De novo PMP2 mutations in families with type 1 CharcotMarieTooth disease

***De novo PMP2* mutations in families with type 1 Charcot-Marie-Tooth disease**

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ABSTRACT:

We performed whole exome sequencing on a patient with Charcot-Marie-Tooth disease type 1 (CMT1) and identified a *de novo* mutation in *PMP2*, the gene that encodes the myelin P2 protein. This mutation (p.Ile52Thr) was passed from the proband to his one affected son, and segregates with clinical and electrophysiological evidence of demyelinating neuropathy. We then screened a cohort of 136 European probands with uncharacterized genetic cause of CMT and identified another family with CMT1 that has a mutation affecting an adjacent amino acid (p.Thr51Pro) that segregates with disease. Our genetic and clinical findings ~~These kindred~~ demonstrate that dominant *PMP2* mutations cause CMT1.

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KEYWORDS: peripheral neuropathy, Charcot-Marie-Tooth disease, CMT, myelin P2 protein, PMP2.

Introduction:

Charcot-Marie-Tooth disease (CMT; also known as Hereditary Motor and Sensory Neuropathy; HMSN) is the name for inherited peripheral neuropathies that are not part of more complex syndromes. With an estimated prevalence of 1 in 2500 persons, CMT/HMSN is ~~one of~~ the most common neuromuscular~~genetic~~ disorder~~diseases~~, and is subdivided according to clinical, electrophysiological, histological, and genetic features [1]. CMT1/HMSN-I is a dominantly inherited demyelinating neuropathy; it is more common than CMT2/HMSN-II, and is characterized by an earlier age of onset (first or second decade of life), nerve conduction velocities (NCVs) less than 38 m/s in upper limb nerves, and segmental demyelination and re-myelination with onion bulb formations in nerve biopsies [2]. CMT2/HMSN-II is also dominantly inherited, but NCVs are greater than 38 m/s, and biopsies mostly show a loss of myelinated axons. Dominantly inherited neuropathies with conduction velocities that fall in between the two forms are called dominant intermediate CMT (CMTDI) [3].

Most CMT1 patients have a *PMP22* (MIM: 601097) duplication; the rest have a mutation in one of four different genes (*MPZ* [MIM: 159440], *LITAF* [MIM: 603795], *EGR2* [MIM:129010], or *NEFL* [MIM: 162280]) or missense *PMP22* mutations [4]. With the exception of *NEFL* (which is primarily expressed in neurons), these genes are robustly expressed in Schwann cells, so that mutations in them are thought to cause demyelination through cell-autonomous effects (the mutations produce their deleterious effects in Schwann cells) [2]. Dominant mutations in 17 different genes cause CMT2; with the exception of *MPZ*, these mutations are thought to produce an axonal neuropathy through their cell-autonomous effects in neurons. While CMT2 is genetically heterogeneous, with many unknown causes yet to be identified, only a few kindred with CMT1 remain unsolved [5]. Here we present two novel *de novo*

PMP2 (MIM: 170715, GenBank: NM_002677) mutations that co-segregate in two families with CMT1.

Materials and Methods:

Protocol approvals and patient consents: IRB approval was obtained from the University of Pennsylvania and the University of Antwerp for these studies. Written informed consent was obtained from each patient who participated.

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Clinical data and sample collection: Each family member was seen by one of the authors (SSS, SWY, JVW, WNL, SK, or AJ) in an outpatient clinic, where clinical neurophysiology was also performed with standard methods. A sural nerve biopsy was performed as part of the family 1 proband's prior diagnostic workup at another institution at age 17 years. We obtained the epoxy blocks, recut them, and processed them with standard electron microscopy techniques~~imaged thin sections with an electron microscope~~.

Whole exome sequencing and analysis: Genomic DNA was isolated from peripheral blood from all participants. Exome DNA was captured using the SureSelect, Human All Exon5 50 Mb kit (Agilent, Stanta Clara, CA) and sequenced on a HiSeq 2000 (Illumina, San Diego, CA). Paired-end reads of 100 bp length were generated and alignment and variant calls were made using BWA [6] and GATK software packages [7]. Data were then imported into GEM.app, a web-based collaborative genome analysis tool [8], where variants were filtered for non-synonymous or splice site variants with <1%? frequency in public databases (not present in NHLBI EVS), conservation (GERP>4, PhastCons Score>0.9, or phyloP Score>1.5) and predicted as damaging in at least one

of the following in silico predictors of mutation consequence (SIFT, PolyPhen 2 HumDiv, Mutation Taster, Mutation Assessor, LRT, or FATHMM). The *PMP2* variant was confirmed by bidirectional Sanger sequencing using forward (TGCAATTGACTTGCCTGAAA) and reverse (AGGAGTGAAACATGGGAGGA) primers.

Cohort screening: A CMT cohort consisting of 136 probands was screened for variants in *PMP2* by Sanger sequencing. The four exons and their exon-intron boundaries were amplified using primers designed in Primer3 v4.0.0 (exon 1: forward 5'-agccttgcaaaactccccag–3', reverse 5'-ttgcttagtcccgacactgc-'3; exon 2: forward 5'-tgtctcaggaggtacactcc-3', reverse 5'-ccactgagcgtaggatgtgg-3'; exon 3: forward 5'-aaaatcttgggtcagtacttga-3' , reverse 5'-tggttccatactgaaagaactgc-3'; exon 4: forward 5'-ctcctctggcccctgtca-3', reverse 5'-actggagactgacatactgat-3'). The PCR products were then purified using ExoSAP-IT (USB, Cleveland, OH) and bidirectionally sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI3730xl DNA Analyzer (Applied ~~b~~Biosystems, Foster City, CA). The acquired sequences were then aligned with SeqManII (DNASTAR Inc., Madison, WI) to the *PMP2* reference sequence (GeneBank accession number CH471068.1, NM_002677, NP_002668.1) obtained from UCSC Genome Browsers ~~(http://genome-euro.ucsc.edu/index.html)~~ GRCh37 (hg19) human genome assembly (http://genome-euro.ucsc.edu/index.html).

Paternity testing and haplotype analysis: To confirm paternity in family 1, segregation of five short tandem repeat polymorphic sites in the HLA locus (D6S265, D6S1552, D6S1517, D6S1260, and MOG-CA) was checked by fragment length analysis using a multiplex fluorescent

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PCR and capillary electrophoresis on an ~~ABI3730~~ ABI3730 analyzer (Applied Biosystems, Foster City, CA) and analyzed with GeneMapper 4.0 software (Applied Biosystems, Foster City, CA) [9]. The same technique was used for haplotype analysis in family 2, with five short tandem repeat polymorphic sites ~~near~~ flanking the *PMP2* locus (D8S2321, D8S1738, D8S275, D8S525, D8S1697).

Mutation Mapping: Our description of the mutations follows HGVS nomenclature guidelines (http://www.hgvs.org/mutnomen) and is based on sequence from RefSeq Transcript NM_002677. The PMP2 protein sequence was aligned using the ClustalW2 sequence alignment tool (http://europepmc.org/abstract/MED/17846036). The PMP protein structure was downloaded from The Protein Data Bank [10] (accession number 2WUT [11]) and viewed and annotated using the PyMol software (www.pymol.org).

Results:

PMP2 mutations in two families with CMT1:

We performed whole-exome sequencing (WES) as an unbiased approach to find the causal mutation in an unsolved family with CMT type 1 (family 1). Prior to his arrival in our clinic, the proband had a commercial test (Athena Diagnostics) for all of the known causes of CMT1 (*PMP22*, *MPZ*, *GJB1* [MIM:304040], *EGR2*, *LITAF* and *NEFL*) as well as *MFN2* (MIM: 608507), *PRX* (MIM: 605725), *SH3TC2* (MIM: 608206), *GDAP1* (MIM: 606598), *GARS* (MIM: 600287), *HSPB1* (MIM: 602195), and *DNM2* (MIM: 602378). All of these were normal except for a synonymous c.234G>A variant in *LITAF*.

Exome sequencing identified 49 candidate variants in the proband that met the filtering

criteria described above (Table S1), one of which was a novel variant c.155T>C in *PMP2* (Figure 1B). This variant was a strong candidate because *PMP2* encodes myelin protein P2, which is a constituent of compact myelin [12]. The proband's parents and his living siblings do not have the disease, and bidirectional Sanger sequencing revealed that they do not carry the variant. Among the three sons of the proband, only the son with CMT1 was found to carry the *PMP2* variant (Figure 1A, 1B). Paternity was confirmed for all living members of the second generation (II.2, II.3, II.4). Segregation analysis combined with confirmation of paternity establishes that this mutation arose *de novo* in the proband and was transmitted as a dominant trait in his progeny.

To provide further genetic evidence for an association between *PMP2* variants and CMT1, we screened a cohort of 136 European probands from families with dominantly inherited CMT of unknown origin for mutations in *PMP2* with Sanger sequencing (104 with CMT1, 14 with CMTDI, 2 with hereditary neuropathy with liability to pressure palsies, 3 with hereditary motor neuropathy, and 12 with unspecified CMT). We identified a c.151A>C variant in *PMP2* in an Austrian family with CMT1. Bidirectional Sanger sequencing showed that the proband, her 2 older affected sisters, and her affected mother carry the c.151A>C variant in *PMP2*. The proband's uncle and two aunts are unaffected and do not carry the mutation. The proband's maternal grandparents are deceased, but showed no signs of neuropathy. Haplotype analysis in all available family members of five STR markers flanking the *PMP2* locus revealed that the c.151A>C variant arose *de novo* in patient II.3 and was transmitted in a dominant fashion in her progeny (Figure S1).

The c.155T>C and c.151A>C variants are not present in the ~7,000 exomes in the GEM.app database, in the NHLBI Exome Variant Server, 1000 genomes, or the Genome Variant Database for Human Disease, nor ExAC. The c.155T>C nucleotide variant is predicted to result

126 in a missense substitution of the isoleucine residue at position 52 with a threonine (p.Ile52Thr or
127 I52T), representing a change from a hydrophobic side chain to a polar side chain. The c.151A>C
128 nucleotide variant is predicted to result in a missense threonine-to-proline substitution of the
129 adjacent amino acid (p.Thr51Pro or T51P), representing a change from a polar uncharged side
130 chain to a structurally rigid side chain. The PMP2 protein sequences from divergent species were
131 aligned using the ClustalW2 sequence alignment tool [13]. Both I52T and I51P lie in a domain of
132 the protein that is well conserved in mammals (Figure 1C), and they are close to the isoleucine
133 residue at position 43 that has been recently proposed as a putative pathogenic mutation in
134 another family with CMT1 (p.Ile43Asn or I43N) [14].

135 The PMP2 crystal structure consists of 10 anti-parallel beta strands, which compose two
136 orthogonal beta pleated sheets that form a beta barrel. This beta barrel is the ligand-binding core
137 surrounded by a hydrophilic surface [15]. The isoleucine residues 43 and 52 occupy the same
138 position on adjacent anti-parallel beta strands in the crystal structure (Figure 1D).

139

140 ***Clinical features of PMP2-associated demyelinating neuropathy in two families:***

141 *Family 1:* The proband of family 1 (Figure 1) was diagnosed by an orthopedic surgeon in
142 his late teens after his feet were examined. At the time, he had a foot drop and reported increased
143 falls. Nerve conduction studies at ages 17 and 32 showed slowed (~20 m/sec) motor and sensory
144 responses with reduced amplitudes. When first seen in our clinic at age 48, he was using molded
145 ankle foot orthoses. Strength, bulk, and tone were normal in the proximal muscles of his arms
146 and legs. There was no movement of his extensor hallucis longus (0/5; MRC scale), severe
147 weakness in ankle dorsiflexion (4-/5), and normal strength in ankle plantar flexion (5/5).
148 Strength was normal in the finger extensors and the ulnar innervated intrinsic hand muscles, but

there was marked weakness (4-/5) and atrophy in his bilateral abductor pollicis brevis. He was areflexic at the ankles, knees, and biceps. Vibratory sensation (scored using a Rydell-Seiffer tuning fork) was absent at the toes, 3 at the ankles, and 6 at the knees. Pinprick sensation was reduced to the calves, but present at the knees. Nerve conduction studies showed absent sensory responses, marked slowing and reduced amplitudes of motor responses (Table 1), and electromyography showed evidence of severe, chronic denervation in distal muscles. His CMT Neuropathy Score (version 2) [16] was 14. A sural nerve biopsy was performed as part of the proband's prior diagnostic workup at another institution at age 17 years. We obtained the epoxy blocks, recut them, and examined sections by light and electron microscopy. The density of myelinated axons was reduced; there were thinly myelinated (presumably re-myelinated) axons, and some examples of onion bulbs (Figure 2).

The proband and his wife first noted that their second son was less nimble and coordinated than his peers at age 3, and subsequently developed difficulty going up stairs and getting up after falls. His exam at age 13 was notable for high arched feet and thin calves. He had decreased bulk in extensor digitorum brevis and intrinsic foot muscles, and reduced strength in extensor hallucis longus (3/5), ankle dorsiflexion (3/5), and ankle eversion (3/5); foot inversion and plantar flexion were normal (5/5). Strength in upper extremities, including in intrinsic hand muscles, was normal. Vibratory sensation was reduced at the toes, ankles, and knees; pinprick, and temperature sensation were decreased below the knees. Reflexes were absent at knees and ankles, and trace in the arms. Sensory responses were absent, and motor responses had mild reduced amplitudes and marked slowing (Table 1); electromyography was not done. His CMT neuropathy score version 2, was 10. The other living members of the family 1 were examined (by SSS or SWY), and showed no evidence of neuropathy; II.1, who was recently deceased, had

no known manifestations of neuropathy.

Family 2: The proband in family 2 (Figure 1) was seen in clinic at age 20 for genetic counseling. At age 5, she was bothered by frequent tripping and distal lower extremity weakness. By age 11, she had developed high arched feet, thin calves, and hammer toes, and required an Achilles tendon lengthening surgery. At age 20, motor and sensory potentials were not detectable in her legs; electrophysiological studies seven years prior had shown absent sensory responses and evidence of chronic denervation (large polyphasic motor units) in the tibialis anterior. The proband reported that her two older sisters (III.1 and III.2), mother (II.3), and niece also had progressive weakness and were diagnosed with CMT. Her eldest sister (III.1) and their mother (II.3) were more mildly affected.

The proband's niece (IV.1) was recently seen in clinic at age 24. Her walking was delayed--she started walking at 18 month--and ~~Subsequently~~ subsequently she had difficulty walking due to pes equinovarus that was surgically corrected at age 8. Since then her motor deficits stabilized and she can walk unassisted for half an hour on flat terrain. She reported no impairment of her hand function. She reported mild numbness in her feet, without neuropathic pain. Her exam was notable for surgically corrected pes equinovarus without additional deformities in her hands or feet (Figure S2). She had severe atrophy of calf, peroneal, and foot muscles, with moderately high arches. Proximal leg muscles were strong, while there was reduced strength in foot inversion (0/5), extensor hallucis longus (0/5), foot dorsiflexion (1/5), and plantar flexion (4/5). Proximal arm muscles were strong, with mild atrophy and weakness (3-4/5) in her interosseous muscles and moderate atrophy and severe weakness (1/5) of abductor pollici~~u~~s brevis. Vibratory and pin prick sensation were reduced in her arms and legs. Deep tendon reflexes were absent in the arms and legs and plantar reflexes were flexor. Nerve

conduction studies showed absent sensory responses and absent median motor response; the unlar motor response had a severely reduced amplitude and was severely slowed (Table 1).

Discussion:

Our description of two *de novo* dominant disease-associated mutations in the PMP2 gene confirm and extend the recent report of a candidate mutation in *PMP2* (I43N) as the cause of CMT1 [14]. The I43N mutation segregated with the CMT phenotype in a family in four individuals. The proband's age of onset was 6 years, and his disease phenotype includes foot deformities, distal atrophy, sensory loss, and loss of deep tendon reflexes. His tibial motor response had reduced amplitude and was severely slowed (18 m/s), and a biopsy showed demyelinating neuropathy with onion bulb formation. Thus, all three reported PMP2-associated mutations share similar ~~both in their~~ clinical and electrophysiological characteristics. Our analysis shows that the three mutations affect amino acids that are predicted to be clustered near to each other in the crystal structure of P2, but how they affect the function of the P2 protein is unknown.

Myelin protein zero (P0, or MPZ), myelin basic protein (P1, or MBP), and peripheral myelin protein 2 (P2, or PMP2) were originally described as the three major proteins in PNS myelin [17]. Like other myelin-related genes, ~~*Pmp2*~~ *PMP2* mRNA expression is regulated by axon-Schwann cell interactions. P2 is a member of the fatty acid binding protein family and plays a role in intracellular trafficking of lipids: P2 binds fatty acids in the cytoplasm and transports them to vesicles and cell membranes, releasing them into the membrane when the protein becomes bound to it [18]. PNS myelin contains much more P2 than does CNS myelin [19,20]; this could account for the lack of clinical findings in our patients that would be typical of leukodystrophies (e.g. spasticity and optic atrophy).

Gonzaga-Jauregui et al. [14] examined the effects of the I43N mutation in a zebrafish model by knocking down the expression of the endogenous P2 (with a morpholino injection) and overexpressing either the wild-type or mutant human *PMP2*. Suppressing the endogenous P2 expression resulted in defective motor axon outgrowth that was rescued by wild-type but not the I43N P2 protein. This functional assay, however, is an experimental test of how gene deficiency affects motor neurons and their developing axons; its relevance as an *in vivo* model for the pathogenicity of the I43N variant in CMT1 is doubtful because (1) it does not mimic the dominant nature of *PMP2* mutation, (2) there is no evidence that axonal outgrowth is affected in human patients with any form of CMT1, including the people with *PMP2* mutations.

It is much more plausible that the I43N, T51P, and the I52T mutants result in a toxic gain of function in myelinating Schwann cells to cause demyelination. *Pmp2*-null mice have a mild phenotype [18], so a dominant-negative mechanism [14] may not be the relevant effect of the I43N, T51P, or I52T mutants. Dominant *SPTLC1* and *SPTLC2* mutations provide an illuminating example. Missense mutations in these genes, both membrane lipid binding proteins, cause a dominantly inherited neuropathy (Hereditary Sensory Neuropathy 1A), likely by causing the misincorporation of glycine and alanine into sphingolipids that are toxic [21,22]. While mutations in at least two domains in *SPTLC1* have been associated with HSAN1A, they cluster in subunits whose mutation allows incorporation of deoxysphingoid bases [23]. With just three candidate mutations identified, we have already observed tight structural clustering that suggests there may be a similar pathogenic mechanism; perhaps the I43N, T51P, or I52T PMP2 mutants alter the lipid binding pocket of P2 and result in aberrant transport of fatty acids and alteration in the lipid composition of compact myelin. This idea fits with prior data showing Schwann cells are very sensitive to perturbations in membrane lipid composition [24].

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Accession Numbers

PMP2 (MIM: 170715, GenBank: NM_002677)

Acknowledgments

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Web Resources

URLs for data presented herein are as follows.

GEM.app: https://genomics.med.miami.edu/
1000 Genomes, http://www.1000genomes.org/
ExAC Browser, http://exac.broadinstitute.org
NHLBI Exome Sequencing Project Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org/
PyMol: (www.pymol.org)

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FIGURE LEGENDS:

Figure 1: *De novo PMP2* mutations segregate with disease in two families with CMT1. In Family 1**,** a c.155T>C mutation segregates with CMT1 and affects a conserved amino acid in the beta barrel of P2. (A) Whole exome sequencing of ~~our~~ proband II.3 in family 1 identified a c.155T>C substitution in *PMP2*. A candidate *PMP2* screen for mutations in families with CMT identified a c.151A>C ~~mutation~~ transversion in family 2. The genotypes of individuals whose DNA was collected and Sanger sequenced is shown below pedigree symbols. In family 1, neither parent has any clinical evidence of neuropathy and neither harbors the mutation. One of the proband's sons is affected (III.2) and harbors the mutation. In Family 2 the c.151A>C mutation segregates with CMT1 and affects a conserved amino acid adjacent to the residue mutated in ~~mutation in~~ Family 1. In family 2, the proband (III.3), her mother (II.3), her two sisters (III.1 and III.2), and two nieces, all show clinical features of neuropathy and all those patients whose samples could be obtained carry the mutation, while the proband's two aunts and one uncle do not have neuropathy and do not carry the mutation. The proband's maternal grandparents are deceased, but did not show any signs of neuropathy. It is not known whether the daughter of the proband is affected or unaffected. (B) Normal Sanger sequencing traces of the *PMP2* gene is shown from the family 1 proband's parents, who are both unaffected, suggesting that the mutation arose *de novo* in our proband, and was passed onto his affected son. Sanger sequencing traces from the proband and her mother show the mutation in family 2, while the proband's unaffected uncle and aunt do not have the mutation. (C) ClustalW2 alignments of the myelin P2 protein from divergent species. Both the I43N, T51P, and I52T CMT1-associated variants disrupt amino acid residues that are conserved in mammals, but are not present in teleosts or invertebrates. (D) The crystal structure of the P2 protein is shown modeled with a bound

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palmitate. Helical structures are shown in light green and beta strands are shown in teal. The three CMT1-associated variants are shown, and they cluster in adjacent positions of beta strand B (I43N) and beta strand C (T51P and I52T).

Figure 2. Sural nerve biopsy from the proband in Family 1. These are digital electron micrographs from the sural nerve biopsy of the proband (patient II.3) at age 17. The density of myelinated axons is reduced, but actively degenerating myelinated axons were not seen. Some of the myelinated axons had myelin sheaths that were inappropriately thin for the axon diameter, and some rudimentary onion bulbs were seen; one of which is indicated in the boxed region. There is increased endoneurial collagen. The inset is a higher magnification image of the boxed region, showing a thinly myelinated axons surrounded by Schwann cell processes. Scale bar: 1 micron.

Supplemental Table 1: A list of variants identified in exome sequencing of the proband after filtering based on the bioinformatics criteria described above.

Supplemental Figure 1: Haplotype analysis demonstrates that the c.151A>C mutation arose *de novo* in family 2. The PMP2 genotype at position c.151 of family members in generations II and III are shown, along with the genotype of 5 surrounding short tandem repeat loci (D8S2321, D8S1738, D8S275, D8S525, D8S1697) to determine the haplotype of relevant family member in this region of chromosome 8. The haplotype containing the mutation (highlighted in blue) is shared in all 3 affected daughters of patient II.3. Two siblings (II.2, II.4)

of the only affected individual in the second generation (II.3) share the same haplotype, but without the variant in *PMP2*. This suggests that this mutation arose *de novo* in individual II.3.

Supplemental Figure 2: Hands and feet of patient IV.1 in family 2 at age 24. Surgically corrected pes equinovarus can be seen bilaterally, no additional deformities in her hands or feet were noted. There is severe atrophy of calf, peroneal, and foot muscles, with moderately high arches. Mild atrophy in her interosseous muscles and moderate atrophy of abductor pollicus brevis were also noted.

Table 1: Nerve conduction studies were performed in patients after they were examined.

	Family 1 II.3 age 46		**Family 1 III.2** age 13		**Family 2 IV.1** age 24		normal values	
SENSORY	µV	m/s	µV	m/s	µV	m/s	µV	m/s
radial	NR	NR	NR	NR	NR	NR	≥15	≥50
median[a]	NR	NR	NR	NR	NR	NR	≥10	≥50
ulnar[a]	NR	NR	ND	ND	NR	NR	≥7	≥50
MOTOR[b]	mV	m/s	mV	m/s	mV	m/s	mV	m/s
peroneal	ND	ND	NR	NR	ND	ND	≥2.0	≥41
median	1.1	17	2.2	12.6	NR	NR	≥4.0	≥49
ulnar	3.3	21	4.1	14.3	1.5	11.7	≥6.0	≥49

[a]Orthodromic; [b]The amplitudes of the distal motor responses are shown; NR: no response; ND: not done

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