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Maternal perinatal undernutrition modifies lactose and serotranferrin in milk: relevance to the programming of metabolic diseases?

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Wattez JS, Delmont A, Bouvet M, Beseme O, Goers S, Delahaye F, Laborie C, Lesage J, Foligné B, Breton C, Metges CC, Vieau D, Pinet F. Maternal perinatal undernutrition modifies lactose and serotranferrin in milk: Relevance to the programming of metabolic diseases? *Am J Physiol Endocrinol Metab* 308: E393–E401, 2015. First published December 30, 2014; doi:10.1152/ajpendo.00452.2014.—A close link between intrauterine growth restriction and development of chronic adult diseases such as obesity, diabetes, and hypertension has been established both in humans and animals. Modification of growth velocity during the early postnatal period (i.e., lactation) may also sensitize to the development of metabolic syndrome in adulthood. This suggests that milk composition may have long-lasting programming/deprogramming metabolic effects in the offspring. We therefore assess the effects of maternal perinatal denutrition on breast milk composition in a food-restricted 50% (FR50) rat model. Monosaccharides and fatty acids were characterized by gas chromatography, and proteins were profiled by surface-enhanced laser desorption/ionization-time-of-flight analysis in milk samples from FR50 and control rat dams. Milk analysis of FR50 rats demonstrated that maternal undernutrition decreases lactose concentration and modulates lipid profile at *postnatal day 10* by increasing the unsaturated fatty acids/saturated fatty acids and diminishes serotransferrin levels at *postnatal day 21*. Our data indicate that maternal perinatal undernutrition modifies milk composition both quantitatively and qualitatively. These modifications by maternal nutrition open new perspectives to identify molecules that could be used in artificial milk to protect from the subsequent development of metabolic diseases.

mass spectrometry; fatty acids; carbohydrates; nutrition; rat

EPIDEMIOLOGICAL STUDIES in humans and experimental data in animals have shown an association between perinatal malnutrition and the occurrence of metabolic disorders in adulthood, including type 2 diabetes, hypertension, or obesity (3). These reports have paved the way for the developmental origins of health and adult disease hypothesis, stating that an environmental change in the perinatal period would program the development and maturation of many tissues, permanently altering the physiological response that ultimately results in the development of metabolic diseases (48). It has also been shown that prenatal malnutrition alters the fetal growth kinetics and increased the incidence of intrauterine growth restriction (IUGR) and macrosomia, which have negative consequences both in humans (17) and in animals (26). In rodents that belong to altricial species, the development of several organs and tissues is delayed and occurs almost exclusively during the postnatal period immediately after birth. The critical pathways involved in metabolic programming mainly develop postnatally but can be modified by both pre- and postnatal environmental manipulation. It is likely that similar principles hold true for humans although the timing of pathway development occurs earlier than in rodents (52). Many data have actually highlighted that maternal nutritional status is partly determinative of the composition of milk secreted. Supplementation of carbohydrates (30), lipids (43), and proteins (49), or conversely by deficiency of nutritive compounds (1, 51), impacts the quantity and quality of milk, as well as the metabolism of the offspring. Breast milk is known to contain many bioactive factors (2) involved in the development and growth as well as in the maturation of the immune system and gut microbiota (13, 27). We have recently shown that cross-fostering by control mothers of IUGR pups prevents the development of hyperphagia, hypertension, and intolerance to glucose observed in perinatally food-restricted adult male rats (53). Interestingly, maternal nutritional status during lactation influences leptin concentration both in milk and in the plasma of pups (53). Because neonatal leptin levels have been demonstrated to play an important neurodevelopmental role (7, 16), our data strongly suggest that the milk composition may participate to the programming of metabolic diseases in adulthood. These observations prompted us to analyze the effects of perinatal global caloric restriction on the composition of breast milk to identify compounds potentially involved in metabolic programming.

MATERIALS AND METHODS

Ethics statement. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (5), European Communities Council Directive of 1986 (86/609/EEC), and approved by the French Departmental Direction of Veterinary Services Committee (DDSV/59-009228). Animal use accreditation by the French Ministry of Agriculture (no. 04860) has been granted to our laboratory for experimentation with rats. All surgery was performed under pentobarbital sodium anesthesia, and all efforts were made to minimize animal suffering.

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Animal studies. Male and virgin mature female Wistar rats were purchased from Charles Rivers Laboratories (L'Arbresle, France) and housed five per cage depending on the sex. Animal rooms were maintained on a 12:12-h dark-light schedule (light on at 0700) and controlled temperature (22 ± 2°C) with free access to food [regular rat chow: total digestible energy 2,900 calories/g (15.1% proteins, 3.1% lipids, 60.9% carbohydrates, 3.9% fibers, 5.1% mineral ash; 11.9% moisture); SAFE D04; UAR, Augy, France] and tap water. After 2 wk of acclimation, 2-mo-old females were mated with a male for one night. If spermatozoa were found in vaginal smears, *day 0* of pregnancy or *embryonic day* (E) *0* was defined. Pregnant rats were then transferred to individual cages and fed ad libitum.

Each pregnant dam was randomly assigned to a control group (CTRL, $n = 11$) and was fed ad libitum during gestation and lactation or to a food-restricted group (FR50, $n = 11$) and received 50% of ad libitum intake determined in a pilot study from E14 until *postnatal day* (PND) *21*, as previously described (37). Food restriction had no effects on the length of gestation, on the litter size, and on the sex ratio of the pups (32). Dams were delivered spontaneously during the night between E21 and E22. At parturition, litter size was adjusted to eight pups per dam (sex ratio 1:1) in both CTRL and FR50 mothers to ensure adequate and standardized nutrition. Thereafter, the pups were kept with their own mothers until weaning.

Milk samples were collected from the same female at two time points (PND10 and -21) and were taken 2 h after having removed the pups from their mothers. An injection of 0.5 ml of Syntocinon (5 IU/ml; Sigma Tau), a synthetic analog of oxytocin, was administered intraperitoneally to lactating females before anesthetizing them using an intraperitoneal injection of 0.2 ml of pentobarbital solution (Ceva Santé Animale). A manual pressure was exercised on the udder, on which a Pasteur pipette was applied, allowing the rise of the milk by capillarity. Milk samples were separated into aliquots and stored at −80°C until use. At the end of the experiment, 38 rat milk samples were collected for the determination of milk composition (CTRL PND10 = 8 samples, FR PND10 = 9, CTRL PND21 = 11, FR50 PND21 = 10).

Global analyses of milk composition. Crude nutrient composition was analyzed as previously described (22). Aliquots (200 μl) of rat milk ($n = 5$ samples/group for each stage) were used to measure dry matter, crude fat and protein content, and lactose concentration. Measurements were made in duplicate. Dry matter was analyzed by drying at 55°C. Crude protein was determined by the Dumas method and nitrogen content using the diary 6.38 conversion factor. Crude fat was determined by a miniaturized version of the Röse-Gottlieb method. Lactose content was determined in isolated whey using HPLC on a Rezex ROA-Organic Acid column (300 × 7.8 mm ID, 8 μm; Phenomenex, Aschaffenburg, Germany).

Determination of monosaccharides and fatty acids in milk. After being thawed, 50 μl of rat milk samples ($n = 38$) were collected in a glass screw tube, and 200 μl of mesoinositol at 100 μg/ml (20 μg) were added as an internal standard with 100 μl of milli-Q water before lyophilization. The dry residue was then subjected to a 24-h methanolysis at 80°C with 500 μl of dry methanolic hydrochloride (0.5 M) for releasing monosaccharides.

For the monosacharides analysis, the samples were cooled to room temperature and then neutralized to pH 6–7 by addition of silver carbonate. The mixture was then *N*-reacetylated overnight at room temperature in the dark by adding 100 μl of acetic anhydride. After centrifugation, each supernatant was collected in a new glass tube before quantification. First, the retention time and area of the most intense peak for each monosaccharide detected by gas chromatography (GC)/mass spectrometry (MS)-electron impact (EI) were selected, and second, the relative ratios between monosaccharide area and internal standard area (mesoinositol) were calculated. On the other hand, the lower methanolic phases containing monosaccharides were combined in another glass tube for evaporation using *N*,*O*-bis trimethylsilyl trifluoroacetamide + 1% trimethylchlorosilane/pyridine (vol/vol, 1:1). Silylation reagent (40 μl) was added to all samples, and the reaction of trimethylsilylation of *O*-methyl glycosides was performed at room temperature for 2 h. This mixture was then diluted with 2 ml of anhydrous n-heptane, before GC analyses. Thereafter, 0.5 μl of this solution was injected into GC/MS-EI.

For the fatty acid analysis, extraction was performed with anhydrous n-heptane (3 × 250 μl), and the upper phases were collected in a glass tube. Next, 200–300 μl of undiluted solution of the derivatized internal standard C17:0 (10 mg of C17:0 were subjected to methanolysis for 30 min at 80°C) were added. Thereafter, 1 μl of this solution was injected into GC/MS-EI. All samples were analyzed by GC/flame ionization detector (FID) and/or GC/MS-EI with GC/MS records performed 3.5 min after the injection. Data were recovered between 35 and 1,000 atomic mass units. Integrations of the peaks were performed on the TIC chromatogram with Xcalibur software (Thermo Fisher Scientific). The internal standards were heptadecanoic acid (C17:0) for fatty acids analysis and mesoinositol for monosaccharides analysis. A mixed standard solution of fatty acid methyl esters (FAMEs) between butyric (C4:0) and nervonic (C24:1n9) acids was commercially purchased (Sigma-Aldrich). Similarly, two mixtures of TMS ethers of monosaccharides were prepared with the same protocol as the samples: one with pentoses and the other one with hexoses. These standard solutions were injected in GC/FID and/or GC/MS-EI. Retention times and mass spectra of each fatty acid and monosaccharide standards were measured and compared with samples. In addition, the mass spectrum obtained by GC/MS for each fatty acid was compared with that of the National Institute of Standards and Technology library. The apparatus GC/MS was used in addition to the GC/FID for confirmation and quantification of identified monosaccharides and quantification of fatty acids. For the relative quantitation of FAMEs by GC/MS-EI, ICIS detection algorithm was used without smoothing of chromatograms. First, the retention time and the area of the chromatographic peaks corresponding to each FAME were identified in the chromatograms extracted at mass-to-charge ratios (*m*/*z*) corresponding to the molecular ion of each FAME. Next, the relative ratios fatty acid area/internal standard area (C17:0) by GC/MS-EI were calculated for each sample.

Surface-enhanced laser desorption/ionization-time-of-flight-mass spectrometry profiling of milk. Surface-enhanced laser desorption/ionization-time-of-flight-mass spectrometry (SELDI-TOF-MS) analysis was used to profile milk protein samples with eight spots format ProteinChip Arrays Q10 (Weak Anion Exchanger), H50 (Reverse Phase), and immobilized metal affinity capture (IMAC) copper (Cu^{2+}) and nickel (Ni^{2+}) (Bio-Rad Laboratories). Arrays were prepared with 5-μl samples diluted with 10 μl of 7 mol/l urea, 2 mol/l thiourea, 4% wt/vol CHAPS in 50 mmol/l Tris, pH 9, and 10-fold in binding buffer, which varied according to array as follows: for Q10, 100 mmol/l Tris, pH 9; for H50, 10% acetonitrile, 0.1% trifluoroacetic acid, and 150 mmol/l NaCl; for IMAC Cu^{2+}, 100 mmol/l copper sulfate; and for IMAC Ni^{2+}, 100 mmol/l nickel sulfate. Each of these samples was tested in duplicate and randomly distributed on arrays, as previously described (18). All data were processed with the ProteinChip Data Manager software (Bio-Rad Laboratories) as previously described (18). For the first step, peaks were automatically detected according to the specified signal-to-noise ratio (4 or 5) and the minimum valley depth (3 or 4), if they were found in at least 10% of all spectra, with an *m*/*z* error of <0.2%. Settings for the second step were a signal-to-noise ratio of 2 and a minimum valley depth of 2. The *m*/*z* range was set between 3,000 and 30,000 for low-mass and between 20,000 and 150,000 for high-mass proteins.

Proteins corresponding to the listed peaks (Table 1) were purified by liquid-phase isoelectric focusing (IEF) via the MicroRotofor cell (Bio-Rad Laboratories), as previously described (19). A 20-μl milk sample was diluted with an IEF buffer solution [7 mol/l urea, 2 mol/l thiourea, 4% CHAPS (wt/vol), and 0.24% Triton X-100], glycerol (5% vol/vol), and ampholytes (pH range 4–6, 1.6% vol/vol). Focusing was performed at room temperature, under a constant power of 1

Table 1. *SELDI peaks selected to be differentially expressed*

PND10		PND21	
Downregulated	Upregulated	Downregulated	Upregulated
5,843†		5,843†	
	6,681		
9,045		9,045	
9,680		9,680	
		10,518	
	12,006		
12,495*		12,495*	
	13,535		13,535
	14,331		14,331
	15,635		15,635
		17,900†	
18,634†		18,634†	
19,717			
			21,733*
	22,192*		22,192*
25,092		25,092	
	28,293*		28,293*
			42,978*
48,288*		48,288*	
		76,251†	
90,585*			
			145,668*
190,442*			

Only peaks expressed as mass-to-charge ratio with statistical difference expression ($P < 0.05$) between control (CTRL) and 50% food restricted (FR50) are indicated. *Peaks are impossible to purify for protein identification according to a previous publication (19). †Peaks were identified by purification and mass spectrometry and summarized in Table 2. SELDI, surface-enhanced laser desorption/ionization; PND, postnatal day.

watt. At the end of IEF, protein fractions from each compartment (200 μl) were harvested quickly to avoid the diffusion of separated proteins, and a two-dimensional (2-D) Clean-Up kit (GE Healthcare) was used to precipitate proteins. Finally, the pellet was suspended in 20 μl of deionized water; 5 μl were used to detect the peaks on ProteinChip NP20 array. Fractions containing the purified peak were separated on a 8% SDS-PAGE (Bio-Rad Laboratories), stained with Coomassie brilliant blue, as described by Neuhoff et al. (38).

Proteins were then identified by an in-gel digestion procedure, as previously described (6), using as MALDI-TOF-MS, a Voyager DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a 337.1-nm nitrogen laser and a delayed extraction facility (125 ms). All spectra were acquired in a positive ion reflector mode at the voltage of 20 kV, with grid-voltage of 61%. Protein identification by peptide mass fingerprinting was conducted by running the MASCOT web searcher (http://www.matrixscience.com/; Matrix Science) against the Swissprot 57-15 (515,203 sequences; 181,334,896 residues) with the following parameters: fixed modifications, carbamidomethyl; variable modifications, oxidation; peptide mass tolerance, ±50 parts/million; peptide charge state, 1+; maximum missed cleavages, 1; taxonomy, rattus.

A sample of 5 μg of antibody against the identified protein, described below, was incubated with 5 μl of milk in 500 μl of immunoprecipitation (IP) buffer (1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.1 mmol/l sodium vanadate, and 0.5% Nonidet P-40 in 10 mmol/l Tris·HCl) at 4°C, as previously described (42). Immunodepleted and undepleted plasmas were compared by SELDI analysis on ProteinChip H50 array; a loss of the peak of interest was visualized compared with untreated milk.

Validation of the milk proteins identified. Quantification of α-lactalbumin (dilution 1:5,000, sc-135344; Santa Cruz Biotechnology), protein-L-isoaspartate (dilution 1:1,000, sc-168915; Santa Cruz Biotechnology), and serotransferrin (dilution 1:70,000, sc-22597; Santa Cruz Biotechnology) was performed by Western blot using the specific antibodies used for verifying the protein identification of, respectively, the *m/z* 17,900, 18,634, and 76,281 peaks.

Proteins from milk (10 μg) were separated by SDS-PAGE (12% acrylamide) and transferred on 0.2 μm nitrocellulose (trans blot Turbo transfer Pack; Bio-Rad Laboratories). Equal total protein loads were confirmed by Ponceau red [0.1% Ponceau (Sigma-Aldrich), 5% acetic acid (vol/vol)] staining of the membranes. Membranes were then washed in TBS-0.1% Tween, saturated in 5% nonfat dry milk (wt/vol), and blotted with serotransferrin-specific antibodies (1/70,000 into 5% milk). Blots were then washed in TBS-0.1% Tween and then incubated with horseradish peroxidase-labeled secondary antibodies for 1 h (1/40,000 in blocking solution). Membranes were then washed five times (10 min/wash). The Chemidoc camera (Bio-Rad) was used for imaging and densitometry analysis after membranes were incubated with enhanced chimioluminescence Western blotting detection reagents (GE Healthcare).

Statistical analysis. All values were expressed as means ± SE. Clusters of all spectra obtained using SELDI-TOF-MS, GC-FID, and GC-MS-EI were subjected to univariate analysis, with a nonparametric test to calculate the *P* value of each cluster and a Mann-Whitney test to compare groups in pairs. Duplicates were averaged before any statistical analysis. A P value <0.05 was considered statistically significant.

RESULTS

Modification of milk composition by food restriction. The percentage of dry matter is higher in milk from FR50 vs. CTRL mothers at PND10 and PND21 (Fig. 1*A*). The same increase is found for the fat content at PND10 and PND21 and for the protein content at PND21. We also observed an increase of protein concentration between PND10 and PND21 in FR50 mothers. In contrast, a reduced lactose concentration is observed in FR50 milk at PND10. However, an increase in the concentration of lactose occurs between PND10 and PND21 in milk from FR50 mothers to reach similar levels in CTRL and FR50 milk at PND21.

Decreased monosaccharides in milk of food-restricted mothers. Five monosaccharide derivatives in the form of *O*-methyl glycoside trimethylsilylated [mannose, galactose, glucose, *N*-acetylgalactosamine (GalNAc), and *N*-acetylglucosamine (GlcNAc)] were quantified in rat milk samples by GC/MS-EI (Figs. 1*B* and 2*A*).

The two major monosaccharides detected in rat milk extracts were galactose and glucose, with decreased levels in FR50 only at PND10 (Fig. 1*B*). Despite low to undetectable levels of GlcNac compared with GalNac in FR50 at PND21, a significant decrease of GalNAc was found in FR50 at PND10 and PND21 while GlcNac was only reduced at PND21. These data show that the decrease in different monosaccharide concentration paralleled that of lactose detected in FR50 mothers at PND10 (Fig. 1*A*).

Modulation of FAMEs ratio in milk of food-restricted mothers. Thirteen fatty acids were quantified in rat milk by GC/MS-EI (Fig. 2*B*), seven linear saturated FAMEs (SFA) (C8:0, C10:0, C12:0, C14:0, C16:0, C18:0 and C20:0), two linear monounsaturated FAMEs (MUFA) (C16:1 and C18:1), two diastereoisomers for stearic acid C18:0 and in smaller quantities, and two linear polyunsaturated FAMEs (PUFA) (C18:2 and C20:4). No saturated odd-chain FAME was detected in these fractions.

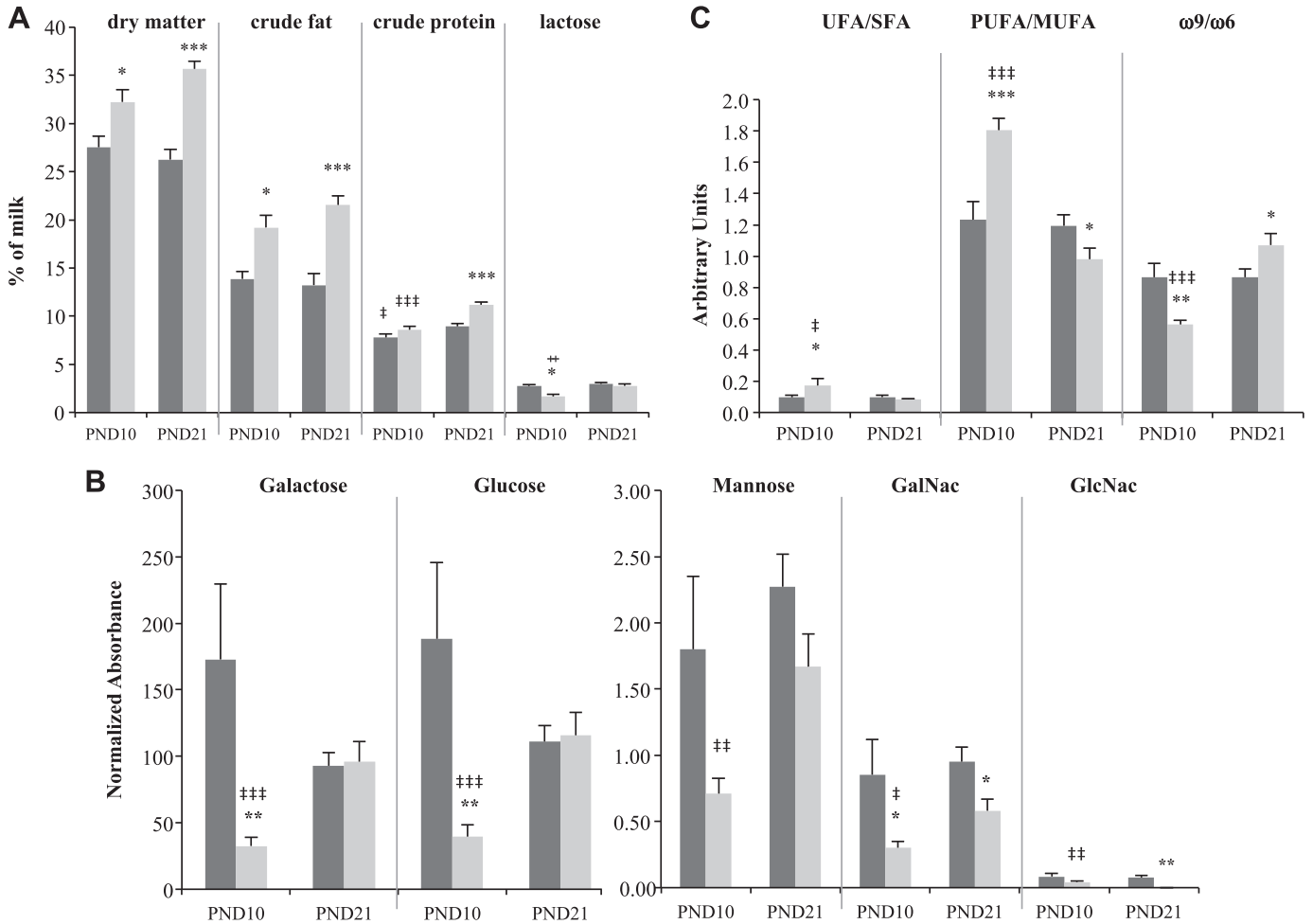


Fig. 1. Global (*A*), monosaccharides (*B*), and fatty acid methyl esters (FAMES) (*C*) composition of milk in 50% food-restricted (FR50, light gray bars) and control (CTRL, dark gray bars) rat mothers at *postnatal day* (PND) *10* and PND21. Values are means ± SE. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, CTRL vs. FR50 at the same stage; $‡P < 0.05$, $‡‡P < 0.01$, and $‡‡‡P < 0.001$, FR50 PND10 vs. FR50 PND21. *A*: $n = 5$ samples/group for each stage; *B* and *C*: CTRL PND10 $n = 8$, FR50 PND10 $n = 9$, CTRL PND21 $n = 11$, FR50 PND21 $n = 10$. GalNac, *N*-acetylgalactosamine; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Some variations in the proportions of FAMEs (affecting those with an aliphatic chain comprising between 16 and 18 carbon atoms) between each set of samples of rat milk extracts were found (Fig. 2*B*).

No significant difference is observed in fatty acid compositions of the rat milk between CTRL and FR50 at PND10 and PND21. However, the same overall profile is observed with a decrease in the concentration of the different FAMEs between PND10 and PND21 (Fig. 2*C*). Indeed, there is a significant increase in the UFA-to-SFA ratio in FR50 milk compared with CTRL milk at PND10 (Fig. 1*C*). This UFA enrichment results in a significant increase in the PUFA-to-MUFA ratio (Fig. 1*C*). Interestingly, FR50 breast milk also exhibits differences in the ω-9-to-ω-6 ratio with a significant decrease at PND10 and a significant increase at PND21 (Fig. 1*C*). Moreover, this ratio was significantly increased in FR50 between PND10 and PND21. However, ω-3 were below the limit of detection.

Identification of serotranferrin. We detected a total of 303 peaks for low-mass proteins (77 for Q10, 64 for H50, 87 for IMAC Cu^{2+}, and 75 for IMAC Ni^{2+}) and 130 peaks for high-mass proteins (41 for Q10, 28 for H50, 28 for IMAC Cu^{2+}, and 33 for IMAC Ni^{2+}) in the four groups of rats. Among these milk proteins, 23 SELDI peaks displayed significant modulation relative to the FR50 group, with 4 peaks being common between PND21 and PND10, 5 being specifically modulated between FR50 and CTRL at PND10, and 5 being specifically modulated between FR50 and CTRL at PND21 (Table 1). Among the peaks that could be purified, four of them have been successfully identified by mass spectrometry and validated by immunodepletion (Table 2). We only present the different steps corresponding to the *m*/*z* 76,251 peak. Purification of the *m*/*z* 76,251 peak by liquid-phase IEF and gel electrophoresis (Fig. 3*A*) combined with mass spectrometry successfully identified the peak as being serotransferrin (Fig. 3*B*). We verified the identification of the serotransferrin peak using a specific polyclonal antibody. Immunodepletion significantly reduced the peak, thereby confirming the identification of the *m*/*z* 76,251 peak as serotransferrin (Fig. 3*C*).

The protein, exhibiting the *m*/*z* 76,251 peak, was significantly downregulated in FR50 at PND21 (Fig. 4, *A* and *B*) with no difference observed at PND10 (Fig. 4*B*).

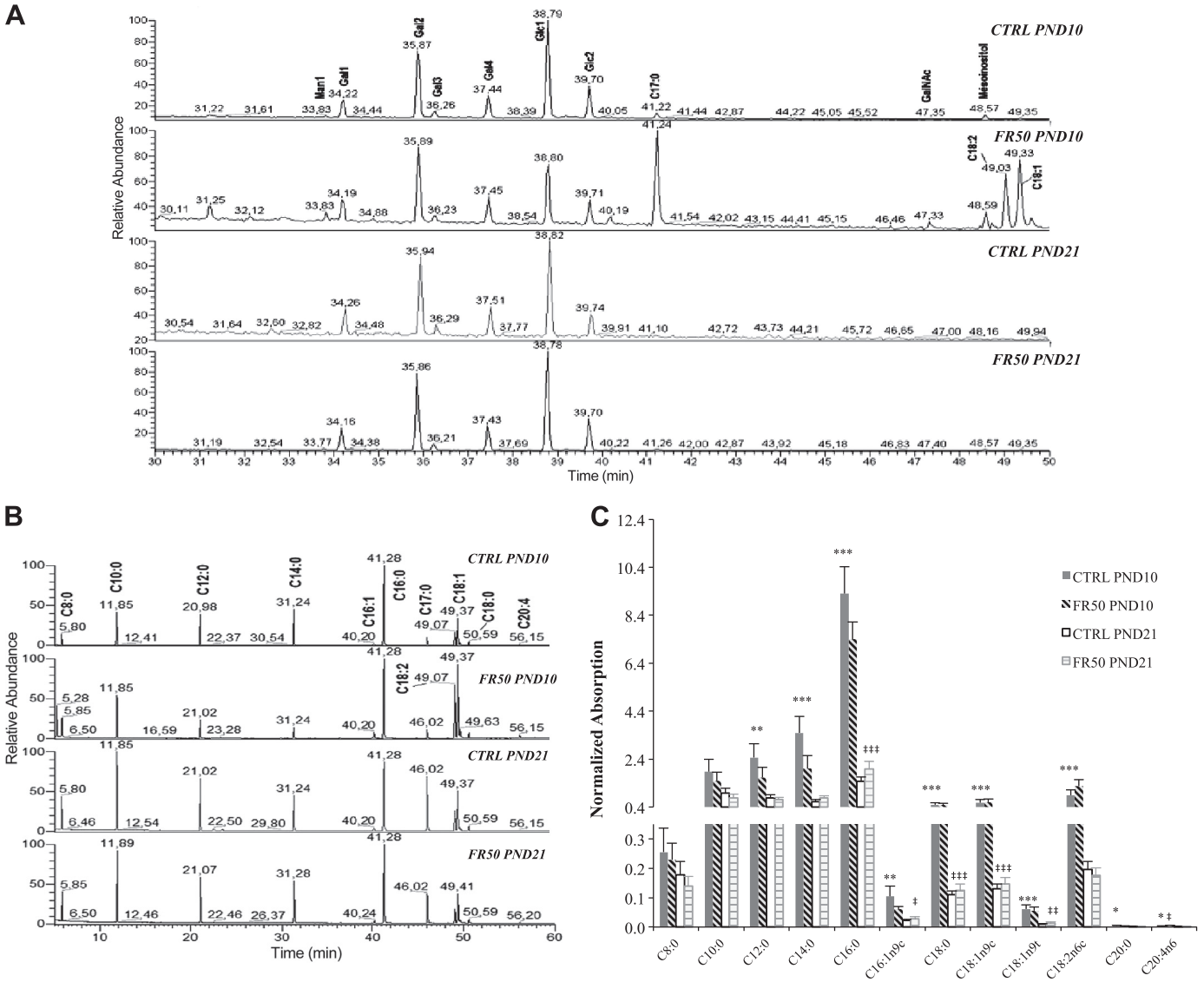


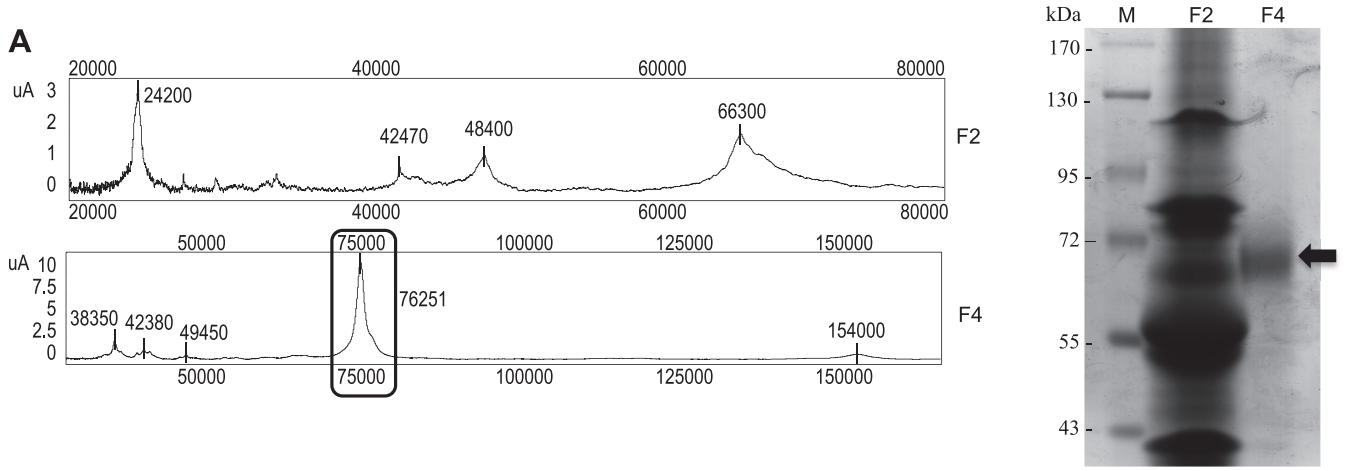
Fig. 2. Chromatograms of monosaccharides (*A*), fatty acids (*B*), and FAMEs (*C*) profiles obtained by gas chromatography (GC)/mass spectrometry (MS)-electron impact (EI) from milk in FR50 and CTRL rat mothers at PND10 and -21. CTRL PND10 $n = 8$, FR50 PND10 $n = 9$, CTRL PND21 $n = 11$, and FR50 PND21 $n = 10$. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, CTRL PND10 vs. CTRL PND21. ‡$P < 0.05$, ‡‡$P < 0.01$, and ‡‡‡$P < 0.001$, FR50 PND10 vs. FR50 PND21.

Quantification of serotransferrin in milk samples. The modulation of serotransferrin observed in SELDI-TOF-MS profiling was confirmed by Western blot (Fig. 4*C*). Indeed, FR50 rats exhibit a similar level as those of CTRL at PND10, whereas it is reduced at PND21. The increase in rate is found between the two stages of development, namely between CTRL at PND10 and PND21 ($P < 0.001$) and FR50 at PND10 and PND21 ($P = 0.007$). These data confirmed those obtained by SELDI-TOF profiling.

Table 2. *SELDI profiles of milk from FR50 and CTRL rat mothers at PND10 and -21*

Peaks (*m/z*)	Accession No.*	Protein Name	Molecular Mass, Da	PND10		PND21	
				CTRL	FR50	CTRL	FR50
5,843	P62986	Ubiquitin-60S ribosomal protein L40	14,728	0.39 ± 0.1	0.20 ± 0.14 ($P = 0.019$)	1.82 ± 1.17	0.67 ± 0.48 ($P < 0.01$)
17,900	P00714	α-Lactalbumin	15,004	ND	ND	1.54 ± 0.25	1.29 ± 0.14 ($P = 0.039$)
18,634	P22062	Protein-L-isoaspartate (D-aspartate *O*-methyltransferase)	17,850	0.53 ± 0.22	0.27 ± 0.16 ($P = 0.019$)	2.31 ± 0.56	1.46 ± 0.57 ($P < 0.01$)
76,251	P12346	Serotransferrin	76,395	ND	ND	0.36 ± 0.11	0.13 ± 0.01 ($P < 0.001$)

Values are means ± SD. *m/z*, Mass-to-charge ratio; ND, not determined. *Accession no. from UniProtKB (release 2014_07 of 09-Jul-14). Mann-Whitney test was used to compare CTRL and FR50 groups at PND10 and PND21.



B

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  1 MRFAVGALLA CAALGLCLAV PDKTVKWCAV SEHENTKCIS FRDHMKTVLP ADGPRLACVK KTSYQDCIKA ISGGEADAIT LDGGWVYDAG
 91 LTPNNLKPVA AEFYGSLEHP QTHYLAVAVV KKGTDFQLNQ LQGKKSCHTG LGRSAGWIIP IGLLFCNLPE PRKPLEKAVA SFFSGSCVPC
181 ADPVAFPQLC QLCPGCGCSP TQPFFGYVGA FKCLRDGGGD VAFVKHTTIF EVLPQKADRD QYELLCLDNT RKPVDQYEDC YLARIPSHAV
271 VARNGDGKED LIWEILKVAQ EHFGKGKSKD FQLFGSPLGK DLLFKDSAFG LLRVPPRMDY RLYLGHSYVT AIRNQREGVC PEGSIDSAPV
361 KWCALSHQER AKCDEWSVSS NGQIECESAE STEDCIDKIV NGEADAMSLD GGHAYIAGQC GLVPVMAENY DISSCTNPQS DVFPKGYYAV
415 AVVKASDSSI NWNNLKGKKS CHTGVDRTAG WNIPMGLLFS RINHCKFDEF FSQGCAPGYK KNSTLCDLCI GPAKCAPNNR EGYNGYTGAF
541 QCLVEKGDVA FVKHQTVLEN TNGKNTAAWA KDLKQEDFQL LCPDGTKKPV TEFATCHLAQ APNHVVVSRK EKAARVSTVL TAQKDLFWKG
631 DKDCTGNFCL FRSSTKDLLF RDDTKCLTKL PEGTTYEEYL GAEYLQAVGN IRKCSTSRLL EACTFHKS
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C

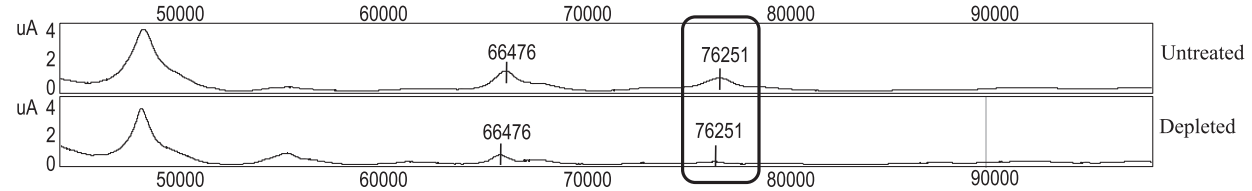

Fig. 3. *A*: purification of the mass-to-charge ratio (*m/z*) 76,251 peak by liquid-phase electrofocusing (pH 7–9) and gel electrophoresis. *B*: peptides identified in serotransferrin sequence. *C*: immunoprecipitation of the *m/z* 76,251 peak with serotransferrin antibody. M, molecular mass marker; F2, fraction 2; F4, fraction 4.

DISCUSSION

The aim of this study was to determine if the composition of the milk was impacted by our model of maternal perinatal undernutrition. Taken together, these results provide new information in milk composition from young undernourished rat mothers (60 days old). Although milk production was not directly measured in the present study, several arguments suggest that maternal perinatal undernutrition also decreases milk quantity: *1*) milk from undernourished mothers is more concentrated and thus less hydrated, reflecting a reduction of milk volume; *2*) cross-fostering of control pups by undernourished mothers also led to reduced kinetic of growth, whereas FR50 pups nursed by control mothers exhibited a weight gain similar to control pups, suggesting that maternal nutrition does not modify feeding behavior in rat neonates (53); and *3*) lactose, the main sugar in most mammalian species, is reduced in the milk of undernourished mothers. It is known to be the primary osmotic agent that attracts water in the milky secretion, and is thus considered as the major determinant of milk volume (36).

Indeed, the biosynthesis of this disaccharide may be limited in part by the amount of glucose that is absolutely essential as energy fuel and as substrate for synthesis of milk lactose both in humans and in animals (35). Thereby, the mammary gland, which does not synthesize glucose, is a very large consumer of glucose during lactation, especially at the onset of lactation. It can capture up to 30% of blood glucose in rats (11) and 35% in humans (50). Thus, a glucose deficiency in these animals could explain the decrease in the production of lactose in FR50 mothers since it has been shown that the synthesis of lactose is partly dependent on the availability of glucose (31, 34). However, the concentration of blood glucose is similar in control and FR50 mothers (data not shown). A decrease of the number of glucose transporters in undernourished mothers may reduce nevertheless glucose flow from the blood to the mammary gland. Because the GLUT family plays an important role to deliver glucose to the site of lactose synthesis (56, 47), it could not be ruled out that a defect of glucose transport in mammary epithelial cells (MEC) of FR50 mothers may be partly responsible for the decreased level of lactose. In addition, undernutrition may impact the production of lactogenic hormones, known to initiate the development of mammary tissue and lactogenesis (39), since it has been reported that lactogenic hormones are able to modulate GLUT expression in vitro in CIT3 mouse MEC (25). The decrease in lactose may also result from the reduction in blood flow to the mammary gland, which

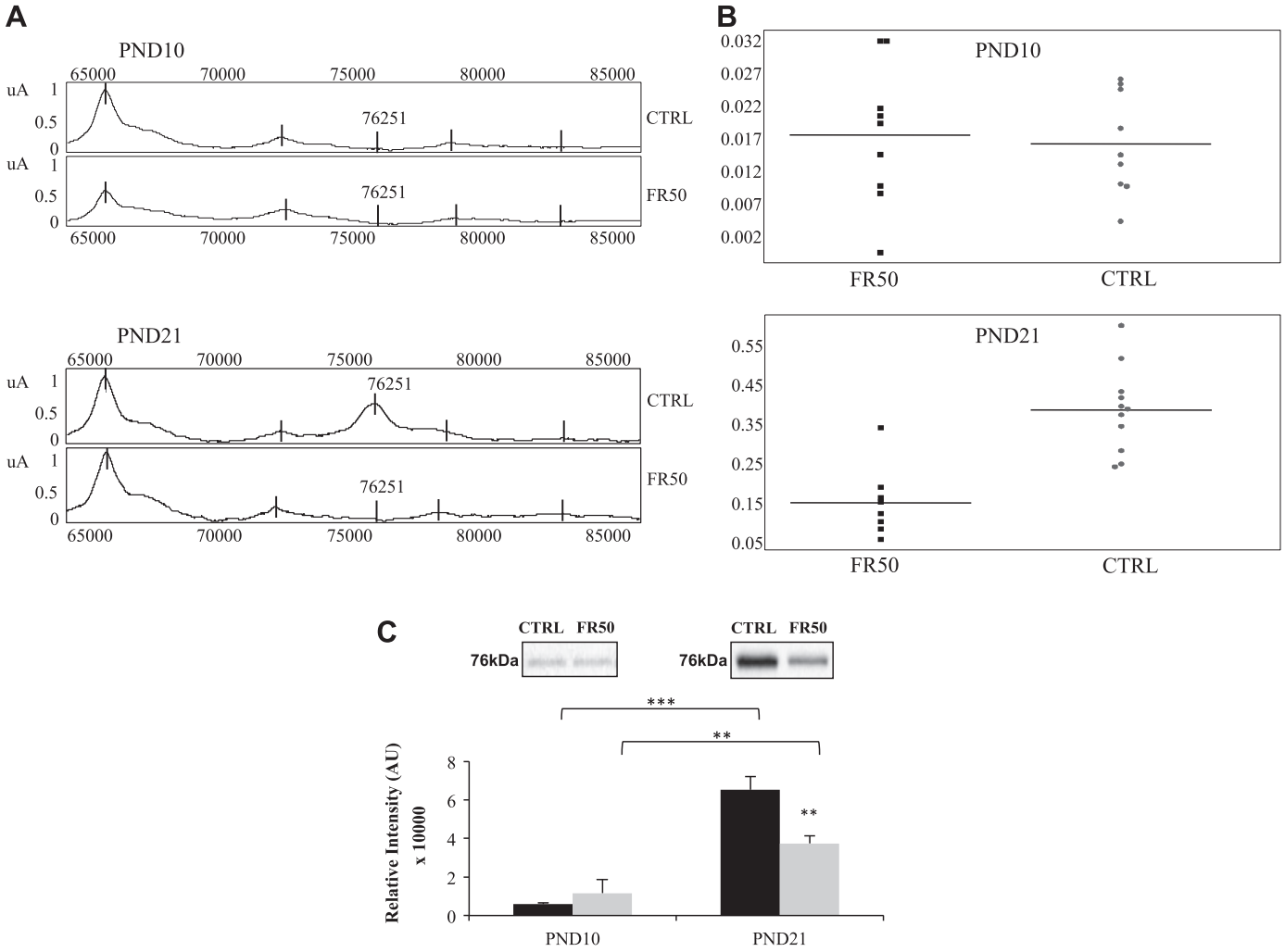

Fig. 4. Profiling (*A*) and quantification (*B*) of the *m/z* 76,251 peak in FR50 and CTRL rat mothers at PND10 and PND21. Representative Western blot of transferrin and quantification (*C*) of transferrin in CTRL (dark gray bars) and FR50 (light gray bars) at PND10 and PND21. Values are means ± SE. $^{**}P < 0.01$ and $^{***}P < 0.001$, CTRL PND10 $n = 8$, FR50 PND10 $n = 9$, CTRL PND21 $n = 11$, and FR50 PND21 $n = 10$.

may limit the availability of glucose to the MEC. Indeed, it has been shown that blood flow is increased in the mammary gland during lactation (15, 54). In contrast, it was shown that both the mammary gland lactation performance and blood flow are decreased in perinatally undernourished mothers, suggesting that the availability of energy substrates can be affected in mammary gland during the undernutrition process (45).

Finally, we could speculate that maternal perinatal undernutrition decreases lactose synthase activity, which is indeed involved in the biosynthesis of lactose. This enzymatic activity actually results from the combination of α-lactalbumin (10) and galactosyltransferase (9). A positive relationship between the concentration of milk lactose and α-lactalbumin has been described, suggesting a limiting role of this protein in the synthesis of lactose (36, 40). Moreover, a reduced amount of α-lactalbumin is observed in food-restricted animals (8, 21, 24).

Our data on milk fatty acid profiling suggest an enrichment in UFA in PND10 FR50, resulting of an increase in PUFA rather than in MUFA. These variations could find their origins in a malfunction of enzymes responsible for the synthesis of fatty acids, such as the desaturases or elongases, which can lead to metabolic disorders (14, 23).

For protein profiling, serotransferrin, also known as transferrin, has first been described for its role in the transport, the absorption, and the use of iron. This protein is also involved in other essential functions such as growth and differentiation of many cell types and is associated with a bacteriostatic effect (4). Although transferrin was initially reported to be produced by the liver (55), it exhibits a wide tissue distribution (33) and is also present in the mammary gland where it could originate from liver or be synthesized in the mammary gland before being secreted into the milk (41, 46). The reduction of transferrin in milk can be caused by food restriction. Indeed, it has been shown that a decrease in vitamin A reduced iron transporters in the mammary gland as well as the expression of the transferrin receptor, resulting in lower concentrations of iron in milk (28), as protein restriction (24). These deficiencies are known to be responsible for severe diseases such as hypertension or anemia (20, 29) but also hippocampal structural alterations leading to psychomotor troubles (12, 44).

Taken together, our data suggest that maternal undernutrition impacts the composition of milk. Interestingly, qualitative modifications induced by the maternal perinatal undernutrition seem to occur in limited milk components. Thus, the combi-

nation of reduced lactose, and transferrin milk, in addition to low levels of leptin highlighted in our previous work (53) as well as changes in lipid profiles may contribute to the increased propensity for metabolic disorders that offspring of undernourished mothers exhibit in adulthood.

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DISCLOSURES

Authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

Author contributions: J.-S.W., D.V., and F.P. conception and design of research; J.-S.W., A.D., M.B., O.B., S.G., and F.D. performed experiments; J.-S.W., A.D., M.B., O.B., S.G., F.D., C.L., J.L., B.F., C.B., C.C.M., D.V., and F.P. analyzed data; J.-S.W., A.D., M.B., O.B., S.G., F.D., C.L., J.L., B.F., C.B., C.C.M., D.V., and F.P. interpreted results of experiments; J.-S.W. prepared figures; J.-S.W., D.V., and F.P. drafted manuscript; J.-S.W., A.D., J.L., B.F., C.B., C.C.M., D.V., and F.P. edited and revised manuscript; J.-S.W., A.D., M.B., O.B., S.G., F.D., C.L., J.L., B.F., C.B., C.C.M., D.V., and F.P. approved final version of manuscript.

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