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SHORT REPORT

# Mutations in the tricarboxylic acid cycle enzyme, aconitase 2, cause either isolated or syndromic optic neuropathy with encephalopathy and cerebellar atrophy

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## ABSTRACT

**Background** Inherited optic neuropathy has been ascribed to mutations in mitochondrial fusion/fission dynamics genes, nuclear and mitochondrial DNA-encoded respiratory enzyme genes or nuclear genes of poorly known mitochondrial function. However, the disease causing gene remains unknown in many families.

**Methods** We used exome sequencing in order to identify the gene responsible for isolated or syndromic optic atrophy in five patients from three independent families.

**Results** We found homozygous or compound heterozygous missense and frameshift mutations in the gene encoding mitochondrial aconitase *(ACO2)*, a tricarboxylic acid cycle enzyme, catalysing interconversion of citrate into isocitrate. Unlike wild type ACO2, all mutant ACO2 proteins failed to complement the respiratory growth of a yeast *aco1*-deletion strain. Retrospective studies using patient-derived cultured skin fibroblasts revealed various degrees of deficiency in ACO2 activity, but also in ACO1 cytosolic activity.

**Conclusions** Our study shows that autosomal recessive *ACO2* mutations can cause either isolated or syndromic optic neuropathy. This observation identifies *ACO2* as the second gene responsible for non-syndromic autosomal recessive optic neuropathies and provides evidence for a genetic overlap between isolated and syndromic forms, giving further support to the view that optic atrophy is a hallmark of defective mitochondrial energy supply.

## INTRODUCTION

In the past few years, enzymopathies of the tricarboxylic acid cycle (TCA) have been reported to cause severe encephalopathies in humans, namely fumarase, succinate dehydrogenase (SDH), α-ketoglutarate dehydrogenase and succinyl-Coenzyme A (CoA) synthetase deficiencies.[1] Clinical features included seizures, muscle weakness, growth and developmental delay. On the other hand, mutations in another TCA enzyme, isocitrate dehydrogenase, have been reported to cause isolated retinitis pigmentosa.[2] Recently, missense mutations in yet another TCA enzyme, mitochondrial aconitase (ACO2), catalysing interconversion of citrate into isocitrate, have been reported in a sibship with infantile-onset encephalopathy, optic nerve involvement and cerebellar atrophy.[3] Hitherto however, no TCA enzyme mutation has been reported in isolated optic neuropathy.

Studying a series of unexplained cases of optic atrophy, we found biallelic *ACO2* mutations in five patients. *ACO2* mutations caused either isolated or syndromic optic neuropathy, irrespective of the residual level of enzyme activity. These observations support the view that optic atrophy is a hallmark of defective mitochondrial energy supply and suggest that extraocular involvement is not related to severity of the enzyme deficiency.

## MATERIALS AND METHODS

### Patients

Patients 1 and 2 were two adult male sibs, born to unrelated parents of French origin and aged 41 years and 36 years, respectively. They presented with decreased visual acuity and pallor of the optic disc at 5 years and 3 years of age, respectively, but no other concerns were noted. In their 20s, they had severely reduced visual acuity (20/150–20/100 for near vision and 20/200 for far vision), paracentral scotoma, red-green dyschromatopsia and marked temporal optic atrophy at the fundus. In their 40s, their optic atrophy remained stable (figure 1A) and isolated. Optical coherence tomography shows marked bilateral reduction in the temporal superior and inferior retinal nerve fibre layers (RNFLs) with preservation of the nasal RNFL. None of them had overt extraocular symptoms.

Patient 3, the first child of first cousin Algerian parents, was born in apparent death with apnoea, bradycardia and major cyanosis (birth weight: 3530 g, height: 50 cm, occipitofrontal head circumference (OFC): 38 cm, Apgar score, 3, 4 and 10). Fundus examination revealed a bilateral oedema of optic disks, but no overt retinal anomalies. Iterative episodes of apnoea, bradycardia, and oxygen desaturation were suggestive of central apnoeas. Brain MRI showed moderate cerebellar atrophy with no

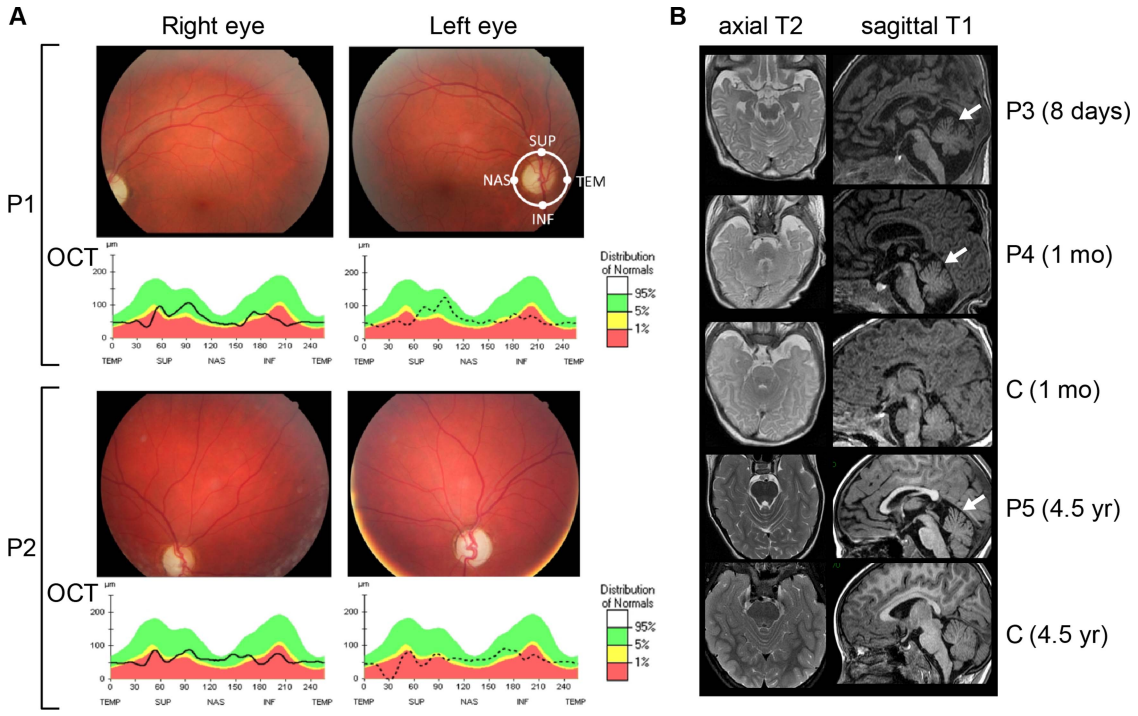


**Figure 1** Fundus images and brain MRI. (A) Marked temporal optic atrophy in two aconitase deficient-adult siblings. Fundus images of patients P1 aged 42 years and P2 aged 37 years, show marked temporal pallor of the optic disc. Consistently, optical coherence tomography (OCT) shows marked bilateral reduction in the temporal superior and inferior retinal nerve fibre layers (RNFL) with preservation of the nasal RNFL. SUP: superior, TEM: temporal; INF: inferior; NAS: nasal. Solid and dotted OCT traces are for the right and left eyes, respectively. (B) Moderate cerebellar vermian atrophy in aconitase deficient patients. Brain MRI of three affected patients and two age-matched controls: Patient 3 and 4 aged 8 days and 1 month respectively, a 1-month-old normal control; patient 5, aged 4.5 years and a 4.5 years old control. Axial T2-weighted images (left column) and sagittal T1- weighted images (right column) showed a moderate superior atrophy of the vermis (arrows) in all affected individuals compared with age-matched controls.

supratentorial anomaly (figure 1B). Metabolic workup revealed severe metabolic acidosis and hyperglycaemia with normal plasma lactate. He fell in a deep reactive coma and died at 57 days of life.

Patient 4, his brother, also presented with iterative episodes of dizziness, trunk hypotonia and twisting of the superior limbs with abnormal eye movements, at birth (weight: 3400 g, height: 53 cm, OFC: 34.5 cm, APGAR score: 10/10). At 7 h, he had bradycardia and apnoeas, requiring intubation and assisted ventilation. Bilateral pallor of optic disks, with extinguished visual-evoked potentials and electroretinograms were noted. EEG at 5 weeks and 6 weeks in patients 3 and 4 showed high amplitude θ waves, bursts of unusual α rhythms, spikes and sharp waves. Brain MRI showed moderate cerebellar atrophy (figure 1B). Metabolic workup was unremarkable. He died of sudden cardiorespiratory arrest at 61 days of life.

Patient 5, a girl, was the only child of unrelated healthy parents (birth weight: 3430 g, height: 51 cm, OFC: 35.5 cm, APGAR score 10 at 1 mn). Her head control was delayed (5 months) and she could not sit unaided until 20 months. At 8 months, she reportedly presented iterative episodes of eye rolling (5–10/day), multidirectional nystagmus and poor eye contact. Head circumference curve slowed down from normal at 8 months to −2 SD at 20 months (46 cm). At that age, she could sit unaided, follow with eyes despite eye saccades, roll over and grasp, but she could not stand. Fundus examination revealed bilateral pallor of the optic disks with altered visually evoked potentials. At 6 years, EEG showed physiological features in awake and sleep conditions. Brain MRI at 4 years showed moderate cerebellar atrophy with no brainstem or supratentorial anomalies (figure 1B). Nerve conduction velocity and extensive metabolic workup were normal, (lactate: 1.3 mM, pyruvate: 115 μM). Enzyme activities and/or assembly of mitochondrial respiratory chain complexes in her skeletal muscle biopsy and cultured skin fibroblasts were normal.

## Methods

Genomic DNA (1 μg) from the patients was subjected to whole exome sequencing.[4] Data were filtered on the basis of autosomal recessive transmission and pedigree structures (see online supplementary table S1).

Skin fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life technologies) medium supplemented with 2.5 mM pyruvate, 200 μM uridine, 100 μg/mL streptomycin, 100 U/mL penicillin and 10% fetal calf serum.

For measurement of ACO2 activity, mitochondria-enriched fractions were prepared as reported[5] and all enzyme activities were measured in potassium phosphate buffer, containing 0.05% Triton-X100. Aconitase activity (ie, consumption of *cis*-aconitate) was coupled to that of native isocitrate dehydrogenase, at 340 nm, in the presence of 0.8 mM Nicotinamide adenine dinucleotide (NAD), 0.8 mM Nicotinamide adenine dinucleotide phosphate (NADP) and 0.10 mM $MgCl_2$. The reaction was initiated by adding 5 mM *cis*-aconitate to the medium, either in absence or in the presence of 100 mM potassium citramalate monohydrate (Sigma Aldrich), a potent inhibitor of ACO2 in citrus fruit.[6] Protein amounts were quantified using Bradford reagent (BioRad).

For Western blot analysis, SDS-Polyacrylamide gele electrophoresis (PAGE) was performed on whole-cell protein extracts

from cultured skin fibroblasts. Immunodetections were performed using the following antibodies: anti-aconitase 1 (Abcam), anti-aconitase 2 (Abcam), anti-β-actin (Abcam) and anti-phosphoglycerate kinase (Invitrogen). Immunoblots were developed using enhanced chemiluminescence (ECL)-based detection system (SuperSignal West Dura, Thermo Scientific) and visualised using a charge-coupled devices (CCD) camera (BioRad).

For RT-qPCR analysis, total RNA was extracted using the RNeasy Mini Kit (Qiagen). Concentration was assessed using the Qubit V.2.0 Fluorometer (Life Technologies) before storage at −80°C. *ACO2* expression was measured by amplifying a 111 bp mRNA fragment using primers: ACO2 forward, 5′-tgcagatcctcatcaaggtcaaa-3′ and ACO2 reverse, 5′-tgagcaggttgttggagatgttat-3′. The results were normalised as described.[7]

*The Saccharomyces cerevisiae* strain BY4743 (*Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YLR304c::kanMX4/YLR304c*) was used for tetrad analysis after transformation with plasmids encoding h*ACO2* or its mutated forms to get chromosomal aconitase haploid knockouts (Δ*aco1*) expressing either the wild type (WT) or the mutated proteins under the phosphoglycerate kinase promoter. Strains harbouring the appropriate plasmids were grown overnight at 30°C in synthetic depleted medium containing 0.67% yeast nitrogen base and 2% glucose supplemented with the appropriate amino acids. Cultures were serially diluted at 10-fold intervals, and 2.5 µL of each was spotted onto glucose or glycerol plates (1% yeast extract, 1% bacto peptone, 2% agar and 2% glucose or 2% glycerol, respectively), which were incubated at 28°C, 34°C and 37°C for 3 days.

## RESULTS

Whole exome sequencing of five patients with isolated or syndromic optic neuropathy suggested that all of the patients were either homozygotes or compound heterozygotes for missense and frameshift mutations in the *ACO2* gene (see online supplementary table S2). Nucleotide changes consistently involved evolutionary conserved residues, predicted to be deleterious by Alamut Mutation Interpretation software (Patients 1 and 2: p.Leu74Val and p.Gly661Arg, Patient 3: p.Gly259Asp, Patient 5: p.Lys736Asn and p.Lys776Asnfs*49, see online supplementary table S2 and supplementary figure S1). Biallelic inheritance of the mutations was confirmed by Sanger sequencing of parental DNA (not shown). All changes but one (p.Leu74Val, rs141772938; MAF 0.003) were absent from all SNP databases.

The total aconitase activity measured on whole cell extracts corresponds to cytosolic aconitase (ACO1) and mitochondrial aconitase (ACO2) activities. Aconitase activity of whole cell extracts and mitochondria-enriched fractions from control fibroblasts was progressively inhibited using increasing concentrations of citramalate (maximum inhibition at 80–100 mM, see online supplementary figure S2A). ACO1 activity remained unchanged even at high concentration of citramalate, demonstrating that citramalate selectively inhibits ACO2 in human cultured skin fibroblasts, as shown in plants.[6] The use of citramalate allowed to directly measure the ACO2 and ACO1 activities in whole cell extracts from control and patients' fibroblasts. ACO2 activity was found to be markedly decreased in cultured skin fibroblasts of patient 3 (5% of residual activity) and patients 1, 2 and 5 (31–66% of residual activity, figure 2A). Interestingly, the citramalate-resistant ACO1 activity was also reduced in all patients (15%, 42%, 61% and 73% of residual activity in patient 3, 5, 1 and 2, respectively).

Normal amount of *ACO2* transcripts was found in fibroblasts of patients 1–3 but was reduced in patient 5, suggesting that her frameshift mutation induced non-sense mediated mRNA decay (see online supplementary figure S2B). Consistently, Western blot analyses showed greatly reduced steady-state levels of ACO2 protein in cultured fibroblasts of patient 5 (figure 2B). This decrease could be related to the reduced stability of paternal *ACO2* transcripts and the destabilising effect of the missense maternal mutation. The ACO2 protein level was also severely reduced in patients' 1–2 fibroblasts, suggesting that the p.Leu74Val and p.Gly661Arg mutations also altered the stability of the protein (figure 2B). Interestingly, the steady-state level of the ACO2 protein was unchanged in patient 3, who exhibited the most severe ACO2 deficiency. ACO1 protein amount was normal in all patients despite reduced enzyme activity (figure 2B).

To definitively demonstrate the pathogenic nature of the identified ACO2 mutations we studied their effect on respiratory growth of a *S. cerevisiae* strain deleted for the mitochondrial aconitase gene *(Δaco1)*. Yeast cells lacking *aco1* rapidly loose mitochondrial DNA and are therefore unable to grow on carbon sources that require functional respiratory chain, such as glycerol. At 28°C only the p.Gly259Asp mutation showed a slightly reduced growth on glycerol when expressed in *Δaco1* (figure 2C). The human WT and p.Gly661Arg ACO2 proteins rescued the growth deficiency of *Δaco1* strain at 34°C but all strains expressing the mutant proteins showed a very clear growth defect under heat stress at 37°C on glycerol. Western immunoblotting on yeast extracts complemented with the different cDNA constructs showed that most constructs were expressed at steady state levels similar or above those of WT ACO2 (figure 2C). Only p.Gly661Arg exhibited lower steady state levels, which can be attributed to decreased protein stability consistent with our observations in patients' fibroblasts. Thus, all identified mutations in *ACO2* resulted in a respiratory growth defect when expressed in *Δaco1* yeast, lending additional support to the pathogenic role of these mutations in isolated or syndromic optic neuropathy.

## DISCUSSION

Studying a series of unexplained optic neuropathies, we report missense and frameshift *ACO2* mutations in five cases of either isolated or syndromic optic atrophy. Isolated, non-progressive optic neuropathy was the only feature in two of five patients. Careful clinical, metabolic and brain MRI investigations failed to detect any extraocular symptoms in those adult patients. Hence, optic neuropathy is a consistent feature and a hallmark of ACO2 mutations. Aconitase deficiency should now be regarded as a possible cause of late-onset, non-progressive optic neuropathy in adulthood. Hitherto, a unique homozygous *ACO2* mutation has been reported in a large inbred sibship suffering from encephalopathy and optic neuropathy.[3] In fact, the patients reported by Spiegel *et al* also presented cerebellar atrophy similar to that of patients 3–5, with cortical atrophy and delayed myelination (see online supplementary table S2).

The pathological nature of the various *ACO2* mutations was demonstrated in yeast as none of the mutations could rescue the growth defect of *Δaco1* yeast on glycerol at 37°C. Moreover, the growth phenotype of the mutant proteins expressed in *Δaco1* yeast showed a good correlation with the severity of the clinical presentation of the patients. Indeed the p.Gly259Asp mutation (patients 3–4) displayed a mild growth defect at 28°C. The p.Lys736Asn (patient 5) and p.Leu74Val mutations (patients 1–2) were unable to complement the growth defect at 34°C whereas the p.Gly661Arg (patients 1–2) displayed growth defect only at 37°C.

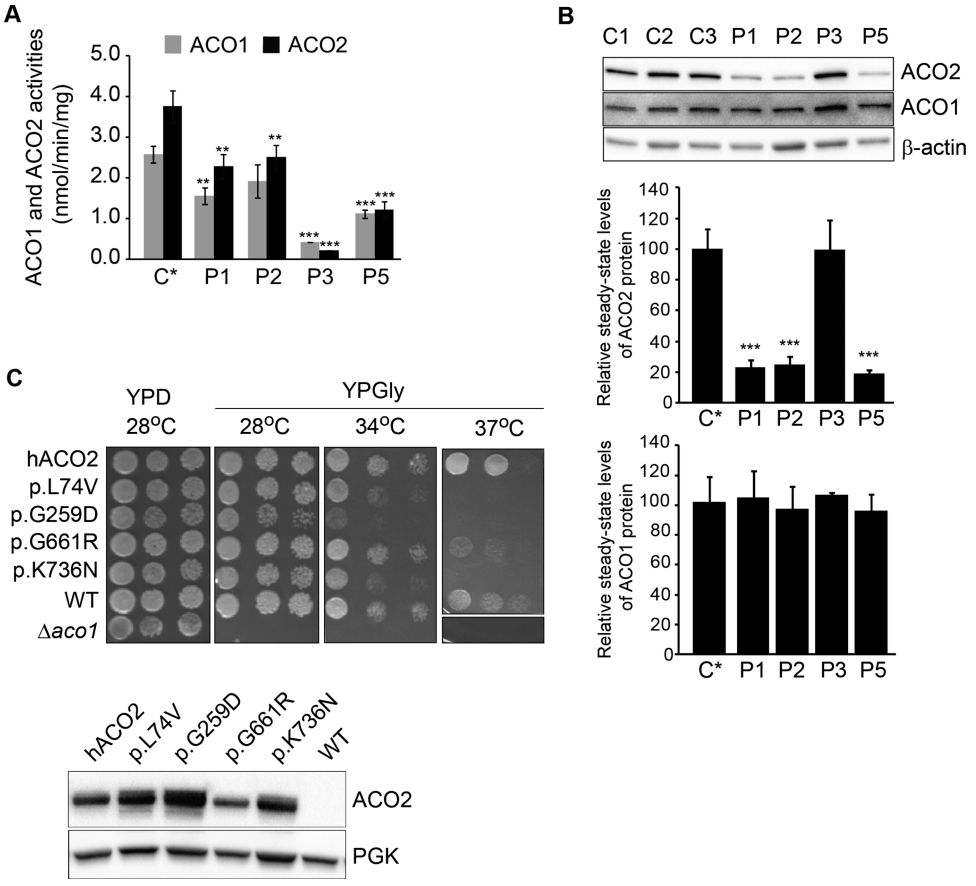


**Figure 2** Aconitase measurements. (A) Aconitase activity in cultured skin fibroblasts of controls and patients. ACO1 and ACO2 activities correspond to citramalate resistant and sensitive aconitase respectively. C* corresponds to the average value of three different control fibroblasts. P1, P2, P3, P5: patients 1, 2, 3 and 5. For all fibroblast lines, enzyme measurement has been done three times at three different passages. (B) Western blot analysis of total protein extracts from cultured skin fibroblasts of patients and controls using antibodies directed against ACO1, ACO2 and β-actin. The histograms indicate the relative steady state levels of ACO1 and ACO2 proteins. C* corresponds to the average value of three different control fibroblasts. (C) Functional complementation of yeast *Δaco1* null mutant. Upper panel: Growth of *Δaco1* null mutant on either glucose (YPD) or glycerol medium (YPGly) was compared. The yeast *Δaco1* null mutant was transformed with the wild type (hACO2) or the various human *ACO2* mutant cDNAs on BFG1 plasmid. The three spots for each experiment correspond to successive dilutions of transformed yeasts (1, 0.1 and 0.01). Lower panel: Human ACO2 protein amount expressed in *Δaco1* null mutant transformed with the wild type (hACO2) or the various human *ACO2* mutant cDNAs.

*ACO2* mutations resulted in defective ACO2 but also ACO1 activities in cultured skin fibroblasts. ACO1 and ACO2 are iron-sulfur proteins and both catalyse conversion of citrate to isocitrate. We could hypothesise that ACO2 deficiency can induce a disturbance that secondarily affects the activity of ACO1 via substrate accumulation, iron-sulfur overload or iron metabolism modification. Future studies will investigate the effect of ACO2 deficiency on iron metabolism in patients carrying mutations in *ACO2*.

TCA enzyme deficiency has been reported to cause severe encephalopathy in humans, namely fumarase, SDH, α-ketoglutarate dehydrogenase and succinyl-CoA synthetase deficiencies. Those phenotypes included seizures, muscle weakness, growth and developmental delay. Retinal defects were occasionally present but were far from being the major concern in those children.

On the other hand, optic neuropathy has been ascribed to mutations in mitochondrial fusion/fission dynamic genes, (*OPA1*, MIM605290[8]; *MFN2*, MIM608507[8]; *DNM1 L*, MIM603850[8]), nuclear or mitochondrial DNA-encoded respiratory chain enzyme genes (*MTND1*, MIM516000; *MTND4*, MIM516003; *MTND5*, MIM516005; *MTND6*, MIM516006; *MTCYB*, MIM516020[9]) or other nuclear genes of known or unknown mitochondrial function (*TIMM8A*, MIM300356[10]; *TMEM126A*, MIM612988[11]; *OPA3*, MIM606580[12]; *OPA8*[13]; *SPG7*, MIM602783,[14] *C19orf12*, MIM614298[15]; C12orf65 MIM 613541[16]; *TSFM*, MIM604723[17]). While isocitrate dehydrogenase (*IDH3*) mutation has been reported to cause isolated retinitis pigmentosa,[2] TCA enzymes have never been hitherto shown to cause isolated optic neuropathy. Whether ACO2 and other TCA enzymes account for other forms of optic neuropathy certainly deserves consideration.

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**Contributors** MDM cloned the human wild type and mutant mitochondrial aconitase cDNA for yeast expressions studies and did all Western blot analysis. AD did the yeast studies. DC did enzyme analysis. XG did Q-RT-PCR experiments. PA-B excluded known genes for optic atrophy. NB did brain MRI. AK, ID, JA, MR, JK and M-CG were in charge of the patients. LH, SG, and CB did experiments and analysed whole exome sequencing. JMR, AR and AM wrote the manuscript.

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**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** Comité Consultatif National d'Ethique.

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