SUPPLEMENTAL DATA:

Sphingomyelins and ceramides with very-long-chain PUFA are excluded from low-density, raft-like domains in differentiating spermatogenic cells

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Running title: Lateral membrane distribution of SM and Cer in germ cells

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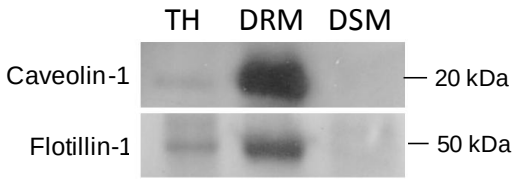
Supplementary Figure S1

A

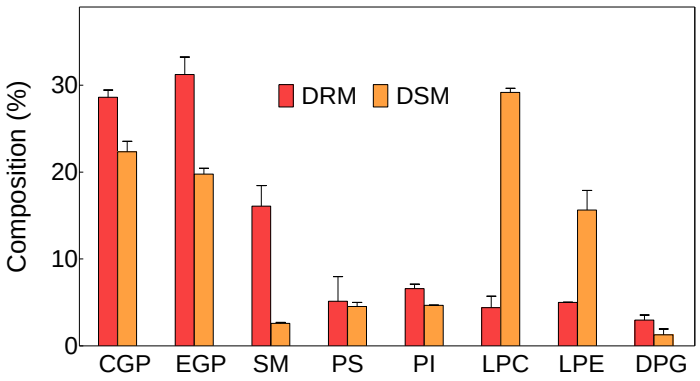
Activity of 5′ nucleotidase in total homogenate, DRM and DSM fractions obtained from total germ cells.

	5´nucleotidase activity	
	(nmol Pi / mg protein protein / min)	*(5´nucleotidase activity / TH* 5´nucleotidase activity)
Total homogenate	3.2 ± 0.3	1.0
DRM	16.1 ± 1.0	5.0
DSM	<2	-

B



C



D

Cholesterol (Chol) levels and Chol / Phospholipid (PL) ratio in total homogenate , DRM and DSM fractions.

	Cholesterol *(pmol / µg protein)*	Chol / PL ratio
Total homogenate	102 ± 14	0.34 ± 0.03
DRM	317.6 ± 40.2	0.42 ± 0.04
DSM	24.8 ± 2.5	0.18 ± 0.03

Figure S1: Characterization of DRMs isolated from spermatogenic cells.
DRMs were isolated from total spermatogenic cells according to the classical method of Brown and Rose (1). The cells were lysed in ice-cold TNE buffer (50 mM Tris-HCl, 70 mM NaCl and 5 mM EDTA; pH 7.4) containing 1% Triton X-100 in the presence of protease inhibitors (inhibitor cocktail, Sigma P-8340) for 20 min on ice. The lysates were

mixed with an equal volume of 80% sucrose in TNE to obtain a final concentration of 40% sucrose. Three ml of cell homogenates were placed at the bottom of ultracentrifuge tubes and overlaid successively with 6 ml of 36% sucrose and 3 ml of 5% sucrose. The gradients were centrifuged for 20 h at 250,000 g at 4°C in the SW41 Beckman rotor of a Beckman Coulter Optima L-90K Ultracentrifuge. The band corresponding to the detergent-resistant membrane fraction (DRM) was collected from the 5-36% sucrose interphase. The fraction containing the detergent-soluble membranes (DSM), remaining under the DRM fraction (≈ 6 ml of 36% and 3 ml 40% sucrose), was also collected for comparative purposes. The DRM fraction was suspended in TNE buffer without detergent and centrifuged for 45 min at 200,000 g at 4 °C in a 90Ti Beckman rotor to remove the excess of sucrose.

Protein and lipid contents were examined in DRM and DSM fractions as described in the main document for the cell membrane fractions.

A. Activity of 5′nucleotidase in the total homogenates from rat spermatogenic cells before membrane fractionation and in the DRM and DSM fractions obtained therefrom. The enzyme activity was measured according to Widnell and Unkeless (2) and it is expressed as mean values ± SD from three samples.

B. Representative immunoblot showing the distribution of caveolin-1 and flotillin-1, two raft/caveolae markers, between DRM and DSM fractions in comparison with the amount present in the starting total homogenates (TH). Equivalent amounts of total protein were used for the electrophoretic runs.

C. Phospholipid composition of the DRM and DSM fractions. After fraction isolation from total rat spermatogenic cells, the phospholipids were extracted, separated into classes and analyzed as described in the main document. The lipid abbreviations correspond to: CGP, choline glycerophospholipids, DPG, diphosphatidylglycerol; EGP, ethanolamine glycerophospholipid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

D. Concentration of cholesterol (Chol) and Chol / total phospholipid (PL) ratio in DRM and DSM in comparison with the starting total homogenate (TH) from total spermatogenic cells.

Comments

Although detergent solubilization has raised criticisms and concerns about discriminating between what is present on a cell membrane and what can be prepared from it in the laboratory (3), it is by far the most widely used tool for protein and lipid analysis of detergent-resistant membrane (DRM) fractions as representative of raft-like domains from plasma membranes. Previous to this study, we had obtained DRM from total spermatogenic cells using Triton X-100 (TX-100). The lipids including SM present in these DRMs showed similar trends as those of the L fraction obtained by the detergent-free procedure described in the main document. Thus, when studied by MALDI-TOF/TOF MS, such SM concentrated species with saturated fatty acids, while most of the rest of the SM species, together, appeared in the detergent-soluble fraction (Supplemental Figure S3). Caveolin-1 and flotillin-1 concentration, 5'nucleotidase activity, cholesterol/phospholipid, and cholesterol/SM ratios satisfied the condition of being all higher in DRMs than in the detergent-soluble fraction Except the low-density rafts, the bulk of the cell membranes were collected together in the detergent-soluble membrane fraction.

The amounts of total lipid phosphorus, total cholesterol and total SM obtained when adding up the detergent resistant + soluble fractions (even including the relatively small amounts present in the residual pellets), were much smaller than in the original cells, suggesting that part of the phospholipids were degraded during DRM isolation. In addition, the phospholipid composition (%) was rather unusual in the DRM fraction (as it showed an unexpectedly high EGP/CGP ratio), and was intensely altered in the detergent-soluble fraction, as it had substantial percentages of lysophospholipids and low percentages of SM. Taken together, these results suggested that different phospholipases (including a sphingomyelinase) became freely active, either during solubilization with TX-100 or during the long hours of centrifugation in the presence of the detergent required to isolate the DRM. In addition, a second type of detergent (the zwitterionic lysoPC and lysoPE) artefactually appeared in the samples to be analyzed.

Another difficulty with TX-100 we experienced is that, being it a non-polar detergent with a low critical micellar concentration, it is not easy to remove from the *lipids* it solubilizes from membrane samples. Its presence in lipid extracts affects polar lipid recovery upon solvent extraction and partition, and interferes with the TLC separation of non-polar lipids (like the ceramides of the present study) for further analysis. Thus, a detergent-free procedure is advantageous if one has the purpose of separating raft-like from non-raft like membrane fractions for lipid analysis. However, DRMs are valuable if the purpose is to study membrane raft domains and its components (e. g., raft-resident lipids and proteins), as it has been extensively demonstrated in the literature.

References

1. Brown, D. A. and J. K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68: 533-544.
2. Widnell, C. C. and J. C. Unkeless. 1968. Partial purification of a lipoprotein with 5'-nucleotidase activity from membranes of rat liver cells. *Proc. Natl. Acad. Sci. U. S. A* 61: 1050-1057.
3. Aureli, M., S. Grassi, S. Sonnino, and A. Prinetti. 2016. Isolation and Analysis of Detergent-Resistant Membrane Fractions. *Methods Mol. Biol.* 1376: 107-131.

Supplementary Figure S2

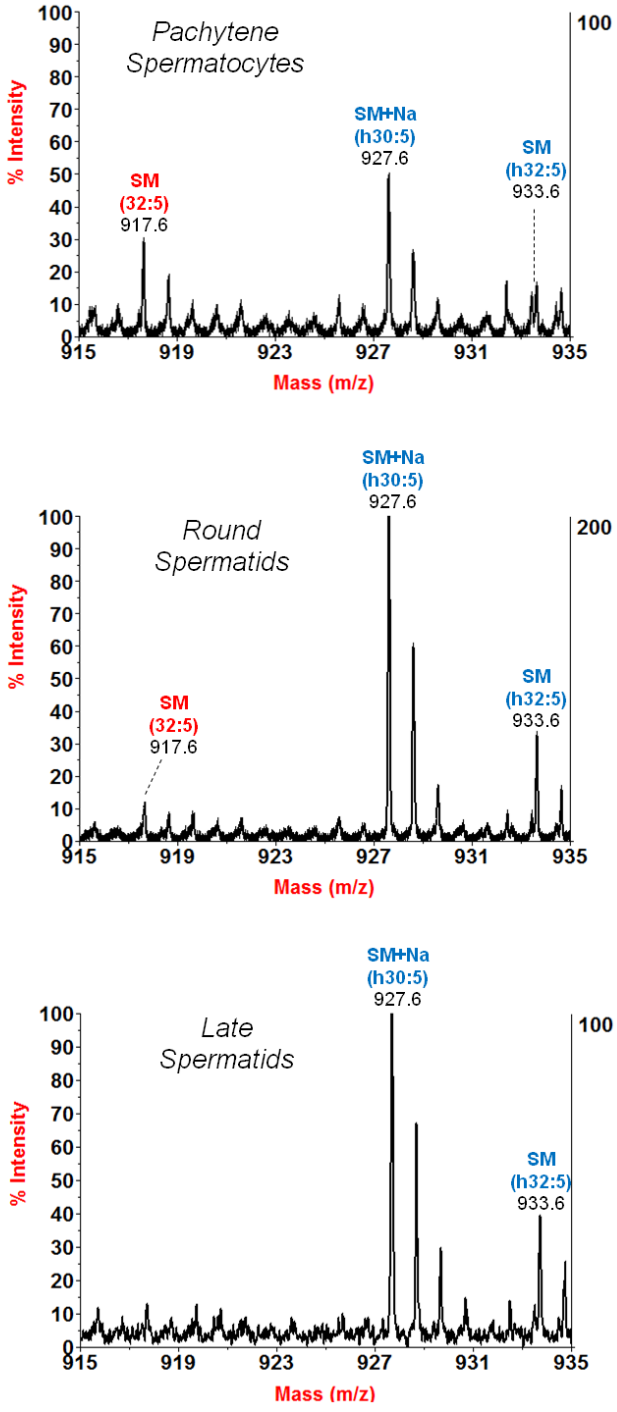


Figure S2: MALDI-TOF mass spectra magnification of the sphingomyelins of the H fraction from spermatogenic cells. A higher amplification of the *m/z* 915-935 region from the mass spectra shown in Figure 6 of the manuscript allowed the detection of SM species containing n-V and h-V with 32 carbon atoms. The peak intensities at *m/z 917.7* and *933.7* correspond to (d18:1/32:5n-6) SM and (d18:1/h32:5n-6) SM, respectively.

Supplementary Figure S3

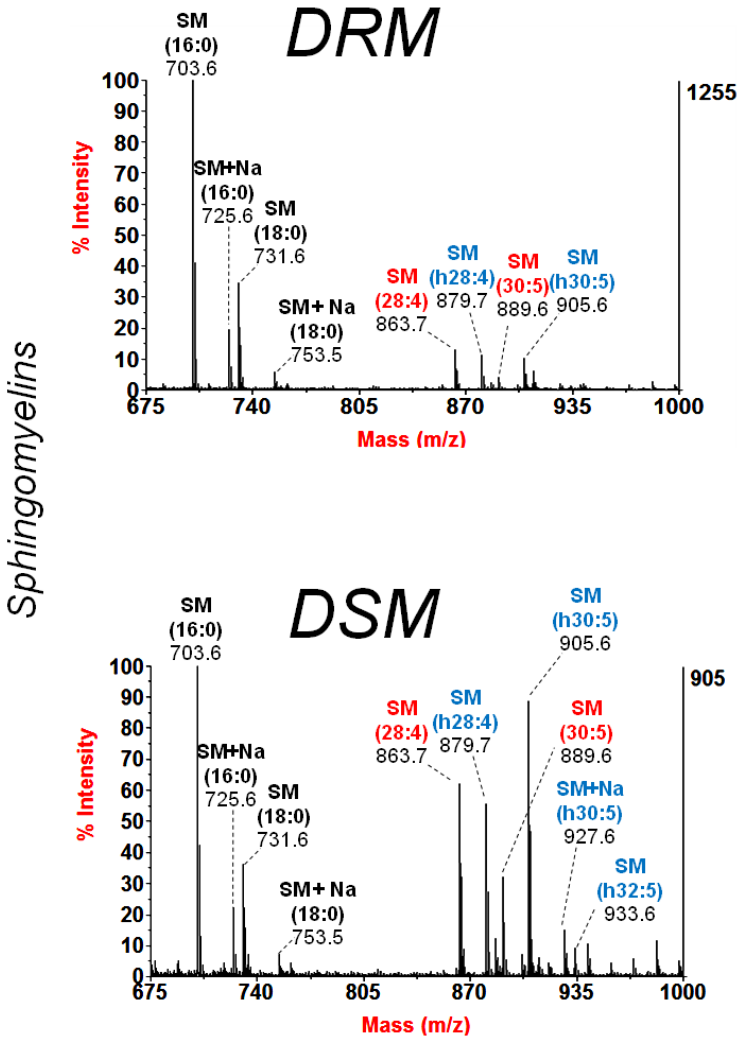


Figure S3: MALDI-TOF mass spectrometric analysis of sphingomyelin (SM) species from detergent-resistant membranes (DRM) and the fraction containing detergent-soluble membranes (DSM) from total spermatogenic cells. DRM and DSM were prepared as explained in the legend of Figure S1. Molecular species of SM were identified by the product ion scan *m/z* $^{+}$*184*. The DRM fraction was rich in the same saturated molecular species of SM that are shown in the manuscript to be present in the L fraction when isolated by a detergent-free procedure. The DSM fraction contained SM with nonhydroxy and 2-hydroxy (h) very long-chain PUFA because, other than the DRM, it contained all of the cell membranes that were solubilized by Triton X-100. Signals at *m/z 863.6 and 889.6* are (d18:1/28:4n-6 + H^+)SM and (d18:1/30:5n-6 + H^+)SM, whereas peaks at *m/z 879.6 and 905.6* are (d18:1/h28:4n-6 + H^+)SM and (d18:1/h30:5n-6 + H^+)SM, respectively. Low intensities from these molecular species were detected in the DRM. Sodium (Na) adducts were observed, such as (d18:1/16:0 + Na^+)SM and (d18:1/h30:5n-6 + Na^+)SM, *m/z 725.6* and *927.6*, respectively. The presence of the detergent in the lipid extracts of the DSM fraction interfered with the TLC isolation of the ceramides and compromised their further analysis.