Supporting Information

Comprehensive and quantitative analysis of polyphosphoinositide species by shotgun lipidomics revealed their alterations in *db/db* mouse brain

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Preparation of Lipid Extracts

Mouse liver, cortex, or spinal cord tissue (5 mg) was homogenized in diluted PBS (0.1x). The protein content of individual homogenate was determined with a BCA assay kit (Pierce, Rockford, IL). Internal standards were added based on the protein content of individual sample. Lipid extraction was performed with three different extraction methods:

(1) Two-step extraction method with addition of IS in the second step

First step: For neutral extraction, the tissue homogenate was extracted with 1 ml chloroform/methanol (1:2, v/v) and vortexed for 30 s for 3-4 times over 10 min at room temperature. After centrifugation (13,000 g, 2 min at 4 °C), the supernatant was carefully removed.

Second step: For acidic extraction, the remaining pellet was resuspended in 1,365 µl of methyl-tert-butyl ether (MTBE)/methanol/2 N HCl (200:60:13, v/v/v) and the internal standards of PPI were added followed by 30 s vortex for every 5 min over 15 min at room temperature. A solution of 0.1 N HCl (250 µl) was added followed by 5 min vortex. The upper organic phase was collected into a new tube after centrifugation (6,500 g, 2 min at 4 °C) for derivatization.

(2) Modified MTBE extraction method under acidic conditions with addition of IS in the first step

First step: Chloroform/methanol (1 ml, 1:2, v/v) and the internal standards of PPI were sequentially added to the homogenate (5 mg) followed by vortex for 30 s for 3-4 times over 10 min at room temperature. After centrifugation (13,000 g, 2 min at 4 °C), the supernatant was carefully removed.

Second step: The remaining pellet was resuspended in 1,365 µl MTBE/methanol/2 N HCl (200:60:13, v/v/v) followed by 30 s vortex for every 5 min over 15 min at room temperature. A solution of 0.1 N HCl (250 µl) was added followed by 5 min vortex. The upper organic phase was collected into a new tube after centrifugation (6,500 g, 2 min at 4 °C) for derivatization.

(3) Modified Folch extraction method under acidic condition with addition of IS in the first step

First step: For neutral extraction, chloroform/methanol (1 ml, 1:2, v/v) and the internal standards of PPI were sequentially added to the homogenate (5 mg) followed by vortex for 30 s for 3-4 times over 10 min at room temperature. After centrifugation (13,000 g, 2 min at 4 °C), the supernatant was carefully removed.

Second step: for acidic extraction, the remaining pellet was resuspended in 750 µl chloroform/methanol/37% HCl (40:80:1, v/v/v) and the internal standards of PPI were added followed by 30 s vortex for every 5 min over 15 min at room temperature. 250 µl cold chloroform and 450 µl cold 0.1M HCl was added followed by 5 min vortex. The lower organic phase was collected into a new tube after centrifugation (6,500 g, 2 min at 4 °C) for derivatization.

Mass Spectrometry of Lipids

A Thermo triple-quadrupole mass spectrometer (Thermo Scientific TSQ Quantiva, San Jose, California) equipped with an automated nanospray device (TriVersa NanoMate, Advion Bioscience Ltd, Ithaca, New York) using Xcalibur software as previously described[1]. Briefly, ionization voltage of 1.15 kV and spray gas pressure of 0.55 psi on the NanoMate apparatus were employed for the MS analyses. The nanospray device was controlled by Chipsoft 8.3.1 software. For individual mass spectrum, a 1-min period of signal averaging in the profile mode was typically employed. For tandem MS experiments, the collision gas (argon) pressure was set at

1.0 mTorr and the collision energy was varied depending on the class of PPI analyzed, and a 2- to 5-min period of signal averaging in the profile mode was used. All the full MS scans and tandem MS scans were automatically acquired by a customized sequence subroutine operated under Xcalibur software.

1. Han, X.; Yang, K.; Gross, R. W., *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2115-2124.

Table S1. The precursor-ion scans and method information for analysis of individual polyphosphoinositide classes

Class	PIS	CE	LOQ	LOD	DR
PI(3)P	389.1/403.1/417.1/431.1	38	0.5	0.15	0.5-500
PI(4)P	389.1/403.1/417.1/431.1	38	0.5	0.15	0.5-500
PI(5)P	389.1/403.1/417.1431.1	38	0.5	0.15	0.5-500
$PI(3,4)P_2$	497.1/511.1/525.1/539.1	40	0.5	0.15	0.5-500
$PI(3,5)P_2$	497.1/511.1/525.1/539.1	40	0.5	0.15	0.5-500
$PI(4,5)P_2$	497.1/511.1/525.1/539.1	40	0.5	0.15	0.5-500
$PI(3,4,5)P_3$	605.1	42	0.1	0.05	0.1-100

CE: Collision Energy (eV); LOQ: limit of quantitation (fmol/μL); LOD: limit of detection (fmol/μL); DR: dynamic range (fmol/μL) which was assessed as demonstrated in Figures 2 and S9.

Table S2. PIP, PIP_2 and PIP_3 species and their levels in mouse liver[a]

	PIP		PIP2		PIP_3	
Species	**WT**	***db/db***	**WT**	***db/db***	**WT**	***db/db***
16:0-18:2			8.7±1.5 (28/0/71)[c]	2.6 ± 0.5 (0/0/100)		
16:0-20:4			17.8±2.8 (43/11/45)	4.2 ± 0.8 (21/7/72)		
16:0:20:3			10.7 ± 1.8 (27/1/73)	2.7 ± 0.4 (0/0/100)		
18:0-18:2			13.3 ± 2.5 (46/2/52)	3.2 ± 0.4 (1/0/99)		
18:1-20:4			8.9 ± 1.36 (19/0/81)	4.1 ± 0.4 (0/0/100)		
18:0-20:4	31.1 ± 6.7 (11/89)[b]	10.1 ± 2.9 (3/97)	146.1 ± 26.6 (94/0/6)	55.6 ± 7.7 (61/3/35)	0.4 ± 0.0	0.1 ± 0.0
18:0-20:3			41.1 ± 7.8 (79/7/14)	18.3 ± 0.9 (60/8/32)	0.4 ± 0.0	0.1 ± 0.0
Total	31.1 ± 6.7 (11/89)	10.1 ± 2.9 (3/97)	246.6 ± 43.8 (48/3/49)	90.8 ± 10.2 (21/3/77)	0.8 ± 0.0	0.3 ± 0.0

a. Lipid levels were determined in comparison to the selected internal standards by using tandem mass spectrometry after methylation as described in the method section and expressed as mean ± SEM in pmol/mg protein from five separate animals.

b. The numbers in the parentheses represent the composition of the isomers of PIP in the order of PI(3)P/(PI(4)P+PI(5)P), which were simulated based on their methylation patterns.

c. The numbers in the parentheses represent the composition of the isomers of PIP_2 in the order of $PI(4,5)P_2/PI(3,4)P_2/PI(3,5)P_2$, which were simulated based on their methylation patterns.

Figure S1 Determination of methylation patterns of PIP_2 classes. Methylation of PIP_2 (1 pmol/μL) was conducted in the presence of trimethylsilyl-diazomethane as described under the method section. Mass spectral analysis of the methylated PIP_2 class as indicated was performed in the presence of a small amount of LiCl in the positive ion mode on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific) as described under the method section.

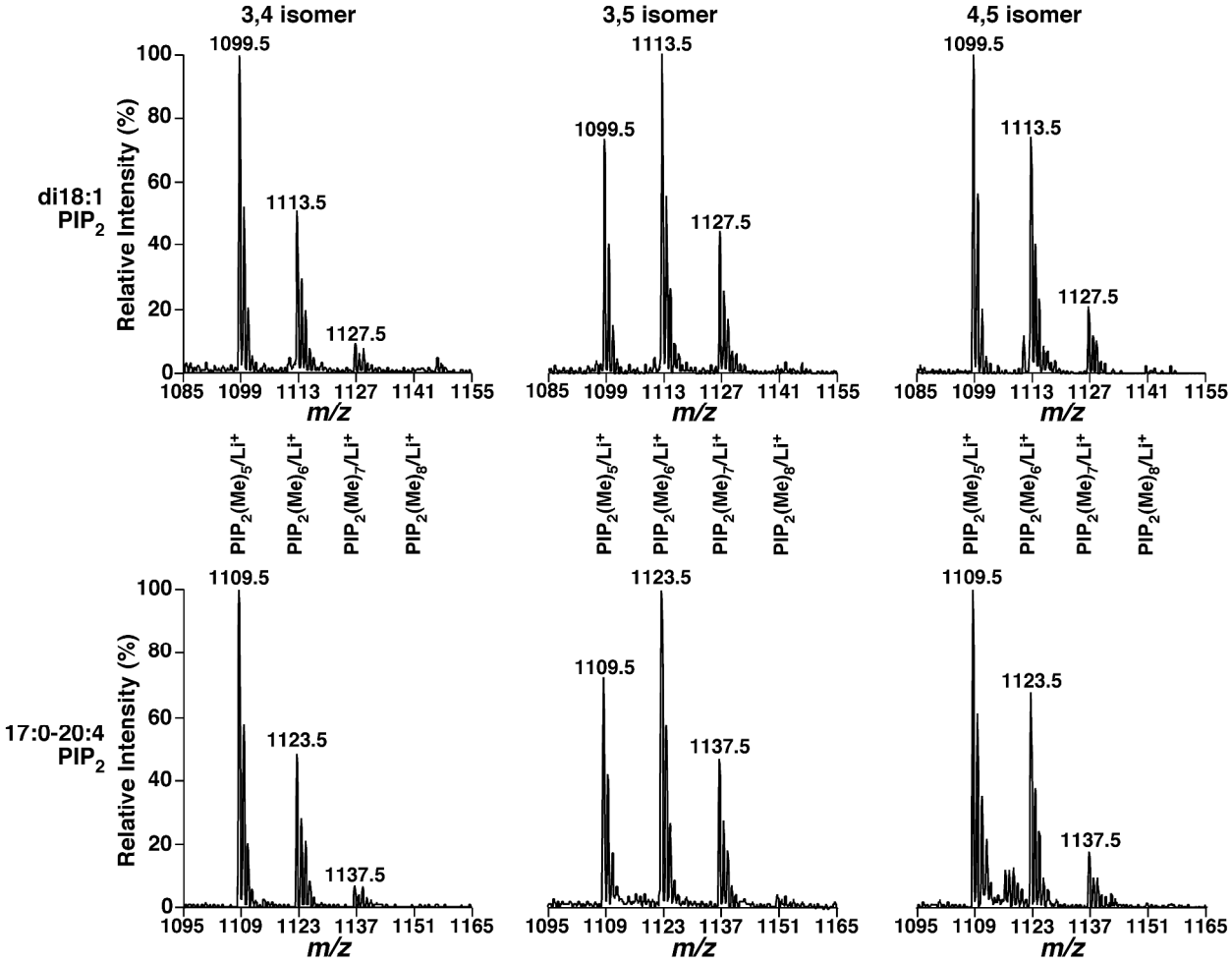

Figure S2 Determination of methylation patterns of PIP classes and the ratios of ion intensities between different PIP classes. Methylation of PIP equimolar mixtures (0.3 pmol/μL each) was conducted in the presence of trimethylsilyl-diazomethane as described under the method section. Mass spectral analysis of the methylated PIP mixtures as indicated was performed in the presence of a small amount of LiCl in the positive ion mode on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific) as described under the method section. (n)/(m) in panels A to F indicates the phosphate positions of di18:1 and 17:0-20:4 PIP species, respectively, which were used to make up the equimolar mixtures for acquiring the mass spectra. xMe in the spectra indicates the ion of PIP species containing "x" methyl groups. Normalized ratios of ion intensities of different PIP classes relative to that of $PI(3)P(Me)_3$ were determined based on the peak intensities displayed in the mass spectra and tabulated in Table 1.

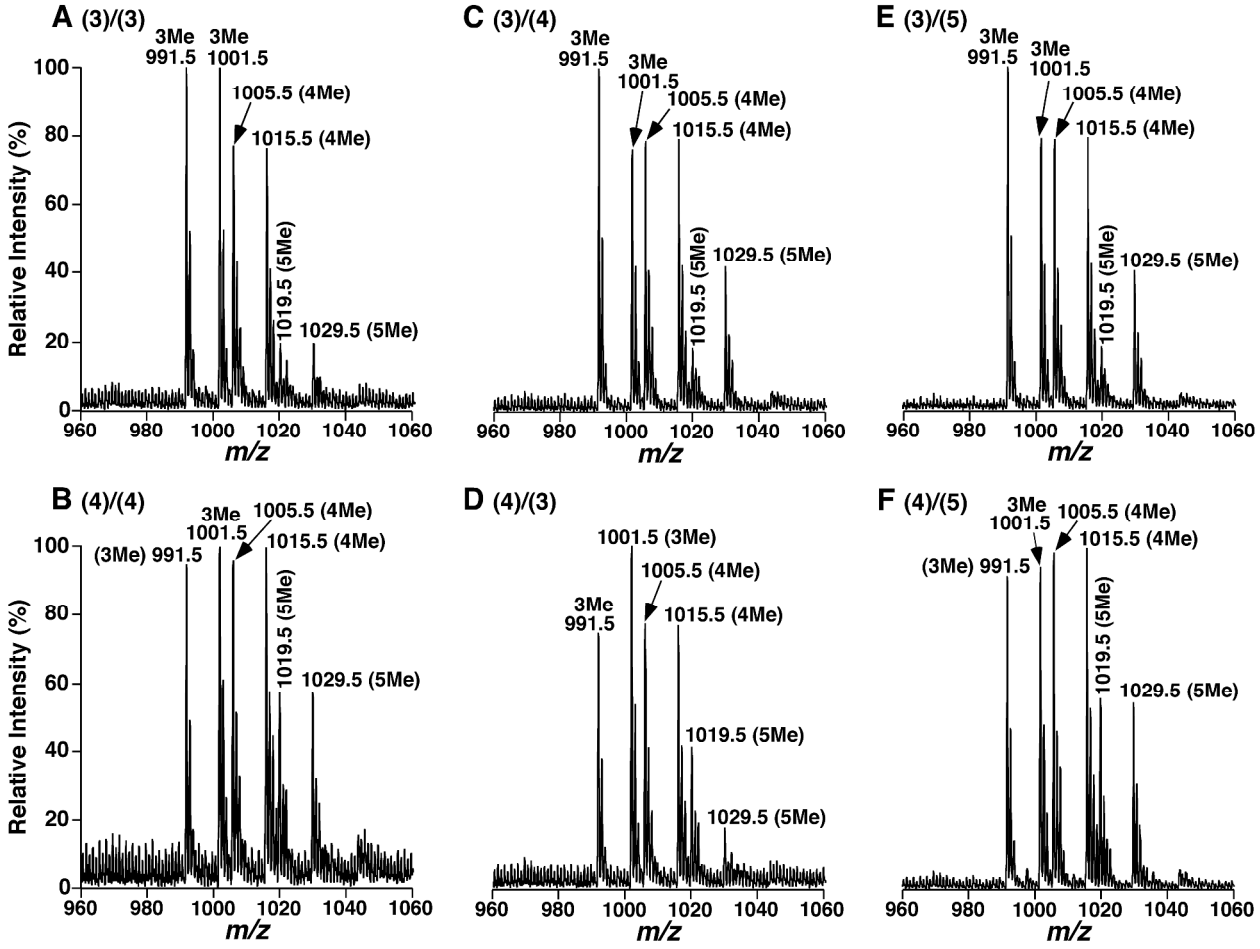

Figure S3 Comparison of mass spectral analyses with protonated, ammoniated and lithiated PIP_2 species. ESI mass spectra of 17:0-20:4 PI(3,4)P_2 (1 pmol/μL) were acquired in the presence of a small amount of LiCl (2000 time diluted of its saturated solution, panel A), formic acid (0.1%, panel B), ammonium acetate (5 mM, panel C), absence of any modifier (panel D) in the positive ion mode on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific).

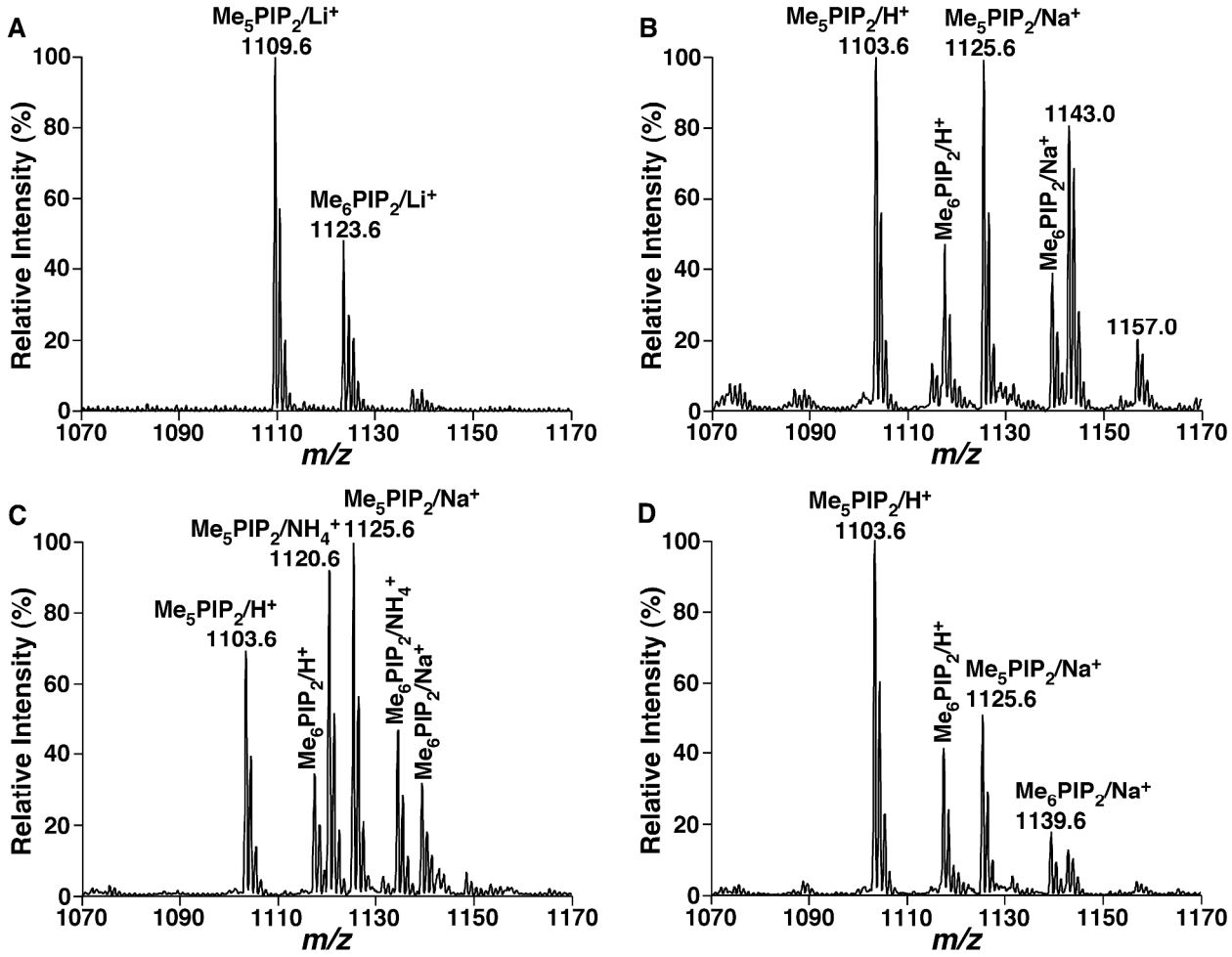

Figure S4 Characterization of methylated PIP_2 species in the positive-ion mode. Standard species of different PIP_2 classes were methylated as described under the section of "Materials and Methods". Lithium adducts of methylated PIP_2 species from different PIP_2 classes were selected and fragmented after CID at CE of 40 eV and collision gas pressure of 1 mTorr on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific). Panels A to D displayed fragmentation patterns resulted from methylated PIP_2 molecular ions containing 5, 6, 7, and 8 methyl groups, respectively. We found these fragmentation patterns were independent of the positions of phosphate isomers.

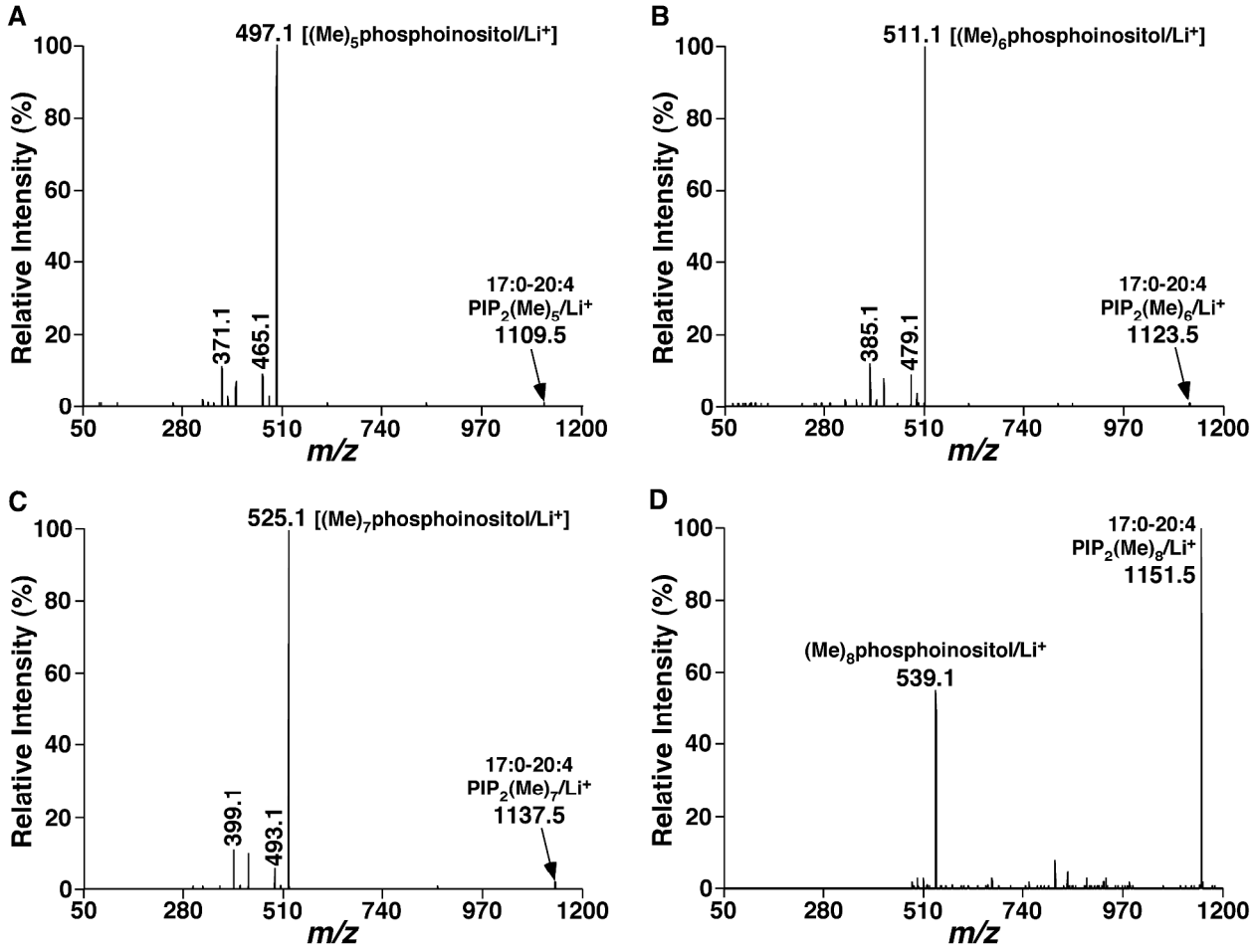

Figure S5 Fragmentation pattern of lithiated PIP species containing different methyl groups. Standard species of different PIP classes were methylated as described under the section of "Materials and Methods". Lithium adducts of methylated PIP species from different PIP classes were fragmented after collision-induced dissociation at collision energy of 38 eV and collision gas pressure of 1 mTorr on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific). Panels A to D displayed fragmentation patterns resulted from methylated PIP molecular ions containing 3, 4, 5, and 6 methyl groups, respectively. We found these fragmentation patterns were independent of the positions of phosphate isomers.

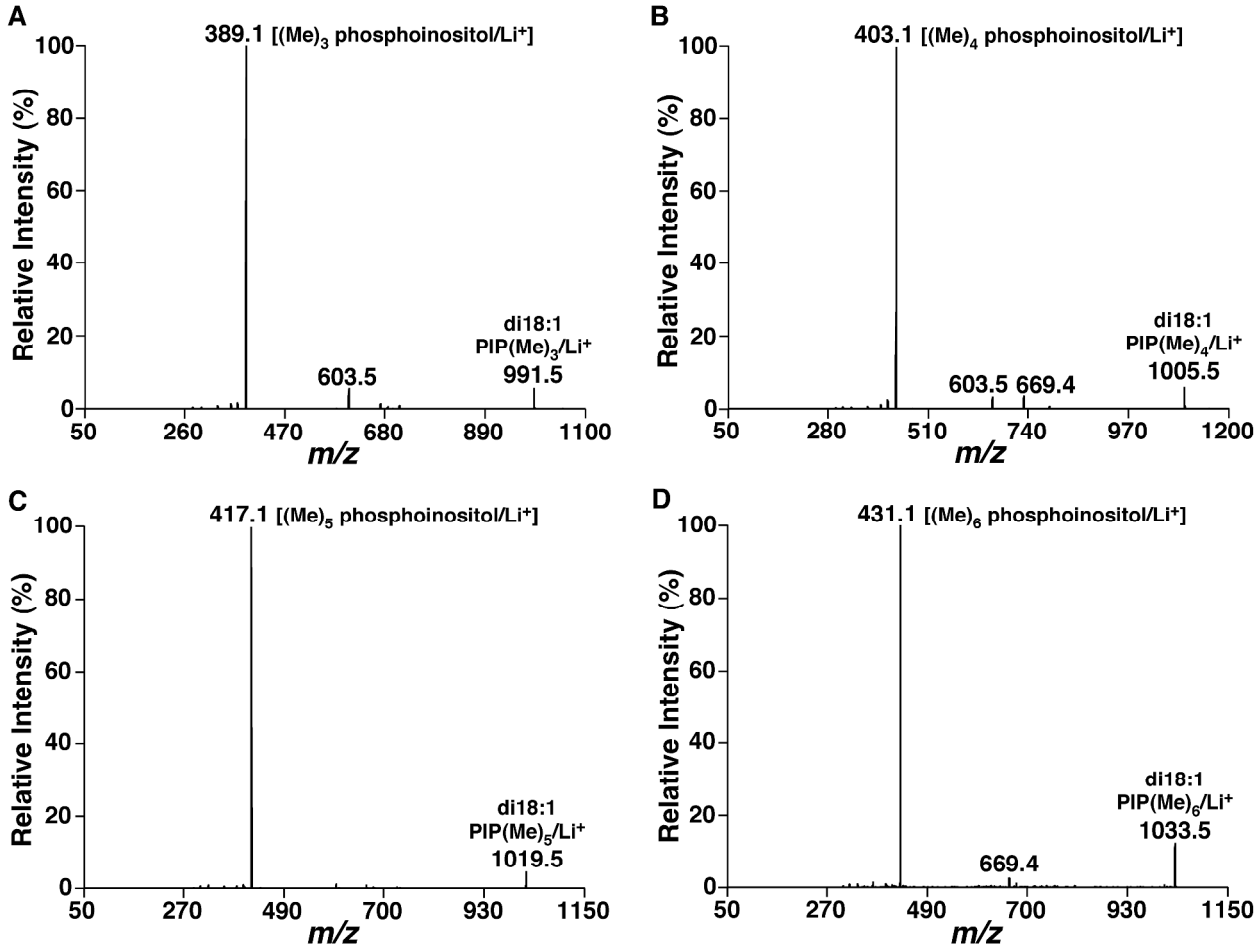

Figure S6 Representative scheme of the methylation reaction of PIP_2 with TMS-diazomethane and their resultant ions in MS and tandem MS analyses. The hydroxyl groups of $PI(4,5)P_2$ (a) were methylated with TMS-diazomethane, which led to producing methylation of 5 to 8 hydroxyl groups present in $PI(4,5)P_2$ to a different degree (b). In methylation, five hydroxyl groups of the phosphates were readily reacted. The highlighted hydroxyl groups on the sugar ring with red were reacted with TMS-diazomethane to a varied degree based on the positions of both hydroxyl groups and phosphates on the sugar ring as indicated in the bottom of panel b and Table 1. Lithium adducts of the methylated $PI(4,5)P_2$ species were readily acquired in the positive-ion mode in the presence of LiCl (c). Unique fragment ions corresponding to the lithiated Me_xPIP_2 (x = 5, 6, 7, 8) head groups were yielded after collision-induced dissociation (CID) (d). These precursor ions were used to quantify the amount of individual ions of PIP_2 and determine the composition of their isomers after simulation using different methylation patterns as listed in Table 1. Demethylation ions were readily yielded from the methylated PIP_2 species in the ion source in the negative ion mode (e). The acyl chain information was acquired from the product ions or two-dimensional mass spectrometric analysis after CID (f).

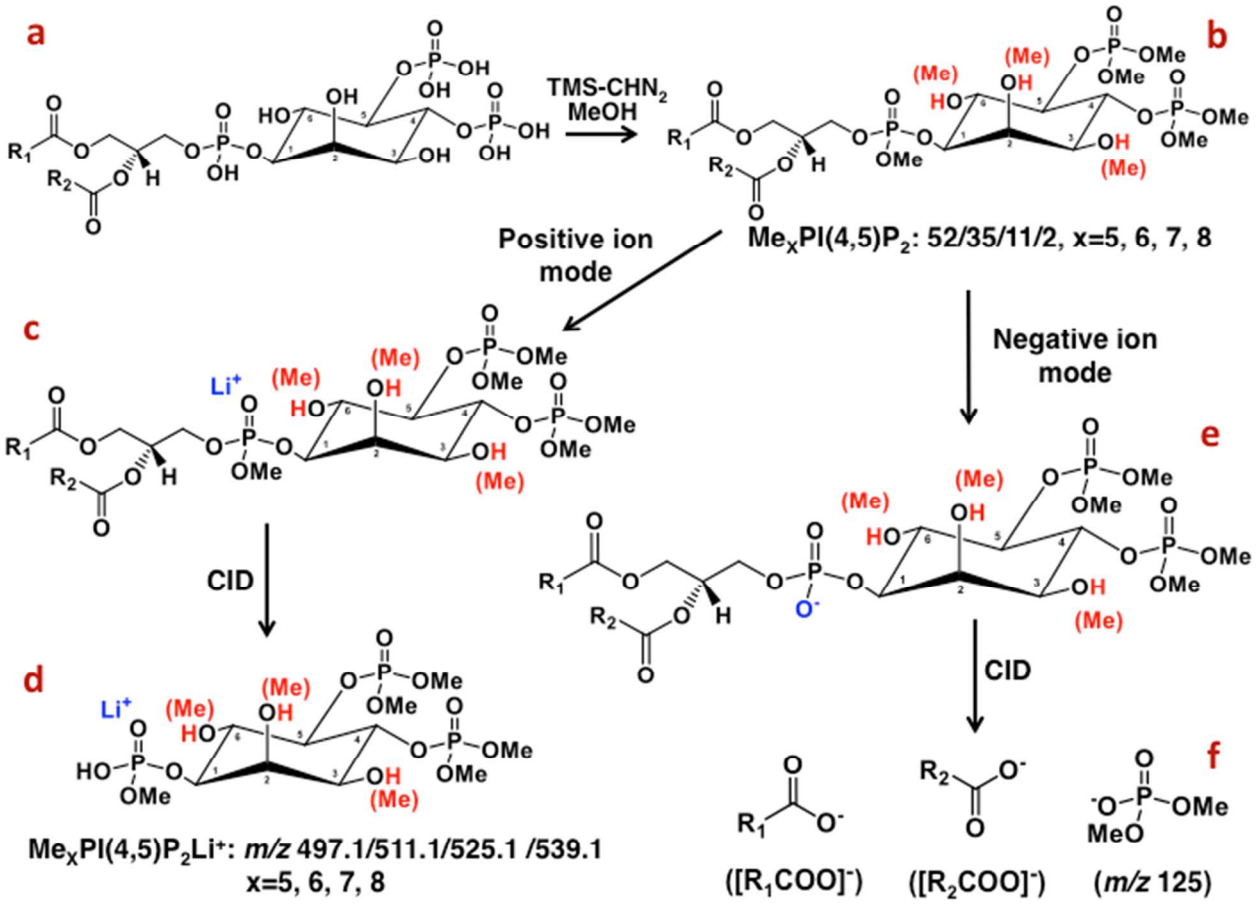

Figure S7 Fragmentation patterns of methylated PPI species in the negative-ion mode. Standard species of different PPI classes were methylated as described under the section of "Methods". Demethylated ions of methylated PIP species from different PIP classes in the negative ion mode were fragmented after collision-induced dissociation at collision energy of 45 eV and collision gas pressure of 1 mTorr on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific). Panels A to H displayed fragmentation patterns resulted from most intense methylated PPI molecular ions. The most abundant fragment ions were those corresponding to fatty acyl carboxylates and the one at *m/z* 125 corresponding to dimethylphosphate (see Figure S6).

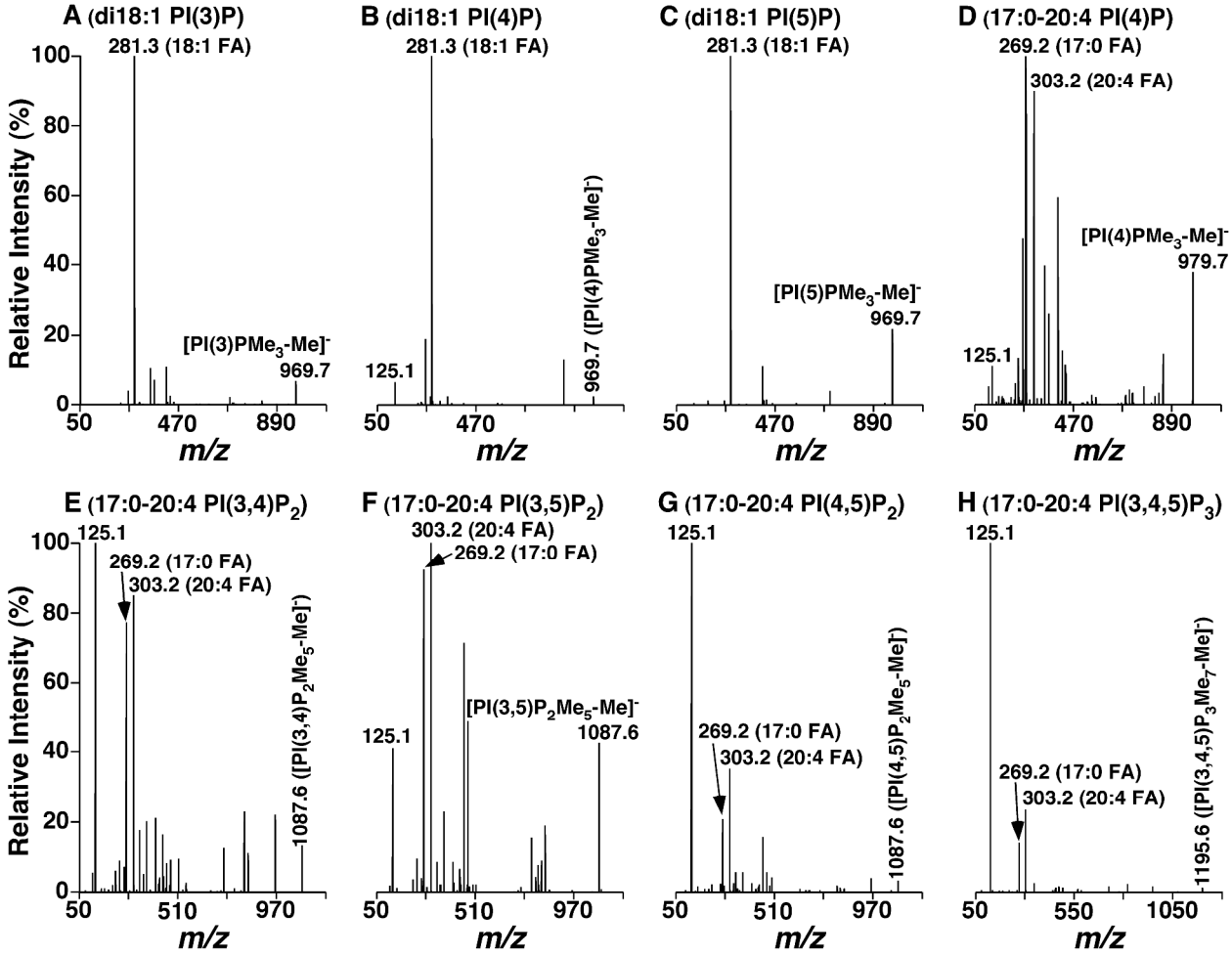

Figure S8 Two-dimensional MS analysis of PPI species present in mouse spinal cord in the negative ion mode. All MS/MS scans for characterization of methylated PPI species in the negative-ion mode were shown in Figure S7. Individual MS and MS/MS traces presented in two-dimensional mass spectrometric analysis were displayed after normalization of the base peaks in the spectra. PIS125 displayed the molecular ions containing dimethylphosphate(s). PIS255, PIS269, PIS281, PIS283, PIS303, PIS305, and PIS331 determined the molecular ions containing 16:0, 17:1, 18:1, 18:0, 20:4, 20:3, and 22:4 fatty acyls, respectively. Identification of individual methylated PPI species from the two-dimensional mass spectrometry was achieved from the crossing peaks. For example, the modest molecular ion at *m/z* 1087.6, as highlighted with a broken line, was crossed with PIS125 (abundant in PIP_2 and PIP_3 fragmentation), PIS255 (16:0 FA), PIS269 (17:0 FA), PIS303 (20:4 FA), and PIS331 (22:4), indicating the presence of 16:0-22:4 Me_6PIP_2 and 17:0-20:4 Me_5PIP_2. The crossed trace peak present in PIS305 corresponds to the ^{13}C isotopic peak of 17:0-20:4 Me_5PIP_2.

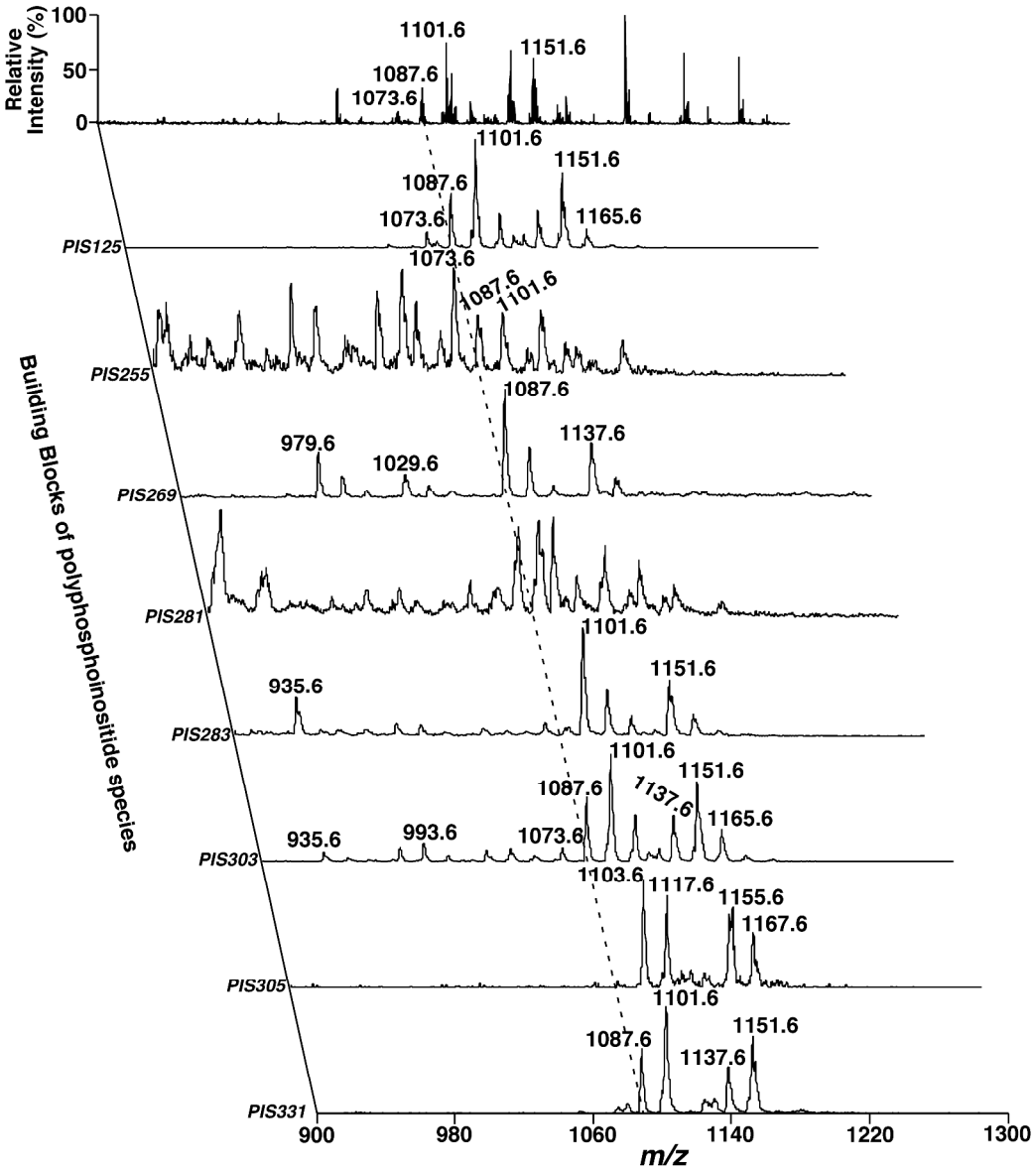

Figure S9 The effects of different fatty acyl chain on ionization efficiency. Mass spectral analyses of different mixtures of di18:1 and 17:0-20:4 PI(3,4)P_2 species as indicated in panels A to G were performed in full mass scan mode on a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific). The linear correction between peak intensity ratio and mass content ratio of the species with a slope of approximately 1 and an intercept of near zero within ±10% of experimental errors indicate the identical ionization efficiency of the species (panel H).

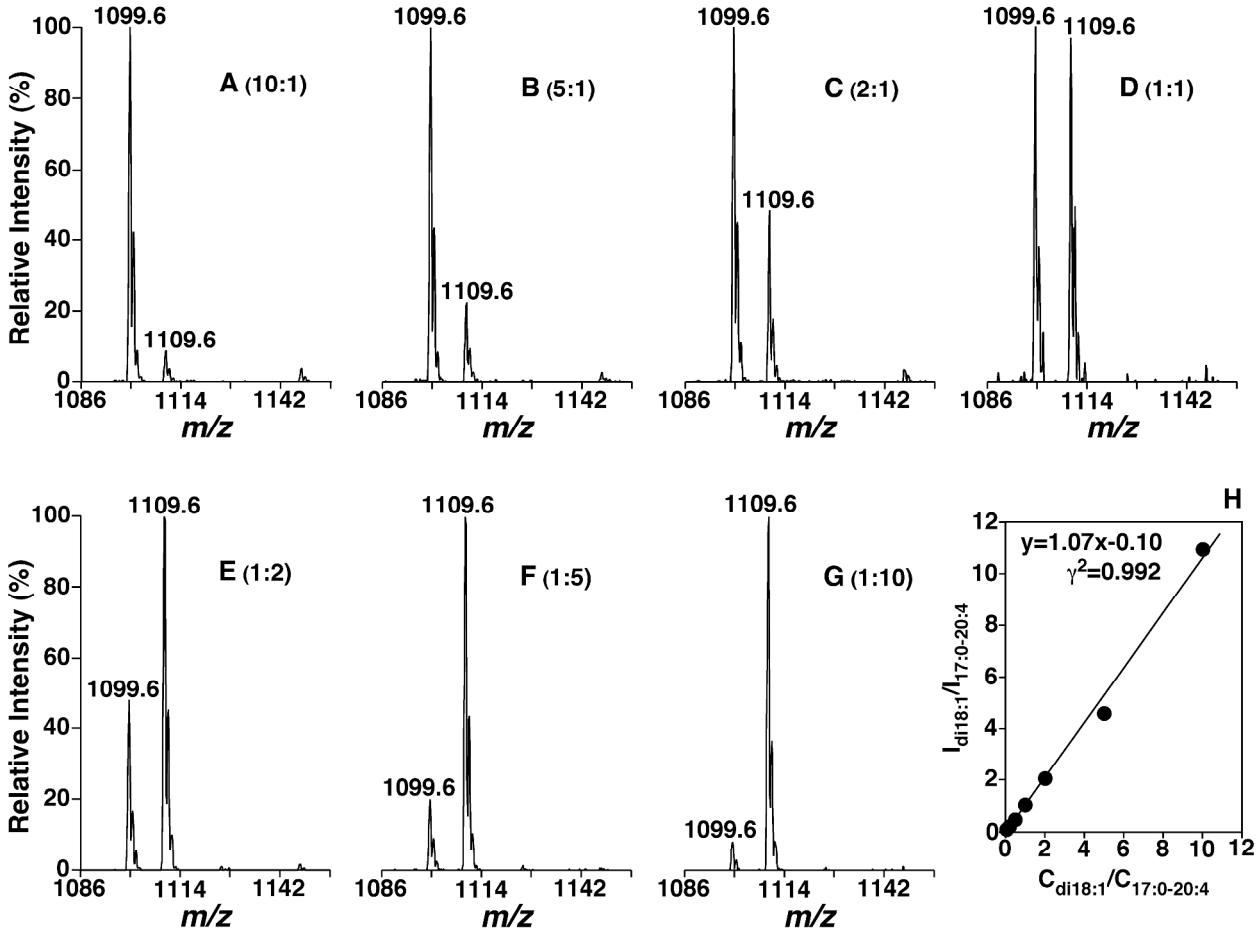

Figure S10 Mass spectral analysis of the profiles of PPI species present in extracts prepared using different approaches. Lipid extraction from mouse spinal cord was conducted with three different approaches and derivatized with TMS-diazomethane as described under the section of "Methods". PIP (panels A to C) and PIP_2 (panels D to F) species present in the discarded solvents from the first step extraction of the two-step extraction approach (panels A and D), the extracts from the second step extraction of the two-step extraction approach (panels B and E), and the extracts in the presence of IS in the first step and prepared with the modified MTBE method (panels C and F), respectively, were analyzed with PIS of lithiated PPI species containing least numbers methyl groups. Panels B and E were displayed after normalization of internal standard ion peaks to those present in Panels C and F, respectively, for comparison. It should be noted that virtually identical profiles of PPI species from the modified Folch procedure (spectra not shown) to those obtained from the modified MTBE procedure (panels C and F) were also obtained.

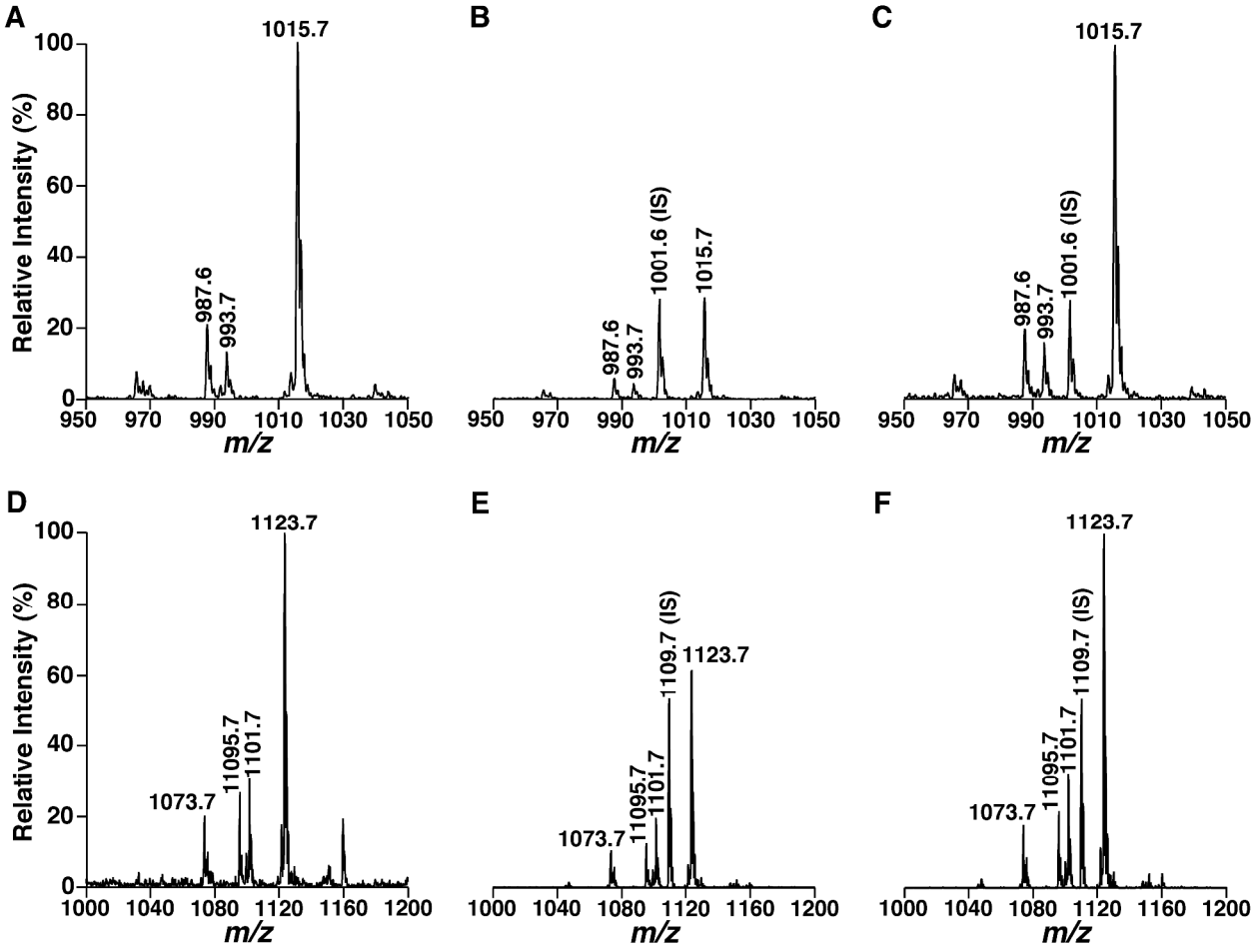

Figure S11 Two-dimensional mass spectral analysis and comparison of PIP_2 species present in hepatic tissue samples of wild type and *db/db* mice. Hepatic lipids of wild type (panel A) and *db/db* (panel B) mice at 4 months of age were prepared in the presence of 17:0-20:4 $PI(4,5)P_2$ (150 pmol/mg protein) as an internal standard (IS) as described under the method section. Two-dimensional ESI MS analyses were performed by precursor-ion scanning (PIS) of all methylated PIP_2 species (i.e., PIS497.1 for Me_5PIP_2, PIS511.1 for Me_6PIP_2, and PIS525.1 for Me_7PIP_2) in the positive-ion mode in the presence of LiCl as described under the method section. For quantification, all the mass spectra were displayed after normalized to the ion peak intensities of IS based on its methylation pattern listed in Table 1. The broken lines highlighted the methylation patterns of the PIP_2 ions. These patterns were used for simulation of phosphate positional isomers as described in the text.