Studies of mice deleted for Sox3 and uc482: relevance to X-linked hypoparathyroidism

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Abstract

Hypoparathyroidism is genetically heterogeneous and characterized by low plasma calcium and parathyroid hormone (PTH) concentrations. X-linked hypoparathyroidism (XLHPT) in two American families is associated with interstitial deletion-insertions involving deletions of chromosome Xq27.1 downstream of SOX3 and insertions of predominantly non-coding DNA from chromosome 2p25.3. These could result in loss, gain, or movement of regulatory elements, which include ultraconserved element uc482, which could alter SOX3 expression. To investigate this, we analysed SOX3 expression in EBV-transformed lymphoblastoid cells from three affected males, three unaffected males, and four carrier females from one XLHPT family. SOX3 expression was similar in all individuals, indicating that the spatiotemporal effect of the interstitial deletion-insertion on SOX3 expression postulated to occur in developing parathyroids did not manifest in lymphoblastoids. Expression of SNTG2, which is duplicated and inserted into the X chromosome, and ATP11C, which is moved telomerically, were also similarly expressed in all individuals. Investigation of male hemizygous (Sox3−/Y and uc482−/Y) and female heterozygous (Sox3+/- and uc482+/-) knockout mice, together with wild-type littermates (male Sox3+/- and uc482+/-, and female Sox3+/- and uc482+/-), revealed Sox3−/Y, Sox3+/−, uc482−/Y, and uc482+/- mice to have normal plasma biochemistry, compared to their respective wild-type littermates. When challenged with a low calcium diet, all mice had hypocalcaemia, and elevated plasma PTH concentrations and alkaline phosphatase activities, and Sox3−/Y, Sox3+/-, uc482−/Y, and uc482+/- mice had similar plasma biochemistry, compared to wild-type littermates. Thus, these results indicate that absence of Sox3 or uc482 at Sox3 does not cause hypoparathyroidism and that XLHPT likely reflects a more complex mechanism.
Introduction

Heritable hypoparathyroidism (HPT) is a genetically heterogeneous disease, characterized biochemically by hypocalcaemia, hyperphosphatemia, low plasma parathyroid hormone (PTH) concentrations and inappropriately normal or high urinary calcium excretion (1). Genetic abnormalities causing HPT may lead to complex congenital syndromes or to an isolated endocrinopathy, for which autosomal dominant, autosomal recessive and X-linked modes of inheritance have been described (OMIM #146200 and %307700) (2). These genetic abnormalities may result in defects in PTH itself, parathyroid gland development, or parathyroid gland function. For example, mutations in PTH cause a lack of functional PTH protein (3), mutations in the parathyroid-specific transcription factor glial cells missing B (GCMB) result in defective parathyroid gland development (4, 5), and gain-of-function mutations in the calcium-sensing receptor (CaSR) may suppress parathyroid gland function in association with hypocalcaemia (6).

X-linked HPT (XLHPT, OMIM %307700) was first reported in 1960 (7) in a large kindred from Missouri (MO), USA and subsequently in a related family also from MO (8, 9, 10), and more recently in a third, unrelated family from Illinois (IL), USA (11). Both families from MO and the family from IL had interstitial deletion-insertions involving deletion of a non-coding region on chromosome Xq27.1 between sex-determining region Y (SRY)-box 3 (SOX3) and adenosine triphosphatase 11C (ATP11C), and an insertion of a larger section emanating from chromosome 2p25.3 (8, 11) (Fig. 1A). In the MO kindred, this consisted of a ~25 kb deletion of non-coding DNA from Xq27.1 ~67 kb downstream of SOX3 and an inverted insertion of ~340 kb containing exons 2–16 of syntrophin gamma 2 (SNTG2). In the IL family, ~1.5 kb was deleted from Xq27.1 ~80 kb downstream of SOX3 and ~47 kb of non-coding DNA was inserted from 2p25.3, which differed from the inserted DNA in the MO kindred (11) (Fig. 1A). Although the 340 kb insertion in the MO kindred contained 15 exons of SNTG2, no open reading frames were present (8), and the 47 kb insertion in the IL family did not contain coding DNA. Similarly, neither of the X chromosome deletions removed coding DNA. We therefore hypothesized that these rearrangements may alter the function of a regulatory element or nearby gene, that affects SOX3 expression, which has been demonstrated in developing mouse parathyroids between embryonic day (E)10.5 and E15.5 (8). SOX3 expression was reported to be altered by a large 774 kb insertion downstream of SOX3 in patients with XX male sex reversal, demonstrating that SOX3 expression may be susceptible to position effects, similar to those reported for SOX9 and SRY (12, 13), although SOX3-coding mutations have not been identified in male patients with HPT (14).

Thus, in the XLHPT families there are three possible mechanisms that could result in such alterations in SOX3
expression (Fig. 1B). Firstly, deletions of X chromosome sequences may result in loss of a repressor or enhancer of SOX3 expression. Secondly, insertions of chromosome 2 sequences may result in insertion of DNA that may repress or enhance SOX3 expression. Thirdly, deletion-insertions, that result in a net gain of DNA on the X chromosome, may move a repressor or enhancer of SOX3 expression further away from SOX3, thereby altering its effect on SOX3 expression, that is, a position effect (Fig. 1B). Gene enhancers and repressors are often evolutionarily conserved, and >60% of ultraconserved (uc) non-coding elements that are conserved in human through to *Takifugu rubripes* (puffer fish), may act as positive enhancers (15). One such element, designated uc482 (or highly conserved non-coding element (HCNE) 8), is located ~400 kb downstream of SOX3 and was reported to be a likely enhancer of Sox3 expression in *Danio rerio* (zebrafish) (16). Furthermore, uc482 is moved further away from SOX3 by both of the deletion-insertions causing XLHPT (Fig. 1B).

To explore the possible roles of SOX3 and uc482 in HPT, we undertook studies to determine the effects of the interstitial deletion-insertion on SOX3 expression in patients with XLHPT and in knock-out mice lacking alleles of Sox3 and uc482.

### Materials and methods

#### Human studies

Venous blood samples were collected after informed consent from individuals and following protocols approved by local and national ethics committees (Multi-centre Research Ethics Committee (London, UK; MREC 02/2/93) and the Human Research Protections Office, Washington University School of Medicine, St. Louis, MO, USA).

#### DNA sequence analysis

DNA was extracted from leukocytes using the Gentra Puregene blood kit (Qiagen) and standard protocols. PCR amplification of DNA across the deletion-insertion boundaries was carried out using primers previously described (8), and Sanger DNA sequence analysis was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and an ABI automated capillary sequencer (Applied Biosystems), as described (4, 8).

### Quantitative real-time PCR (qRT-PCR)

Lymphocytes were Epstein-Barr virus (EBV)-transformed to generate a lymphoblastoid cell line as previously described (3), and mRNA extracted using the Oligotex mRNA mini kit (Qiagen) and standard protocols. cDNA was generated using Quantscript reverse transcriptase (Qiagen), and quantitative reverse transcriptase (qRT)-PCRs performed using the QuantiTect SYBR Green PCR kit (Qiagen) in quadruplicate for each individual, and SOX3, SNTG2, ATP11C, and GAPDH specific primers, using a Rotorgene 5 (Qiagen), as described (17). Expression of genes was normalized to GAPDH expression and analyzed by the comparative $\Delta\Delta C_T$ method (18).

#### Mouse studies

All animal studies were approved by the University of Oxford Ethical Review Committee and were licensed (project license numbers PPL 30/2241, PPL 30/2739 and PPL 30/3251) under the Animals (Scientific Procedures) Act 1986, issued by the UK Government Home Office. Sox3-knockout mice were maintained on the MF1 outbred background (19) and uc482-knockout mice were maintained on the C57Bl/6 background (20). Mice were genotyped using DNA extracted from ear biopsies followed by PCR assay. PCR primers for Sox3 genotyping were WT F: 5′-TCGGGTTGGTGGGAGAGGTTAT-3′; WT R: 5′-GTGGGTTGTGCGCTCAGTGG-3′; KO F: 5′-CACGGCGAGCCTGTCAATCAGG-3′, KO R: 5′-TTGATGCCTTCTTTTGTGTCG-3′; and for uc482 genotyping were For: 5′-GGAAATGGCGCCAGCTCAAG-3′; WT R: 5′-TACCGAAGATCTAACCTTGGGTCG-3′; KO R: 5′-TGGAATCTGTGCAGCTTCTTCCA-3′. After weaning at age 21 days, mice were fed either a synthetic control diet (Dyets Inc., Bethlehem, PA, USA) containing 0.95% calcium and 4.50 iU/g vitamin D$_3$ or a synthetic low-calcium diet containing 0.001% calcium and 0.0 iU/g vitamin D$_3$ (Dyets Inc.), as previously described (21). The low calcium/no vitamin D$_3$ diet has previously been shown to unmask defects in the ability of the parathyroid glands to respond to deficiencies in dietary calcium, which may not be evident when calcium is abundant (21). Food and water were allowed *ad libitum*. Mice were killed and blood was collected from the jugular vein for plasma analysis using an AU400 clinical chemistry analyser (Olympus), and plasma calcium was corrected for albumin (Corr. Ca) using the formula: Corr. Ca = Ca (mmol/L) – ((Alb (g/L) − 30) × 0.017), as described previously (22).
PTH concentrations were measured using an ELISA for mouse intact PTH (Immutopics, Quidel, San Diego, CA, USA), as described previously (22).

**Histological studies**

To study parathyroids of the knockout mice, the trachea, thyroid, and parathyroids were dissected en bloc and fixed for 24 h in 10% neutral buffered formalin. Paraffin sections (6 μm thick) were dewaxed and stained with haematoxylin and eosin (H&E) using standard techniques. H&E stained sections were viewed using an Eclipse E400 light microscope (Nikon) and images acquired using NIS-elements BR2.30 imaging software (Nikon). An estimation of parathyroid gland size was performed by measuring the largest cross-sectional area of the parathyroid gland in serial sections from each mouse, as described previously (21, 23), and corrected for body weight.

**Statistical analyses**

For human qRT-PCR analyses, data were analyzed using one-way ANOVA, and all data are represented as mean ± s.d. fold change relative to unaffected males. For animal studies, data were analyzed using unpaired Student’s t-test. All data are represented as mean ± s.d., and for plasma biochemical results, significances are reported after Bonferroni correction for multiple comparisons.

**Results**

**Expression of SOX3, ATP11C and SNTG2 in patients with XLHPT**

PCR analysis using primers, previously described (8), that spanned the telomeric breakpoint, was used to confirm the presence or absence of the X chromosome deletion-insertion in three affected males, four carrier females, and three unaffected related males from the MO kindred (Fig. 2A). Thus, individuals II.5, II.6 and III.1, who are unaffected males with normocalcaemia, were confirmed to have only the wild-type (normal) allele; individuals IV.1, IV.2 and IV.3, who are affected males with hypocalcaemia due to XLHPT, were confirmed to have only the mutant allele; and individuals II.2, II.3, II.4 and III.2, who are carrier females with normocalcaemia, were confirmed to be heterozygous in having wild-type and mutant alleles (Fig. 2A). Parathyroid glands from XLHPT patients (7), and normal individuals were not available to study the expression of SOX3 and ATP11C, which flank the interstitial deletion-insertion involving chromosomes Xq27.1 and 2p25.3, and the inserted region of SNTG2 in the XLHPT MO kindred (Fig. 1). Lymphoblastoid cell mRNA was therefore used to undertake qRT-PCR analysis of SOX3, ATP11C, and SNTG2 expression using mRNA obtained from EBV-lymphoblastoids from unaffected males (unaff) (n = 3), affected males (aff) (n = 3) and carrier females (carriers) (n = 4), (n = 4 replicates for each individual). Data are displayed as mean ± s.d. relative to expression in unaffected males.

![Figure 2](https://ec.bioscientifica.com)

**Figure 2**

SOX3, SNTG2, and ATP11C expression in XLHPT family members. (A) Confirmation of the Xq27.1-2p25.3 deletion-insertion by PCR using primers spanning the telomeric breakpoint (Fig. 1) in four carrier females (circles with dots), and three affected males (filled squares) from the MO kindred. Three unaffected related males (open squares) in whom the Xq27.1-2p25.3 insert is not present are also shown. The PCR primers were designed to yield a wild-type (WT) product of 237 bp in size, and a mutant (M) product of 321 bp in size, S = size marker. (B) Quantitative reverse transcriptase (qRT)-PCR analyses of SOX3, ATP11C, and SNTG2 expression using mRNA obtained from EBV-lymphoblastoids from unaffected males (unaff) (n = 3), affected males (aff) (n = 3) and carrier females (carriers) (n = 4), (n = 4 replicates for each individual). Data are displayed as mean ± s.d. relative to expression in unaffected males.
in lymphoblastoid cells, which are not representative of parathyroid cells. We therefore studied mouse knockout models deleted for Sox3 and uc482.

**Mice deleted for Sox3 have normal plasma calcium and PTH concentrations**

Mice were genotyped (Fig. 3A), and wild-type (Sox3+/Y) and hemizygous (Sox3−/Y) male adult mice, and wild-type (Sox3+/Y) and heterozygous (Sox3−/+Y) female adult mice were found to have normal body weights and plasma biochemistry at ages 6 and 28 weeks (Table 1). Thus, corrected calcium concentrations in plasma were similar in: Sox3+/Y mice compared to Sox3−/Y mice (3.08 ± 0.11 mmol/L vs 3.05 ± 0.15 mmol/L at 6 weeks, and 2.83 ± 0.19 mmol/L vs 2.79 ± 0.12 mmol/L at 28 weeks, n=10–21); and in Sox3−/+ mice compared to Sox3+/− mice (3.02 ± 0.08 mmol/L vs 3.06 ± 0.10 mmol/L at 6 weeks, and 2.65 ± 0.12 mmol/L vs 2.73 ± 0.11 mmol/L at 28 weeks, n=9–25). Plasma PTH concentrations were also similar in: Sox3+/Y and Sox3−/Y mice (11.8 ± 7.6 pmol/L vs 12.8 ± 10.4 pmol/L at 6 weeks, and 41.8 ± 31.4 pmol/L vs 44.8 ± 34.1 pmol/L at 28 weeks)

![Figure 3](https://ec.bioscientifica.com)

**Figure 3**

Plasma biochemistry and parathyroid gland sizes in wild-type and mutant Sox3 mice consuming either control or low calcium and vitamin D diets. (A) Genotyping of wild-type (Sox3+/Y) and hemizygous (Sox3−/Y) male, and wild-type (Sox3+/Y) and heterozygous (Sox3−/+Y) female mice. S: size marker; B: blank; WT: wild-type band; mut: mutant band. (B) Plasma corrected calcium; (C) plasma PTH; (D) plasma alkaline phosphatase (ALP) activity; (E) plasma urea; (F) plasma creatinine; (G) parathyroid gland area. Wild-type males (+/Y) and mutant males (−/Y) are shown by the dark grey and light grey solid bars, respectively. Wild-type females (+/+) and mutant carrier females (+/−) are shown by the dark grey and grey hatch solid bars, respectively. Data are displayed as mean ± s.d. *P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.001 for mice on the low calcium and vitamin D (low Ca2+ and vit D) diet compared to mice of the same genotype on the normal diet. (H) Representative histological images used for quantification of parathyroid gland area. Pt: parathyroid; Th: thyroid; SM: strap muscle. Scale bars = 200 µm.
35.9±34.1 pmol/L at 28 weeks, n=14–22); and in Sox3+/− and Sox3−/− mice (15.3±1.8 pmol/L vs 9.9±1.06 pmol/L at 6 weeks, and 35.6±23.8 pmol/L vs 24.8±19.1 pmol/L at 28 weeks, n=12–24) (Table 1). In addition, the plasma phosphate, urea, creatinine and albumin concentrations, and alkaline phosphate activity were similar in wild-type and mutant Sox3 mice at 6 and 28 weeks of age (Table 1).

The standard control mouse chow contains 0.95% calcium and 4.5 IU/g vitamin D3, which represent levels that are ~150-fold and ~30-fold higher, respectively, than those recommended for daily intake in humans (21). Furthermore, these high dietary contents of calcium and vitamin D have been reported to mask hypocalcaemia in mice with a different cause of HPT (21). We therefore challenged Sox3+/+, Sox3−/−, Sox3+/−, and Sox3−/− mice with a diet low in calcium (0.001%) and lacking in vitamin D3 (low diet) from weaning until 60 days old. Wild-type male and female mice on the low diet had significantly lower plasma corrected calcium concentrations than those on the control diet (mean±s.d.) (low diet versus control diet for Sox3+/+ = 1.96±0.24 mmol/L vs 3.08±0.11 mmol/L, P < 0.001; and Sox3−/− = 2.01±0.2 mmol/L vs 3.02±0.08 mmol/L, P < 0.001, n=16–25) (Fig. 3B). These lower plasma corrected calcium concentrations were not due to lower plasma albumin concentrations when on the low diets, as these plasma albumin concentrations were similar (Sox3+/+ = 32.70±0.35 g/L, Sox3−/− = 32.58±0.24 g/L, Sox3+/Y = 31.50±0.34 g/L and Sox3−/Y = 32.40±0.40 g/L), on low diet to those observed in mice on the control diet (Table 1). This hypocalcaemia was associated with significant increases in plasma PTH concentrations (Sox3+/+ = 150.1±46.3 pmol/L vs 11.8±7.6 pmol/L, P < 0.001; and Sox3−/− = 157.8±47.6 pmol/L vs 15.3±10.6 pmol/L, P < 0.001, n=15–24) (Fig. 3C) and alkaline phosphatase (AP) activities (Sox3+/+ = 668.1±161.9 U/L vs 258.7±47.2 U/L, P < 0.001; Sox3−/− = 820.8±234.4 U/L vs 296.5±58.4 U/L, P < 0.001, n=16–25) (Fig. 3D). Wild-type mice on the low diet, compared to those on the control diet, also had significantly lower plasma urea concentrations (Sox3+/+ = 5.98±0.91 mmol/L vs 7.85±1.24 mmol/L, P < 0.001; and Sox3−/− = 5.69±0.99 mmol/L vs 6.90±1.13 mmol/L, P = 0.042, n=18–25) (Fig. 3E) and creatinine concentrations (Sox3+/+ = 14.3±0.4 µmol/L vs 17.3±0.88 µmol/L, P < 0.001; and Sox3−/− = 14.9±1.11 µmol/L vs 17.7±0.9 µmol/L, P < 0.001, n=17–23) (Fig. 3F).

Similar to wild-type mice, mutant male (Sox3+/−) and heterozygote female (Sox3+/−) mice on the low diet also had significantly lower plasma corrected calcium concentrations than those on the control diet (Sox3+/− = 1.93±0.2 mmol/L vs 3.05±0.15 mmol/L, P < 0.001; and Sox3−/− = 1.91±0.14 mmol/L vs 3.06±0.10 mmol/L, P < 0.001, n=13–24) (Fig. 3B). This was associated with significantly higher plasma: PTH concentrations (Sox3+/− = 162.6±37.6 pmol/L vs 12.8±10.4 pmol/L, P < 0.001; and Sox3−/− = 187.5±28.6 pmol/L vs 9.9±10.6 pmol/L, P < 0.001, n=13–21) (Fig. 3C); and ALP

Table 1 Plasma biochemistry in 6- and 28-week-old wild-type and mutant Sox3 mice consuming the control diet.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sox3+/+</td>
<td>n = 19–22</td>
<td>n = 23–25</td>
</tr>
<tr>
<td>Sox3−/−</td>
<td>n = 14–18</td>
<td>n = 12–15</td>
</tr>
<tr>
<td>Age: 6 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>11.85±3.49</td>
<td>12.05±2.30</td>
</tr>
<tr>
<td>Corr. Ca (mmol/L)</td>
<td>3.08±0.11</td>
<td>3.02±0.08</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>11.8±7.6</td>
<td>15.3±11.8</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>4.24±0.76</td>
<td>4.07±0.62</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>258.7±47.2</td>
<td>296.5±58.4</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>7.85±1.24</td>
<td>6.90±1.13</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>17.3±0.8</td>
<td>17.7±0.9</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>30.5±1.7</td>
<td>31.7±2.0</td>
</tr>
<tr>
<td>Age: 28 weeks</td>
<td>n = 10–14</td>
<td>n = 8–12</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>47.51±6.81</td>
<td>32.16±1.66</td>
</tr>
<tr>
<td>Corr. Ca (mmol/L)</td>
<td>2.83±0.19</td>
<td>2.65±0.12</td>
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<tr>
<td>PTH (pmol/L)</td>
<td>41.8±31.4</td>
<td>35.6±23.8</td>
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<tr>
<td>Phosphate (mmol/L)</td>
<td>3.33±0.49</td>
<td>2.97±0.21</td>
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<tr>
<td>ALP (U/L)</td>
<td>81±15</td>
<td>98±24</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>8.3±1.12</td>
<td>7.2±1.4</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>15.9±0.9</td>
<td>16.1±1.7</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>29.5±2.2</td>
<td>32.2±1.5</td>
</tr>
</tbody>
</table>

Mean ± s.d., values are shown. Wild-type males (Sox3+/+), mutant males (Sox3−/−), wild-type females (Sox3+/−), and mutant carrier females (Sox3+/−). There were no significant differences between knockout and wild-type littermates at the same age. ALP, alkaline phosphatase; Corr. Ca, corrected calcium; PTH, parathyroid hormone.
activities (Sox3−/Y = 700.3 ± 183.2 U/L vs 270.2 ± 49.4 U/L, P < 0.001; Sox3+/− = 906.4 ± 217.2 U/L vs 282.4 ± 48.4 U/L, P < 0.001, n = 12–22) (Fig. 3D). Sox3−/Y and Sox3+/− mice on the low diet also had significantly lower plasma: urea concentrations (Sox3−/Y = 6.6 ± 0.92 mmol/L vs 7.83 ± 0.87 mmol/L, P < 0.001; and Sox3+/− = 5.88 ± 0.92 mmol/L vs 7.10 ± 0.74 mmol/L, P < 0.018, n = 13–25) (Fig. 3E); and creatinine concentrations (Sox3−/Y = 12.2 ± 1.3 μmol/L vs 16.8 ± 1.4 μmol/L, P < 0.001; and Sox3+/− = 15.3 ± 1.3 μmol/L vs 17.7 ± 1.6 μmol/L, P < 0.002, n = 12–23) (Fig. 3F). The plasma corrected calcium and PTH concentrations in Sox3−/Y and Sox3+/− mice on the low diet were not statistically different to the wild-type mice on the low diet, indicating that Sox3−/Y and Sox3+/− mice have normal parathyroid function (Fig. 3B and C). Plasma ALP activities and urea and creatinine concentrations were also similar in wild-type mice compared to Sox3−/Y and Sox3+/− mice on the low calcium diet (Fig. 3D, E and F).

Histological analysis of parathyroid gland size from wild-type and mutant Sox3 mice revealed that parathyroid gland areas in Sox3−/Y and Sox3+/− mice were significantly greater, in keeping with diet-induced secondary hyperparathyroidism, in mice on the low diet, when compared to the control diet (Sox3−/Y = 3885 ± 1012 μm²/g vs 1824 ± 656 μm²/g, P = 0.007; and Sox3+/− = 4672 ± 1914 μm²/g vs 2539 ± 684 μm²/g, P = 0.040, n = 4); however, there was no difference between Sox3−/Y and Sox3+/− mice on each diet (normal diet P = 0.091; low diet P = 0.247) (Fig. 3G and H). Parathyroid gland sizes in Sox3+/− and Sox3+/− mice were similar on the low diet compared to the control diet (Sox3+/− = 4065 ± 1178 μm²/g vs 2775 ± 1244 μm²/g, P = 0.091; and Sox3+/− = 4542 ± 1664 μm²/g vs 2684 ± 1010 μm²/g, P = 0.052, n = 4), and there was no difference between Sox+/− and Sox−/− mice on each diet (normal diet P = 0.457; low diet P = 0.328) (Fig. 3G). Thus, Sox3−/Y and Sox3+/− mice appear to have normal parathyroid function and normal responses to hypocalcaemia, and loss of Sox3 does not result in smaller parathyroids or HPT.

Mice deleted for ultraconserved element uc482 have normal plasma calcium and PTH concentrations

Mice were genotyped (Fig. 4A) and wild-type (uc482+/Y) and hemizygous (uc482−/Y) male adult mice, and wild-type (uc482+/−) and heterozygous (uc482+/−) female adult mice were found to have normal plasma biochemistry at the ages of 8 weeks and 28 weeks (Table 2). Thus, plasma corrected calcium concentrations were similar in uc482+/− mice compared to uc482−/Y mice (2.95 ± 0.08 mmol/L vs 2.98 ± 0.10 mmol/L at 8 weeks, and 2.76 ± 0.08 mmol/L vs 2.83 ± 0.13 mmol/L at 28 weeks, n = 10–24); and in uc482+/− mice compared to uc482−/− mice (2.80 ± 0.07 mmol/L vs 2.84 ± 0.12 mmol/L at 8 weeks, and 2.70 ± 0.05 mmol/L vs 2.72 ± 0.12 mmol/L at 28 weeks, n = 7–42) (Table 2). Plasma PTH concentrations were also similar in uc482+/− and uc482−/− mice (12.9 ± 9.3 pmol/L vs 12.1 ± 9.0 pmol/L at 8 weeks, and 19.9 ± 16.7 pmol/L vs 26.7 ± 15.3 pmol/L at 28 weeks, n = 14–20); and in uc482+/− and uc482−/− mice (11.1 ± 10.4 pmol/L vs 23.5 ± 24.3 pmol/L at 8 weeks, and 18.4 ± 20.2 pmol/L vs 13.6 ± 12.0 pmol/L at 28 weeks, n = 9–19) (Table 2). In addition, the plasma phosphate, urea, creatinine and albumin concentrations, and alkaline phosphatase activities were similar in wild-type and mutant uc482 mice at 8 weeks and 28 weeks of age (Table 2).

When challenged with the low diet for 60 days, plasma corrected calcium concentrations were significantly lower in: male wild-type (uc482+/Y) and mutant hemizygous (uc482−/Y) mice (uc482−/Y = 2.01 ± 0.17 mmol/L vs 2.95 ± 0.08 mmol/L, P < 0.0001; and uc482−/Y = 1.90 ± 0.27 mmol/L vs 2.98 ± 0.10 mmol/L, P < 0.0001, n = 10–24); and in female wild-type (uc482+/−) and heterozygous (uc482+/−) mice (uc482+/− = 1.92 ± 0.11 mmol/L vs 2.80 ± 0.07 mmol/L, P < 0.0001; and uc482+/− = 1.93 ± 0.17 mmol/L vs 2.84 ± 0.12 mmol/L, P < 0.0001, n = 14–42) (Fig. 4B). These lower plasma corrected calcium concentrations were not due to lower plasma albumin concentrations when on the low diets, as these plasma albumin concentrations were similar (uc482+/− = 30.44 ± 0.34 g/L, uc482−/Y = 30.73 ± 0.26 g/L, uc482+/− = 30.63 ± 0.38 g/L and uc482−/Y = 30.41 ± 0.70 g/L), on low diet to those observed in mice on the control diet (Table 2). This was associated with significantly higher plasma PTH concentrations in uc482−/Y and uc482+/− mice (uc482−/Y = 194.6 ± 48.2 pmol/L vs 12.9 ± 9.3 pmol/L, P < 0.0001; and uc482+/− = 194.0 ± 36.3 pmol/L vs 12.1 ± 9.0 pmol/L, P < 0.0001, n = 10–20); and in uc482+/− and uc482−/− mice (uc482+/− = 211.3 ± 26.4 pmol/L vs 11.1 ± 10.4 pmol/L, P < 0.0001; and uc482−/− = 226.3 ± 54.4 pmol/L vs 23.5 ± 24.3 pmol/L, P < 0.0001, n = 9–19) (Fig. 4C). Plasma ALP activities were also significantly higher in: uc482−/Y and uc482+/− mice (uc482−/Y = 1062.7 ± 233.1 U/L vs 280.7 ± 53.9 U/L, P < 0.0001; and uc482+/− = 1123.0 ± 125.4 U/L vs 279.8 ± 36.8 U/L, P < 0.0001, n = 17–29); and in uc482+/− and uc482−/− mice (uc482+/− = 1189.9 ± 211.0 U/L vs 286.8 ± 41.1 U/L, P < 0.0001; and uc482−/− = 1133.7 ± 346.5 U/L vs 293.8 ± 40.1 U/L, P < 0.0001, n = 13–40) (Fig. 4D). The plasma corrected calcium and PTH concentrations,
and plasma ALP activities in uc482+/Y and uc482+/− mice on the low diet were similar to those of the uc482+/+ and uc482+/− mice, respectively, on the control diet, indicating that uc482+/Y and uc482+/− mice have normal parathyroid function (Fig. 4B, C and D). There were no differences in plasma urea or creatinine concentrations between uc482+/Y, uc482+/−, uc482+/+ or uc482−/− mice on the low diet compared to the control diet (Fig. 4E and F).

Histological analysis of parathyroid gland size from wild-type and mutant uc482 mice revealed that parathyroid gland areas in all genotypes were significantly larger, in keeping with diet-induced secondary hyperparathyroidism, in mice on the low diet compared to the control diet (uc482+/Y = 7697 ± 1596 µm²/g vs 3545 ± 824 µm²/g, P = 0.002; uc482−/− = 7887 ± 1016 µm²/g vs 3797 ± 802 µm²/g, P < 0.001; uc482+/− = 7525 ± 1780 µm²/g vs 4220 ± 1348 µm²/g, P = 0.013; and uc482−/− = 7693 ± 276 µm²/g vs 5123 ± 782 µm²/g, P = 0.004, n = 4) (Fig. 4G, H). There were no differences between uc482+/Y and uc482−/− mice, or between uc482+/− and
Table 2  Plasma biochemistry in 8 and 28 week old wild-type and mutant uc482 mice consuming the control diet.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Males</th>
<th></th>
<th></th>
<th>Females</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Age: 8 weeks</td>
<td>uc482+/+</td>
<td>uc482−/−</td>
<td></td>
<td>uc482+/+</td>
<td>uc482−/−</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>(n = 17–25)</td>
<td></td>
<td></td>
<td>(n = 9–15)</td>
<td></td>
<td></td>
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<tr>
<td>PTH (pmol/L)</td>
<td>12.9 ± 9.3</td>
<td>12.1 ± 9.0</td>
<td></td>
<td>11.1 ± 10.4</td>
<td></td>
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<tr>
<td>Phosphate (mmol/L)</td>
<td>4.10 ± 0.70</td>
<td>4.17 ± 0.39</td>
<td></td>
<td>4.01 ± 0.54</td>
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<tr>
<td>ALP (U/L)</td>
<td>280.7 ± 53.9</td>
<td>279.8 ± 36.8</td>
<td></td>
<td>286.8 ± 41.1</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>6.29 ± 0.80</td>
<td>6.16 ± 0.79</td>
<td></td>
<td>6.63 ± 1.04</td>
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<tr>
<td>Creatinine (µmol/L)</td>
<td>28.8 ± 22.3</td>
<td>28.63 ± 2.20</td>
<td></td>
<td>28.43 ± 1.55</td>
<td></td>
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<tr>
<td>Albumin (g/L)</td>
<td>29.8 ± 1.5</td>
<td>29.0 ± 1.3</td>
<td></td>
<td>32.0 ± 1.9</td>
<td></td>
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<tr>
<td>Age: 28 weeks</td>
<td>uc482+/Y</td>
<td>uc482−/Y</td>
<td></td>
<td>uc482+/Y</td>
<td>uc482−/Y</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>(n = 8–14)</td>
<td></td>
<td></td>
<td>(n = 11–15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>19.9 ± 16.7</td>
<td>26.7 ± 15.3</td>
<td></td>
<td>18.4 ± 20.2</td>
<td></td>
<td></td>
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<tr>
<td>Phosphate (mmol/L)</td>
<td>3.55 ± 0.55</td>
<td>3.71 ± 0.66</td>
<td></td>
<td>3.20 ± 0.54</td>
<td></td>
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<tr>
<td>ALP (U/L)</td>
<td>69.7 ± 7.5</td>
<td>71.0 ± 7.0</td>
<td></td>
<td>79.7 ± 8.5</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>10.04 ± 1.04</td>
<td>9.23 ± 1.21</td>
<td></td>
<td>8.46 ± 0.83</td>
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<td></td>
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<tr>
<td>Creatinine (µmol/L)</td>
<td>33.8 ± 2.2</td>
<td>35.3 ± 2.2</td>
<td></td>
<td>33.4 ± 1.7</td>
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<tr>
<td>Albumin (g/L)</td>
<td>30.5 ± 1.3</td>
<td>31.1 ± 1.3</td>
<td></td>
<td>31.5 ± 1.8</td>
<td></td>
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</table>

Mean ± s.d. values are shown. Wild-type males (uc482+/+), mutant males (uc482−/−), wild-type females (uc482+/+), and mutant carrier females (uc482+/Y). There were no significant differences between knock-out and wild-type littermates at the same age. ALP, alkaline phosphatase; Corr. Ca, corrected calcium; PTH, parathyroid hormone.

uc482−/− mice, either on the control diet, or the low diet (uc482+/Y vs uc482−/−) control diet P = 0.338 and low diet P = 0.424; and uc482+/+ vs uc482−/− control diet P = 0.145 and low diet P = 0.432). Thus, uc482−/− and uc482+/− mice appear to have normal parathyroid gland function and normal responses to hypocalcaemia, and loss of uc482 does not result in smaller parathyroids or HPT.

Discussion

The molecular and cellular effects of the interstitial deletion-insertion involving chromosomes Xq27.1 and 2p25.3 in two families with XLHPT and their effects on SOX3 expression in the etiology of HPT remain to be elucidated (Fig. 1). Elucidation of the mechanism(s) may also have relevance to other disorders, as interstitial deletion-insertions involving deletions at the same locus on Xq27.1, but insertions from other autosomes, have been described in other diseases (7, 24, 25, 26) (Fig. 5).

For example, three families with X-linked congenital generalized hypertrichosis (CGH) have been reported to have deletion-insertions comprising: a Chinese family with a ~1.3 kb Xq27.1 deletion and a ~125.5 kb 5q35.3 insertion containing intronic sequence from within collagen type 23, alpha 1 (COL23A1); a Mexican family with a 7 bp deletion and a ~300 kb 4q31.22-31.23 inverted insertion containing protein arginine methyltransferase 10 (PRMT10), transmembrane protein 184C (TMEM184C), and parts of Rho GTPase activating protein 10 (ARHGAPIO) and endothelin receptor type A (EDNRA); and another Mexican family with a 2 bp Xq27.1 deletion and a ~389 kb 6p21.2 insertion containing Dishevelled-associated activator of morphogenesis 2 (DAAM2) and kinesin family member 6 (KIP6) and a 56 bp 3q21.1 insertion containing part of family with sequence similarity 162 member A (FAM162A) (27, 28, 29) (Fig. 5). There are two distinguishing clinical features in these CGH families. In the Chinese family, the presence of spina bifida in the proband and scoliosis in 4 family members implicates a role for SOX3, which has been linked to neural tube defects in man (24, 25), chicken (26), Drosophila (30) and zebrafish (31). In the Mexican family with the 6p21.2 and 3q21.1 insertions, several members also had dental anomalies. The disease mechanism in this family was postulated to be a position effect on fibroblast growth factor 13 (FGF13), which is found in developing hair follicles, dental mesenchyme and in developing tooth buds, was detected in patient keratinocytes (27). By contrast, a patient with XX male sex reversal was found to have a 4 bp deletion at the same locus on Xq27.1 and a ~774 kb 1q25.2-25.3 insertion containing 6 complete genes (acetyl-CoA binding domain containing 6 (ACBD6), xenotropic and polytropic retrovirus receptor 1 (XPR1), unknown gene KIAA1614,
Chromosomal rearrangements associated with XX sex reversal and hypertrichosis. A patient with XX sex reversal was found to have an interstitial deletion-insertion involving chromosome Xq27.1 and chromosome 1q25.3 (11). One family from China and two unrelated families from Mexico with hypertrichosis have been reported to have interstitial deletion-insertions involving chromosome Xq27.1 and chromosomes 5q35.3, 4q31, and 6p21.2, respectively (23, 24, 25). Dotted lines denote the location of the XLHPT interstitial deletion-insertions.

Combined, these studies demonstrate that diverse phenotypes can result from similar sized insertions into the same Xq27.1 locus, and that similar phenotypes (e.g. CGH) can result from insertions from different autosomes, highlighting the complexity underlying long-range gene regulation and the effects of chromosomal rearrangements in disease. Indeed, Hi-C chromatin interaction analyses have revealed that the alterations of genomic repressors or enhancers can affect long-range gene expression, and thus it is feasible that these deletion-insertions of Xq27.1 may affect genes that are nearby SOX3, but which exert their effects on different distant genes. For example, MIR505 located downstream of SOX3 (Figs 1 and 5) encodes a microRNA that inhibits: neural tube formation by targeting fibroblast growth factor 18 (FGF18) located on chromosome 5q35.3 (32); and cell growth and endothelial migration by targeting mitogen-activated protein kinase kinase kinase 3 (MAP3K3) through the AKT-NF-kB pathway whose genes are located on chromosomes 17q23.3 (MAP3K3), 14q32.33 (AKT serine/threonine kinase 1 (AKT)), 10q24.31 (component of inhibitor of nuclear factor kappa B kinase complex (IKK1)), 8p11.21 (inhibitor of nuclear factor kappa B kinase subunit beta (IKK2)), 4q24 (nuclear factor kappa b subunit 1 (NFKB1)), and 11q13.1 (RELA proto-oncogene, NF-KB subunit (RELA)) (33). Thus, altered expression of MIR505 could be involved in the occurrence of spina bifida in the proband from the Chinese family with CGH that is associated with a deletion of Xq27.1 and insertion of 5q35.3 (29), although it also remains possible that it is the deletion-insertion associated disruption of SOX3 itself that is responsible for spina bifida, as SOX3 dysfunction has been reported to be linked to neural tube defects in human (24, 25). Different mechanisms are likely to be involved in the phenotypes of the other CGH families with insertions of 4q31.22-31.23 and 6p21.2 (27, 28), and the XX male sex reversal patient, with insertion of 1q25.2-25.3, who did not have neural tube defects (12). Thus, it seems that different types of insertions in the same locus can affect expression in other genes and thereby result in different phenotypes. Plasma biochemistry has not been reported in CGH or XX male sex reversal, and thus any co-occurrence of HPT and associated hypocalcaemia in these patients remains unknown, although such occurrence of HPT in these patients would seem unlikely as hypocalcaemic seizures are likely to have been recognized and also as XLHPT patients do not have hypertrichrosis or XX male sex reversal.

In the two XLHPT families, the two deletions within Xq27.1 are of different sizes but do overlap, whilst the two insertions which both originate from 2p25.3 do not overlap, and both deletion-insertions lead to a net gain of DNA of differing amounts (~315 kb and ~46 kb), resulting in the same phenotype. This comparison may help to elucidate the mechanism(s) by which the deletion-insertion alters SOX3 expression (Fig. 1B). First, this may be due to loss of an enhancer or repressor from the shared missing segment of the X chromosome.
This is possible because there is overlap such that the 1.5 kb deletion in the IL family is contained within the 25 kb deletion from the MO family, but unlikely because the small 1.5 kb region does not contain any ultraconserved sequences. Second, this may be due to gain of an enhancer or repressor within the inserted DNA. This is possible because most of the inserted DNA is non-coding, but unlikely because the two inserted regions in the two families do not overlap, and there is a low probability that two different insertions would both result in the same subtle effect on timing of SOX3 expression in the developing parathyroid glands, as is postulated in these two families. Third, the deletion-insertion may move an enhancer or repressor away from SOX3. This is possible because both deletion-insertions cause net gain of DNA. One such regulatory element, which has been shown to be a likely enhancer of gene expression in zebrafish, is uc482 (16), although uc482 has not been shown directly to affect SOX3 expression and our studies reveal that mice lacking uc482 do not develop HPT (Fig. 4 and Table 2). However, there are also 4 other ultraconserved elements in the region between SOX3 and uc482, all of which would also be affected by the interstitial deletion-insertions (16), so a combinatorial loss of these elements may be required to alter SOX3 expression and cause HPT. Finally, the deletion-insertion may disrupt a topological associated domain (TAD), which represents a fundamental three-dimensional evolutionarily conserved genomic structural unit that directs regulatory elements to their promoters and insulates them from other genes (34, 35). Examination for the locations of TAD boundaries on the X chromosome in human non-parathyroid cell types using Hi-C chromatin interaction data did not identify TAD boundaries between the SOX3 and ATP11C genes, with the nearest TAD boundary being located approximately 135 kb upstream of SOX3 (36). It is therefore unlikely that the deletion/insertion involving chromosomes Xq27.1 and 2p25.3 altered an existing TAD boundary on the X chromosome, although the possibility that the inserted chromatin may have resulted in the generation of a new TAD boundary to alter SOX3 expression cannot be excluded.

SOX3 expression was not altered by the Xq27.1/2p25.3 deletion-insertion in EBV-lymphoblastoid cells from our XLHPT patients (Fig. 2), and this may or may not reflect the situation in developing parathyroid cells. However, absence of SOX3 expression that would occur in parathyroids of mutant mice with deletion of Sox3 did not result in hypocalcemia or HPT in Sox3+/− mice (Fig. 3 and Table 1). Thus, this result indicates that complete loss of SOX3 expression is unlikely to cause HPT, and that XLHPT is likely to be due to a more complex mechanism.

Nevertheless, a role for SOX3 in parathyroid development is highly likely as SOx3 expression has been detected in developing mouse parathyroid glands between E10.5 and E15.5 (8). However, the absence of any decrease in parathyroid size or development of HPT in mice lacking Sox3, even when challenged with a low calcium diet lacking vitamin D, indicates that loss of Sox3 alone is not sufficient to cause HPT and that other SOX family transcription factors may compensate for loss of SOX3. Indeed, SOX proteins, which are arranged into groups designated A-H, compensate for each other within the same group. For example, two members of the SOXF group, SOX17 and SOX18, compensate for each other in developing vascular endothelial cells (37). SOX3 is a member of the SOXB1 group, which also includes SOX1 and SOX2, and all three members share significant protein sequence homology, which is at its greatest (~95%) within the high mobility group (HMG) DNA-binding domain (38). Furthermore, these SOXB1 members have overlapping expression patterns in the CNS (38), and despite expression of Sox3 throughout the developing CNS, Sox3 null mice develop specific defects only in the hippocampus, corpus callosum and hypothalamus, and it is therefore postulated that partial compensation by SOX1 and/or SOX2 may prevent a more severe neurological phenotype in Sox3 null mice (19, 39). Indeed, redundancy between SOX2 and SOX3 has been shown for morphogenesis of the second pharyngeal arch during development in mice (39). The related SOXB1 factor, SOX19B, also acts redundantly with SOX3 in restricting WNT signalling to the organiser during CNS development and dorsoventral patterning (40).

The absence of HPT in Sox3+/− and Sox3+/− mice indicates that loss of Sox3 expression is not the underlying mechanism in XLHPT. Altered expression of Sox3 in three other ways could potentially be considered as possible mechanisms for XLHPT. First, the deletion-insertions may result in Sox3 overexpression. However, this seems unlikely, as patients with SOX3 duplications have been reported to have hypopituitarism, mental retardation, and XX male sex reversal, which are not features of XLHPT (24, 41, 42). The other two possible mechanisms are that Sox3 could either be expressed inappropriately early in the developing pharyngeal pouches, or that there might be inappropriate continued expression after the window in which Sox3 would normally be expressed. Sox3 expression in the mouse is detected in developing parathyroid glands at E10.5, E13.5, and E15.5, but not...
at E18.5, indicating that the timing of Sox3 expression is likely to be important, as has been shown for other SOX proteins during organogenesis. For example, SRY expression in somatic cells of the male genital ridge has a critical expression window of only ~6 hours, during which its expression must reach a certain threshold level to trigger Sertoli cell differentiation (43). Consistent with this hypothesis, we could not demonstrate altered Sox3 expression in lymphoblastoid cells from XLHPT patients, which might not be representative of SOX3 time and/or tissue specific expression, as for example in the parathyroid glands. Investigation of a temporospatial effect on Sox3 expression in developing parathyroid glands is not possible in XLHPT patients, and will require a mouse model engineered to harbour the appropriate chromosomal rearrangements.

The lower plasma urea and creatinine concentrations in the wild-type and mutant Sox3 mice, which are on a MF1 outbred background when on the low calcium and vitamin D diet (low diet) is interesting and likely to be a strain specific response to this dietary challenge. Thus, the plasma urea and creatinine concentrations did not decrease in response to the challenge with the low calcium and vitamin D diet, in the wild-type and mutant uc482 mice (Fig. 4E and F), which are on a C57Bl/6 background, and in previously reported wild-type and mutant Gata3 mice, which were on a FVB/N background, (21). The findings of a decreased plasma urea and creatinine concentrations in both wild-type and mutant Sox3 mice, but not in wild-type or mutant uc482 and Gata3 mice, indicate that these decreased plasma concentrations are not mutant dependent, but instead are strain-specific and may involve modifier genes that influence urea and creatinine metabolism. In addition, the observed changes in creatinine metabolism due to low vitamin D in the MF1 strain (Fig. 3) are similar to that reported in healthy populations (without chronic kidney diseases) from Taiwan and Spain, in whom low dietary vitamin D was found to correlate with low plasma creatinine concentrations (44, 45).

In conclusion, XLHPT does not result from the absence of expression of SOX3 or absence of the regulatory element uc482. Instead, the interstitial deletion-insertions likely have other effects on the temporospatial expression of SOX3 in the developing parathyroid glands.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

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References
3 Parkinson DB & Thakker RV. A donor splice site mutation in the parathyroid hormone gene is associated with autosomal recessive hypoparathyroidism. Nature Genetics 1992 1 149–152. (https://doi.org/10.1038/ng0592-149)
9 Mumma S, Whyte MP, Thakker RV, Buetow KH & Schlessinger D. mtDNA analysis shows common ancestry in two kindreds with


14 Nesbit MA, Bowl MR, Harding B, Schlessinger D, Whyte MP & Thakker RV. X-linked hypoparathyroidism region on Xq27 is evolutionarily conserved with regions on 3q26 and 13q34 and contains a novel P-type ATPase. *Genomics* 2004 84 1060–1070. (https://doi.org/10.1016/j.ygeno.2004.08.003)


