common diseases, such as OA. Further studies are needed to determine whether TUFT1 variants associate to cartilage phenotypes on population level and to elucidate the precise biological function of tuftelin in chondrogenesis and mineralization of cartilage.

Materials and methods

Subjects

This study was approved by the Ethical Committee of the Northern Ostrobothnia Hospital District and a written consent was obtained from the study participants. Nissi et al. (2011) reported a Finnish family where early-onset MTP I OA is inherited in an autosomal dominant manner [15]. Thirteen out of 52 family members were affected (Fig 1). The mean age of onset was 26, ranging from 12 to 51. The radiological findings are described in detail by Nissi et al. [15]. Briefly, the X-ray images of the affected family members revealed typical OA related findings in the first MTP joint including joint space narrowing, subchondral sclerosis, osteophyte and cyst formation. Otherwise the other joints of the feet were healthy and there was no evidence of erosive lesions. No other deformities in the skeletal body were detected and large joints were not affected even in the older family members. Affected family members were otherwise healthy and had normal body height and weight. Also, individuals participating to the present study did not display any self-reported dental phenotype.
All the family members were contacted and interviewed. Four patients (II-4, III-1, III-3, III-12) having symptomatic and radiographic bilateral OA in addition to five asymptomatic family members (III-2, III-5, III-6, III-10, III-19) were willing to participate to the study.

Whole exome sequencing
Three affected family members (II-4, III-1 and III-12) were selected for exome sequencing. In addition, two unrelated individuals were exome sequenced and used as control samples in the variant filtering steps. Blood samples were obtained from the participants and DNA was extracted using standard protocols. Exome sequencing and variant calling were done at the Institute for Molecular Medicine Finland (FIMM). Exonic sequences were enriched using the NimbleGen SeqCap EZ Human Exome Library v2.0 (Roche, NimbleGen, Inc., Madison, USA), and sequencing was done using a High Seq 2000 platform (Illumina, San Diego, USA). To ensure the quality of the variants, the exome data were taken through the FIMM bioinformatics pipeline [44].

Variant annotation and filtering
Exome sequence data was filtered based on three assumptions. The causal variant was assumed to fit to the autosomal dominant model of inheritance as appeared in the pedigree. Furthermore, the causal variant(s) was/were presumed to be \textit{de novo} or rare, since to our knowledge no similar families were previously described in the literature. Penetrance was assumed to be 100\%, and therefore all the individuals having the disease genotype were presumed to be symptomatic. Variant filtering was done in R by selecting the variants shared by the affected family members and then excluding the variants found in the two unrelated control samples. Next, the variants previously not annotated into the Single Nucleotide Polymorphism Database 135 (dbSNP135), rare variants according to the 1000 Genomes Project (minor allele frequency \leq 0.01) and variants with a record in dbSNP but with unknown frequency were annotated using ANNOVAR [45]. Exonic variants with harmful prediction by SIFT [46], PolyPhen 2 [47] or MutationTaster [48] algorithms and variants located at splice sites were considered harmful and chosen for further validation.

Variant validation using Sanger sequencing
The selected variants were genotyped in all family members with DNA available using Sanger sequencing. Primers used in Sanger sequencing were designed using Primer3 v0.4.0 [49]. Primer sequences and PCR conditions are available on request. Purified PCR products were sequenced using ABI3500xL Genetic Analyzer and BigDye Terminator vs.1.1 reagents (Life Technologies, Carlsbad, USA). The results were analyzed using Variant Reporter version 2.0 (Life Technologies, Carlsbad, USA).

Detection of CNVs
Detection of CNVs from whole exome data was done using a read count based CNV caller cnMOPS [50]. CNVs regions shared by the samples were annotated using Database of Genomic Variants [51] to identify common CNVs.

\textit{TUFT1} constructs
A \textit{TUFT1} cDNA clone was obtained from GenScript and cloned into pcDNA3.1 (-) expression vector (Invitrogen, Carlsbad, USA) using BamHI and XbaI restriction enzymes. The \textit{TUFT1} variant c.524C>T was generated using QuikChange Site-Directed Mutagenesis Kit
(Stratagene, San Diego, USA) according to the manufacturer’s instructions. All sequences were confirmed by capillary sequencing.

**Cell culture and transfections**

HEK293 cells were cultured and maintained in DMEM with 10% FBS and plated in 10cm-plates at a density of 2 x 10^6 cells/plate for protein analyses and in 6-well plates at a density of 3.5 x 10^5 cells/well for real-time qPCR analyses 24h prior to transient transfections. Transfections for protein and qPCR analysis were performed using 5.6 μg DNA (wt or mutant TUFT1 construct) and 40 μl FuGENE HD transfection reagent (Promega, Madison, USA) or 2 μg DNA and 14 μl FuGENE HD transfection reagent, respectively.

Mouse ATDC5 cells are widely used to model of chondrogenic differentiation and subsequent mineralization [52,53]. We applied a model for ATDC5 cells developed by Newton et al. allowing us to study chondrogenesis and ECM mineralization in a 15-day period [54]. ATDC5 cells (Sigma, St Louis, USA) were cultured in maintenance medium DMEM/F-12 (1:1) with GlutaMAX I (Gibco, Paisley, Scotland) containing 5% FBS, 1% sodium pyruvate, 0,1% penicillin (Sigma, St Louis, USA) and 0,01% Fungizone (Cambrex Bio Science, Walkersville, MD). For stable transfections 3.5 x 10^5 cells/well were plated on six-well plates and transfected at a ratio of 7:2 FuGENE HD transfection reagent (Promega, Madison, USA) to 2 μl of DNA according to the manufacturer’s instructions. Empty pcDNA3.1 (-) was used as a control. After 24h the medium was replaced with fresh maintenance medium supplemented with 500 μg/ml Geneticin, G418 (Sigma, St Louis, USA). The selection medium was changed every second or third day until all of the cells without the neomycin resistance gene on the on a separate control plate were killed. Cell death was evaluated using light microscopy (Leica Microsystems, Wetzlar, Germany). A mixed population of stable G418 transfectants was expanded and cultured in differentiation medium (maintenance medium supplemented with 1x insulin-transferrin-selenium, 500 μg/ml Geneticin). The cells were plated in 24-well plates at a density of 5.0 x 10^4 cells/ml. After the cells had reached confluency, 50 μg/ml ascorbic acid and 10 mM βGP were added and the cells were incubated in a humidified atmosphere (37˚C, 5% CO2) for 15 days.

During differentiation chondrocytes are expected to form mineralizing cartilage nodules that can be detected as opaque deposits in microscopy images [54,55]. Nodule formation of ATDC5 was determined, and pictures were taken after 15 days of differentiation using EVOS fluorescence microscope.

**Real-time qPCR**

Preceding to qPCR, total RNA was extracted from cells using an E.Z.N.A Total RNA Kit (Omega Bio-Tek, Norcross, USA) with RNase-free DNase (Omega Bio-Tek, Norcross, USA) treatment and cDNA was synthesized using 1 μg RNA per sample using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA). The effect of the c.524C>T on TUFT1 expression was studied in HEK293 cells at one time point from three duplicate samples using an iTaq Universal SYBR Green Supermix kit (Bio-Rad, Hercules, USA) according to the manufacturer’s instructions in a CFX96 Real-Time System (Bio-Rad, Hercules, USA). ΔCt was determined by using ACTB (beta actin) and B2M (beta-2-microglobulin) as reference genes.

In chondrogenic differentiation mesenchymal progenitor cells proliferate and differentiate into chondrocytes. During this step, chondrocytes express Sox9 transcription factor and number of cartilage matrix genes, including Col2a1 and Agc1. This is followed by endochondral ossification when chondrocytes lose their differentiated phenotype, and became hypertrophic and mature chondrocytes. In this phase cells start to express matrix metallopeptidase 13, a
cartilage degrading enzyme encoded by Mmp13, alkaline phosphatase (Alpl) which is an important enzyme for mineralization, runt related transcription factor 2 (Runx2) involved in regulation of skeletal gene expression and type X collagen (Col10a1), a specific marker for hypertrophic chondrocytes (reviewed in [56] and [57]). To analyze the expression of these chondrogenic and hypertrophic markers in chondrocytes, a real-time qPCR was carried out on four samples in duplicate on days 8, 12 and 15 and three technical repeats were performed. The gene expression levels were determined by the comparative C\textsubscript{T} \( \left(2^{\Delta \Delta C_{T}}\right) \) method [58] using Hprt (hypoxanthine guanine phosphoribosyl transferase) and Ppia (peptidylprolyl isomerase A) as reference genes.

Oligonucleotide primers used in real-time qPCR amplification are listed in S4 Table. For each primer no template controls were included in duplicate.

**Preparation of cell lysates and Western blotting**

After 48h of culturing, the growth media of HEK293 cells transiently transfected with wt or mutant TUFT1 were collected (cell medium fraction, CM) and the cells were washed with ice cold phosphate buffered saline (PBS). Buffer 1 (10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% TritonX-100, 1 mM DTT, 1 × protease inhibitor (cOmplete ULTRA Tablets, mini, Roche, Mannheim, Germany)) was added to the plates and the cells were scraped off. Next, the cells were incubated 30 min with constant agitation at 4°C. The samples were centrifuged and the supernatants were collected (cytosolic fraction, CF). The pellet was washed with PBS and the cells were resuspended in buffer 2 (20 mM Hepes (pH 7.9), 400 mM NaCl, 0.25 mM EGTA, 1.5 mM MgCl\textsubscript{2}, 10% glycerol, 0.5 mM DTT, 1 × protease inhibitor (cOmplete ULTRA Tablets, mini, Roche, Mannheim, Germany)) and incubated a further 20 min with constant agitation at 4°C. The samples were centrifuged and supernatants collected (nuclear fraction, NF).

To analyze the effect of the c.524C>T on tuftelin expression and localization, 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared. For electrophoresis 1 μg of total protein was analyzed under reducing conditions. Proteins separated on the gels were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, USA), which were blocked with 1×TBS (50 mM Tris-Cl, 150 mM NaCl, pH 7.5) with 0.05% Tween 20 (Merck, Darmstadt, Germany) containing 5% non-fat dry milk and probed with the primary antibody (1:2000 Anti-TUFT1 (86–100) antibody produced in rabbit (Sigma, St Louis, USA) in 1xTBS). Anti-beta Tubulin produced in rabbit (Abcam, Cambridge, UK) was used as a loading control. To visualize the immune complex 1:10 000 Anti-Rabbit IgG—Peroxidase antibody produced in goat in 1xTBS (Sigma, St Louis, USA) was used. The imaging of Western blot membranes was performed using Luminescent Image Analyzer LAS-3000 (FUJI-FILM Medical Systems, USA) and the signal densities were quantified using ImageJ 1.47v (National Institutes of Health, USA).

**Histochemical staining of ATDC5 cells**

Calcium and proteoglycan content of mixed populations of ATDC5-ctrl, ATDC5-wtTUFT1, and ATDC5-mutTUFT1 cells were determined by staining the cell layers with Alizarin red stain (Sigma, St Louis, USA) or Alcian blue stain (Sigma, St Louis, USA), respectively. Cells were grown 15 days after their mineralization state was analyzed. To evaluate calcium concentration, ATDC5 cells were fixed with 4% paraformaldehyde (PFA) for 5 min at 4°C, washed with PBS, stained with 2% Alizarin red (pH 4.2) for 5 min in room temperature, washed with distilled water and bound dye was extracted with 10% cetylpyridium chloride for 10 min. Optical density (OD) of the samples was determined at 570 nm by spectrophotometry.
Proteoglycan content was analyzed by washing ATDC5 cells with PBS, fixing with 95% methanol for 20 min, staining with 1% Alcian blue 8GX (Sigma, St Louis, USA) in 0.1M HCl overnight and rinsing with distilled water. Cell cultures were extracted with 6M guanidine-HCl for 6h at room temperature and the released dye was evaluated by measuring the OD at 630 nm by spectrophotometer.

Statistical analysis
Differences in TUFT1 expression in HEK293 cells as well as in ECM mineralization characterized by Alizarin red Alcian blue stainings were analyzed using Student’s t-test. Differences in marker gene expressions between ATDC5-ctrl, ATDC5-wtTUFT1, and ATDC5-mutTUFT1 cells were determined using repeated measurements analysis of variance (ANOVA) followed by Tukey’s Honest Significant Difference (HSD) post hoc test. Prior to ANOVA and Tukey HSD, fold changes were log(x+1) transformed. P-values smaller than 0.05 were considered statistically significant in all the analyses. Statistical analyses were performed using Microsoft Excel 2013 (Student’s t-test for TUFT1 expression in HEK293 cells) and R version 3.2.2. (repeated measurement ANOVA, Tukey HSD and Student’s t-test for ECM mineralization).

Supporting information
S1 Fig. The original uncropped Western blot image. 1 μg of total protein was used in the preparation of Western blot. Anti-TUFT1 and Anti-beta Tubulin primary antibodies and Anti-Rabbit IgG—Peroxidase secondary antibody were used in the experiment. (TIF)

S1 Table. Variants selected for validation by Sanger sequencing. (DOCX)

S2 Table. Expression of marker genes for chondrocyte differentiation and hypertrophy (real-time qPCR): Repeated measurements ANOVA. Measurements were done separately for ATDC5-ctrl, ATDC5-wtTUFT1 and ATDC5-mutTUFT1 cell populations (CELL) at three time points (DAY). CELL:DAY interaction indicates whether the relative gene expression level changes over time in differing manner in the three cell populations. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001. (DOCX)

S3 Table. Expression of marker genes for chondrocyte differentiation and hypertrophy (real-time qPCR): Tukey HSD. Pairwise comparisons of the marker gene expression levels in the three cell populations at the three time points. (DOCX)

S4 Table. Primers used in qPCR analyses. (DOCX)

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Author Contributions
Conceptualization: MM RN MT ES JI.
Formal analysis: ES MT MW SS.

Funding acquisition: MM.

Investigation: ES MT MW SS VA LR.

Methodology: MT ES LR.

Project administration: MM MT ES.

Resources: RN JI.

Supervision: MM LR.

Visualization: MT ES.

Writing – original draft: MT ES MW SS.

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