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Defects of the endoplasmic reticulum and changes to lipid droplet size in mammary epithelial cells due to *miR-30b-5p* overexpression are correlated to a reduction in *Atlastin 2* expression

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ABSTRACT

During lactation, mammary epithelial cells secrete fat in the form of milk fat globules that originate from intracellular lipid droplets. These droplets may form *de novo* from the endoplasmic reticulum or be derived from existing lipid droplets; they then either grow because enzymes of triacylglycerol synthesis relocate from the reticulum to their surface, or due to fusion and fission with other droplets. The overexpression of *miR-30b-5p* in the developing mouse mammary gland impairs lactation, which includes an increase in lipid droplet size. This study was performed to understand the origin of this defect affecting lipid droplets observed in transgenic mice.

Electron microscopy analyses revealed a fragmented and discontinued tubular network of endoplasmic reticulum in the mammary epithelial cells of transgenic mice. The milk fatty acid composition was modified, with lower levels of medium-chain saturated fatty acids and a proportional increase in long-chain monounsaturated fatty acids in transgenic *versus* wild-type mice. Further, investigations of microRNA targets revealed a significant downregulation of ATLASTIN 2 (a GTPase described as playing a key role in lipid droplet formation) due to *miR-30b-5p* overexpression.

Our results suggest that the increase in lipid droplet size observed in the mammary epithelial cells of transgenic mice might result from changes to lipid droplet formation and secretion because of direct modifications to *Atl2* expression and indirect changes to endoplasmic reticulum morphology resulting from the overexpression of *miR-30b-5p*.

Introduction

Mammary epithelial cells (MEC) secrete fat in the form of milk fat globules (MFG) which originate from intracellular lipid droplets (LD), the principal fat storage structures in eukaryotic cells. These organelles are dynamic, containing neutral lipids (predominantly sterol esters and triacylglycerols (TG)) at their core, which are surrounded by specific proteins involved in lipid metabolism and a monolayer of amphipathic lipids (phospholipids and cholesterol). LD may have two origins: from existing LD or resulting from the development of *de novo* LD in the endoplasmic reticulum (ER). Two pathways may regulate LD size. One well-described pathway involves regulation of the cellular triglyceride content in cells. The local synthesis of TG at their surface induces LD growth, requiring a cellular trafficking pathway to deliver the enzymes necessary for TG synthesis to the LD. A second mechanism involves the fusion and fission of intracellular LD with other droplets [1]. Although fusion has been demonstrated *in vivo* in the murine mammary gland [2] the regulatory mechanisms governing this process still need to be determined. The phospholipid composition of the membrane [1] or specific proteins on the surface of intercellular LD may regulate fusion, as has been shown for murine MEC [3]. In

Abbreviations: MEC, mammary epithelial cell; LD, lipid droplet; ER, endoplasmic reticulum; TG, triacylglycerol; FA, fatty acid.

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bovine MEC cultures, Cohen et al. [4] recently demonstrated that LD size is determined by fusion, independent of the cellular triglyceride composition.

The re-localisation of enzymes from the ER to the surface of droplets [5] may induce mature and nascent LD. The dominant model is the formation of LD from an accumulation of neutral lipids and cholesterol between the two leaflets of the ER membrane [6], and links have been established between LD and the ER [7] which participate in localising TG enzymes to the LD surface [8,9]. Consistent with this, factors that maintain ER structure such as ATLASTIN, a GTPase that mediates membrane fusion to connect ER tubules, play a crucial role in regulating the size of LD [10].

Member of the highly conserved *miR-30* family, *miR-30b-5p* plays an important role in the biology of the mammary gland. Its deregulation impairs lactation, reduces the size of alveolar lumens and causes LD defects [11]. Indeed, when compared to wild-type mice, fewer and larger LD are observed in the mammary tissue of transgenic mice overexpressing *miR-30b-5p* in their MEC.

The aim of this study was therefore to understand the origin of the LD defects observed in the mammary glands of *miR-30b* transgenic mice through the deep phenotyping of mammary glands and the analysis of milk fatty acids (FA).

Materials and methods

Animal experiments and sample collection

miR-30b transgenic mice were first described by Le Guillou et al. [11]. This mouse line has since been maintained on an FVN/B background in a controlled environment. All experiments for the present study that involved animals were performed in strict accordance with the guidelines on the human care of animals and the Code for Methods and Welfare Considerations in Behavioural Research with Animals (EU Directive 2010/63/EU). They further complied with the recommendations of the French "Commission de Génie Génétique" (Permit # 1730 (10.03.2016)) which also approved the study. After mating, the days of plug detection and delivery were designated as day-0 of pregnancy and lactation, respectively. Day-0 of involution was induced by removing the pups from the dams after a suckling period which ranged from 8 to 15 days. To sample for RNA and for protein analyses, the lymph nodes of the abdominal mammary glands were removed before tissue homogenisation. The mothers were separated from their pups for 4 h, then stimulated with an injection of oxytocin and anaesthetised before milking, as previously described [12]. Milk samples were collected on day-12 of lactation by hand stripping and capillary rising in a Pasteur pipette into a sterile tube, kept on ice and stored at −80 °C.

Lipid analyses

The proportions of FA methyl esters (FAME) in milk fat samples were measured using the creamatocrit method, as previously described [13]. Briefly, the skim fraction of whole milk samples (75 μl in glass capillary tubes) was separated using centrifugation (12,000 rpm for 12 min). The percentage cream length in the tubes was determined in order to calculate the fat content (g/100 mL). The FA composition was analysed after one-step extraction and methylation of the milk fat samples. The FAME profile was determined using gas chromatography and the chromatographic peaks were identified as previously described [14].

Quantitative PCR analyses

mRNA were quantified by RT-qPCR as previously described [11] and in triplicate for five wild-type and five transgenic animals. *Atlastin 2 (Atl2)* gene expression was studied using the following primer set: forward primer: 5′-CACGTCTCGTCTACGACCTC-3′ and reverse primer: 5′-GGTCATCTTCATGGGCGAGA-3'. *Transforming Growth Factor Beta Regulator 4 (Tbrg4)* mRNA, used as the housekeeping gene, was quantified using the following primer set: forward primer: 5′-GAGACAGAGCTGCACACGGTT-3′ and reverse primer: 5′-ATGTGGACCAATTTCTGGAAGGT-3'. The comparison of *Atl2* expression levels (normalised for *Tbrg4*) between wild-type and transgenic mice was conducted using the Delta-Delta Ct method ($2^{-\Delta\Delta Ct}$) [15]. RT-PCR quantifications of microRNA were carried out using TaqMan MicroRNA Expression Assays (Applied Biosystems), as previously described [11].

Transmission electron microscopy analyses

The fixation of mammary glands was performed at room temperature, using 2% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.2, for 1 h. Contrast was obtained with 0.5% Oolong Tea Extract (OTE) in cacodylate buffer on samples post-fixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, gradually dehydrated in ethanol (30%–100%) and substituted in a mix of ethanol-epon, then embedded in Epon (Delta microscopie).

After collection on 200 mesh copper grids, thin sections (70 nm) were counterstained with lead citrate and then examined with a Hitachi HT7700 electron microscope operated at 80 kV (Elexience). Image acquisition was performed using a charge-coupled device camera (AMT). The surfaces of LD were measured on biological images using the Fiji open-source platform [16].

Protein analyses

Mammary glands from three wild-type and three transgenic mice were homogenised separately in 1.6 mL cold lysis buffer (0.32 M saccharose, 0.5% deoxycholic acid (Sigma Aldrich), 0.5% IGEPAL CA-630 (ICN Biomedicals)), and centrifuged at 2000 rpm for 10 min at 4 °C. Protein-containing supernatants were collected and kept on ice or stored at −80 °C until use. Quick Start Bradford protein 1x dye reagent (Bio-Rad Laboratories), with a bovine serum albumin standard, was used to determine the protein concentrations. After denaturation, 80 μg of each sample were run on 12.5% SDS-Page gel, electrotransferred onto a nylon Hybond C membrane (Amersham, GE Healthcare) and immunoblotted with anti-Atl2 antibody (NBP1-98394, Novus Biologicals) diluted 1:1000, and anti-β-Actin antibody (A5441, Sigma-Aldrich) diluted 1:10,000. Molecular weight was determined using the PageRuler™ Prestained Protein Ladder (#26616, ThermoFisher Scientific). Immunoreactivity was visualised by chemiluminescence (ChemiDoc™ Touch Imaging System, Bio-Rad).

Calculations and statistical analyses

Protein expression was quantified from the results of Western blots using Bio-Rad Image Lab™ 5.2.1 software (Bio-Rad Laboratories). The means and standard errors of different FA classes were calculated for each group. Medians, quartiles and minimum and maximum values were processed for the RT-qPCR expression analyses.

Differences in results between the wild-type and transgenic mice were compared using nonparametric Mann and Whitney statistical analyses, with Microsoft Excel (Microsoft) software. Tests results were considered to be statistically significant when p-values were higher than 0.05.

Results

The overexpression of miR-30b-5p in MEC results in morphological alterations to the ER structure

To evaluate the effects of *miR-30b-5p* overexpression on the structure of MEC, electron microscopy studies were performed on mammary gland samples collected at lactation day-12. The MEC contained numerous LD extending from the basal to the apical poles of the cells. LD in the MEC of transgenic mice were larger than those seen in wild-type mice (Fig. 1A). The MEC of lactating mice contained an extensive network of regularly arranged ER compartments, consistent with its role in the mechanism of milk component secretion. However, the ER in the MEC of transgenic did not display a regular parallel arrangement as is normally found; its tubular network was indeed fragmented and discontinuous (Fig. 1B). These observations showed that the overexpression of *miR-30b-5p* in MEC altered ER morphology.

The overexpression of miR-30b-5p in MEC modifies milk FA composition

The size of LD in MEC prior to their secretion into milk determines the size of the MFG secreted, which in turn is tightly linked to its lipid, FA and protein contents, thus determining milk composition [4]. The milk FA composition was therefore determined in order to obtain indirect information on the composition of LD which influence their size. While the level of polyunsaturated FA was not modified, a reduction in saturated FA (−8.7%) and an increase in monounsaturated FA (+29.5%) were observed in the milk of transgenic mice *versus* wild-type mice (Table 1). Changes to saturated FA mainly resulted in a significant reduction in medium-chain myristic acid (C14:0) and to a lesser extent in medium-chain lauric acid (C12:0) in the milk of transgenic mice (Supplementary Table 1). Modifications to monounsaturated FA were mainly due to an increase in long-chain FA, the most important of these being oleic acid (*cis-9* C18:1), then palmitoleic acid (*cis-9* C16:1, co-eluted with heptadecanoic acid (C17:0 *anteiso*)) and *cis*-vaccenic acid (*cis-11* C18:1). Thus the overexpression of *miR-30b-5p* in the mammary gland modified the FA composition of milk in transgenic mice.

Table 1
Overexpression of *miR-30b-5p* in MEC modifies the milk FA composition. Milk from wild-type (WT) and *miR-30b* transgenic (Tg) mice are distinctive for their proportions of saturated and monounsaturated FA. Mean values with their standard errors (SEM). P: P-value of Mann and Whitney statistical analysis. ns: non-significant, p-value > 0.05. #: total number of individual samples analysed.

FA class (g/100 g FA)	WT	Tg	SEM	P	#
Σ Saturated FA	67.50	61.64	5.34	0.016	16
Σ Monounsaturated FA	18.84	24.39	4.58	0.012	16
Σ Polyunsaturated FA	12.99	13.45	2.16	ns	16
Σ Conjugated Linoleic Acid	0.10	0.08	0.03	ns	12
Σ Branched FA (*iso & anteiso*)	0.09	0.09	0.04	ns	13

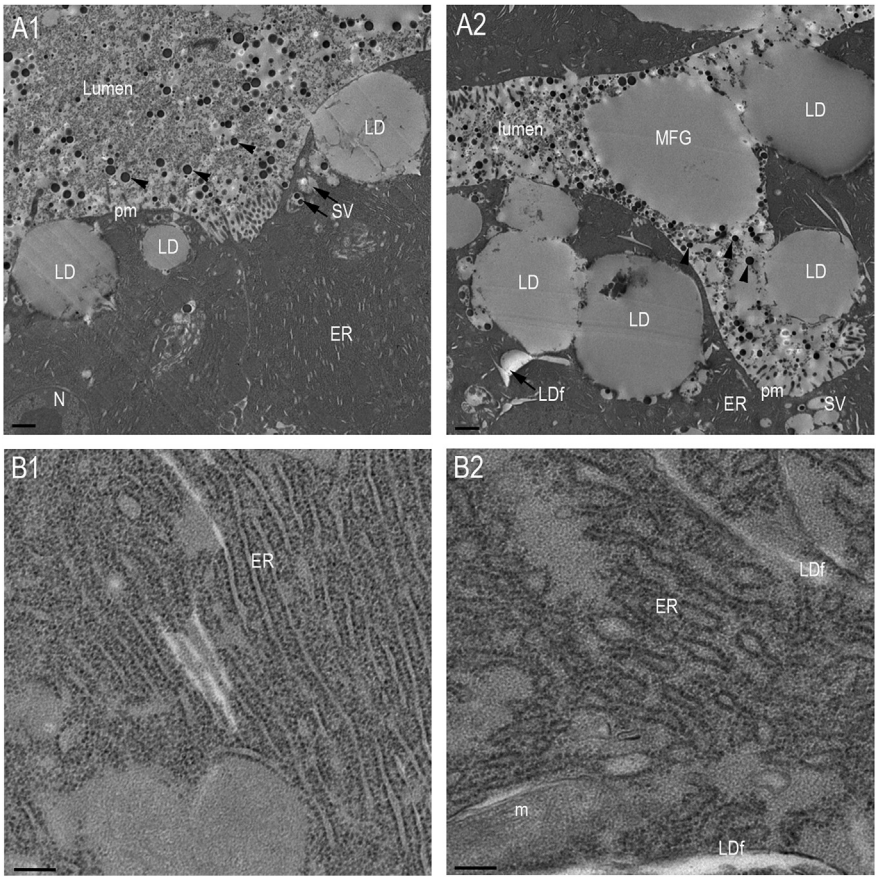

Fig. 1. Overexpression of *miR-30b-5p* in MEC during lactation leads to an increase in LD size (A) and fragmentation of the ER (B). Transmission electron microscopy images of lactating mammary gland (day-12) from wild-type (A1, B1) and *miR-30b* transgenic (A2, B2) mice. LD, Lipid droplet; LDf, Lipid droplet in formation; SV, secretory vesicle with casein micelles (arrows); N, nucleus; pm, plasma membrane; ER, Endoplasmic Reticulum; m, mitochondria; MFG, Milk Fat Globule. Scale bars: 100 nm (A1, A2) and 200 nm (B1, B2).

The overexpression of miR-30b-5p provokes a downregulation of Atl2 gene expression

Using a microarray approach, transcriptomic analyses were performed to determine the genes differentially expressed in the mammary gland of transgenic mice compared to wild-type mice, at lactation day-12 [11]. Among the 220 differentially expressed genes, one - *Atl2* - is a member of the ATLASTIN GTPase group described as playing a key role in LD formation [10]. Working in *C. elegans,* Klemm et al. [10] demonstrated a role for ATLASTIN in the fusion of ER membranes and in regulating LD size. In the mammary gland of wild-type mice, the expression of *Atl2* was significantly stronger at the mid-lactation stage when compared to the end of pregnancy and involution stages (Supplementary Fig. 1). The *Atl2* gene contains features of the *miR-30b-5p* target. Computational algorithms to predict miRNA targets, such as Diana microT-CDS, have described *Atl2* as one of the direct targets of *miR-30b-5p*, with two complementary sites of the seed sequence present in its 3′UTR (Fig. 2A). Our previous transcriptomic analysis showed that its expression was downregulated in the mammary gland of transgenic mice when compared to wild-type mice at lactation day-12 (Fold change = −1.41; adjusted p-value = 0.033) [11]. Here, this result was confirmed by the RT-qPCR approach, revealing a reduction of around 20% (p = 0.032) in *Atl2* gene expression in the mammary gland of transgenic mice *versus* wild-type mice (Fig. 2B). Western blot analysis also showed a decrease at the protein level, with a 40% reduction (p = 0.049) in ATLASTIN 2 in the mammary tissue of transgenic *versus* wild-type mice (Fig. 2C).

Discussion

During this study, we revealed modifications to the FA composition of milk from mice overexpressing *miR-30b-5p*. These changes were dependent on the length of the FA [17]. During lactation, MEC synthesise triglycerides using long-chain FA from the blood, whereas short- and medium-chain FA are synthesised *de novo* from glucose and acetate. *miR-30b-5p* overexpression did not affect the percentages of short-chain FA but modified those of medium and long-chain FA. However, the expression of genes involved in FA absorption from plasma or in *de novo* synthesis [18] was not modified in the mammary gland of transgenic mice at lactation day-12 [11]. The mechanisms underlying FA biosynthesis did not appear to be responsible for these changes to the lipid composition of milk.

Secretion also impacts the milk lipid composition, depending on whether LD develop in the ER and/or accumulate in MEC. Electron microscopy studies showed that the ER is tightly associated with LD and a physical coupling of the two organelles is a prerequisite for LD expansion [8,10,19,20]. Here, the overexpression of *miR-30b-5p* in the mammary gland altered the ER structure which could therefore be linked to the larger size and different quantities of LD.

The size of MFG is tightly associated with their composition and determined by their formation and secretion processes [21]. Saturated FA levels are known to be higher in small MFG than in large MFG [22]. In transgenic mice, the significant reduction in saturated FA and the proportional rise in monounsaturated FA in milk were consistent with the larger size of LD observed in MEC. Moreover, *in vitro* studies have shown that treating cells with oleic acid, in

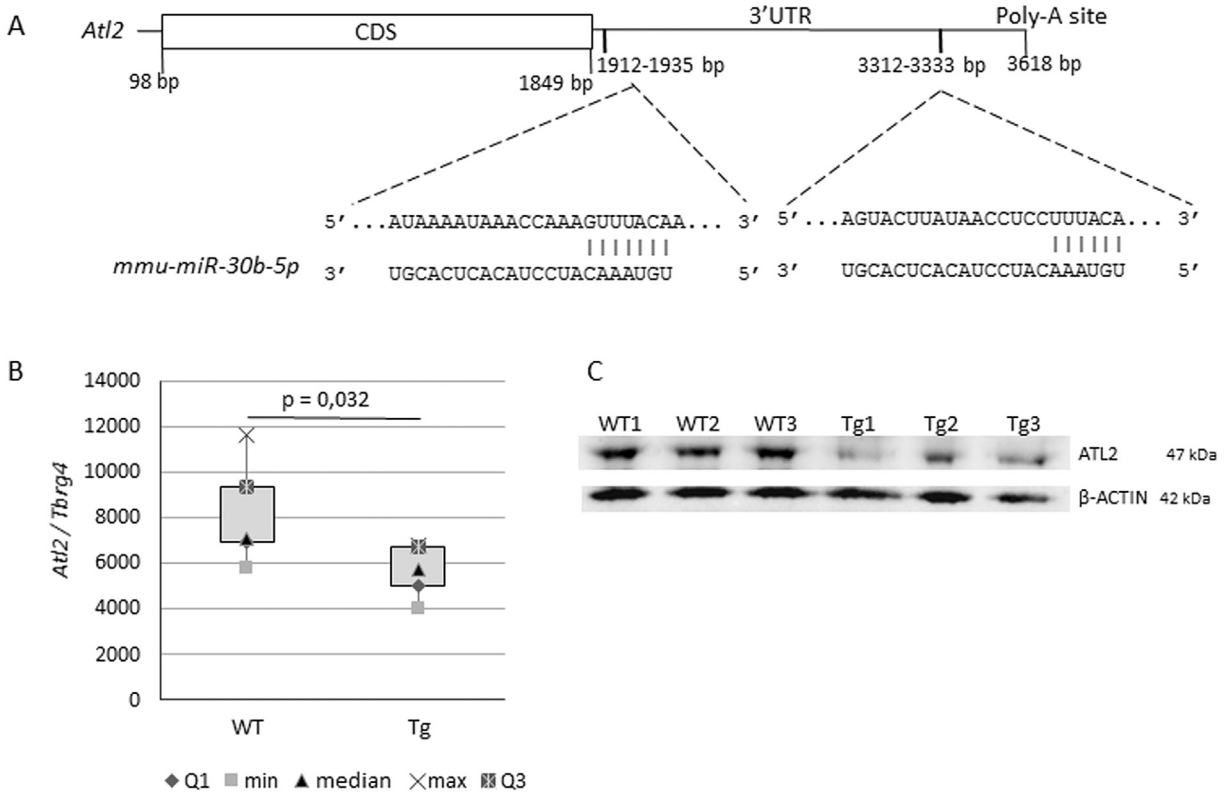

Fig. 2. The overexpression of *miR-30b-5p* leads to a significant reduction in the expression of *Atl2* in the mammary gland of transgenic mice (Tg) compared to wild-type mice (WT) at lactation day-12. A. Two regions of the *Atl2* 3′UTR murine sequence are targeted by *miR-30b-5p*. Positions on the transcript variant 1 mRNA (GenBank Accession ID NM_019717.3). **B.** *Atl2* expression, measured by RT-qPCR, relative to *Tbrg4* housekeeping gene expression. The box-plot diagram represents the medians of *Atl2* expression in five individuals per group, with the first (Q1) and third (Q3) quartile ranges of data, and the minimum and maximum values. p: p-value of Mann-Whitney analysis. **C.** Western blot analysis of ATL2 in the mammary gland of three wild-type (WT1, WT2, WT3) and three transgenic (Tg1, Tg2, Tg3) lactating mice.

particular, increased the number of large LD when compared to other FA treatments and controls [4,23]. The significant rise in oleic acid levels in the milk of transgenic mice (Supplemental Table 1) could be linked to the larger LD observed during our study. These modifications to the milk FA composition may be associated with defective LD biogenesis.

The key genes involved in the formation, growth or fusion of LD, as described by Wilfling et al. [5] and Yang et al. [24] in their reviews, are not deregulated in the mammary gland of transgenic mice. However, *miR-30b-5p* overexpression downregulated the *Atl2* gene, an ATLASTIN GTPase described as playing a crucial role in regulating the size of LD [10]. This gene bears features of the *miR-30b-5p* target, such as the presence of two complementary seed sequences in its 3′UTR and an anti-correlated expression profile of *miR-30b-5p* between the end of pregnancy through different lactation stages [11].

During our study we were able to show that the overexpression of *miR-30b-5p* in MEC during lactation led to a significant reduction in ATLASTIN 2 protein levels and visible fragmentation of the ER. ATLASTIN is required to both form and maintain the ER network [25] by mediating the membrane fusion required for its maintenance and by stabilising newly formed tubular junctions and the membrane curvature [26,27]. Moreover, Hu et al. [26,28] and Orso et al. [29] showed in *S. cerevisiae* yeast and in the Drosophila *D. melanogaster*, respectively, that a lack of ATLASTIN fusion activity caused ER morphology defects. In fact, ATLASTIN depletion inhibits tubular interconnections in the ER, resulting in its fragmentation and discontinuity of its network, made up of unbranched tubules. By repressing *Atl2* expression, *miR-30b-5p* may alter the integrity of the ER network within MEC.

Furthermore, ATLASTIN can regulate LD size through its membrane fusion activity [10] so that the LD can continue to grow substantially during the secretion process [30]. Therefore, the increase in LD size observed in the MEC of transgenic lactating mice could have resulted from changes to LD formation and secretion, due to the combination of a direct impact (the modification of *Atl2* expression) and an indirect impact (ER defects) on the overexpression of *miR-30b-5p*.

miR-30b belongs to the *miR-30* family that contains five members. Recent studies have shown that this family plays an important regulatory role in the development of different tissues and organs. In a recent review, Mao et al. [31] proposed that this microRNA family is crucial to adipogenesis and participates in the regulation of brown adipose tissue function, so it may be a potential new actor regulating lipid metabolism. The results described here seem to confirm this hypothesis.

In conclusion, we were able to show that the overexpression of *miR-30b-5p* in the mammary gland provokes *Atl2* gene deregulation which influences the integrity of the ER and the size of LD.

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Transparency document

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Appendix A. Supplementary data

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