Mutations in *MAP3K7* that alter the activity of the TAK1 signalling complex cause frontometaphyseal dysplasia

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Abstract

Frontometaphyseal dysplasia (FMD) is a progressive, sclerosing skeletal dysplasia affecting the long bones and skull. The cause of FMD in some individuals is gain-of-function mutations in *FLNA*, although how these mutations result in a hyperostotic phenotype remains unknown. Approximately one half of individuals with FMD have no identified mutation in *FLNA* and are phenotypically very similar to individuals with *FLNA* mutations except for an increased tendency to form keloid scars. Using whole exome sequencing and targeted Sanger sequencing in 19 FMD individuals with no identifiable *FLNA* mutation, we identified mutations in two genes - *MAP3K7,* encoding transforming growth factor β (TGFβ)-activated kinase (TAK1) and *TAB2,* encoding TAK1-associated binding protein 2 (TAB2). Four mutations were found in *MAP3K7*, including one highly recurrent (*n*=15) *de novo* mutation (c.1454C>T, p.Pro485Leu) proximal to the coiled-coil domain of TAK1, and three missense mutations affecting the kinase domain (c.208G>C, p.Glu70Gln; c.299T>A, p.Val100Glu and c.502G>C, p.Gly168Arg). Notably these latter three subjects had a milder FMD phenotype. A further *de novo* mutation was found in *TAB2* (c.1705G>A, p.Glu569Lys). The recurrent mutation does not destabilize TAK1, or impair its ability to homodimerise or bind TAB2, but it does increase TAK1 autophosphorylation and alters the activity of more than one signalling pathway regulated by the TAK1 kinase complex. These findings show that dysregulation of the TAK1 complex produces a close phenocopy of FMD caused by *FLNA* mutations. Furthermore they suggest that the pathogenesis of some of the filaminopathies caused by *FLNA* mutations may be mediated by misregulation of signalling co-ordinated through the TAK1 signalling complex.

Introduction

Frontometaphyseal dysplasia (FMD; MIM 305620) is a progressive, sclerosing skeletal dysplasia characterised by supraorbital hyperostosis, under-modelling of the long bones, small and large joint contractures and other extra-skeletal developmental abnormalities, most notably of the cardiorespiratory system and genitourinary tract.[1] An X-linked form of FMD has been clearly defined and in this condition males manifest most prominently although females can express a milder phenotype that includes hyperostosis and limb anomalies.[2,3] The severity of the disorder in males can extend from normal stature with near-normal adaptive functioning to perinatal lethality, principally due to the consequences of the extraskeletal malformations.[3] The hyperostosis observed in FMD is not associated with bone fragility and the excess cortical bone has the histological appearance of mature lamellar bone.

Gain-of-function mutations in *FLNA* (MIM 300017), located on the X chromosome and encoding the actin binding protein filamin A, account for FMD in ~50% of individuals.[3] This form of the condition is allelic to a spectrum of related phenotypes known as the otopalatodigital spectrum disorders (OPDSD).[2,4] The mutations leading to OPDSDs are principally missense and small in-frame deletions and insertions that are clustered throughout the coding regions of *FLNA*, with some being highly recurrent.[2,5,6] Many of these mutations, including some leading to FMD,[3] result in the substitution of residues in the N-terminal actin binding domain of the protein. The effect of FMD-associated mutations on the function of this domain has not been explicitly addressed but a recurrent missense mutation leading to the allelic disorder otopalatodigital syndrome type 2 (OPD2; MIM 304120) confers enhanced avidity for actin.[7] How this activity results in malformations in addition to hyperostosis is unknown, but the mechanism could relate to one or more of the many and varied biochemical functions of filamin A that include engagement with integrin-mediated cell-cell adhesion,[8-10] cytoskeletal remodelling,[11,12] cell spreading and migration,[13] mechanotransduction[14,15] and influencing many cell signalling pathways through physical interactions with a multiplicity of second messenger proteins.[16]

Around 50% of individuals with a diagnosis of FMD have no demonstrable mutation in *FLNA*.[3,17] These individuals are almost indistinguishable phenotypically from those with *FLNA*-mutation positive FMD[3,17] with one notable difference being the preponderance for individuals who do not have an identifiable *FLNA* mutation to develop keloid scarring. Unlike X-linked FMD, the severity of the phenotype does not vary between the sexes although females are more likely to develop keloid, similar to the presentation of this sporadically-arising cutaneous condition.[17] The majority of individuals with the FMD phenotype not explained by a *FLNA* mutation are isolated cases in their families; only rare instances of vertical transmission of the phenotype have been reported.[3,17,18]

Here we analyse 19 individuals with FMD who do not have an identifiable *FLNA* mutation, and find mutations in two genes encoding components of the transforming growth factor β (TGFβ)-activated kinase (TAK1) signalling complex in all subjects. Strikingly, 15 individuals have a recurrent mutation in *MAP3K7* (MIM 602614), which encodes for TAK1. We show that these mutations result in enhanced TAK1 autophosphorylation and alteration of MAPK and NF-κB signal transduction emanating from this protein complex. Three further individuals have missense mutations in the region of *MAP3K7* that encodes the kinase domain. These mutations produce a notably milder phenotype which suggests a domain-specific phenotype-genotype correlation in this form of FMD. These results place TAK1 as a key regulator of several cell signalling pathways that co-ordinately regulate osteogenesis.

Materials and Methods

Subject Ascertainment and Ethical Approval

Individuals with FMD were recruited by physician-initiated referral and consented to participate under approved protocols MEC/08/08/094 and 13/STH/56 (Health and Disability Ethics Committee, New Zealand). Subjects were diagnosed with FMD based on clinical and radiological assessment according to published criteria.[3] Sanger sequencing of *FLNA* did not detect any mutations. Where possible, DNA was also collected from parents and siblings and familial relationships were confirmed as declared by the examination of the segregation of six unlinked microsatellite markers.

Whole exome sequencing

Identification of candidate genetic variants was initially performed using exome sequence data from parents-proband trios for four simplex individuals (01, 02, 05 and 17). Further Sanger and exome sequencing was carried out on samples from the remaining 15 subjects. Genomic DNA was extracted from peripheral blood using standard protocols. Parental DNA was available from 10 families.

For three trios (subjects 01, 02 and 05, and their parents), exome enrichment was performed using the Agilent SureSelect Human All Exon V4+UTRs capture kit (Agilent, CA, USA), and paired-end sequencing (generating 100 basepair reads) was performed on the Illumina (CA, USA) HiSeq2000 sequencing platform. Sequencing data were aligned to human genome assembly GRCh37 using BWA (MEM algorithm) v0.7.12. Realignment around indels, marking of duplicate reads, and recalibration of base quality scores, was undertaken using tools from Picard v1.140 (Broad Institute, MA, USA) and the Genome Analysis Toolkit (GATK) v3.4-46 (Broad Institute). Individual variant calling was undertaken with GATK HaplotypeCaller followed by multisample genotyping and variant quality score recalibration. Gene context annotation was added using SnpEff v4.1L (SnpEff, MA, USA), and allele frequencies were obtained from the 1000 genomes phase 1, ESP6500 and ExAC

using GATK VariantAnnotator. Sequential filtering of the multisample vcf file was undertaken using GATK SelectVariants and SnpSift v4.1L (SnpEff). Subsequently this protocol was carried out on individual 14. For samples from subjects 17, 18 and 19 and their respective parents, exome enrichment was performed using Illumina TruSeq Exome Enrichment kit (Illumina). Massively parallel sequencing was performed using the Illumina HiSeq2000 platform generating 100 basepair paired-end reads. Demultiplexing, base calling, alignment, variant calling and annotation of these trios was performed as described previously.[19] For all individuals analysed by exome sequencing platform artefacts were removed and good quality, rare variants (minor allele frequency <0.001) in coding regions (including splice sites and 5' and 3' UTRs) were retained (Table S4). *De novo* variants (present in the affected child but not in either parent) were then identified for each individual. Candidate mutations, and their *de novo* status, were confirmed by Sanger sequencing in subjects and their parents. Samples from subject 12 and their parents were exome sequenced utilising a slightly different protocol and analytical pipeline that has been described previously.[20] Directed Sanger sequencing of the two candidate genes was performed in 12 further individuals (for primer sequences see Table S1).

cDNA preparation and RT-PCR

Primary dermal fibroblasts from individuals with *MAP3K7*-mutation positive FMD were cultured in DMEM (Gibco, ThermoFisher), plus 10% FBS (Moregate, Hamilton, NZ) and 1% streptomycin and penicillin (Gibco). RNA was extracted using Trizol reagent (ThermoFisher) and the Nucleospin RNA kit (Machery-Nagel, Duren, Germany) according to kit protocols. cDNA was reverse-transcribed from total RNA using the SuperScriptIII kit (ThermoFisher) according to the kit protocol. PCR amplification and Sanger sequencing was carried out using primers listed in Table S1.

Generation of expression constructs

The full coding sequence of *MAP3K7* (variant A[21]; RefSeq NM_003188.3) was amplified from HEK293 cDNA using primer pair 1 (for all primer sequences see Table S2) and cloned into pcDNA3.1 using the TOPO cloning kit (ThermoFisher). This transcript was selected for these studies

since (a) it represents the form that contains exons encoding sequences subject to mutation in the disorder under study and (b) it represents the isoform studied by the vast majority of investigations into the properties of this gene. Primer pairs 2, 3 and 4 were used to sub-clone *MAP3K7* into EcoRI and XbaI restriction digested pCMV-Myc, pCMV-HA and p3xFLAG-CMV using the Gibson assembly kit (New England Biolabs, MA, USA). *TAB2* (RefSeq NM_015093.5) was amplified from HEK293 cDNA using primer pair 4 and cloned into pcDNA3.1 using TOPO cloning. Primer pair 5 was utilized for the sub-cloning of *TAB2* into EcoRI and XbaI digested pCMV-HA using the Gibson assembly. *TAB1* (pT7-FLAG) was a kind gift from K. Matsumoto (Nagoya University). Mutagenesis in *MAP3K7* was carried out using overlapping amplicons containing the mutagenized base, followed by Gibson Assembly.

Co-immunoprecipitation

For competitive co-immunoprecipitation of TAK1 and TAB2, HEK293FT cells were cultured in DMEM (Gibco) plus 10% FBS (Moregate) and co-transfected with one of the following: (1) pCMV-HA TAB2 (800 ng), pCMV-MYC TAK1 (10 ng) and p3XFLAG TAK1 (10 ng), (2) pCMV-HA TAB2 (800 ng), pCMV-MYC TAK1 (10 ng) and p3XFLAG TAK1 p.Pro485Leu (10 ng), (3) pCMV-MYC TAK1 (10 ng) and p3XFLAG TAK1 (10 ng), (4) pCMV-MYC TAK1 (10 ng) and p3XFLAG TAK1 p.Pro485Leu (10 ng) using Lipofectamine2000 (ThermoFisher) in a 24 well plate. For analysis of TAK1 dimerization by competitive co-immunoprecipitation, HEK293FT cells were co-transfected with one of the following: (1) pCMV-HA TAK1 (250 ng), pCMV-MYC TAK1 (250 ng) and p3XFLAG TAK1 (250 ng), or (2) pCMV-HA TAK1 (250 ng), pCMV-MYC TAK1 (250 ng) and p3XFLAG TAK1 p.Pro485Leu (250 ng) using the same protocol. After 20 hours cells were lysed in 1xPBS, 1% Triton X^{100} with protease inhibitor (cOmplete mini, Roche, Basel, Switzerland). Lysates were clarified by centrifugation and 30 µl retained for input samples. To 150 µl supernatant, 2 µl of mouse anti-HA (H3663, Sigma Aldrich) was added and samples mixed at 4ºC for 4 hours. 50 µl of washed Protein G Dynabeads (ThermoFisher) were added to each sample and incubated for a further 30 minutes at 4ºC, followed by three 1 ml washes in PBS. Protein was eluted in SDS sample buffer, and samples resolved by 7% SDS-PAGE, followed by transfer to a nitrocellulose membrane.

Membranes were blocked in 5% milk (PBS) then incubated with either mouse anti-HA (H3663, Sigma Aldrich; 1:5000), mouse anti-MYC (631206, Takara/Clontech, CA, USA; 1:5000) or mouse anti-FLAG (F1804, Sigma Aldrich; 1:7000), followed by secondary goat anti-mouse IRDye 800CW (926-32210, Li-COR, NE, USA; 1:25000). Membranes were scanned on a Li-COR Odyssey Clx, and bands quantified using Li-COR Image Studio Lite software.

Luciferase assays

HEK293 cells were cultured as previously described and transfected in a 24 well plate with 100 ng of plasmid DNA per well using Lipofectamine2000. Transfections were harvested 20 hours later in passive lysis buffer and assayed for Renilla and firefly luciferase activities using a Synergy2 multi-mode reader (BioTek, VT, USA) and injector module, which delivered the respective luciferase assay buffers (Promega, WI, USA). MAPK activity was assayed using the pFR-luc and pFA-ELK1 (Pathdetect vectors, Agilent), which detect MAPK phosphorylation of the GAL4-ELK1 fusion protein and subsequent transcription from the pFR-luc reporter. NF-kB activity was measured from the reporter pNF-κBREluc (pGL4.32[luc2P/NF-kB-RE/Hygro]; Promega). DNA constructs were transfected with the following amounts (ng/well): pFRluc (10), pFA-ELK1 (10), pNF-κBREluc (5), pSVminRL (10-45), pTAK1 and mutants (10), pHA-TAB2 and mutant (40), pCR3.1 (as empty vector) (10-50).

Western blotting

For TAK1 stability, HEK293FT cells were transfected with 20 ng p3XFLAG TAK1 (WT, p.Pro485Leu or p.Gly168Arg) and treated with 85 μg/ml cycloheximide in DMSO or equivalent DMSO control after 24 hours. For quantification of phospho-TAK1, HEK293FT cells were transfected with 40 ng pCMV-HA TAB2 (WT or p.Glu569Lys), 20 ng pT7-FLAG TAB1 and 20 ng p3XFLAG TAK1 (WT and mutants) with Lipofectamine2000 in a 24 well plate. Transfections for phospho-p38 Western blots were carried out as for luciferase assays. After 24 hours cells were lysed in 1xPBS, 1% TritonX^{100} with protease inhibitor; supernatant was cleared by centrifugation. Samples were denatured in 2.3% SDS and 1% DTT buffer. Proteins were resolved by electrophoresis on 8%

polyacrylamide gels, followed by transfer to nitrocellulose membrane. Membranes were blocked in 5% milk (PBS) or Odyssey PBS buffer (LiCor) and probed for 1 hour to overnight with anti-FLAG (F1804, Sigma Aldrich; 1:7000), anti-Myc (631206, Takara/Clontech; 1:5000), anti-phospho-TAK1 (Thr187, CST4536, Cell Signalling Technologies, MA, USA; 1:1500), anti-p38 (CST9212, Cell Signalling Technologies; 1:2000) or anti-phospho-p38 (Thr180/Tyr182, CST9216, Cell Signalling Technologies; 1:4000). Detection was with secondary goat anti-mouse IRDye 800CW (926-32210, Li-COR; 1:25000) and goat anti-rabbit IRDye 700CW (926-68071, Li-COR; 1:25000) antibodies as described above.

Statistical analysis

Quantitative data from Western blots and luciferase assays was imported into excel, and scaled and normalized to appropriate controls. Two-way, unpaired t-tests were carried out and critical p-values were Bonferroni corrected (* ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

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Results

Individuals with FMD with and without *FLNA* mutations are phenotypically similar

We ascertained a cohort of 19 individuals with FMD without *FLNA* mutations. Clinically, they are very similar to FMD individuals with *FLNA* mutations apart from an increased incidence of keloid formation[17,18] (Figure 1, Table 1). Core features of the phenotype include prominent supraorbital ridges, hyperostosis, downslanting palpebral fissures, hypertelorism and a wide nasal bridge. The chin is typically small and pointed (Figure 1A). Progressive contractures of the joints are common, especially the fingers and wrists (Figure 1B). Radiographs show a dense skull, especially the frontal bone and skull base (Figure 1C). The long bones of the hands and feet are undermodeled, frequently with sclerotic cortices (Figure 1D). Subjects often present with mild to severe scoliosis and have undermodelled, deformed ribs, sometimes with a 'coat-hanger' configuration. The long bones have dense cortices and splaying of the metaphyses (Figure 1D). Three subjects had a somewhat milder skeletal phenotype (07, 18 and 19; Table 1) and individual 07 lacked a number of facial characteristics but still had prominent supraorbital ridges. Of the nineteen individuals listed in Table 1, eight (2 male; 6 female) exhibit keloid scarring that is occasionally progressive and severe and has the potential to form either spontaneously or after surgical trauma (Figure 1E).

Mutations in *MAP3K7* and *TAB2* cause FMD

Whole exome sequencing in family trios identified three individuals (01, 02, and 05) with the same *de novo* missense mutation, c.1454C>T, in *MAP3K7* (NM_003188.3; Table 2). The subjects each had no more than three non-synonymous, validated *de novo* mutations in total across their respective exomes (Tables S3-4). The recurrent mutation is predicted to substitute a proline to a leucine at position 485 (p.Pro485Leu) of the protein product of *MAP3K7*, TAK1. Subsequently, a further individual (subject 12) was independently found to have the same *de novo* mutation following exome analysis.

Sequencing of another family trio (subject 17, Figure 1A, right panel) demonstrated a *de novo* missense variant, c.1705G>A, in *TAB2* (MIM 605101, NM_015093.5) which encodes the binding-partner of TAK1, TAK1 binding protein 2 (TAB2).[22] This variant substitutes a glutamic acid to a

lysine at position 569 (p.Glu569Lys) of TAB2 and was confirmed by Sanger sequencing. This individual had one other *de novo* variant in *EFHC1*; NM_018100.3; c.674C>G, p.Thr225Ser. Missense mutations in this region of *EFHC1* are associated with juvenile absence or myoclonic epilepsy[23,24]. Because individual 17 has no history of seizures and because *TAB2* is known to directly interact with and stabilize TAK1[22], the *TAB2* mutation was prioritised for further analysis.

Further exome analysis and targeted Sanger sequencing of the exons of *MAP3K7* and *TAB2* in the remaining unsolved individuals found that another eleven individuals have the recurrent c.1454C>T *MAP3K7* mutation and a further subject (07) was found to have novel *de novo* mutation in *MAP3K7* c.502G>C predicting the substitution p.Gly168Arg in TAK1 (Table 2). In addition, two unrelated individuals (18 and 19) who share the FMD phenotype with their respective mothers were found to have missense mutations in *MAP3K7*: c.208G>A and c.299T>A, predicting the substitutions p.Glu70Gln and p.Val100Glu, respectively. Both of these mutations were shown to have been inherited from the proband's affected mother. In both instances maternal grandparental samples were not available to ascertain if they had arisen *de novo* or not.

The phenotype of individuals with the recurrent c.1454C>T mutation is not noticeably different from subject 17 with the missense mutation in *TAB2* (Table 1; Figure 1A, right panel). The presentation of subject 07 with the p.Gly168Arg TAK1 substitution is however milder than the rest of the cohort. Individuals 18 and 19 (p.Glu70Gln and p.Val100Glu respectively) have a typical facial appearance for FMD however the skeletal presentation is milder (Table 1). Only individuals with the TAK1 p.Pro485Leu substitution developed keloid scarring (Table 1).

Together we found 4 variants in *MAP3K7* to be causative of FMD in a total of 18 individuals. In the single remaining individual in this cohort a missense mutation was identified in *TAB2,* which encodes for a protein that interacts directly with TAK1, the protein specified by *MAP3K7.*

TAK1 and _TAB2_ mutations predict substitutions in functionally relevant, conserved domains

Our genetic studies identified five different missense mutations affecting two proteins that form part of the TAK1 complex. *MAP3K7* encodes TAK1, a MAP-3-kinase and the core enzymatic component of a multiprotein complex that is a hub for the control of many signalling pathways[25]. *TAB2* encodes TAB2 a scaffolding protein necessary for the activation of some of signalling properties of TAK1[22] although it has not been shown to possess enzymatic activity itself.

The TAK1 protein is comprised of an N-terminal kinase domain, a poorly characterised 'linker region', and a C-terminal coiled-coil domain[25,26] (Figure 2). Pro485 in TAK1, which is substituted to a leucine in 15 unrelated individuals with FMD, is phylogenetically highly conserved with the residue being invariant in both vertebrates and invertebrates including *C.elegans* (genomic evolutionary rate profiling [GERP] score of 5.79, Figure 2A). The substitution occurs within the C-terminus of TAK1 immediately N-terminal to the coiled-coil domain within a region that mediates interactions with TAB2[27] and that may also constitute a homodimerisation interface[28] (Figure 2B). A crystal structure is available for the N-terminal kinase domain[29] but not for the linker region or C-terminus, and therefore the structural consequences of substituting Pro485 cannot be ascertained. Variant effect prediction software (PolyPhen 2, Mutation Taster and SIFT) estimates that the recurrent p.Pro485Leu substitution is likely to be deleterious (PolyPhen score 0.992, probably damaging; Mutation Taster score 0.999, disease causing; SIFT score 0, damaging).

Similarly, the p.Glu70Gln, p.Val100Glu and p.Gly168Arg substitutions lie within a stretch of 120 almost completely conserved residues in 7 vertebrates. A glutamine at position 70 is conserved to *Drosophila* (GERP score, 5.22) and a valine at position 100 is conserved in the 7 vertebrate homologs analysed (GERP score, 5.67; Figure 2A). Both are predicted to be disease causing with PolyPhen scores of 0.995 and 0.951 respectively (probably damaging), Mutation Taster scores of 0.999 (disease causing) and SIFT scores of 0 (damaging). A glycine at position 168 is conserved in *Drosophila* (GERP score, 5.18; Figure 2A). PolyPhen (0.802, possibly damaging), Mutation Taster (0.999, disease causing) and SIFT (0, damaging) predicting this substitution to be disease causing. These three substitutions lie within the conserved kinase domain of TAK1 (Figure 2B). Predictions based on the published crystal structure[29] (TAK1-TAB1 fusion protein, Protein Data Bank Accession: 2EVA)

of this region of the protein suggest that the p.Gly168Arg substitution would disrupt a tightly constrained β-hairpin turn that is required to order the active site of the kinase domain (Figure 2C). Glu70 is close to an important loop which sits over the active site of the TAK1 enzyme, however substitution to a glutamine is conservative and it may establish similar interactions within the structure. Val100 is located in a hydrophobic pocket with Ile65, Phe74, Arg71 and Leu97, and substitution to a glutamic acid could be destabilising to this region (Figure 2C).

Predicting the effect of the TAB2 variant (p.Glu569Lys) is constrained by limited data on this protein. The substitution has a PolyPhen2 score of 0.989 (probably damaging), Mutation Taster score of 0.999 (disease causing) and a SIFT score of 0 (damaging) suggesting the potential for pathogenicity. Glu569 lies in an area of high conservation in vertebrates (GERP score, 5.06; Figure 2A) but no tertiary structure for this protein is available. Similar to TAK1, TAB2 has a C-terminal coiled-coil domain within which the TAK1 binding domain resides.[22,27] The p.Glu569Lys substitution is located 5 residues N-terminal to this mapped TAK1 binding interface (residues 574-693, Figure 2B).

To evaluate the consequences of the recurrent MAP3K7 c.1454C>T mutation at the level of the transcript, cDNA was prepared from cultured primary fibroblasts obtained from subjects 02, 13 and 16 (*MAP3K7* c.1454C>T) and RT-PCR followed by Sanger sequencing was performed. Sequence chromatograms demonstrated the persistence of the mutant allele in all instances and no additional RT-PCR products were observed (data not shown). An RNA source for subjects 07, 17, 18 and 19 was not available to test these alternative mutations in a similar manner. Together these genetic, bioinformatic and phylogenetic data constitute conclusive evidence that mutations in *MAP3K7* cause frontometaphyseal dysplasia.

To explore the mechanism by which mutations confer this phenotype we first considered a hypothesis that invokes haploinsufficiency for the pathogenesis of this form of FMD. Individuals with haploinsufficiency encompassing the *MAP3K7* locus[30] and mice with conditional knock-out of *Map3k7* in the skeleton, do not have an osteosclerotic phenotype[31,32], suggesting that the phenotype arising from the *MAP3K7* mutations described here is not a result of haploinsufficiency. To further test the hypothesis that the mutations in *MAP3K7* do not exhibit their pathogenic effect by affecting

the stability of the TAK1 protein, we performed quantitative western blots on lysates prepared from cycloheximide-treated cells transfected with expression constructs specifying either WT TAK1 ($TAK1^{WT}$), TAK1 with the p.Pro485Leu ($TAK1^{p.Pro485Leu}$) or the p.Gly168Arg substitutions ($TAK1^{p.Gly168Arg}$). There was no difference in protein stability between mutant TAK1 and wild type protein up to 8 hours post cycloheximide treatment (Figure 2D) indicating that the pathogenic effect of these alleles is unlikely to relate to destabilization of the protein product. Similarly, individuals with haploinsufficiency of *TAB2* have no skeletal dysplasia and instead present with cardiac malformations[33] and *Tab2* knock-out mice die *in-utero* of liver degeneration.[34] These data therefore suggest that the phenotype of the individual described here with a missense mutation at this locus is unlikely to arise from a haploinsufficient mechanism.

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The p.Pro485Leu substitution does not affect TAK1/TAB2 binding or TAK1 homodimerisation

A parsimonious hypothesis for the mechanism leading to FMD in these individuals would be that causative mutations disrupt the interaction between TAK1 and TAB2 since both the recurrent mutation and the substitution in TAB2 lie within (or very close to) the interface formed by both proteins.[27] Competitive co-immunoprecipitation was used to examine the relative TAB2 binding affinity of p.Pro485Leu in the presence of WT TAK1 using a transient transfection protocol in HEK293FT cells. Two differentially tagged TAK1 proteins, with one construct specifying the p.Pro485Leu substitution and the other wild type, were transfected alongside TAB2 and immunoprecipitation was performed to measure the relative ability of these proteins to bind TAB2. No significant difference is observed between $TAK1^{WT}$ and $TAK1^{p.Pro485Leu}$ in their ability to bind TAB2 ($p = 0.59$; unpaired t-test, Figure 3A), indicating that this interaction is physically unimpaired by the presence of the p.Pro485Leu substitution in TAK1.

A second hypothesis relates to the possibility that TAK1 homodimerises[35] and that this activity impacts on regulation of the signalling functions of the TAK1 complex. The TAK1 homodimerisation interface has been mapped within the coiled-coil domain to a region that is adjacent to, but not

overlapping Pro485.[35] We tested the capacity of TAK1^{WT} to dimerise with TAK1$^{p.Pro485Leu}$ using competitive co-immunoprecipitation. Again, no significant difference ($p = 0.37$; unpaired t-test) is noted between the formation of TAK1$^{WT/WT}$ homodimers and TAK1$^{p.Pro485Leu/WT}$ heterodimers, suggesting that the p.Pro485Leu substitution does not impair this activity (Figure S2).

TAK1$^{p.Pro485Leu}$ and TAK1$^{p.Gly168Arg}$ increase TAK1 autophosphorylation

In the absence of a clear mechanism relating the p.Pro485Leu substitution to mapped protein-protein interactions within the C-terminus of TAK1, we sought evidence that this substitution altered the kinase activity of this protein. Upon activation, TAK1 is sequentially autophosphorylated within its kinase domain[36] starting at Ser192 and followed by Thr178, Thr187 and Thr184 which in turn triggers the phosphorylation of a number of downstream effectors.[36-38] In addition to being scaffolded by TAB2, signalling through the TAK1 complex is also dependent on a second interacting factor TAB1 (TAK1 associated binding protein 1; MIM 602615).[26] To test if the mutations leading to FMD affect TAK1 autophosphorylation *MAP3K7* constructs specifying TAK1^{WT}, TAK1$^{p.Pro485Leu}$ and TAK1$^{p.Gly168Arg}$ proteins were expressed in HEK293FT cells alongside constructs encoding its activating proteins TAB1 and TAB2. Under these conditions, quantitative Western analysis revealed that both TAK1$^{p.Pro485Leu}$ and TAK1$^{p.Gly168Arg}$ are significantly more phosphorylated at Thr187 compared to TAK1^{WT} ($p = 0.00014$ and $p = 0.00010$ respectively; unpaired t-tests; Figure 3B). Consistent with the established facultative requirement for the co-activator TAB1 in mediating TAK1 autophosphorylation, no difference in the phosphorylation of TAK1 mutants is observed when TAK1 was expressed with TAB2 alone (Figure S3A).

The p.Pro485Leu substitution alters signalling downstream of the TAK1 complex

Enhanced autophosphorylation of TAK1 in the presence of substitutions that lead to FMD suggests that signalling pathways downstream of this complex should exhibit altered activity. Multiple signalling pathways are activated downstream of TAK1, including JNK[39], p38 MAPK[40] and NF-κB[41]

(Figure 4A). We first employed a luciferase reporter assay (Pathdetect, Agilent) that measures a global readout for transcriptional activation mediated by several MAPK targets including ERK, p38 and JNK. A significantly enhanced activation of the reporter is observed for the TAK1$^{p.Pro485Leu}$ compared to TAK1^{WT} (Figure 4B). Since this reporter system detects activity mediated via a number of MAPK pathway effectors, and considering the documented specific role for p-p38 downstream of TAK1 in the maintenance of bone mineralisation in the mouse[31], phosphorylated p38 (p-p38) was also assayed by western blot (Figure 4C). These data indicate that the TAK1$^{p.Pro485Leu}$ construct results in a substantial increase in phosphorylation of p38 compared to that mediated by TAK1^{WT}. Notably however, neither MAPK signalling nor p-p38 are increased when TAK1$^{p.Gly168Arg}$ is expressed under the same experimental conditions (Figure 4B-C), despite the significant increase in autophosphorylation noted in the TAK1$^{p.Gly168Arg}$ protein.

The second signalling pathway relevant to skeletogenesis that we evaluated was that mediated by NF-κB[32,42]. NF-κB is essential for RANK-L mediated osteoclast differentiation[43,44] whereas its activation inhibits osteoblastogenesis.[42] Using a luciferase reporter sensitive to activation of this pathway, both the TAK1$^{p.Pro485Leu}$ and the TAK1$^{p.Gly168Arg}$ (Figure 4D) substitutions confer significantly reduced reporter activity in a transient transfection assay (Figure 4D). Together these results indicate that signalling emanating from the TAK1 complex is altered by TAK1$^{p.Pro485Leu}$, but that the degree and quality of this disturbance is not exactly mirrored by the TAK1$^{p.Gly168Arg}$ substitution even though both variants enhance the autophosphorylation of the enzyme and the phenotypic consequences in the human of both variants are similar.

The p.Glu569Lys substitution in TAB2 alters MAPK signalling

The observation of a single instance of a *de novo* missense variant in *TAB2* in an individual with FMD does not, in itself, constitute sufficiently strong evidence for pathogenicity. However the location of this variant close to the mapped binding interface for TAB2 and TAK1 in an individual who demonstrates a phenotype that is indistinguishable from individuals with *MAP3K7*-associated FMD presents a strong *a priori* case for further investigation. We first questioned if equivalent alterations in

signalling were conferred by the *de novo* mutation in *TAB2* as were observed in expression assays using the TAK1$^{p.Pro485Leu}$ construct. We found that the presence of the TAB2$^{p.Glu569Lys}$ substitution alters neither TAK1 autophosphorylation (Figure 3B) nor NF-κB signalling activity (Figure 4D). There is however a clear increase in MAPK luciferase reporter activity and an increased p-p38/p38 ratio (Figure 4B and C) indicating that, despite a lack of enhanced autophosphorylation of TAK1, this variant in TAB2 has a strong effect on the activity of downstream components of the MAPK pathway.

Other substituting residues at TAK1 Pro485 alter TAK1 activity

Clinically only the proline to leucine substitution is observed at position 485 in the TAK1 protein in this cohort of individuals with FMD. Other mutations at this codon however may occur and lead to similar biochemical and clinical consequences. The likelihood of this may be dependent on the mode of gain-of-function and whether this primarily relates to the substitution of Pro485 and/or the identity of the substituting residue. We therefore examined the effect of other substitutions at the Pro485 codon to test the possibility that they may also confer a gain-of-function. Both p.Pro485Arg and p.Pro485Ala substitutions did enhance autophosphorylation (Figure S3B) and also had a variable effect on luciferase-based reporter assays for MAPK and NF-κB transcriptional targets. Substitution to an amino acid with a bulky and/or charged side-chain (p.Pro485Arg) conferred more alteration in these signalling pathways than more conservative missense substitutions (e.g. p.Pro485Ala and p.Pro485Ser). Although TAK1$^{p.Pro485Arg}$ shows no increase in MAPK activity (Figure S4A), it does show a significant increase in p-p38 when measured on immunoblot, in addition to significantly decreased NF-κB activity (Figure S4B and C). Substitutions to residues with smaller and less polar side-chains (Ser and Ala) result in no difference in signalling as assayed either through reporter assays or p38 phosphorylation, except for a small but significant increase in p-p38 with TAK1$^{p.Pro485Ser}$ (Figure S4B). Overall, the changes in signalling function are smaller in magnitude than those conferred by the clinically observed substitution to leucine. We conclude that the identity of the substituting amino acid at this position does influence TAK1 function and that these substitutions also hold the potential to be disease-causing.

Discussion

Until now mutations in only one gene, *FLNA*, have been shown to cause FMD.[4] Here, we have used a combination of exome and targeted Sanger sequencing to reveal four mutations in *MAP3K7* to be the cause of FMD in 18 unrelated individuals and another variant in the gene encoding the interacting protein TAB2 to be likely causative of the condition in a further individual. A specific mutation introducing a p.Pro485Leu substitution at the boundary of the C-terminal coiled-coil domain of the MAP-3-kinase, TAK1, was present in 15 unrelated individuals from diverse ethnic backgrounds. In all instances where parental samples were available (n=6), this mutation had arisen *de novo*. A second *de novo* mutation predicting the substitution p.Gly168Arg was found in the highly conserved kinase domain of TAK1 in one individual. Two further missense mutations leading to the substitutions p.Glu70Gln and p.Val100Glu were found in another two subjects, both inherited from a similarly affected parent. We propose that the X-linked form of FMD be henceforth referred to as FMD1 to underscore its inheritance pattern, and that the autosomal dominant phenotype described here caused by mutations in *MAP3K7*, the gene encoding TAK1, be denoted as FMD2. Although the *de novo* variant observed in TAB2 is an observation confined to a single individual, the functional relationship that this protein has with TAK1 and the biochemical activity it confers on the TAK1 signalling complex present a strong, but still provisional, case that it also be considered causative of FMD. For this reason we suggest that *TAB2* will eventually be shown to represent a third causative locus and that this form of FMD will be denoted FMD3.

Phenotypically there may be some differences between FMD1, FMD2 and *TAB2*-related FMD. Subjects with substitutions in the kinase domain of TAK1 have a notably milder phenotype than those with TAK1 p.Pro485Leu substitutions, *TAB2*- or *FLNA*- associated FMD, suggesting a phenotype-genotype correlation. We noted that the individuals with the recurrent substitution in TAK1 have a higher incidence of cleft palate (5/16 – 31% subjects compared to 9% reported in FMD1).[3,17,45] We also previously reported a higher incidence of keloid scarring in individuals with FMD2 caused by TAK1 p.Pro485Leu.[17] This is notable because keloid is a rare phenotypic manifestation in Mendelian disorders and the data presented here may increase the understanding of the pathogenesis of

spontaneous keloid formation. Keloid may only very occasionally appear as a feature of FMD1,[3] but a recently described novel filaminopathy caused by a specific missense mutation in *FLNA* (NM_001110556.1; c.4726G>A, p.Gly1576Arg) is also characterised by keloid scarring, joint contractures and, heart and kidney abnormalities. As such it is reminiscent of FMD without its skeletal manifestations.[46,47] Therefore it is possible that TAK1 and filamin A operate in the same pathway to promote keloid formation, a possibility consistent with data that indicates that activation of the TGFβ pathway, upstream of the MAP-kinases p38, ERK and JNK, has an important role in keloid pathogenesis. Small-molecule inhibition of p38, ERK and JNK in cultured keloid fibroblasts impairs collagen accumulation and keloid development after TGFβ stimulation[48] and therefore attention to therapeutic targets for keloid could usefully be focused on the pathway which links filamin A, TAK1, TGFβ and MAP-kinases.

FMD2 is primarily caused by TAK1 p.Pro485Leu in the cohort presented here (15/18 individuals). The finding of a highly recurrent mutation is consistent with a gain-of-function mechanism. Although instances of highly recurrent mutations can be the result of a paternal-age effect,[49,50] we only demonstrated a modest signal for this once we tabulated the age of the fathers at the birth of the TAK1$^{p.Pro485Leu}$ individuals (Table 2).[49] A more salient observation is that the *MAP3K7* mutation occurs at a hypermutable CpG dinucleotide and therefore the observation of a C>T transition is expected to be more common than other mutations at this site.[51,52] We predict that other substitutions at the Pro485 codon will exist and be associated with FMD phenotypes but have not been identified in this study, perhaps because of an ascertainment bias or because of the small number of individuals studied here. It may be relevant to note that a less significant effect on downstream signalling is observed when Pro485 is replaced with residues other than leucine and so if individuals do exist with alternative residues replacing Pro485, they may present with a milder phenotype.

The structural and biochemical reasons for why TAK1$^{p.Pro485Leu}$ is specifically implicated in this gain-of-function remain enigmatic. The TAK1$^{p.Pro485Leu}$ substitution does not impair TAB2 binding, homodimerisation or TAK1 stability. We note that Pro485 lies within a consensus sequence for prolyl hydroxylation (LXXLAP) as represented in other proteins[53-55] but efforts to demonstrate that this site

553 is subject to this form of modification using mass spectroscopy, peptide substrates and co-expression
554 with prolyl hydroxylases failed to reveal any evidence to support this hypothesis (data not shown).
555 Furthermore, substitution with residues other than leucine at this site did not invariably confer the
556 same effect on autophosphorylation or signalling.

557 TAK1 is a MAP-3-kinase.[38] On activation it binds its co-activators TAB1 and TAB2, leading to
558 autophosphorylation, triggering downstream phosphorylation cascades.[38] The *de novo* TAK1
559 substitutions p.Pro485Leu and p.Gly168Arg significantly increase TAK1 autophosphorylation. The
560 activated TAK1 complex is able to coordinate signalling in a number of different pathways, including
561 the regulation of both osteoblast differentiation and activity.[31,56] The p38 pathway is especially critical
562 because p38 phosphorylates and activates RUNX2, the master differentiation factor for osteoblasts,
563 and DLX5, a transcription factor which stimulates the expression of the osteoblast genes *IBSP*
564 (integrin-binding sialoprotein) and *SP7* (osterix).[31,56] TAK1$^{p.Pro485Leu}$ leads to increased
565 phosphorylation of p38 and an enhanced ability to activate a generic MAPK transcriptional reporter.
566 The TAK1$^{p.Gly168Arg}$ substitutions did not show the same ability to activate downstream MAPK
567 cascades; this could reflect the noticeably milder phenotype in this individual and the lack of
568 sensitivity of our assays to detect subtle changes in signalling outputs. The *TAB2* substitution
569 p.Glu569Lys also increases MAPK signalling and enhances phosphorylation of p38, although these
570 effects are not associated with activation of TAK1 via detectably enhanced autophosphorylation.

571 Not only is p38 essential for osteoblast differentiation[31] it is also critical for the proper differentiation
572 of osteoclasts in response to RANK-L.[57] Therefore a gain-of-function in p38 signalling in both cell
573 types is predicted to result in both increased osteoblast differentiation and activity, and increased
574 osteoclast differentiation. Further evidence that p38 is a central, but not necessarily exclusive,
575 mediator of TAK1-directed osteogenesis, is the observation that the osteopenia observed in mice with
576 *MAP3K7* deleted in the osteoblastic lineage is attributable to reduced p38 activation.[31]

577 The signalling output of TAK1 is complex and multifarious and also includes regulation of NF-κB.[58]
578 While MAPK signalling promotes the development of both the osteoblastic and osteoclastic lineage,
579 NF-κB has opposing effects. Its activation by inflammatory cytokines is generally inhibitory for

osteoblastogenesis[42,59] but essential for osteoclastogenesis stimulated by RANK/RANK-L.[43,44] Mice with an osteoclast-specific knock-out of TAK1 have an osteopetrotic phenotype because of decreased NF-κB activation, demonstrating that TAK1 is necessary for proper osteoclast differentiation.[32] Reduced NF-κB signalling could therefore promote hyperostosis by acting in concert in both of these cell types. Our data indicate that the TAK1$^{p.Pro485Leu}$ and TAK1$^{p.Gly168Arg}$ variants are associated with diminished NF-κB signalling, which is also the effect observed in an osteoclast specific-knockout of *Map3k7* in mice. Hence, we conclude that activity conferred by TAK1 substitutions cannot be attributed to a simple biochemical gain-of-function mechanism across all affected pathways since it is clear that the TAK1-complex variants alter multiple outputs, in different directions. This may be especially important for TAK1$^{p.Gly168Arg}$ which does not increase MAPK signalling output but does decrease NF-κB activity. This could still, therefore, disrupt the overall ratio of MAPK to NF-κB signalling and cause a milder FMD phenotype. Additionally our *in vitro* data indicate that the TAB2$^{p.Glu569Lys}$ substitution confers no difference in NF-κB signalling but still results in a florid FMD phenotype. Overall this suggests that the combinatorial effect of these variants on all signalling pathways involved will likely only be definitively established once they can be evaluated in bone tissue from animal models of this disorder.

The TAB2$^{p.Glu569Lys}$ substitution does not increase TAK1 autophosphorylation, nor does it decrease NF-κB signalling output. TAB2 has a number of TAK1-independent functions and therefore the phenotype arising from this substitution could be attributed to these activities. For instance TAB2 has been shown to localise in the nucleus with transcriptional repressors N-CoR and HDAC3 where it acts to repress gene-targets of NF-κB; the complex is exported out of the nucleus on stimulation of the TAK1 complex.[60] Additionally TAB2 has been shown to bind Smad7 downstream of TGFβ activation which blocks TAK1-TAB2-TRAF2 complex assembly.[61] Taken together, these data suggest that TAB2 acts to repress signalling mediated by TAK1 but it is unknown whether TAB2 may also act to promote MAPK functions, although our results indicate that the Glu569Lys substitution does confer such activity.

As well as influencing major developmental mechanisms in the skeleton, TAK1 has a headline role governing the innate immune response and inflammation.[41] The TAK1 complex is activated by inflammatory cytokines (e.g. TNFα[41] and IL-1[62]), and triggers downstream signalling responses to these stimuli such as activation of NF-κB.[41,63] We may expect that individuals with gain-of-function TAK1 signalling may present with inflammatory phenotypes, however this is not a clinically apparent aspect of the FMD2 phenotype. Focused study from this point could reveal hitherto unexpected subclinical anomalies in this disorder.

The TAK1 signalling complex is a broadly dispersed signalling hub in multiple tissues. Our observation that alteration of its signalling functions via a specific mutational mechanism hints at levels of regulation of this complex that remain uncharacterised. Although the phenotype of FMD extends to tissues other than bone, the pronounced effect that these mutations have on the mineralisation of the skeleton suggests that understanding the function of this signalling hub in this tissue could produce new insights and therapeutic options for conditions characterised by osteopenia.

Supplemental Data

The supplemental data contains 4 Figures and 4 Tables

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Web Resources

MIM: http://www.ncbi.nlm.nih.gov/omim

631 BWA: http://bio-bwa.sourceforge.net/

632 Picard tools: http://broadinstitute.github.io/picard/

633 GATK: https://www.broadinstitute.org/gatk/

634 ExAC: https://www.exac.broadinstitute.org/gatk/

635 ESP6500: http://evs.gs.washington.edu/EVS/

636 1000genomes: http://www.1000genomes.org/

637 ClustalOmega: http://www.ebi.ac.uk/Tools/msa/clustalo/

638 PolyPhen2: http://genetics.bwh.harvard.edu/pph2/

639 Mutation Taster: http://www.mutationtaster.org/

640 SIFT: http://sift.jcvi.org/

641 GERP: http://mendel.stanford.edu/SidowLab/downloads/gerp/

642 Protein databank: http://www.rcsb.org/pdb/home/home.do

643 Ensembl Genome Browser: http://asia.ensembl.org/index.html

644 DECIPHER: https://decipher.sanger.ac.uk/

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879 **Table 1: Clinical and Radiographic Characteristics of FMD cases**

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		Clinical features												Radiological features					
Subject	Sex	Supraorbital ridges	Small chin	Hearing loss	Hypertelorism	Downslanting palpebral fissures	Wide nasal bridge	Cleft palate/bifid uvula[a]	Congenital stridor/subglottic stenosis	Hydronephrosis	Scoliosis	Intellectual disability	Keloid	Cervical vertebral fusion	Elbow contractures/dislocated radial head	Flared metaphyses	Digital and wrist contractures	Under-modelled phalanges	Broad thumbs and fingers
01	M	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
02	M	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+
03	M	+	-	+	+	+	+	-	U	U	+	+	-	U	+	U	+	U	+
04	M	+	-	+	+	+	+	U	U	U	U	-	-	U	+	U	+	U	U
05	M	+	+	+	+	+	+	-	-	-	+	-	+	-	+	+	+	+	+
06	M	+	+	U	+	+	+	-	-	-	+	+	-	-	+	+	+	+	+
07	M	+	-	-	+	-	+	-	-	-	(+)	-	-	+	+	-	+	+	-
08[b]	M	+	+	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+
09	F	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
10[b]	F	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+
11[b]	F	+	-	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+
12	F	+	+	+	+	+	+	+	+	-	-	(+)	-	U	+	U	+	U	+
13[b]	F	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+
14	F	+	+	U	+	+	+	-	U	U	U	-	-	+	+	U	+	U	+
15	F	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+
16[b]	F	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+
17	F	+	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	+	+
18[b]	F	+	+	+	+	+	+	-	U	U	+	-	-	-	U	-	-	+	U
19	F	+	+	-	+	+	+	-	-	-	+	-	-		+	+	+	U	(+)

881 +, present; -, absent; U, unknown; [a] including submucous cleft palate; [b] reported in ref.17 or 21.

882 Table 2: Mutations in *MAP3K7* and *TAB2*, paternal age at birth and ancestry of individuals with FMD

Subject	Gene	DNA	Protein	Inheritance	Paternal age at birth of proband (years)	Ancestry
01	*MAP3K7*	c.1454C>T	p.Pro485Leu	De novo	33	Italian
02	*MAP3K7*	c.1454C>T	p.Pro485Leu	De novo	40	English
03	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	36	Austrian
04	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	38	Australian - European
05	*MAP3K7*	c.1454C>T	p.Pro485Leu	De novo	26	Scottish
06	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	32	Swiss
08	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	28	Korean
09	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	18	Hispanic
10	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	46	Turkish
11	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	47	Mexican
12	*MAP3K7*	c.1454C>T	p.Pro485Leu	De novo	35	German
13	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	26	English
14	*MAP3K7*	c.1454C>T	p.Pro485Leu	De novo	41	German
15	*MAP3K7*	c.1454C>T	p.Pro485Leu	U	30	Dutch
16	*MAP3K7*	c.1454C>T	p.Pro485Leu	De novo	40	Australian - European
18	*MAP3K7*	c.208G>C	p.Glu70Gln	Dominant	U	French - Canadian
19	*MAP3K7*	c.299T>A	p.Val100Glu	Dominant	U	Hungarian
07	*MAP3K7*	c.502G>C	p.Gly168Arg	De novo	28	Brazilian
17	*TAB2*	c.1705G>A	p.Glu569Lys	De novo	U	Australian - European
				Mean	34.4	
				SD	7.9	

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884 U, unknown; SD, standard deviation

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