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Frequent intragenic microdeletions of elastin in familial supravalvular aortic stenosis

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ABSTRACT

Background: Supravalvular aortic stenosis (SVAS) is a congenital heart disease affecting approximately 1:25,000 live births. SVAS may occur sporadically, be inherited in an autosomal dominant manner, or be associated with Williams-Beuren syndrome, a complex developmental disorder caused by a microdeletion of chromosome 7q11.23. *ELN* on 7q11.23, which encodes elastin, is the only known gene to be recurrently mutated in less than half of SVAS patients.
Methods: Whole-exome sequencing (WES) was performed for seven familial SVAS families to identify other causative gene mutations of SVAS.
Results: Three truncating mutations and three intragenic deletions affecting *ELN* were identified, yielding a diagnostic efficiency of 6/7 (85%). The deletions, which explained 3/7 of the present cohort, spanned 1–29 exons, which might be missed in the course of mutational analysis targeting point mutations. The presence of such deletions was validated by both WES-based copy number estimation and multiplex ligation-dependent probe amplification analyses, and their pathogenicity was reinforced by co-segregation with clinical presentations.
Conclusions: The majority of familial SVAS patients appear to carry *ELN* mutations, which strongly indicates that elastin is the most important causative gene for SVAS. The frequency of intragenic deletions highlights the need for quantitative tests to analyze *ELN* for efficient genetic diagnosis of SVAS.

1. Introduction

Supravalvular aortic stenosis (SVAS; MIM #185500) is a congenital heart disease affecting approximately 1:25,000 live births [1]. Congenital narrowing of the lumen of the ascending aorta or peripheral pulmonary arteries provokes increased resistance to blood flow and causes elevated ventricular pressure and hypertrophy resulting in heart failure. Peripheral pulmonary stenosis (PPS) is known to occasionally coexist with SVAS [2]. Approximately 30% to 50% of patients with SVAS have Williams-Beuren Syndrome (WBS; MIM #194050) [2–4], which is a complex genetic disorder caused by 7q11.23 microdeletion and characterized by growth failure, a characteristic facial appearance (so-called "Elfin face"), mental retardation, and SVAS [5].

On the other hand, Eisenberg et al. first reported non-syndromic "familial SVAS" with autosomal dominant inheritance in 1964 [3], accounting for 20% of SVAS cases (approximately 1:125,000 live births) [6]. These patients showed normal intelligence and lacked the dysmorphic features of WBS. Genetic analysis including linkage analysis identified *ELN*, which encodes elastin, as a causative gene of non-syndromic familial SVAS [1,7–17]. In harmony with the genetic findings, luminal obstruction of the aorta was shown in a transgenic mouse model carrying homozygous or heterozygous elastin gene deletion [18,19].

Metcalfe et al. sequenced *ELN* exons of patients with non-syndromic SVAS, which showed truncating mutations in 35 cases, but no causative variants were found in the remaining 64 patients (of which 8 were familial cases) [20]. Micale et al. also investigated *ELN* gene mutations in 14 familial and 10 sporadic cases of SVAS, resulting in 7 novel mutations, including 5 frameshift and 2 donor splice site mutations, but found no *ELN* gene abnormality in the remaining 17 cases [21]. Therefore, less than half of the cases could be explained by *ELN* mutations, whereas it still remains unclear whether *ELN* could explain the remaining cases

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Abbreviations

SVAS	supravalvular aortic stenosis
WES	whole-exome sequencing
PPS	peripheral pulmonary stenosis
WBS	Williams-Beuren syndrome
FISH	fluorescence in situ hybridization
MLPA	Multiplex ligation-dependent probe amplification

with SVAS, or there are unidentified causative genes. In this study, whole-exome sequencing (WES) was performed with careful assessment of *ELN* mutations, including copy number analysis, to elucidate the genetic background of SVAS.

2. Methods

2.1. Sample collection

This study included seven families of Japanese ancestry with autosomal dominant inheritance of SVAS. There was no developmental delay or dysmorphic features suggestive of WBS or positive fluorescence in situ hybridization (FISH) on 7q11.23 in any family members. The vascular malformation (SVAS and PPS) was diagnosed if the sinotubular junction of the aorta was smaller than the diameter of the aortic annulus and significant pressure gradients were measurable by echocardiogram and/or angiographically [6]. Written, informed consent was obtained from patients or their parents, and whole blood or saliva was collected. Saliva samples were collected using an Oragene DNA self-collection kit (DNA Genotek, Ottawa, Canada). Genomic DNA was extracted from whole blood or saliva using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine (approval number 2015–0032).

2.2. Whole-exome sequencing analysis

Exome capture was performed on each proband using SureSelect Human All Exon V5 (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. Generated libraries were sequenced on a HiSeq 2500 platform (Illumina, San Diego, CA). Sequence data were analyzed using an in-house pipeline [22]. Briefly, reads were aligned to UCSC build hg19 reference genome using the Burrows-Wheeler Aligner [23]. Picard tools (http://broadinstitute.github.io/picard) were utilized to remove PCR duplicates. Variants were called using VarScan2, where a variant allele frequency of >0.20 was used as a cutoff [24]. ANNOVAR was used together with in-house scripts to annotate genetic variants [25]. The average depth of coverage across the whole exome for each sample achieved was 111.14 (range 90.68 to 127.66), and the number of mutations found per sample ranged from 25,534 to 25,920.

2.3. Mutational analysis

Mutational analysis to define each variant's pathogenicity was essentially based on the latest release of the American College of Medical Genetics (ACMG) guideline [26]. Briefly, variants outside of coding regions and common variants with >1% minor allele frequency in the National Heart, Lung, and Blood Institute ESP (Exome Sequencing Project) 6500 [27], 1000 Genomes Project [28], ExAC (Exome Aggregation Consortium) [29], HGVD (Human Genetic Variation Database) [30], or the in-house database were excluded. Variants expected to cause the disorders (eg, missense variants with reported pathogenicity and nonsense, frameshift insertion/deletion, and splice-site variants on genes known to cause a disease by inactivation) were validated by Sanger sequencing using PrimeSTAR GXL DNA polymerase (Takara, Shiga, Japan) and the Big Dye Terminator 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Waltham, MA) with ABI PRISM 3130xL (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table S1.

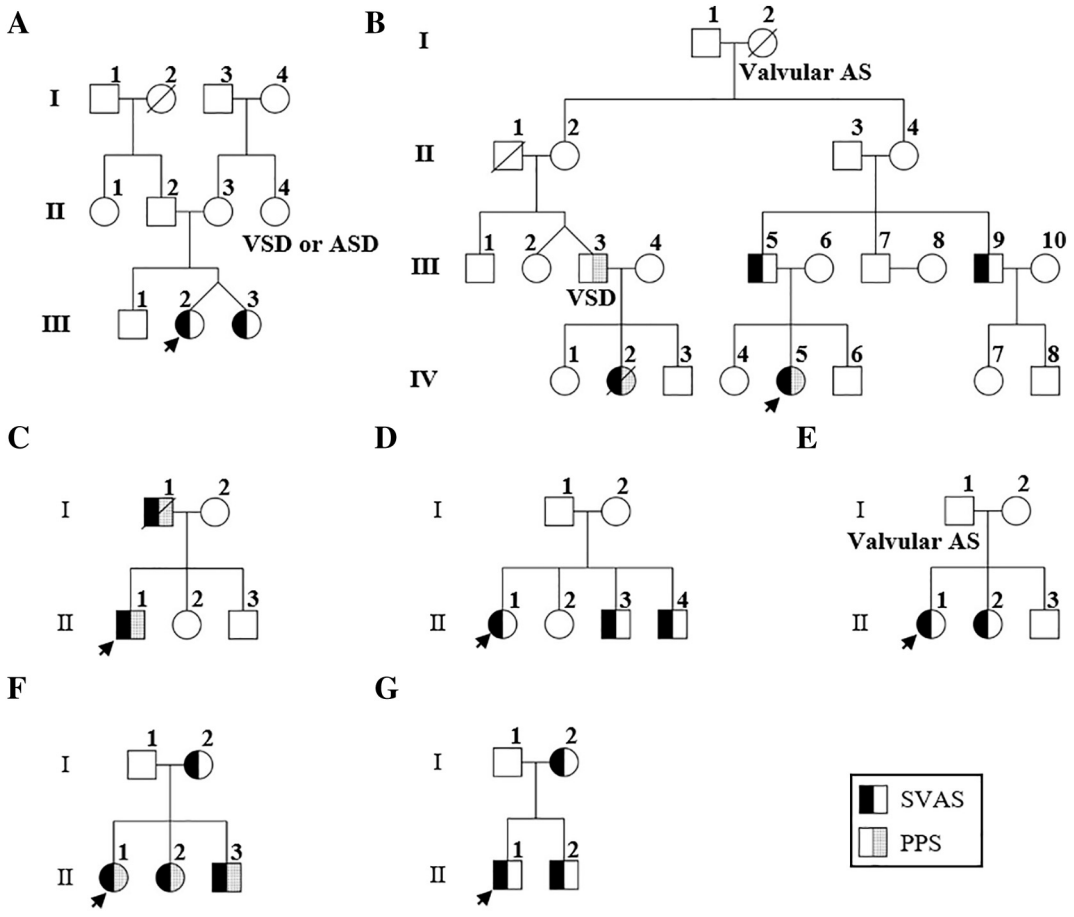

Fig. 1. Pedigree chart of families with familial supravalvular aortic stenosis Arrows indicate probands for whom whole-exome sequencing was performed. SVAS, supravalvular aortic stenosis; PPS, peripheral pulmonary stenosis; VSD, ventricular septal defect; ASD, atrial septal defect; AS, aortic stenosis.

2.4. Copy number analysis

Copy number analysis was performed by comparing the number of reads conveying each exon normalized by the mean depth of the entire sample with that of unrelated normal DNA samples, as we have previously shown [22]. Exons of normalized coverage >3 standard deviations (SDs) or less than −3 SDs from the mean coverage of reference samples were considered to be candidates for copy number variants. Multiplex ligation-dependent probe amplification (MLPA) according to the manufacturer's protocol with the SALSA MLPA P029-WBS probemix (MRC Holland, Amsterdam, Netherlands), which includes 10 exons of the *ELN* gene (Exon 1, 3, 4, 6, 9, 16, 20, 26, 27 and 33), was performed to validate candidate exonic deletions detected in the *ELN* gene by WES analysis. MLPA analysis software Coffalyser (MRC Holland) was used to identify CNVs.

3. Results

WES-based detection of point mutations and copy number alterations was performed in seven families of Japanese ancestry with SVAS showing an autosomal dominant mode of inheritance (Fig. 1). Three heterozygous pathogenic mutations in *ELN* (c.370delT, p.Ser124Leufs*13 in family A, c.572-1G > A splice site mutation affecting the acceptor of exon 12 in family B, and c.218_219insTG, p.Gly74Valfs*49 in family C) were identified. All of these mutations were novel, and they were validated on all available family members by Sanger sequencing (Fig. S1). Mutations were present

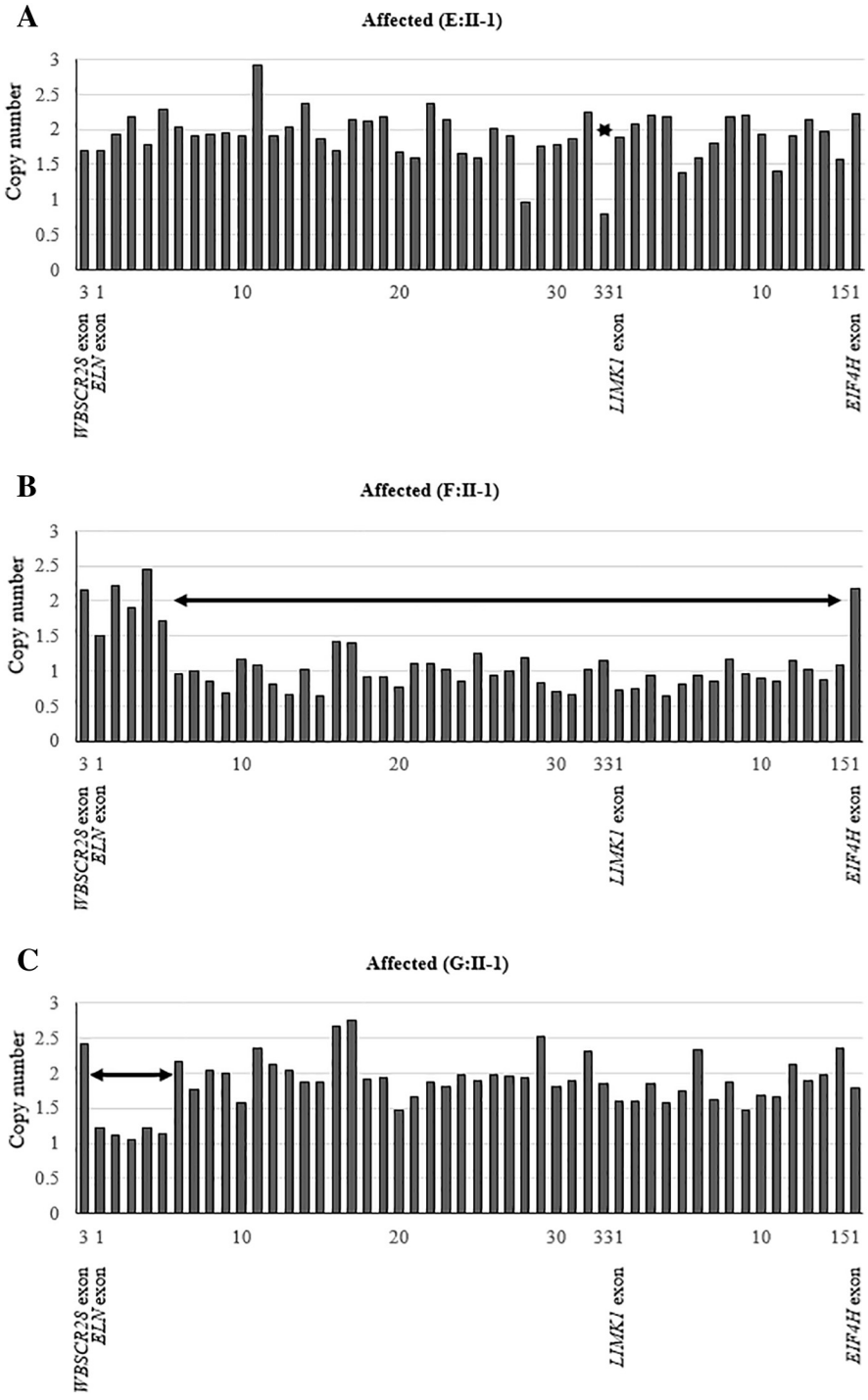

Fig. 2. Copy-number analysis The estimated copy number of each exon based on the number of reads in each exon in whole-exome sequencing. Each bar represents an exon, and the vertical axis represents the estimated copy number. Arrows indicate estimated deleted regions.

Table 1
Phenotype and segregation of *ELN* mutations.

Family	Subject	Phenotype	*ELN* mutation
A	II-2	–	–
	II-3	–	c.370delT, p.Ser124Leufs*13
	III-1	–	–
	III-2	SVAS	c.370delT, p.Ser124Leufs*13
	III-3	SVAS	c.370delT, p.Ser124Leufs*13
B	III-3	PPS, VSD	c.572-1G > A splice site
	III-5	SVAS	c.572-1G > A splice site
	III-6	–	–
	IV-4	–	–
	IV-5	SVAS, PPS	c.572-1G > A splice site
	IV-6	–	–
C	I-2	–	–
	II-1	SVAS, PPS	c.218_219insTG, p.Gly74Valfs*49
	II-2	–	–
	II-3	–	c.218_219insTG, p.Gly74Valfs*49
D	II-1	SVAS	–
E	I-1	Valvular AS	Microdeletion (exon 33)
	I-2	–	–
	II-1	SVAS	Microdeletion (exon 33)
	II-2	SVAS	Microdeletion (exon 33)
	II-3	–	–
F	I-1	–	–
	I-2	SVAS	Microdeletion (exon 5–33)
	II-1	SVAS, PPS	Microdeletion (exon 5–33)
	II-2	SVAS, PPS	Microdeletion (exon 5–33)
	II-3	SVAS, PPS	Microdeletion (exon 5–33)
G	I-1	–	–
	I-2	SVAS	Microdeletion (exon 1–5)
	II-1	SVAS	Microdeletion (exon 1–5)
	II-2	SVAS	Microdeletion (exon 1–5)

All mutations were heterozygous.
SVAS, supravalvular aortic stenosis; PPS, peripheral pulmonary stenosis; VSD, ventricular septal defect; AS, aortic stenosis.

in all patients and several family members without SVAS, indicating incomplete penetrance (Table 1).

Copy number aberrations in *ELN* were identified in three other families, all of which were deletions (exon 33 in family E, exons 5–33 in family F, and exons 1–5 in family G, Fig. 2). Such deletions were validated by MLPA (Fig. S2). All microdeletions showed complete co-segregation with clinical symptoms (Table 1).

In the remaining family D, no diagnostic mutations associated with SVAS could be identified in the proband (D:II-1) either by WES or MLPA (Table S2, Fig. S3).

4. Discussion

Pathogenic mutations or deletions in *ELN* gene were identified in six of seven families with autosomal dominant inheritance of SVAS, including three novel point mutations and three intragenic deletions. These findings suggest that intragenic deletions in *ELN* gene could explain the genetic cause in half of so-far unexplained cases with familial SVAS in Japan. Updated by these findings, a comprehensive list of reported pathogenic SVAS mutations is provided (Fig. 3) [7–9,12,17,20,21,31–38].

ELN encodes elastin, which is expressed in various tissues and organs, including smooth muscle cells of the great arteries, and contributes to tissue elasticity [39,40]. The molecular mechanism of the pathogenesis of SVAS is not fully elucidated. However, considering accumulating knowledge from patients and transgenic mice [18,19,21,41], it seems likely that mutations of *ELN* impair vascular elasticity, and increased shear stress in the vascular wall could result in SVAS [39,40].

The microdeletions of *ELN* gene shown in the present study were not identified with existing FISH probes for WBS. There are a few case reports showing that microdeletions of *ELN* gene are the cause of SVAS [8,9]. The present finding raises the necessity to investigate the

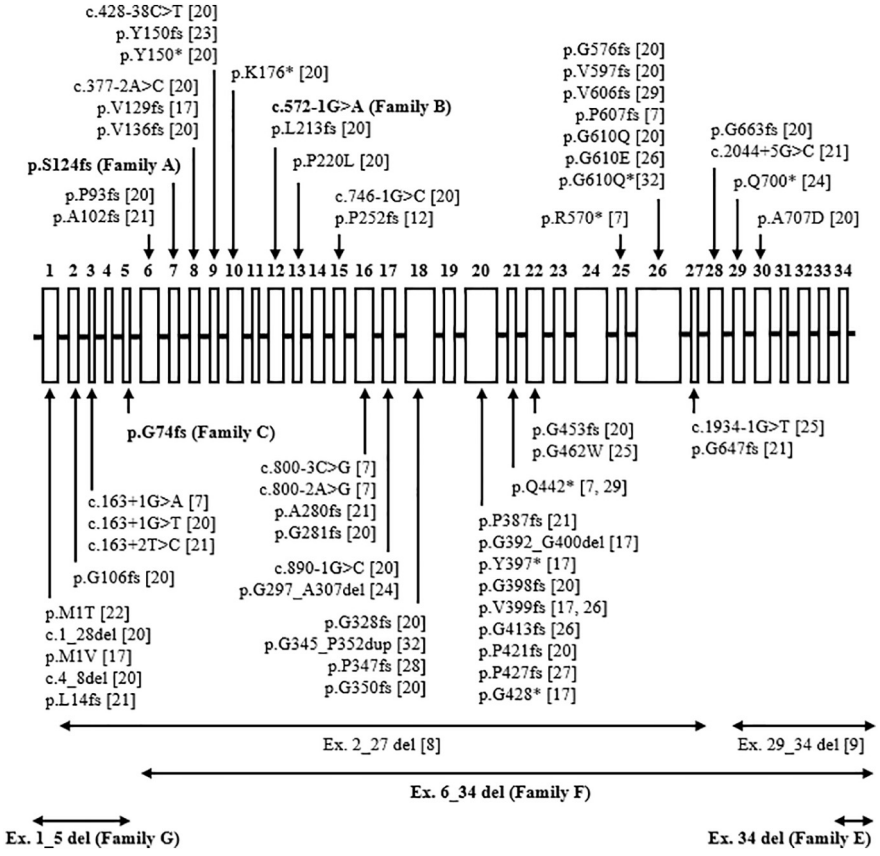

Fig. 3. *ELN* cDNA showing the exons and mutations detected This figure summarizes previously reported and newly identified mutations for familial and sporadic SVAS. The numbers above open boxes indicate the exon numbers. The present findings are shown in bold letters.

exon-spanning deletions affecting *ELN* using MLPA, array-CGH, or other methods to establish sufficient coverage for its mutations.

The present analysis showed co-segregation of symptoms and *ELN* mutations in the majority of analyzed individuals. The three mutations identified in this study (c.370delT in family A, c.572-1G > A splice site in family B, and c.218_219insTG in family C) were highly pathogenic truncating mutations that result in premature stop codons (PTCs). A number of PTC mutations have actually been shown to be substrates of *ELN* mRNA insufficiency through nonsense-mediated decay in previous studies [17,21,35].

A highly variable phenotype within families with SVAS has been reported for large studies with many families with point mutations [20,21], and even for a family with apparently damaging 30 kb deletion involving multiple exons [7], ranging from asymptomatic mutation carrier to severe stenosis with multiple arteries. There were also two asymptomatic persons carrying *ELN* mutations (A:II-3 and C:II-3) in the present study. Factors affecting the variability of cardiovascular phenotypes in patients with *ELN* mutation are not yet fully understood, and there has as yet been no clear genotype-phenotype correlation reported for SVAS. Our comprehensive list of reported pathogenic SVAS mutations and deletions showed a universal distribution of variants over the entire *ELN* gene with no significant hotspot.

The primary mechanism for the pathogenesis of SVAS is proposed to be haploinsufficiency of *ELN*, as hemizygosity of *ELN* is established as the mechanism of SVAS in WBS [5]. Incomplete penetrance and a broad range in severity of cardiovascular phenotype are also seen in patients with WBS in whom one copy of *ELN* gene is totally lost [42,43]. Among possible causes affecting the severity of symptoms, the effect of the mutations in the remaining allele of *ELN* is very limited, as only two rare missense changes were identified through exon sequencing of 49 patients with WBS [44]. Currently, there is no definite explanation for the phenotypic variability associated with *ELN* mutations.

The present study showed that microdeletions of *ELN* gene could account for additional cases, around a half of previously unexplained cases, of familial SVAS, which would strengthen the causative role of *ELN* mutations in this disease entity. Therefore, quantitative genetic tests such as MLPA or array-CGH of *ELN* gene should be performed to genetically diagnose patients with familial SVAS to obtain satisfactory sensitivity. Further investigations of a larger cohort and so-far unexplained cases will be needed to elucidate the remaining molecular pathogenetic mechanisms of SVAS.

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Authors' contributions

All authors developed the concept and designed the research; S.H., Y.O., and M.T. performed the experiments; S.H., Y.O., M.T., H.I., H.K., and T.K. analyzed the data; all authors interpreted the results of the experiments; S.H., Y.O., and T.K. prepared the figures; S.H., Y.O., M.T., H.I., Y.F., H.K., S.K., Y.T., and T.K. drafted the manuscript; S.H., Y.O., and T.K. edited the manuscript; all authors approved the final version of the manuscript.

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Competing interests

The authors report no relationships that could be construed as a conflict of interest.

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