



# Deletion of sterol O-acyltransferase 2 (SOAT2) function in mice deficient in lysosomal acid lipase (LAL) dramatically reduces esterified cholesterol sequestration in the small intestine and liver

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## ABSTRACT

Sterol O-acyltransferase 2 (SOAT2), also known as ACAT2, is the major cholesterol esterifying enzyme in the liver and small intestine (SI). Esterified cholesterol (EC) carried in certain classes of plasma lipoproteins is hydrolyzed by lysosomal acid lipase (LAL) when they are cleared from the circulation. Loss-of-function mutations in LIPA, the gene that encodes LAL, result in Wolman disease (WD) or cholesteryl ester storage disease (CESD). Hepatomegaly and a massive increase in tissue EC levels are hallmark features of both disorders. While these conditions can be corrected with enzyme replacement therapy, the question arose as to what effect the loss of SOAT2 function might have on tissue EC sequestration in LAL-deficient mice. When weaned at 21 days, *Lal*<sup>-/-</sup>:*Soat2*<sup>+/+</sup> mice had a whole liver cholesterol content (mg/organ) of 24.7 mg vs 1.9 mg in *Lal*<sup>+/+</sup>:*Soat2*<sup>+/+</sup> littermates, with almost all the excess sterol being esterified. Over the next 31 days, liver cholesterol content in the *Lal*<sup>-/-</sup>:*Soat2*<sup>+/+</sup> mice increased to 145 ± 2 mg but to only 29 ± 2 mg in their *Lal*<sup>-/-</sup>:*Soat2*<sup>-/-</sup> littermates. The level of EC accumulation in the SI of the *Lal*<sup>-/-</sup>:*Soat2*<sup>-/-</sup> mice was also much less than in their *Lal*<sup>-/-</sup>:*Soat2*<sup>+/+</sup> littermates. In addition, there was a >70% reduction in plasma transaminase activities in the *Lal*<sup>-/-</sup>:*Soat2*<sup>-/-</sup> mice. These studies illustrate how the severity of disease in a mouse model for CESD can be substantially ameliorated by elimination of SOAT2 function.

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## 1. Introduction

In mammals, most of the cholesterol present in the major organ systems is unesterified [1]. Exceptions are the adrenal glands and plasma [1,2]. Several organs are capable of generating esterified cholesterol (EC) through the action of either sterol O-acyltransferase 1 (SOAT1) (also known as ACAT1) which is present in steroidogenic tissues, kidneys, sebaceous glands and macrophages, or SOAT2 (ACAT2) which is expressed predominantly in the liver and small intestine [3,4]. The roles that both SOAT1 and SOAT2

**Abbreviations:** ALT, alanine aminotransferase; AST, aspartate aminotransferase; EC, esterified cholesterol; ERT, enzyme replacement therapy; LAL, lysosomal acid lipase; LIPA, gene that encodes LAL; NPC1L1, Niemann-Pick C1-Like1; SI, small intestine; SOAT2, sterol O-acyltransferase 2; TAG, triacylglycerol; TC, total cholesterol; UC, unesterified cholesterol.

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play in the formation of cholesteryl esters and the pathogenesis of atherosclerosis have made these enzymes, particularly SOAT2, key targets for pharmacological intervention [5–8].

Atherosclerosis is not the only disease in which tissue EC accumulation is a causative factor. When various classes of lipoproteins such as low density lipoproteins and chylomicron remnants are cleared from the circulation through receptor-mediated and bulk-phase endocytosis, their cholesteryl esters and triacylglycerols are hydrolyzed by lysosomal acid lipase (LAL) [9]. Mutations in LIPA, the gene that encodes LAL, result in either Wolman disease (WD), or cholesteryl ester storage disease (CESD). Whereas WD is a severe, early onset illness caused by complete loss of LAL activity, CESD is a milder, later-onset disease resulting from partial LAL deficiency [10]. Hepatomegaly and a massive increase in tissue EC levels are hallmark features of both disorders. A spontaneous rat model for Wolman disease was described in 1990 [11], and subsequently a mouse model for CESD was generated and characterized [12,13]. These models have been used for the development and testing of an enzyme replacement therapy (ERT) for this disorder [14–16]. This therapy is also being evaluated in humans [17,18].

A recent review summarized the treatment modalities used thus far in patients with CESD [19]. In one case, a statin was used in combination with the cholesterol absorption inhibitor, ezetimibe [20]. We found that in LAL-deficient mice, the addition of ezetimibe to their diet starting at the time they were weaned, resulted in a marked reduction in hepatic EC content and improved liver function [21]. This benefit was attributed primarily to a diminished delivery of intestinally-derived EC to the liver, with a resultant fall in the mass of EC entrapped in the lysosomes. In the current studies we used the CESD mouse model to investigate the extent to which the progression of disease stemming from the absence of LAL might slow in the face of a concurrent loss of SOAT2 function.

## 2. Materials and methods

### 2.1. Animals and diets

$Lal^{+/-}$  breeding stock were obtained from the laboratory of Drs. Grabowski and Du at the Children's Hospital Research Foundation in Cincinnati [12,13]. These mice were of the FVB/N strain and were used to generate  $Lal^{+/+}$  and matching  $Lal^{-/-}$  offspring for study at 21 and 93 days of age. In 2003 we purchased  $Soat2^{+/-}$  mice (on a BL/6:129S4 background) from the Jackson Laboratory, Bar Harbor, ME. The background was shifted to BL/6:129/Sv over the course of more than eight generations.  $Lal^{+/-}$  and  $Soat2^{-/-}$  mice were used to generate  $Lal^{+/-};Soat2^{+/-}$  breeding stock that in turn were bred to obtain offspring of the four genotypes ( $Lal^{+/+};Soat2^{+/+}$ ,  $Lal^{-/-};Soat2^{+/+}$ ,  $Lal^{+/+};Soat2^{-/-}$  and  $Lal^{-/-};Soat2^{-/-}$ ) needed for the current studies. Across all litters approximately equal numbers of mice of these four genotypes were obtained, with similar numbers of males and females for each genotype. Only the males were used in the present study. Their age on the day of study averaged  $52 \pm 1$  days. The preceding study with 21- and 93-day old  $Lal^{+/+}$  and  $Lal^{-/-}$  mice also used only males. Litters were genotyped at 18–20 days, and weaned at 21 days onto a low-cholesterol, low-fat, rodent chow diet as described [21]. All mice were group-housed in a light-cycled room and were studied in the fed state toward the end of the dark phase of the lighting cycle. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

### 2.2. Quantitation of total, esterified and unesterified cholesterol in tissues, and liver transaminase activities

After exsanguination of the mice, the liver and entire small intestine were removed, rinsed, blotted and weighed. Aliquots of liver and the whole small intestine were placed in chloroform:methanol (2:1 v/v) for measurement of the esterified (EC) and unesterified cholesterol (UC) fractions [22,23]. All cholesterol quantitation was done using gas chromatography [24]. Plasma total cholesterol concentrations were expressed mg/dl. For the liver and small intestine, the total cholesterol concentration was expressed as mg/g tissue. To obtain whole organ cholesterol content (mg/organ), the total cholesterol concentration was multiplied by the respective whole organ weight. Plasma ALT and AST activities (units/L) were measured by a commercial laboratory.

### 2.3. Quantitation of liver triacylglycerol content

Hepatic total triacylglycerol concentrations (mg/g) were measured using a method that combines column chromatography and an enzymatic colorimetric assay [25]. The initial extracts of liver tissue contained [ $^{14}C$ ]triolein (American Radiolabeled Chemi-

cals, Inc., St. Louis, MO) to correct for the procedural losses. These values and liver weight were used to determine whole liver triacylglycerol content (mg/organ).

### 2.4. Analysis of data

Values are mean  $\pm$  SEM for the specified number of animals. GraphPad Prism software, version 6.02, (GraphPad, San Diego, CA) was used to perform all statistical analyses. Depending on the design of each experiment, differences between mean values were tested for statistical significance ( $P < 0.05$ ) by an unpaired two-tailed Student's *t*-test, or a one-way analysis of variance.

## 3. Results

The data in Table 1 demonstrate multiple striking differences between  $Lal^{-/-}$  mice and their  $Lal^{+/+}$  littermates, starting at the time of weaning (21 days), and at about 10 weeks later when they were young adults. Genotypic differences in absolute and relative liver weights, already apparent at weaning, were highly pronounced in the 93-day old mice. Comparatively, there were only marginal genotypic differences in small intestine weight at these two age points. The most distinctive feature of the  $Lal^{-/-}$  mice was their dramatic increase in hepatic esterified cholesterol (EC) concentrations (mg/g), which, at 93-days, were more than 100-fold higher in the  $Lal^{-/-}$  mice vs their  $Lal^{+/+}$  littermates. The increases in both liver mass and hepatic EC concentration combined, resulted in a whole liver cholesterol content (mg/organ) in the  $Lal^{-/-}$  mice that was 80-fold more than in the  $Lal^{+/+}$  controls. In the 21-day old mice, the EC concentration in the small intestine of the mutants exceeded that in their wildtype littermates by 8.3-fold. Although the concentrations of EC and UC were not determined in the small intestine of the 93-day old mice, the total content of cholesterol in the intestine of the  $Lal^{-/-}$  mice at that age exceeded that in their  $Lal^{+/+}$  littermates by 3.4-fold. In the 93-day-old  $Lal^{-/-}$  mice, plasma ALT activities were elevated 20.5-fold compared to their age matched  $Lal^{+/+}$  littermates.

From the data in Table 1, it was clear that even at weaning, there was a substantial buildup of EC in the livers and small intestines of the  $Lal^{-/-}$  mice. This progressed to extremely high levels by 93 days of age, with pronounced hepatic dysfunction being evident. Therefore, it was decided that, for the purpose of measuring the impact of SOAT2 deletion on disease progression in the LAL-deficient mice, we would study the  $Lal^{-/-};Soat2^{-/-}$  mice and their wildtype, SOAT2-deficient, and LAL-deficient littermates when they were 52 days old. This age point was about midway between weaning and 93 days of age. As shown in Fig. 1A and B, respectively, the final body weights and small intestine weights did not vary significantly amongst the four genotypes. However, there were profound differences in intestinal EC concentrations as a function of genotype (Fig. 1C). Consistent with our previous findings [23], the EC level in the small intestine of wildtype and  $Soat2^{-/-}$  mice was very low. In the mice deficient in both LAL and SOAT2, the increment in the intestinal EC concentration was less than half of that seen in their littermates deficient in LAL only. The intestinal UC concentrations changed little with genotype other than a marginal rise in the  $Lal^{-/-};Soat2^{-/-}$  mice (Fig. 1D). Although intestinal TAG levels increase considerably in the LAL-deficient mouse [13], this parameter was not measured in the present study. Plasma total cholesterol concentrations were measured although the data are not illustrated. The values, given as mg/dl, were as follows:  $Lal^{+/+};Soat2^{+/+}$  ( $116 \pm 6.5$ ),  $Lal^{+/+};Soat2^{-/-}$  ( $115 \pm 6.2$ ),  $Lal^{-/-};Soat2^{+/+}$  ( $103 \pm 2.2$ ), and  $Lal^{-/-};Soat2^{-/-}$  ( $101 \pm 8.6$ ).

The data for the livers from the same mice that were used for the intestinal measurements are presented in Fig. 2. The deletion

**Table 1**  
Characteristics of male lysosomal acid lipase-deficient mice at weaning and in early adulthood

Parameters	Average age (days)			
	21 ± 0.1	93 ± 1	93 ± 1	93 ± 1
LAL genotype	+/+	-/-	+/+	-/-
SOAT2 genotype	+/+	+/+	+/+	+/+
Body weight (g)	10.5 ± 1.5	11.6 ± 0.4	30.1 ± 0.5	28.9 ± 0.8
Liver weight (g)	0.53 ± 0.09	0.77 ± 0.04	1.33 ± 0.02	4.82 ± 0.18*
Relative liver weight (% bw)	5.0 ± 0.1	6.6 ± 0.2*	4.4 ± 0.1	16.8 ± 1.0*
Small intestine weight (g)	0.42 ± 0.05	0.62 ± 0.02*	1.02 ± 0.04	1.31 ± 0.10
Hepatic esterified cholesterol concentration (mg/g)	1.6 ± 0.4	28.4 ± 0.4*	0.4 ± 0.04	45.8 ± 2.8*
Hepatic unesterified cholesterol concentration (mg/g)	2.2 ± 0.1	2.3 ± 0.2	2.0 ± 0.03	5.1 ± 0.2*
Whole-liver cholesterol content (mg/organ)	1.9 ± 0.1	24.7 ± 0.7*	3.2 ± 0.1	255.6 ± 9.2*
Small intestine esterified cholesterol concentration (mg/g)	0.16 ± 0.02	1.34 ± 0.05*	nm	nm
Small intestine unesterified cholesterol concentration (mg/g)	3.0 ± 0.1	2.6 ± 0.1	nm	nm
Whole-small intestine cholesterol content (mg/organ)	1.3 ± 0.1	2.5 ± 0.1*	2.6 ± 0.1	8.8 ± 0.4*
Plasma ALT activity (units/L)	34 ± 3	48 ± 9	37 ± 1	759 ± 152*

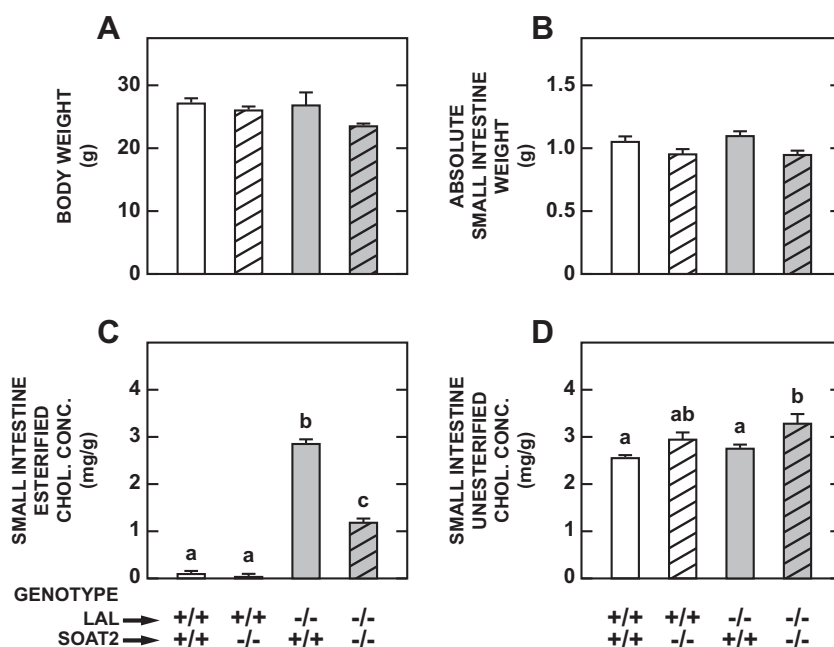
All mice were weaned at 21 days of age and thereafter were fed ad libitum a basal rodent chow diet.

They were studied in a fed state.

Values are mean ± 1 SEM of data from 5 to 7 mice per group.

nm-not measured.

\*  $p < 0.05$  compared to corresponding  $Lal^{+/+};Soat2^{+/+}$  mice of the same age.



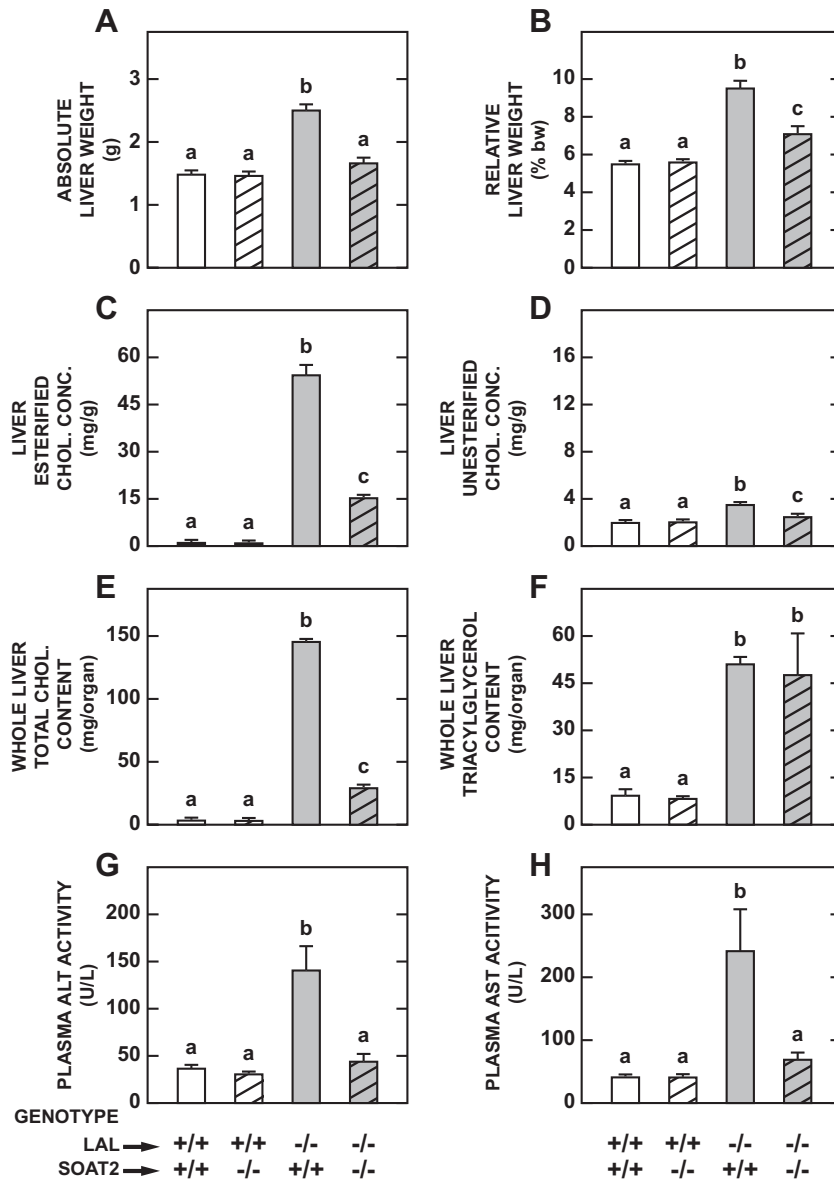
**Fig. 1.** Loss of the cholesterol-esterifying enzyme SOAT2 in LAL-deficient mice markedly reduced the concentration of esterified cholesterol in the small intestine. Body weight (A), small intestine weight (B), and intestinal concentrations of EC (C) and UC (D) were measured in male mice deficient in either SOAT2, LAL, or both SOAT2 and LAL, at 52 days of age. They were maintained since weaning on a basal rodent chow diet and were in the fed state at the time of study. Values are the mean ± SEM of data from 5 mice in each group except for the  $Lal^{-/-};Soat2^{+/+}$  mice were  $n = 4$ . Different letters denote statistically significant values ( $p < 0.05$ ) as determined by a one-way analysis of variance.

of SOAT2 activity in the  $Lal^{-/-}$  mice resulted in a marked reduction in the degree of hepatomegaly as shown by the absolute and relative weights for the liver (Fig. 2A and B, respectively). There was a dramatic reduction in hepatic EC concentrations in the  $Lal^{-/-};Soat2^{-/-}$  mice vs their  $Lal^{-/-};Soat2^{+/+}$  littermates (Fig. 2C). In contrast, there were only marginal shifts in the UC concentration in the liver, with the small increase seen in the  $Lal^{-/-};Soat2^{+/+}$  mice being partially reversed by the loss of SOAT2 activity (Fig. 2D). The most striking change was seen in the data for whole liver total cholesterol content (Fig. 2E). Here, the content in the mice deficient in both LAL and SOAT2 fell to only 20% of that seen in the mice deficient in LAL only. It is important to note that the liver TC content in the 52-day old  $Lal^{-/-};Soat2^{-/-}$  mice (29.0 mg/organ) was essentially about what it was in the LAL-deficient mice at 21-days

(24.7 mg) (Table 1). Although the deletion of SOAT2 greatly diminished EC sequestration in the livers of the mice lacking LAL, it had no effect on the content of triacylglycerol in the liver (Fig. 2F). Finally, the profound reduction in liver cholesterol content in the  $Lal^{-/-};Soat2^{-/-}$  mice was accompanied by a decisive improvement in liver function as measured by the plasma activities of ALT (Fig. 2G) and AST (Fig. 2H).

#### 4. Discussion

Given the role that SOAT2 plays in generating the esterified cholesterol that is contained in very low density lipoproteins secreted by the liver into the circulation, and in chylomicrons delivered into the lymph from the small intestine [26], it seemed important to



**Fig. 2.** Loss of SOAT2 activity in LAL-deficient mice results in a dramatic reduction in whole liver cholesterol content that reflects a decrease in liver mass, together with a decisive fall in the esterified cholesterol concentration. Absolute (A) and relative (B) liver weights, concentrations in liver of EC (C) and UC (D), whole liver contents of cholesterol (E) and triacylglycerol (F), and plasma activities of ALT (G) and AST (H) in male mice deficient in either SOAT2, LAL, or both SOAT2 and LAL, at 52 days of age. These data were obtained from the same mice used for the small intestine measurements (Fig. 1). Values are the mean  $\pm$  SEM of data from 5 mice in each group except for the *Lal*<sup>-/-</sup>:*Soat2*<sup>+/+</sup> mice were *n* = 4. Different letters denote statistically significant values (*p* < 0.05) as determined by a one-way analysis of variance.

investigate the extent to which deletion of SOAT2 might lessen the amount of EC entrapped in the liver and small intestine of the LAL-deficient mouse. The effect was much more dramatic than was anticipated, particularly for the liver. Several of the findings presented here are particularly noteworthy. One of these pertains to the data showing that, even at the time of weaning, the hepatic EC concentration in *Lal*<sup>-/-</sup>:*Soat2*<sup>+/+</sup> mice was already elevated almost 18-fold compared to that in *Lal*<sup>+/+</sup>:*Soat2*<sup>+/+</sup> littermates. This raises the intriguing question of whether at birth the *Lal*<sup>-/-</sup>:*Soat2*<sup>+/+</sup> mice already have a substantial elevation in hepatic EC levels, and if so, what might be found in newborn pups deficient in both LAL and SOAT2. A related question is whether the ablation of SOAT2 function in *Lal*<sup>-/-</sup> mice would continue to have a beneficial impact on the liver and small intestine at ages well beyond 52 days, and ultimately on their highly variable lifespan [27].

Another finding warranting comment concerns the lack of change in hepatic TAG levels in the *Lal*<sup>-/-</sup>:*Soat2*<sup>-/-</sup> mice (Fig. 2F).

Here it should be pointed out that, while suppression of SOAT2 activity in a mouse model with dietary cholesterol-associated steatosis enhances hepatic TAG mobilization [28], in that instance the excess TAG is present in cytoplasmic lipid droplets and not sequestered in the lysosomal compartment as it is in LAL deficiency. Studies using enzyme replacement therapy in the CESD mouse model have demonstrated a decisive reduction in hepatic TAG content, even in animals with advanced disease [14,16].

There are many interconnections in cholesterol movement and processing between the small intestine and liver that occur continually [23,24,26]. Thus perhaps the most important question raised by these new findings is the extent to which the benefit resulting from global deletion of SOAT2 in LAL deficiency stems from the loss of enzyme activity in the liver vs the small intestine. Studies with liver and small intestine-selective SOAT2 deficient mice have demonstrated that, in both models, there is prevention of diet-induced cholesterol accumulation in the liver and blood [29]. Newly pub-



lished work using low density lipoprotein receptor-deficient (*Ldlr*<sup>-/-</sup>) mice carrying liver or intestine-specific deletion of SOAT2 shows that although EC from both the intestine and liver contribute to the development of atherosclerosis, the *Ldlr*<sup>-/-</sup> mice with liver-specific deletion of SOAT2 had less aortic EC accumulation and smaller aortic lesions than the *Ldlr*<sup>-/-</sup> mice with intestine-specific SOAT2 deletion [30]. Presumably, the use of LAL-deficient mice with selective deletion of SOAT2 in either the liver or small intestine will establish whether the progression of CESD is driven more by SOAT2 activity in one of these organs than the other. Irrespective of what is determined from such models, we conclude from the current studies that testing of one of the new SOAT2 selective inhibitors [5,8] in this mouse model for CESD might reveal the potential of such agents for the management of this disorder.

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