Supplementary Information

Legends for Supplementary Figures

Fig. S1. SM restores the binding of VacA to the plasma membrane of SMase C-pretreated AZ-521 cells in a dose-dependent manner.

AZ-521 cells were incubated in the absence or presence SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold medium to remove SMase C. The cells were then incubated on ice in the absence or presence of pre-chilled SM at the indicated concentrations, and then further incubated with Alexa Fluor 488-labeled VacA (50 nM) for 1 h on ice, and then analyzed for cell binding using flow cytometry. Binding data were normalized relative to the binding of VacA of cells that had been mock pretreated (incubated only with PBS pH 7.2) prior to incubation with VacA (50 nM). These data were combined from three independent experiments. Statistical significance was calculated for differences in VacA binding between cells pretreated with SMase C followed by SM and cells that had been pretreated with SMase C only.

Fig. S2. The binding of VacA to SM-depleted AZ-521 cells is not restored by other common lipids.

AZ-521 cells were incubated with SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold complete medium to remove SMase C. The cells were then incubated on ice in the absence or presence of pre-chilled SM, PC, PE, PI, or cholesterol (100 μM) followed by incubation with prechilled Alexa Fluor 488-labeled VacA (50 nM) for 1 h on ice and then analyzed for cell binding using flow cytometry. Binding data were normalized relative to the binding of VacA of cells that had been mock pretreated (incubated only with PBS pH 7.2) prior to incubation with VacA (50 nM). These data were combined from three independent experiments Statistical significance was calculated for differences in VacA binding between cells pretreated with SMase C followed by lipid and cells that had been pretreated with SMase C only.

Fig. S3. SM confers AZ-521 cell sensitivity to VacA.

AZ-521 cells were incubated in the absence or presence SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold complete medium to remove SMase C. The cells were then incubated with pre-chilled VacA at the indicated concentrations for 1 h on ice, and then further incubated at 37 °C under 5% CO_2. After 24 h, cellular vacuolation was determined using the neutral red assay. Vacuolation data were normalized relative to the neutral red uptake of cells that had been mock pretreated (incubated only with PBS pH 7.2) prior to incubation with VacA (200 nM). Statistical significance at each VacA concentration was calculated for differences

in vacuolation between mock pre-treated cells and SMase C pre-treated cells. * indicates $P<0.05$.

Fig. S4. SM add-back restores the sensitivity of SMase C-pretreated AZ-521 cells to VacA.

AZ-521 cells were incubated in the presence of SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold medium to remove SMase C. The cells were then incubated on ice in the absence or presence of pre-chilled SM 50 μM, and then further incubated with VacA at the indicated concentrations for 1 h on ice, and then further incubated at 37 °C under 5% CO_2. After 24 h, cellular vacuolation was determined using the neutral red assay. Vacuolation data were normalized relative to the neutral red uptake of cells that had been mock pretreated (incubated only with PBS pH 7.2) prior to incubation with VacA (100 nM). Statistical significance at each VacA concentration was calculated for differences in vacuolation between cells enriched in plasma membrane SM (indicated by "+SM addback") and cells depleted in plasma membrane SM (indicated by "-SM addback").

Fig. S5. SM restores the sensitivity of SMase C-pretreated AZ-521 cells to VacA in a dose-dependent manner.

AZ-521 cells were incubated in the absence or presence SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold complete medium to remove SMase C. The cells were then incubated on ice in

the absence or presence of pre-chilled SM at the indicated concentrations, and then further incubated with VacA (50 nM) for 1 h on ice, and then further incubated at 37 °C under 5% CO_2. After 24 h, cellular vacuolation was determined using the neutral red assay. Vacuolation data were normalized relative to the neutral red uptake of cells that had been mock pretreated (incubated only with PBS pH 7.2) prior to incubation with VacA (100 nM). Statistical significance was calculated for differences in vacuolation between cells pretreated with SMase C followed by SM and cells that had been pretreated with SMase C only.

Fig. S6. The sensitivity of SM-depleted AZ-521 cells to VacA is not restored by other common lipids.

AZ-521 cells were incubated in the absence or presence of SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold complete medium to remove SMase C. The cells were then incubated on ice in the absence or presence of pre-chilled SM, PC, PE, PI, cholesterol at the indicated concentrations followed by incubation with prechilled VacA (50 nM) for 1 h on ice. The cells were then further incubated at 37 °C under 5% CO_2. After 24 h, cellular vacuolation was determined using the neutral red assay, and total protein using the Coomassie assay. Vacuolation data were normalized relative to the neutral red uptake into cells that had been mock pretreated (incubated only with PBS pH 7.2) prior to incubation with VacA (50 nM). Statistical significance was calculated for differences in vacuolation between cells pretreated with

SMase C followed by the indicated lipid and cells that had been pretreated with SMase C only.

Fig. S7. Cellular SM levels in AZ-521 cells incubated with acyl chain length variants of SM.

AZ-521 cells were incubated with SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold complete medium to remove SMase C. The cells were then incubated for 30 min on ice in the absence or presence of pre-chilled C2-, C6-, C12-, C18-SM and membrane extracted SM (25 μM). Total cellular SM content (nmol SM/mg of total protein) was quantified as described under "Experimental Procedures." SM incorporated was calculated by subtracting the SM content in cells treated only with SMase C from cells pretreated with SMase C and enriched with the indicated SM variant. The results are rendered as the SM incorporated in SMase C cells incubated with C2-SM, C6-SM, C12-SM, or C18-SM, relative to the SM incorporated in SMase C cells incubated with membrane extracted SM (labeled as "membrane-extracted SM"). Statistical significance was calculated for differences in relative SM incorporated in cells supplemented with C2-SM, C6-SM, C12-SM, or C18-SM and cells supplemented with membrane extracted SM.

Fig. S8. The trafficking of VacA to Lamp1 enriched intracellular vesicles is sensitive to variation in SM acyl chain length.

AZ-521 cells were incubated with SMase C (50 mU/mL) for 1 h at 37 °C, and then chilled on ice. The cells were washed with ice-cold complete medium to remove SMase C. The cells were then incubated for 30 min on ice in the absence or presence of pre-chilled C2- or C18-SM (25 μM). The cells were further incubated with prechilled Alexa Fluor 647 labeled VacA (50 nM) for 1 h on ice. Cells were washed 1 time with prechilled, complete medium plus unlabeled VacA (50 nM), and then further incubated at 37 °C with the addition of pre-warmed complete medium containing unlabeled VacA (50 nM). After 2 h, the cells were fixed, permeabilized, and stained for Lamp1 by incubating with anti-Lamp-1 antibodies followed by incubation with secondary antibodies conjugated to Alexa Fluor 488. The cells were evaluated for VacA colocalization with Lamp-1 using fluorescence/DIC microscopy. Within the larger merged image of VacA and Lamp1, the areas within the dashed white box were enlarged as solid white boxes located in the lower, right hand corners of the merged images, to illustrate VacA (red puncta), Lamp1 (green puncta), or VacA co-localized with Lamp1 (pseudo yellow-colored puncta). The white puncta in the smaller, upper black and white images illustrate, as labeled VacA or Lamp1. The smaller, bottom panels are the DIC images provided to illustrate the overall shape of the cell(s), and include the black scale bars. The colocalization index was determined for VacA with Lamp1 in cells enriched in C2-SM, cells enriched in C18-SM, or un-pretreated cells. These data were combined from three independent experiments. Statistical significance was calculated for differences in the colocalization between VacA and Lamp-1 in cells enriched in either C2-SM or

C18-SM and control cells that had not been pretreated prior to incubation with VacA.