

1 **Identification of a novel loss-of-function *PHEX* mutation, Ala720Ser, in a sporadic case of adult-**  
2 **onset hypophosphatemic osteomalacia**

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1 **Abstract**

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4 Adults presenting with sporadic hypophosphatemia and elevations in circulating fibroblast growth  
5 factor-23 (FGF23) concentrations are usually investigated for an acquired disorder of FGF23 excess  
6 such as tumor induced osteomalacia (TIO). However, in some cases the underlying tumor is not  
7 detected, and such patients may harbor other causes of FGF23 excess. Indeed, coding-region and  
8 3'UTR mutations of phosphate-regulating neutral endopeptidase (*PHEX*), which encodes a cell-  
9 surface protein that regulates circulating FGF23 concentrations, can lead to alterations in phosphate  
10 homeostasis, which are not detected until adulthood. Here, we report an adult female who presented  
11 with hypophosphatemic osteomalacia and raised serum FGF23 concentrations. The patient and her  
12 parents, who were her only first-degree relatives, had no history of rickets. The patient was thus  
13 suspected of having TIO. However, no tumor had been identified following extensive localization  
14 studies. Mutational analysis of the *PHEX* coding-region and 3'UTR was undertaken, and this revealed  
15 the patient to be heterozygous for a novel germline *PHEX* mutation (c.2158G>T; p.Ala720Ser). *In*  
16 *vitro* studies involving the expression of WT and mutant PHEX proteins in HEK293 cells  
17 demonstrated the Ala720Ser mutation to impair trafficking of PHEX, with <20% of the mutant  
18 protein being expressed at the cell surface, compared to >80% cell surface expression for WT PHEX  
19 (p<0.05). Thus, our studies have identified a pathogenic *PHEX* mutation in a sporadic case of adult-  
20 onset hypophosphatemic osteomalacia, and these findings highlight a role for *PHEX* gene analysis in  
21 some cases of suspected TIO, particularly when no tumor has been identified.

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23 **Key words:** FGF23, PHEX, X-linked, hypophosphatemia, osteomalacia, tumor

24

25 **Abbreviations:** ALP, alkaline phosphatase; ESR, erythrocyte sedimentation rate; EVS, Exome  
26 Variant Server; EXAC, Exome Aggregation Consortium; FDG, <sup>18</sup>fluorodeoxyglucose; FGF23,  
27 fibroblast growth factor-23; PHEX, phosphate-regulating neutral endopeptidase; PsA, psoriatic  
28 arthritis; TIO, tumor induced osteomalacia; TmP/GFR, Tubular maximum of phosphate/glomerular  
29 filtration rate; WT, wild-type; XLH, X-linked hypophosphatemia.

## 1 **1. Introduction**

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3 The circulating concentration of phosphate is regulated by fibroblast growth factor-23 (FGF23),  
4 which is an osteocyte-derived hormone that influences proximal renal tubular phosphate reabsorption  
5 and the renal synthesis of 1,25-dihydroxyvitamin D (1). Primary disorders of FGF23 excess are  
6 characterized by renal tubular phosphate wasting and low serum 1,25-dihydroxyvitamin D  
7 concentrations, which lead to hypophosphatemia and impaired skeletal mineralization (1, 2). The  
8 most common inherited cause of FGF23 excess is X-linked hypophosphatemia (XLH; OMIM  
9 #307800), which has a prevalence of 1:20,000 (3), and is caused by loss-of-function mutations  
10 affecting the *PHEX* gene on chromosome Xp22.1 (4-7). *PHEX* encodes the phosphate-regulating  
11 neutral endopeptidase, which is a cell-surface protein expressed in osteocytes, osteoblasts and  
12 odontoblasts; and considered to play a role in inhibiting FGF23 synthesis (1). XLH is in general a  
13 highly penetrant X-linked dominant disorder characterized by childhood rickets, which is  
14 unresponsive to physiological doses of vitamin D, and occurs in association with growth retardation  
15 and dental abnormalities (5, 8). However, XLH can also mimic a sporadic or X-linked recessive form  
16 of rickets, which is characterized by a mild clinical phenotype, and caused by a mutation within the  
17 *PHEX* 3'-UTR region (9). In contrast to XLH, which generally manifests in the second year of life  
18 when affected individuals begin weight-bearing, patients presenting in adulthood with  
19 hypophosphatemia and elevated serum FGF-23 concentrations, in the absence of any family history of  
20 rickets, are usually investigated for an underlying acquired cause such as tumor induced osteomalacia  
21 (TIO) (10). This paraneoplastic disorder is most commonly caused by the ectopic secretion of FGF23  
22 from benign mesenchymal tumors (11, 12). The diagnosis of TIO is often difficult as the causative  
23 mesenchymal tumors are generally small and occur in any soft tissue or bone (13). Indeed, despite  
24 extensive tumor localization studies, which may span several years and involve a range of imaging  
25 modalities such as whole body MRI, octreotide scintigraphy and <sup>18</sup>fluorodeoxyglucose PET/CT  
26 (FDG-PET/CT) (14), the underlying cause of the FGF23 excess is often not established. Here, we  
27 report a previously well patient with no known family history of rickets, who presented with  
28 hypophosphatemic osteomalacia and raised serum FGF23 concentrations in adulthood. She was

1 suspected as having TIO, but no tumor was detected. However, mutational analysis identified a novel  
2 germline loss-of-function *PHEX* mutation, and these findings suggest that *PHEX* mutations may  
3 account for some cases of sporadic adult-onset hypophosphatemic osteomalacia.

## 4 5 **2. Case Report**

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7 A previously well 43-year-old woman presented with widespread psoriasis in association with a 12-  
8 month history of pain and stiffness affecting the lumbar back, hips and feet; and swelling of the  
9 metacarpophalangeal joints. She was not on any regular medications, did not take any vitamins or  
10 tonics, and had not altered her diet. She was diagnosed with a late-onset form of psoriatic arthritis  
11 (PsA) (presenting at >40 years), which accounts for ~30% of all PsA cases (15). She had a  
12 persistently raised erythrocyte sedimentation rate (ESR), ranging from 26-81 mm/hr (normal 2-19  
13 mm/hr), which is observed in ~50% of PsA patients (16). However, her symptoms did not improve  
14 following treatment with methotrexate. Plain radiography identified Looser zones affecting the  
15 femora (Fig. 1A), and she was assumed to also have vitamin D deficient osteomalacia, and  
16 commenced on ergocalciferol 250 micrograms weekly. However, her symptoms persisted, and serum  
17 biochemistry, which was measured on a random (non-fasting) sample, following three months of  
18 treatment with ergocalciferol revealed a low phosphate of 0.43 mmol/L (normal 0.70-1.40 mmol/L),  
19 normal concentrations of albumin-adjusted calcium and creatinine, borderline elevation of alkaline  
20 phosphatase (ALP) activity, adequate 25-hydroxyvitamin D of 72.4 nmol/L (29.0 ng/mL) and raised  
21 parathyroid hormone concentration (Table 1). Tubular maximum of phosphate/glomerular filtration  
22 rate (TmP/GFR) was low at 0.40 mmol/L (normal 0.80-1.35 mmol/L), consistent with a renal tubular  
23 phosphate loss. No alterations in serum electrolytes or urate concentrations were noted (Table 1).  
24 Moreover, urinary glucose was not detected, and the urinary concentrations of amino acids and retinol  
25 binding protein were not elevated, thus indicating that the patient did not have a generalised  
26 disturbance of proximal renal tubular function. Serum 1,25-dihydroxyvitamin D was inappropriately  
27 normal, given the hypophosphatemia; at 98 pmol/L (normal 43-144 pmol/L). Serum FGF23, which  
28 was measured using the human C-terminal FGF23 ELISA (Immutopics) (17), was elevated at 779

1 RU/mL (normal <100 RU/mL). These findings were consistent with FGF23-mediated  
2 hypophosphatemia. She had no childhood history of rickets, and the onset of her hypophosphatemia  
3 was not known, as serum biochemical profiling had not been previously undertaken. Moreover, it was  
4 uncertain whether there was a family history of rickets as she had no children or siblings. However,  
5 her parents were not known to be of short stature or affected by any musculoskeletal disorders. She  
6 had a history of dental abscesses, which were attributed to dental trauma as a child. Her height was  
7 150 cm (4 feet and 11 inches), which is within the normal range for women of her ethnicity (Middle  
8 Eastern origin) and corresponds to the 12<sup>th</sup> height centile. No disproportionate lower limb shortening  
9 was noted, and the upper and lower segment heights were 70cm and 80cm, respectively. No frontal  
10 bossing or other skeletal deformities were detected on examination. Mild enthesopathic changes  
11 affecting the ischial tuberosities, and an incidental finding of L5 spina bifida, were noted on a review  
12 of her plain radiographs (Fig. 1A). No abnormalities were detected on technetium 99m skeletal  
13 scintigraphy. She had no known acquired causes of FGF23 excess, such as being treated with iron  
14 infusions or having undergone a renal transplant (18, 19). Investigations for TIO, which included  
15 whole body MRI, octreotide scintigraphy and FDG-PET/CT did not detect an underlying tumor. She  
16 was commenced on oral phosphate (500 mg elemental phosphorus 2-3 times daily) and alfacalcidol  
17 250 ng daily, which improved the hypophosphatemia and normalised the ALP activity (Fig. 1B).  
18 However, she has remained symptomatic and her serum C-terminal FGF23 concentrations have been  
19 persistently elevated (Fig. 1B). This patient has been assessed over a period of eight years with serial  
20 imaging studies for presumed TIO, and no causative tumor has been identified.

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## 1 **3. Methods**

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### 3 *3.1 Genetic analysis*

4 All genetic analyses were performed by the Department of Molecular Genetics at the Royal Devon  
5 and Exeter Hospital, UK. PCR and Sanger sequence analysis of all 22 exons of the *PHEX* gene was  
6 performed using leukocyte DNA. PCR primer sequences are available on request. *PHEX* gene dosage  
7 analysis was assessed by multiple ligation-dependent probe amplification (MLPA) using MRC  
8 Holland kit P2223-B1. Subsequent analysis of the *DMP1*, *ENPP1*, *FGF23*, *PHEX* and *SLC34A3*  
9 genes was undertaken by targeted next generation sequencing (Agilent custom capture v6/Illumina  
10 NextSeq500). All the coding regions and exon/intron boundaries (50 bp upstream to 10 bp  
11 downstream of each exon) were analysed for these five genes and also included the 3'UTR region of  
12 the *PHEX* gene for the detection of the reported c.\*231A>G mutation (9). Publicly accessible  
13 databases including the Exome Variant Server (EVS) (<http://evs.gs.washington.edu/EVS/>) and the  
14 Exome Aggregation Consortium (EXAC) (<http://exac.broadinstitute.org/>), *PHEX* mutation database  
15 'PHEXdb' (<http://www.phexdb.mcgill.ca/>) and HGMD Pro >([https://portal.biobase-](https://portal.biobase-international.com/hgmd/pro/start.php)  
16 [international.com/hgmd/pro/start.php](https://portal.biobase-international.com/hgmd/pro/start.php)) were examined for the presence of any detected sequence  
17 variants. *PHEX* ortholog protein sequences were aligned using ClustalOmega  
18 (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) (20).

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### 20 *3.2 Cellular analysis of PHEX protein expression*

21 HEK293 cells were cultured in DMEM (Sigma) supplemented with 10% FBS (Invitrogen). Cells were  
22 split into 12-well plates, and transfected using Lipofectamine 2000 (Invitrogen) and vectors encoding  
23 either the full-length wild-type (WT) human *PHEX* (Source Bioscience; clone accession: KJ891794)  
24 or mutant *PHEX* (GeneArt, Invitrogen; mutation: c.2158G>T) or an empty pCS3 vector, as described  
25 (21, 22). Cells were lysed for western blotting or fixed for immunostaining 48h following  
26 transfections. HEK293 cells were lysed using RIPA buffer and denatured in Laemmli sample buffer  
27 (21). Protein separation and western blot were performed, as described (22). An anti-*PHEX* rabbit  
28 polyclonal antibody (Abcam, ab96072) was used at 1:500 dilution. Secondary HRP-conjugated

1 antibody (anti-rabbit; Cell Signalling) was used at 1:2000 dilution. Immune complexes were  
2 visualised by chemiluminescence using ECL kit (Thermo Fisher Scientific). Ponceau S staining (*Po-*  
3 *S, Sigma Co.*) was used to visualise the loaded protein. HEK293 cells were fixed in 4%  
4 paraformaldehyde in PBS, and immunostaining performed, as described (22). To assess for PHEX  
5 and endoplasmic reticulum (ER) co-immunostaining, cells were permeabilised with 0.5% Triton X-  
6 100. Immunostaining was performed using anti-PHEX (1:500; Abcam; ab96072), anti-Na-K-ATPase  
7 (1:100; 610992, BD Bioscience) or anti-calnexin (1:100; 610523, BD Bioscience) antibodies; and  
8 using secondary anti-mouse AlexaFluor-488 and anti-rabbit AlexaFluor-594 antibodies (Invitrogen).  
9 Cells were visualised using a Zeiss fluorescent microscope. Colocalisation quantification was  
10 performed using BioimageX (23). The percentage of PHEX immunostaining at the plasma membrane  
11 or ER was quantified using a minimum of six slides from at least four separate experiments, and  
12 compared between WT and mutant-expressing cells using the Student's t-test.

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#### 4. Results

18 DNA sequence analysis of the *PHEX* coding regions and adjacent splice sites identified a novel  
19 heterozygous G-to-T transversion at nucleotide c.2158 in exon 22 in the patient (Fig. 1C). This G-to-T  
20 transversion (GCA to TCA) resulted in a missense substitution, p.Ala720Ser, of the PHEX protein  
21 (Fig. 1D). The absence of this DNA sequence abnormality in >6500 exomes from the EVS cohort and  
22 >60,700 exomes from the ExAC cohort, together with evolutionary conservation of the Ala720  
23 residue in vertebrate PHEX orthologs (Figure 1E), indicated that the Ala720Ser abnormality likely  
24 represented a pathogenic *PHEX* mutation rather than a benign polymorphic variant. No alterations in  
25 *PHEX* gene dosage or in the *PHEX* 3'UTR were identified. Moreover, analysis of the *DMP1*, *ENPP*,  
26 *FGF23* and *SLC34A3* genes, which are involved in phosphate homeostasis and have been associated  
27 with FGF23-mediated hypophosphatemia (1, 18), did not reveal any abnormalities.

28 PHEX proteins that harbor missense mutations have previously been shown to be sequestered  
29 intracellularly (3), and we therefore investigated whether the Ala720Ser mutation may impair the  
30 expression and cellular processing of PHEX by *in vitro* transient transfection of WT (Ala720) or

1 mutant (Ser720) *PHEX* full-length cDNA constructs in HEK293 cells. Western blot analysis of whole  
2 cell lysates obtained from transfected HEK293 cells demonstrated similar levels of expression of WT  
3 and mutant *PHEX* proteins, whereas, cells transfected with an empty vector (control) were shown to  
4 not express *PHEX* (Fig. 2A). Immunofluorescence analysis of permeabilised and non-permeabilised  
5 cells was undertaken to determine the cellular localization of WT and mutant *PHEX* proteins (Fig.  
6 2B-C). A localisation analysis of non-permeabilised cells revealed that ~80% of the total cellular  
7 amount of WT *PHEX* was localised at the plasma membrane (Fig. 2B and 2D). Whereas, in  
8 permeabilised cells, less than 20% of WT *PHEX* was localised in the ER (Figure 2C-D). In contrast,  
9 only ~20% of the mutant Ser720 *PHEX* protein was localised at the plasma membrane in non-  
10 permeabilised cells (Fig. 2B and 2D), whereas greater than 60% of mutant *PHEX* was associated with  
11 the ER (Fig. 2C-D). These findings indicate impaired trafficking and ER retention of the mutant  
12 Ser720 *PHEX* protein.

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## 14 **5. Discussion**

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16 Our studies have identified a pathogenic *PHEX* mutation in a patient with elevated circulating FGF23  
17 concentrations and hypophosphatemic osteomalacia that first manifested in adulthood. Although,  
18 *PHEX* mutations are occasionally detected in osteomalacic adults (24), and even in asymptomatic  
19 adults (25), such cases usually arise within a kindred known to be affected with XLH. In contrast, the  
20 patient reported here did not have a known family history of rickets or osteomalacia, which indicates  
21 that her adult-onset XLH had likely occurred sporadically. It is of note that this patient was also  
22 diagnosed with PsA, which is an inflammatory musculoskeletal disease characterised by features such  
23 as arthritis, dactylitis, psoriatic skin disease and nail dystrophy (26). Moreover, PsA has been  
24 associated with elevated serum FGF23 concentrations (27), and this may potentially have contributed  
25 to the FGF23 excess in this patient. Furthermore, she was found to have enthesopathic changes on  
26 plain radiography. Such findings have been reported in >65% of XLH patients (28) and in 30-50% of  
27 PsA patients (26), and thus the cause of the enthesopathy in this patient who is affected with both of  
28 these conditions, remains to be elucidated. In addition, she had a history of dental abscesses that



1 began in childhood and were attributed to prior trauma, but which may potentially have represented  
2 an early manifestation of XLH. Indeed, dental abscesses are a common feature of XLH in children  
3 and have been reported to affect the primary dentition of 25% of XLH patients (29).

4 The missense Ala720Ser mutation identified in this case involved the substitution of a WT  
5 non-polar alanine residue with a mutant polar serine residue, and this was predicted to result in  
6 misfolding and retention of the mutant PHEX protein within the ER (3). Indeed, >50% of XLH-  
7 causing missense PHEX mutations, which includes another mutation affecting codon 720 of the  
8 *PHEX* gene (Ala720Thr), have previously been shown to impair trafficking of the mutant PHEX  
9 protein to the plasma membrane (3). Our *in vitro* studies revealed the Ala720Ser mutation to partially  
10 abrogate cell surface expression of the PHEX protein, and these milder pathogenic effects may  
11 explain why the patient became symptomatic only in adulthood. Another contributing factor to the  
12 milder clinical phenotype may have been cellular mosaicism arising from skewed X-inactivation of  
13 the mutant *PHEX* gene (30). Although it should be noted that such skewing has not been reported in  
14 peripheral blood cells obtained from females with XLH (31), and it remains to be elucidated whether  
15 preferential inactivation of the mutant *PHEX* gene may occur in FGF23-secreting cells such as  
16 osteocytes. Some females with XLH have been reported to have an absence of skeletal disease, and  
17 the only manifestation may be asymptomatic hypophosphatemia (25). Similarly, a recent study of  
18 XLH caused by a *PHEX* 3'-UTR mutation included an assessment of the affected mothers, and their  
19 only consistent phenotype was a mild reduction in TmP/GFR, which was not associated with  
20 substantial hypophosphatemia or skeletal abnormalities (9). The findings of these previous studies and  
21 the present report highlight that *PHEX* mutations in females may not present until adulthood or could  
22 potentially go unnoticed throughout adult life (9, 25).

23 The present case illustrates the challenge of investigating hypophosphatemic patients with  
24 demonstrable FGF23 excess in the absence of a known family history of rickets or osteomalacia. Such  
25 patients are usually suspected of harboring an acquired disorder such as TIO (18), and may undergo  
26 radiological investigations over several years to detect the underlying tumor (14, 32). However,  
27 despite these imaging studies, the causative tumor has been reported to not be identified in 25-60% of  
28 patients with FGF23-mediated adult-onset hypophosphatemic osteomalacia (12, 14, 33), thus

1 indicating that some patients may harbor an alternate etiology for their mineral disorder. Our findings  
2 highlight that a monogenic cause of FGF23 excess should be considered in such cases, even in the  
3 absence of a relevant family history, and that *PHEX* gene analysis may have utility in the  
4 investigation of patients with suspected TIO, particularly when the underlying tumor has not been  
5 identified. Appropriate diagnosis in such cases will prevent unnecessary radiological investigations,  
6 although treatment with phosphate and active vitamin D may not fully alleviate symptoms. Whether  
7 anti-FGF23 antibody treatment (34) would be beneficial in such patients remains to be investigated.

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10 **Author's role:**

11  
12 Study design: FMH and WDF. Study conduct: FMH. Data collection: KG-W, AT, RM, N-JR, MS,  
13 AS. Data analysis and interpretation: KG-W, AT, RM, N-JR, MS, AS. Drafting manuscript: KG-W,  
14 AT, WDF, FMH: Approving final version of manuscript: all authors. FMH takes responsibility for the  
15 integrity of the data analysis.

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17 **Disclosure statement:**

18  
19 FMH has received honoraria from Shire Pharmaceuticals. WDF has received educational awards from  
20 Alexion and Shire; and speaker fees from Alexion, Shire, Lilly, Roche, Seimens and Abbott; and been  
21 on Advisory Boards for Alexion, Shire, Internis and Stirling Anglian Pharmaceuticals.

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- 42
- 43

1 **Figure legends**

2

3 **Figure 1.** Clinical findings and *PHEX* mutational analysis. (A) Pelvic and proximal femoral  
4 radiographs showing bilateral cortical lucencies of the proximal medial femoral diaphysis with  
5 associated focal cortical thickening (yellow arrows), representing an insufficiency-type fracture or  
6 “Looser zone”. Mild enthesopathic changes affecting the ischial tuberosities (red arrowheads) and an  
7 incidental finding of L5 spina bifida (black arrow) are also noted. (B) Graphs showing serum  
8 concentrations of phosphate (Pi), alkaline phosphatase (ALP) and fibroblast growth factor-23  
9 (FGF23) over an 8-year period. Boxes above graphs indicate periods of treatment with ergocalciferol  
10 (D2), and with oral phosphate and alfacalcidol. (C) DNA sequence analysis showing a heterozygous  
11 G-to-T transversion at nucleotide c.2158 (red arrow) of the *PHEX* gene. (D) This sequence  
12 abnormality was predicted to lead to a missense amino acid substitution of Ala to Ser at codon 720.  
13 (E) Multiple protein sequence alignment of *PHEX* orthologs. The WT Ala720 (A) residues are shown  
14 in black, and the mutant Ser720 (S) residue is shown in red. Conserved residues are shaded grey.

15

16 **Figure 2.** Cellular localization of WT and mutant *PHEX*. (A) Western blot showing elevated levels of  
17 *PHEX* protein following transfection of HEK293 cells with a vector encoding WT or mutant *PHEX*  
18 as compared to cells transfected with an empty vector. (B) Immunofluorescence of non-permeabilised  
19 HEK293 cells showing the co-localisation of *PHEX* (red) with Na-K-ATPase, which is a plasma  
20 membrane-associated protein (green). (C) Immunofluorescence of permeabilised HEK293 cells  
21 showing the co-localisation of *PHEX* (red) with calnexin, which is an ER-associated protein (green).  
22 (D) Quantification of co-localisation of WT or mutant *PHEX* protein with plasma membrane or ER-  
23 associated proteins in HEK293 cells. \* $p < 0.05$ ; bars show standard deviation.

24

1 **Table 1.** Serum biochemical parameters at presentation.

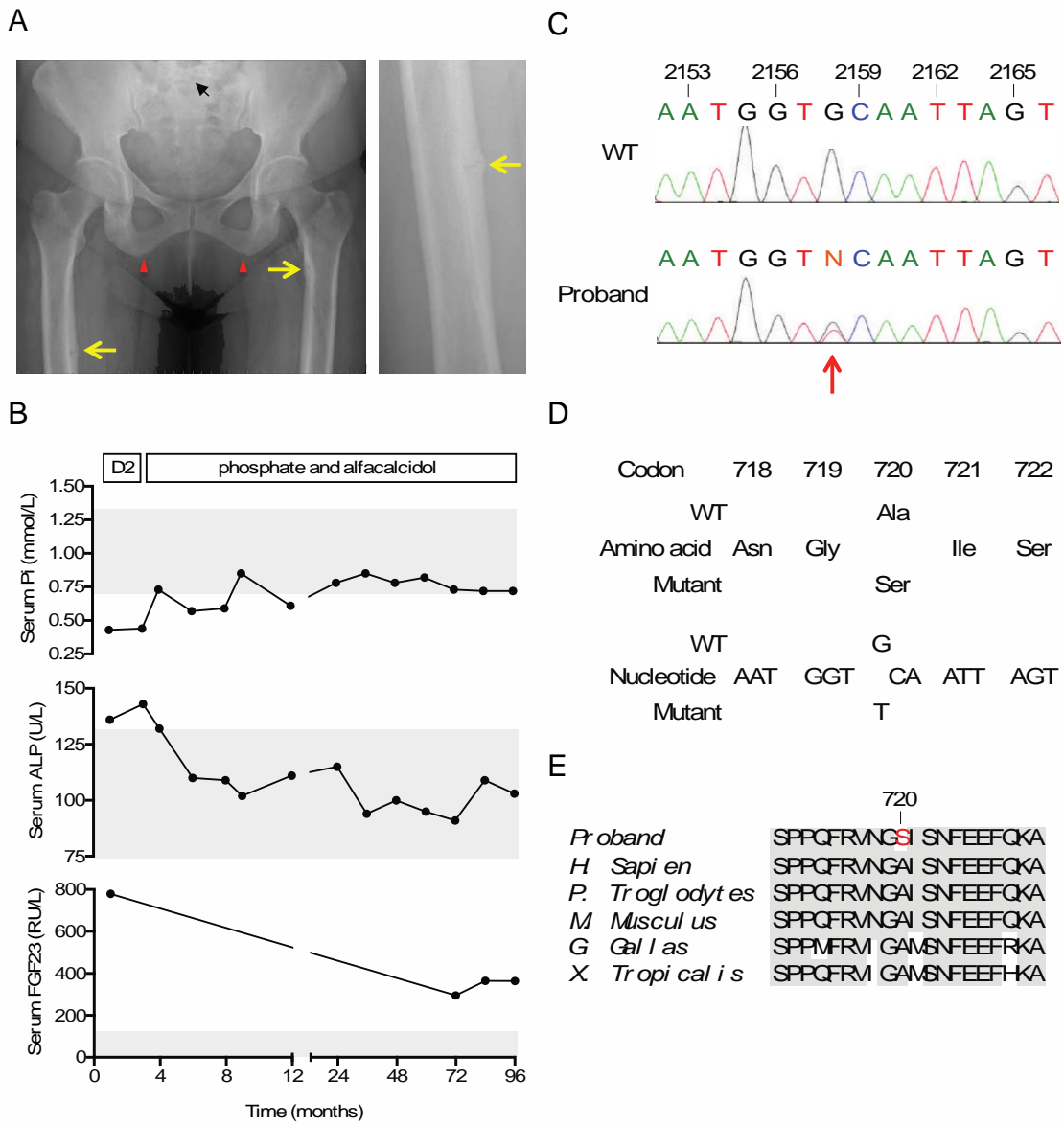
2

<b>Parameter</b>	<b>Value</b>	<b>Reference range</b>
Sodium (mmol/L)	136	135-145
Potassium (mmol/L)	3.7	3.5-5.0
Creatinine (μmol/L)	57	54-145
Albumin-adjusted calcium (mmol/L)	2.41	2.20-2.60
Phosphate (mmol/L)	0.43	0.70-1.40
Alkaline phosphatase (U/L)	136	30-130
Urate (μmol/L)	237	140-360
Parathyroid hormone (pmol/L)	11	1.1-6.9
25-hydroxyvitamin D (nmol/L)	72.4	Adequate >50
1,25-dihydroxyvitamin D (pmol/L)	98	43-144
FGF23 (RU/mL)	779	<100

3

4

Figure 1

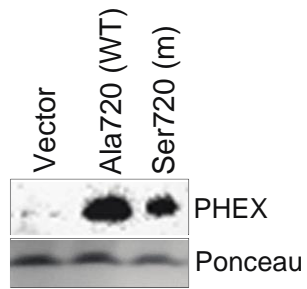


1

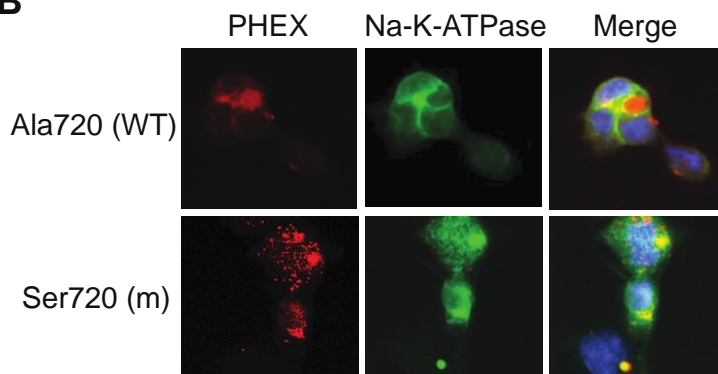
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**Figure 2**

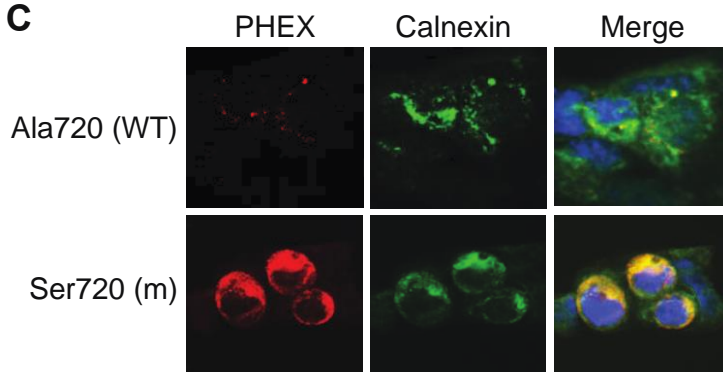
**A**



**B**



**C**



**D**

