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Partial loss of function of the GHRH Receptor leads to mild Growth Hormone Deficiency

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Abstract

Introduction Recessive mutations in \textit{GHRHR} are associated with severe isolated GH deficiency (IGHD), with a final height in untreated patients of 130cm±10cm (-7.2±1.6SDS; males) and 114±0.7cm (-8.3±0.1SDS; females). Objective We hypothesised that a consanguineous Pakistani family with IGHD in 3 siblings (2 males, 1 female) would have mutations in \textit{GH1} or \textit{GHRHR}. Results Two novel homozygous missense variants [\textit{c.11G>A} (p.R4Q), \textit{c.236C>T} (p.P79L)] at conserved residues were identified in all 3 siblings. Both were absent from control databases, aside from pR4Q appearing once in heterozygous form in the ExAc Browser. The brothers were diagnosed with GHD at 9.8 and 6.0 years (height SDS: -2.24 and -1.23 respectively), with a peak GH of 2.9 μg/l with low IGF-1/IGFBP3. Their sister presented at 16 years with classic GHD (peak GH <0.1μg/l, IGF-1<3.3mmol/L) and attained an untreated \textbf{near-adult height} of 144 cm (-3.0 SDS); the tallest untreated patient with \textit{GHRHR} mutations reported. An unrelated Pakistani female IGHD patient was also compound homozygous. All patients had a small anterior pituitary on MRI. Functional analysis revealed a 50% reduction in maximal cAMP response to stimulation with GHRH by the p.R4Q/p.P79L double mutant receptor, with a 100 fold increase in EC50. Conclusion We report the first co-existence of two novel compound homozygous \textit{GHRHR} variants in 2 unrelated pedigrees associated with a partial loss of function. Surprisingly, the patients have a relatively mild IGHD phenotype. Analysis revealed that the pP79L mutation is associated with the compromise in function, with the residual partial activity explaining the mild phenotype.
Introduction

The gene encoding the growth hormone releasing hormone receptor (GHRHR) is 15.51kb in length and incorporates 13 exons on chromosome 7p14. It encodes a G-protein coupled receptor (423aa) and is expressed on the somatotroph cells of the anterior pituitary (1). Its ligand growth hormone releasing hormone (GHRH), released from the hypothalamus, stimulates the synthesis and release of growth hormone (GH; encoded by GHI) upon binding in the presence of the pituitary-specific transcription factor POU1F1 (PIT1) (2,3). GH in turn binds to receptors on the liver and generates insulin-like growth factor 1 (IGF1) and insulin-like growth factor binding protein 3 (IGFBP3), thereby promoting growth.

Consistent with their role in growth regulation, mutations in GHRHR, GHI and SOX3 are implicated in the etiology of isolated growth hormone deficiency (IGHD) (4), and the pathway was recently implicated in the GHD phenotype observed in the autosomal dominant disorder pseudohypoparathyroidism type 1b (5). Autosomal recessive mutations occurring in the GHRHR gene have been implicated in severe IGHD Type 1B, also known as Sindh dwarfism (6,7). Reported aberrations in GHRHR have included missense, splice (8), nonsense (9,10), microdeletion and promoter mutations (11,12). Many have been shown to specifically affect cAMP production, for example GHRHR (p.K329E), which fails to show a cAMP response after treatment with GHRH (13). All mutations described to date have shown a complete loss of function.

Severe IGHD Type 1B was initially described in pedigrees from the Indian subcontinent (14) and Brazil (15). Interestingly the phenotype is usually not that of classic IGHD in that affected patients have minimal facial hypoplasia and no microphallus, but do manifest anterior pituitary hypoplasia (APH) on their magnetic
resonance imaging (MRI) (3). However, growth failure is severe with proportionate
dwarfism and pubertal delay, and biochemically, the patients have low GH and IGF1
concentrations with otherwise normal pituitary function. To date, reported height in
untreated patients with a GHRHR mutation is on average 130 ± 10cm (-7.2 ± 1.6SDS)
in males and 114 ± 0.7cm (-8.3 ± 0.1SDS) in females (16).

Previous studies in our cohort of IGHD patients (n=224) revealed GHRHR
mutations in 3.7% of cases (15 patients from 7 pedigrees). All were familial cases,
predominantly from the South East Asian community, manifesting severe growth
failure with the vast majority showing APH on their MRI (7). In this manuscript, we
report the presence of two homozygous variants in GHRHR in consanguineous
pedigrees with a relatively mild GHD phenotype, and present functional data that
reveal the first partial loss of function mutation in GHRHR. Additionally, an
independent patient with the identical variants was also identified, suggesting the
presence of a founder effect.

Materials and Methods

Patients

DNA was extracted from blood samples taken from two consanguineous
pedigrees with IGHD. Ethical committee approval was obtained from the Institute of
Child Health/Great Ormond Street Hospital for Children Joint Research Ethics
Committee and informed written consent was obtained from patients and/or parents.

Direct Sequencing Analysis

Three siblings with IGHD from Pedigree 1 and a separate patient from
Pedigree 2 were screened for GH1 and GHRHR mutations. The coding region of these
genes consists of 5 exons in GH1 and 13 exons in GHRHR. These were amplified by
PCR on an Eppendorf Thermocycler over 35 cycles with primers designed using the
Primer3 program (available at http://frodo.wi.mit.edu/primer3) flanking each of the
exons in the coding regions of the genes. PCR products were treated with MicroClean
reagent (Web Scientific, cat # 2MCL-10) according to manufacturer’s instructions
and then sequenced using BigDye v1.1 sequencing chemistry (Applied Biosystems)
and analysed on a 3730X1 DNA Analyzer (Applied Biosystems/Hitachi, Japan, cat #
625-0020). Details of the PCR conditions are available upon request including the
primer sequences, product sizes and annealing temperatures. For any mutations
identified, control databases were consulted as follows: Exome Variant Server
(Evs.gs.washington.edu/EVS/) (EVS), 1000 Genomes (www.1000genomes.org), an
in-house panel of 200 ethnically matched controls, and the Exome Aggregation
Consortium (ExAC Browser) (http://exac.broadinstitute.org/).

**Molecular modelling**

The RasMol prediction model database was used to build a 3D annotated
model of the GHRHR wild type and mutant proteins respectively, to analyse and
compare protein folding and structure.

**Functional analysis:**

An expression vector was obtained encoding full-length wild-type GHRHR
cloned into pcDNA3.1 (Source Bioscience). Detected mutations p.R4Q, p.P79L and
the double mutant p.R4Q/p.P79L were introduced into the sequence using the
QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies UK LTD).
Vectors were transfected into HEK293 cells (American Type Culture Collection)
cultured in DMEM supplemented with 10% foetal bovine serum, 100U/ml penicillin,
100µg/ml streptomycin and 1% non-essential amino acids at 37°C in a humidified 5% CO₂ incubator. Approximately 1x10⁶ cells were transfected with 1.2 µg Glosensor 22F (Promega, Madison, WI, USA) and 1.2 µg GHRHR using Polyjet transfection reagent (SignaGen laboratories, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Cells were plated in a white 96-well dish at a density of approximately 35,000 cells per well and the following day media replaced with Leibovitz’s L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 2mM luciferin (Promega). After equilibration at 25°C, the basal luciferase activity was measured on a Glomax luminometer (Promega) and cells were then stimulated with various concentrations of GHRH 1-44 (Bachem, Bubendorf, Switzerland) and the luciferase response monitored approximately every 3 minutes over a period of at least 60 minutes. Response to GHRH was calculated as the area under the curve for the time period of measurement after correction for background activity from unstimulated cells.

Results

Patient phenotypes:

Patient IV.1

The proband was a male born at term (birth weight 3.6 kg) to a consanguineous Pakistani family (Figure 1A), and first presented at the age of 4 years with bilateral undescended testes, micropenis and a hypoplastic scrotum. There was no history of neonatal hypoglycemia or jaundice, he had no dysmorphic features, and at presentation his height was 100.7cm (-0.73 SDS) with a weight of 14.8kg (-1.09 SDS). At the age of 4.2 years he had an acceptable testosterone response to a 3-day human chorionic gonadotrophin (hCG) stimulation test, rising from 0.4 to 4.8nmol/L.
and basal gonadotrophins in the pre-pubertal range (LH <0.7 U/L, FSH 1.0 U/L).

Following hCG stimulation, testes were bilaterally palpable; however, later examination revealed impalpable testes and he received a further 6-week course of bilateral orchidopexies at the age of 8.2 years. Between the ages of 4-7 years, he grew steadily with a growth velocity of 5.0-5.5 cm/year (-1.34 to -1.09 SDS), but by the age of 8.5 years, his height was 119.7cm (-1.91 SDS) and his growth velocity had slowed to 2.3 cm/year (-4.1 SDS). A glucagon stimulation test performed at the age of 9.8 years (Ht 123.8cm, -2.24 SDS) showed a low peak GH (2.9µg/L) with otherwise normal pituitary function. He commenced treatment with recombinant human (rh) GH around the age of 10 years (mean dose 1mg/m²/day), progressed normally through puberty and attained a normal adult height of 170.4 cm (-0.65 SDS (Table 1); mid-parental height of 169.2 cm, -0.8 SDS) (Figure 2A). Retesting at the end of growth demonstrated persisting GHD with a low IGF1 (6.9 nmol/l; range 29.4-117.4), an undetectable peak GH (<0.1 µg/L) (Table 2) to insulin tolerance test, and otherwise normal pituitary function. A pituitary MRI confirmed APH (Figure 2D) and he remained on adult rhGH replacement (0.6mg/day).

**Patient IV.2**

The younger male sibling (Figure 1A) of patient IV.1 first presented at the age of 1.5 years with bilateral undescended testes, micropenis and a hypoplastic scrotum. He was born at term with a birth weight of 3.64 kg and there was no history of neonatal problems. At presentation he had a height of 79.6 cm (-0.5 SDS) with a weight of 9.8 kg (-1.48 SDS) and no dysmorphic features. A 3-day and 3-week HCG stimulation test showed normal testosterone responses (11.1 nmol/l and 18.7 nmol/l
respectively), with baseline gonadotrophins in the prepubertal range (LH <0.7U/L, FSH 1.7U/L); both testes were visualised in the inguinal canal. By the age of 2 years, he had a further 6-week hCG treatment course with good response in terms of testicular descent. However, at the age of 4 years, he had left testicular torsion with subsequent orchidectomy and right orchidopexy. By the age of 6 years his height was 110.2 cm (-1.21 SDS) and his growth velocity had slowed to 3.6cm/year (-2.63 SDS) (Figure 2B). Glucagon stimulation test at that time confirmed GHD with a peak GH of 2.9µg/l and a low IGF1 (18 ng/ml; normal range (NR) 45-321 ng/ml) and IGFBP3 (1.24 mg/l; range 1.86-4.39) (Table 2) with otherwise normal pituitary function and APH on MRI (Figure 2E). Treatment with rhGH was commenced at the age of 6.5 years with an excellent response. By the age of 14.6 years, he had progressed into puberty with a height of 173.3cm (+1.02 SDS) and subsequently decided to stop rhGH. He has decided not to attend any further clinics.

Patient IV.3

The female sibling of patients IV.1 and IV.2 (Figure 1A) first presented at age 16 years with short stature (height 144cm, -3.0 SDS). She had already attained menarche and had a clinical phenotype suggestive of untreated GHD (abdominal fat deposition, a high pitched voice and frontal bossing). She had an undetectable IGF1 (<3.3 nmol/L), undetectable peak GH to insulin tolerance test (<0.1 µg/l) (Table 2), a low bone mineral density (-2.5 Z scores in lumbar spine) and APH on MRI (Figure 2F). She was commenced on adult rhGH replacement (0.6mg/day) and reached a final height of 146.3cm (-2.7 SDS) (Table 1). She remains overweight, with acanthosis nigricans suggestive of insulin insensitivity (HOMA-IR of 3.1, peak insulin to oral glucose load of 143 mU/L, with a 2 hour blood glucose of 5.1 mmol/L).
Patient II.1

A female patient (unrelated to pedigree I) born to a consanguineous Pakistani pedigree (Figure 1B) with a birth weight of 3.32 kg, presented at age 6 years with short stature [height 104.3 cm (-1.8 SDS), weight 19.4 kg (-0.34 SDS)], poor growth with a growth velocity of 3.3 cm/year (-3 SDS), and APH on her MRI (Figure 2G). Biochemical testing revealed GH deficiency, with a peak GH to glucagon testing of 1.1µg/l, an IGF1 of 17 ng/ml (NR 45-321 ng/ml) and an IGFBP3 of 1.52 mg/L (NR 1.862-4.399 mg/L) (Table 2). At the age of 6 years she failed to respond to a GHRH test, and was subsequently commenced on rhGH treatment at a dose of 0.65mg/m$^2$/day (Figure 2C). She underwent spontaneous puberty and there were no concerns regarding her physical development. She has achieved a final height of 166 cm (+0.66 SDS) (Table 1). Her father’s cousin has two daughters that are on GH treatment for short stature (DNA not available).

Mutational analysis

Following direct sequencing analysis of three siblings (pedigree I) and an unrelated female patient (pedigree II) with IGHD, two homozygous variants were identified in the GHRHR gene in all four patients. The first was a novel homozygous missense variant in exon 1 (c.11G>A) (Figure 1Ci) resulting in the substitution of arginine by glutamine (p.R4Q). The second was a novel homozygous missense variant in exon 3 (c.236C>T) (Figure 1Cii) resulting in the substitution of proline by leucine (p.P79L). Neither of these changes were identified on control databases.
including Exome Variant Server, 1000 genomes and the ExAc Browser, nor in 200 ethnically-matched controls, with the exception of p.R4Q being present once on the ExAc browser in heterozygous form out of a total of 20,396 control alleles. Both p.R4Q and p.P79L have not been previously described and both are located within a highly conserved region between species (Figure 1D). All four patients were also screened for mutations in GH1 and were negative.

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**Protein modelling**

Molecular modelling predicts that the GHRHR p.P79L variant will disrupt a disulphide bridge, thus destabilising the protein. In addition, the protein prediction model Polyphen2 ([http://genetics.bwh.harvard.edu/pph2/](http://genetics.bwh.harvard.edu/pph2/)) predicted p.P79L to be functionally deleterious. Moreover, the crystal structure of a glucagon-like peptide-1 in complex with the extracellular domain of its receptor (likely to have the same structure as the GHRHR extracellular domain) shows that residues close to p.P79 interact with the ligand. Therefore, even if the mutant protein were to fold correctly without the disulphide bond in place (or with a weak disulphide bridge), the mutation is still predicted to disrupt the ligand-binding region.

It was not possible to model the R4Q mutant as the model did not extend far enough into the N-terminus. This region is in the signal peptide and is outside the hydrophobic region shown to be required for function (17). Additionally, the arginine or glutamine at position 4 (p.R4Q) have identical scores for signal peptide prediction (SignalIP4.1).

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**Luciferase assays**
Functional analysis was performed by monitoring cAMP responses of cells expressing wild-type and mutant GHRHR to varying concentrations of GHRH, and demonstrated that the double p.R4Q/p.P79L mutation had a significantly reduced maximal activity to 52.0+/−4.6% of wild-type GHRHR (p<0.001; Figure 3), with a reduction in affinity for the GHRH1-44 ligand (EC50 p.R4Q/p.P79L 113x10⁻¹¹ +/- 1.51x10⁻¹¹ vs WT 1.12x10⁻¹¹ +/- 0.21x10⁻¹¹, p<0.001). Analysis of GHRHR protein with individual mutations demonstrated that p.P79L is responsible for both the reduction in activity (55.3+/−4.4% of wild-type, p<0.001) and the altered affinity (EC50 113x10⁻¹¹ +/- 1.51x10⁻¹¹, p<0.001 vs WT; non-significant difference vs p.R4Q/pP79L) (Figure 3). The single p.R4Q mutation had no significant effect on either maximal activity (p = 0.65) or EC50 (p = 0.9) compared with wild type GHRHR (Figure 3). Western analysis of cell extracts demonstrated no significant difference in the expression levels of the various forms of GHRHR (data not shown).

Discussion

We report two novel homozygous GHRHR variants in three siblings (Pedigree I; IV.1-IV.3), and in an unrelated patient (Pedigree 2; II.1), from consanguineous families from the South East Asian community, suggesting a possible founder effect. Pedigree I (incorporating patients IV.1-IV.3) is multiply consanguineous, with the parents of the probands being first cousins. Despite all patients having IGHD and APH on their MRI, the combined effect of these variants is variable in terms of height deficit, and the patients’ phenotypes are mild compared to previous reports, with presentation in mid-childhood. Indeed, the untreated female patient from pedigree I presented much later, after she had almost completed her growth, and reached a near-adult height of 144cm (-3.0 SDS). Compared to the mean of ~114cm in the literature,
this is the tallest untreated height reported for a patient with a GHRHR mutation to our knowledge. Subsequent treatment with an adult replacement dose of rhGH resulted in an improvement in her final height to 146.3 cm (-2.7 SDS). Surprisingly, the clinical presentation of the two brothers within the same pedigree with bilateral undescended testes, hypoplastic scrotum and micropenis was suggestive of hypogonadotropic hypogonadism, although endocrine testing confirmed that the gonadal axis was intact and they progressed normally through puberty, with normalization of phallic size after commencement of rhGH treatment. The older brother and sister are now treated with adult GH replacement therapy.

The asymptomatic mother of patients IV.1-IV.3 was a heterozygous carrier of both variants, and the father is also expected to be a carrier, although his DNA is unavailable. The presence of these two homozygous variants in the two ostensibly unrelated families raises the possibility that pedigrees I and II are distantly related or may originate from the same area in South-East Asia.

Apart from a single report (18), patients with GHRHR mutations do not have neonatal hypoglycemia and in all reports to date they are reported to have normal genitalia. This is the first report of male patients with GHRHR mutations presenting with a micropenis and bilateral undescended testes. The mechanism underlying this presentation is unknown.

A number of previously reported missense GHRHR mutations (p.H137L, p.L144H, p.A176V, p.A222E, p.F242C, p.K329E) have been shown to result in correct surface expression of the receptor but reduced ability to bind to GHRH, thereby impairing intracellular signalling and stimulation of GH secretion (19,13,20). However, a missense mutation (p.V10G) within the signal peptide has been shown to affect the correct processing of the receptor and results in incomplete cleavage of the
signal peptide with failure of the mutant GHRHR receptor to translocate to the cell surface (17). The first variant, p.R4Q in exon 1, results in the substitution of a strongly basic arginine residue by a neutral glutamine residue. Despite our p.R4Q variant being located in the signal peptide, when arginine is substituted by tryptophan (p.R4W) at position 4 there is unaltered function, and this is consistent with our functional data whereby the p.R4Q variant appears to retain function (17).

The second variant, p.P79L in exon 3 results in the substitution of a proline residue by leucine. Proline is known to be essential for protein folding (21); therefore its loss at this highly conserved position will likely affect protein conformation, which supports our protein prediction model for p.P79L. The functional assays performed further support this and conclude that the p.P79L mutation alters the binding affinity and activity of GHRHR, and is thus the likely cause of the GHD observed in patients IV.1-IV.3 and II.1. Therefore the 50% reduction in the maximal cAMP response to stimulation with GHRH observed by the p.R4Q/p.P79L double mutant receptor is most likely due to this pathogenic p.P79L mutation alone rather than the combination of both p.R4Q and p.P79L (Figure 3). Our studies do not rule out the possibility that the p.R4Q variant may be contributory in some way to the mild phenotype.

**Conclusion**

We report the presence of two novel homozygous variants in *GHRHR* in a pedigree, and an unrelated patient with IGHD, suggesting a possible founder effect of these variants in patients with IGHD originating from a certain area of South-East Asia. The initial phenotype of all patients appears to be relatively mild, despite the presence of the two variants in the same gene. We show here the importance of performing functional studies in this highly unusual scenario where two variants are
present in compound homozygosity in affected individuals. All previously reported
GHRHR mutations have been associated with complete loss of function. Our
functional studies have shown that the novel p.P79L variant is pathogenic with what
appears to be a partial loss of function, and is most likely the cause of the unusually
mild form of IGHD in all four patients. Additionally, the female sibling in pedigree 1
has the tallest recorded height for an untreated patient with a GHRHR mutation, and
our data therefore suggest the possibility that rare patients with “idiopathic” short
stature may manifest mild genetic forms of GHD and reach the target height range for
the family without treatment.

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**Figures**

**Figure 1.** (A) Consanguineous Pakistani pedigree with IGHD. This family tree shows two male probands in Pedigree 1 and their affected sister (shaded black squares and a shaded circle respectively). The double lines represent consanguinity, with the parents of the affected patients being first cousins. The generations within the family are indicated by roman numerals. (B) Pedigree II with IGHD. This family consists of one affected female (shaded black circle) and her unaffected sister, born to first
cousin parents. (C) Two GHRHR mutations associated with IGHD phenotypes. A novel homozygous missense mutation, c.11G>A causing a p.R4Q substitution, was identified in exon 1 (‘(i)’ - shown as ‘N’ and indicated by arrow) and a homozygous missense mutation; c.236C>T, causing a p.P79L substitution, was found in exon 3 ((ii)’ – shown by ‘N’ and indicated by arrow) in three siblings from pedigree I and in an unrelated female patient from pedigree II. (D) Highly conserved residues across multiple species. GHRHR protein sequences spanning both amino acids that are substituted in the patients. The p.R4 and p.P79 are represented in green and show high conservation between multiple species. Any spanning amino acid residues that differ from the reference human sequence are highlighted in red.

Figure 2. (A-C) Growth charts of Patients IV.1, IV.2 and II.1. (A) Growth of patient IV.1 with GH treatment commencing at ten years of age. (B) Growth of patient IV.2 with GH treatment commencing at seven years of age (C) Growth of patient II.1 with GH treatment commencing at six years of age. (D-G) Pituitary MRI scan of patients IV.1, IV.2, IV.3 and II.1 respectively, presenting with a small anterior pituitary (indicated by the arrows).

Figure 3. Functional analysis of mutant GHRHR proteins. Transfection of HEK293 cells with wild-type or mutant GHRHR demonstrating the effects of mutations on GHRHR responses to stimulation with ligand. Transfected cells were stimulated with varying concentrations of GHRH and receptor activation monitored by cAMP accumulation in the cells (evaluated by cotransfection with the cAMP sensor Glosensor). Values shown are the mean+/− SE of three independent transfection reactions, with the data normalised to the maximal response of the wild-type receptor for each assay. ***:
p<0.001 for both EC50 and maximum cAMP level, n.s.: not significant, one-way ANOVA, with Tukey post-hoc test.

Table 1. Auxological parameters of affected patients.

Table 2. Endocrine data from Pedigrees I and II. Endocrine values relative to age and MRI results for all patients: IV.1, IV.2, IV.3 and II.1.
Table 1: Auxology on patients IV.1, IV.2, IV.3 and II.1

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Table 2: Endocrine testing for patients IV.1, IV.2, IV.3 and II.1

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<td>16.3</td>
<td>&lt;0.1</td>
<td>6.9 (nmol/L; NR 29.4-117.4)</td>
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<td>1.29</td>
<td>221</td>
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<td>2.1</td>
<td>221</td>
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<td>&lt;0.7</td>
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<td>2.9</td>
<td>18 (NR 45-321)</td>
<td>1.24 (NR 1.86-4.39)</td>
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<td>&lt;0.7</td>
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<td>3.3 (nmol/L; NR 30.8-129.5)</td>
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