Figure legends for Supplemental Figures

Supplemental Figure 1. Intracellular localization of recombinant hSK1 constructs as assessed by sucrose density gradient fractionation. Total membranes from HeLa cells were fractionated on a discontinuous sucrose density gradient as detailed. **(A)** Equal volumes of each fraction were resolved using SDS-PAGE and immunoblotted with antibodies specific for intracellular markers. Intracellular markers represented here are α-ATPase for plasma membrane (PM); α-calnexin for endoplasmic reticulum (ER); α-Tom20 for mitochondria (Mito); and α-LDH for cytosol. α-SK1 antibody was used to probe for recombinant hSK1 constructs. **(B-F)** Following sucrose density gradient fractionation of total membranes from transiently transfected HeLa cells, Western blot analysis was performed as described and densitometry readings were used to generate distribution profiles. Profiles consist of an overlay of distribution curves for SK1 (filled circles), the PM marker, ATPase (open circles), or the ER marker, calnexin (filled triangles). SKwt (wild-type recombinant hSK1); S225A (phosphorylation-deficient mutant of hSK1); Cb5 (ER-targeted SK1 with cytochrome b5 C-terminus); PL16 (ER-targeted SK1 with polyleucine tail); Lck (PM-targeted SK1 with myristoylation/palmitoylation motif).

Supplemental Figure 2. Lck-SK is localized to the plasma membrane and Cb5-SK is localized to the endoplasmic reticulum. HeLa cells were transfected with either Lck-SK **(A)** or Cb5-SK **(B)** and fractionated as indicated in the legend for Supplementary Figure 1 with the modification that the sucrose gradients were centrifuged for 1 hour rather than 2.5 hours. This results in the plasma membrane marker sedimenting as a single peak rather than

a bimodal distribution. Densitometry of the resulting immunoblots was normalized to the fraction with the greatest signal. Profiles consist of an overlay of distribution curves for SK1 constructs (filled circles), the PM marker, ATPase (open circles), or the ER marker, calnexin (filled triangles).

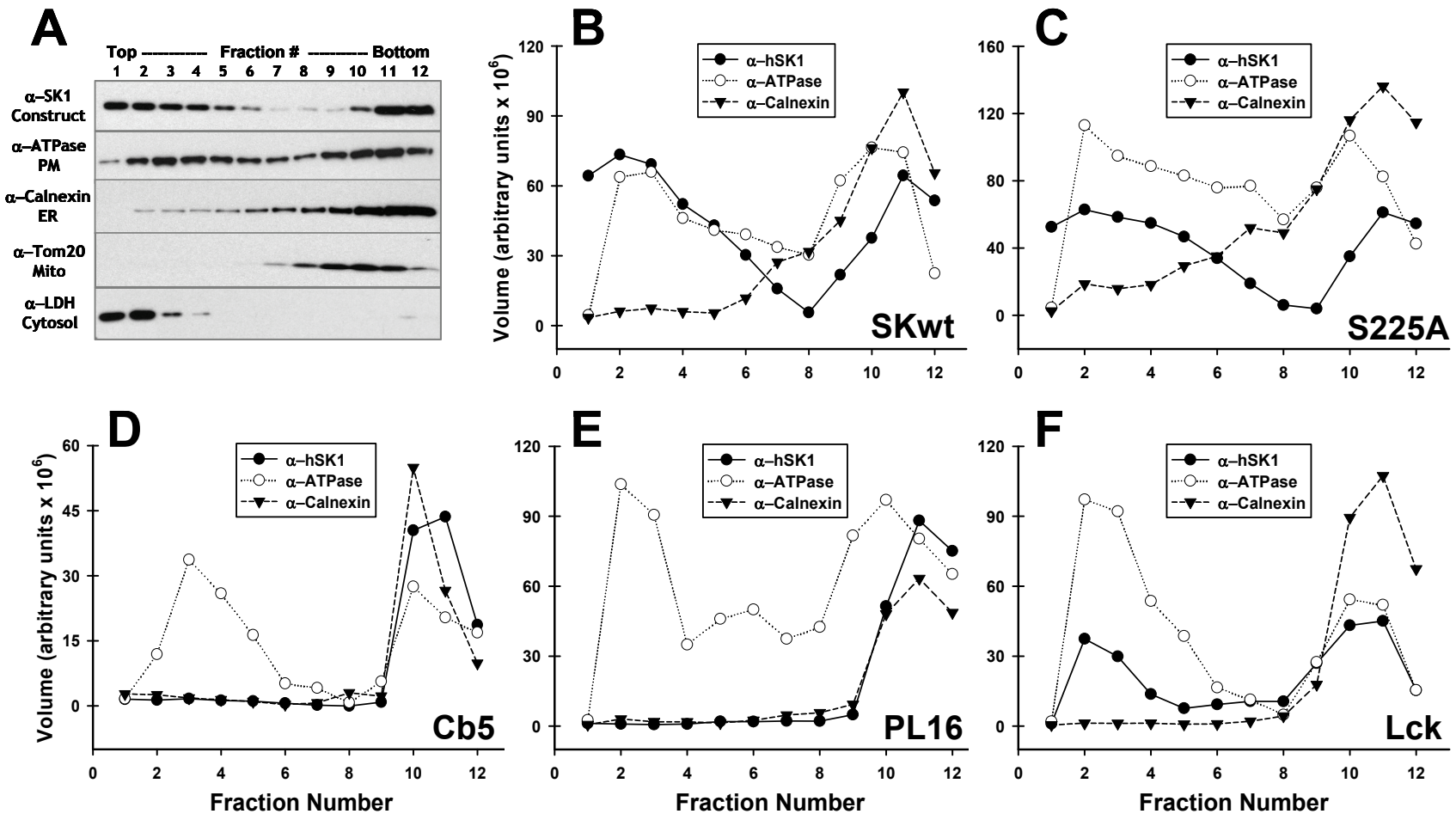
Supplemental Figure 3. Membrane-targeted constructs of hSK1 are inserted in the membrane and activity is preserved. HeLa cells were transfected with either SK1wt, the PM-targeted construct, Lck, or the ER-targeted constructs, PL16 and Cb5. 24 hours post-transfection, cells were harvested and total membranes were collected using centrifugation. **(A)** An aliquot of each membrane fraction was subjected to alkaline treatment for 30 minutes then pelleted again (AM). Comparable volumes of each fraction were resolved using SDS-PAGE, then Western blot analysis was performed using α-hSK1 along with markers for ER membrane (α-calnexin), mitochondrial membrane (α-Tom20), and cytosol (α-LDH). For each panel (left to right), bands represent whole cell lysate (L), cytosol (C), total membranes (M) and alkaline-treated membranes (AM). **(B)** Comparable volumes of cytosol (black bars) and total membrane fractions (gray bars) for each construct were subjected to *in vitro* kinase assays and specific activity was calculated (pmol S1P/min/mg protein). Values represent % of total SK1 activity for each fraction as means ± S.D. (n=6).

Supplemental Figure 4. Activity of sphingosine kinase constructs with sphingosine or dihydrosphingosine as substrates. **(A)** Sphingosine kinase activity was measured for lysates from either vector transfected cells or cells transfected with the indicated SK1 constructs. Assays were performed with either sphingosine or dihydrosphingosine at 50μM

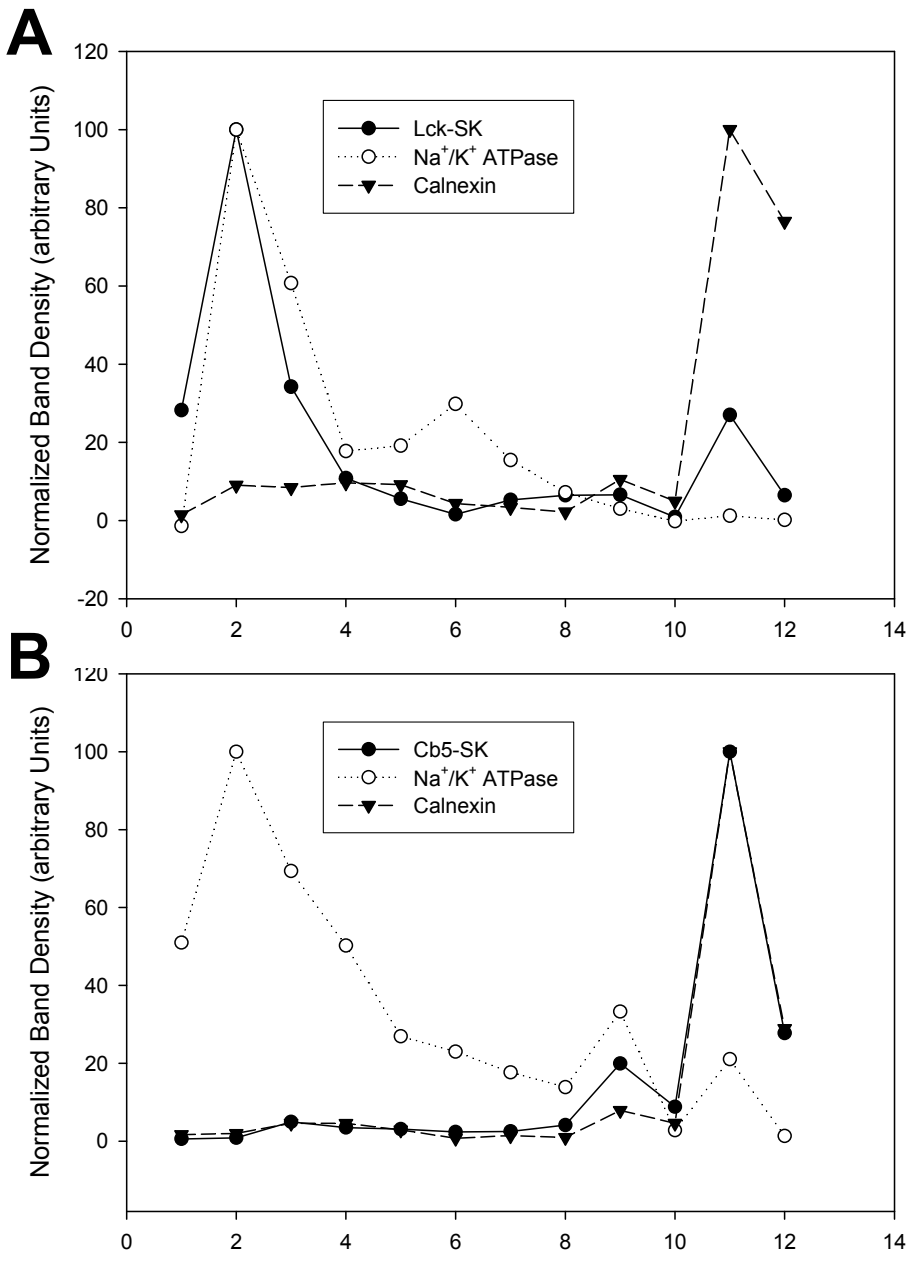
as substrates as described. Shown are the means ± S.D. of three replicates. Essentially identical results were obtained at 10μM substrate concentrations (not shown). **(B)** Expression levels of sphingosine kinase constructs. One μg of total lysate protein from transfected cells used in the enzymatic assays depicted above was subjected to SDS/PAGE and immunoblotting using an anti-sphingosine kinase antibody as described in Methods.

Supplemental Figure 5. Effect of siRNA knockdown of sphingosine-1-phosphate lyase (SPL) and sphingosine-1-phosphatases (SPP1/2) on mass levels of sphingolipids in cells with endogenous levels of SK1. HeLa cells were transfected with siRNA against the indicated genes 48 hours prior to lipid extraction and sphingolipid analysis as described. Shown are the means ± S.D. (n=4). **(A)** Sphingosine-1-phosphate (solid bars) and dihydrosphingosine-1-phosphate (gray bars). **(B)** Sphingosine (solid bars) and dihydrosphingosine (gray bars). **(C)** Total ceramides (solid bars) and total dihydroceramides (gray bars). Statistical analysis was performed using an unpaired Student's *t* test comparing samples treated with scrambled oligonucleotides to the indicated siRNAs. *$p \leq 0.05$, **$p \leq 0.01$.

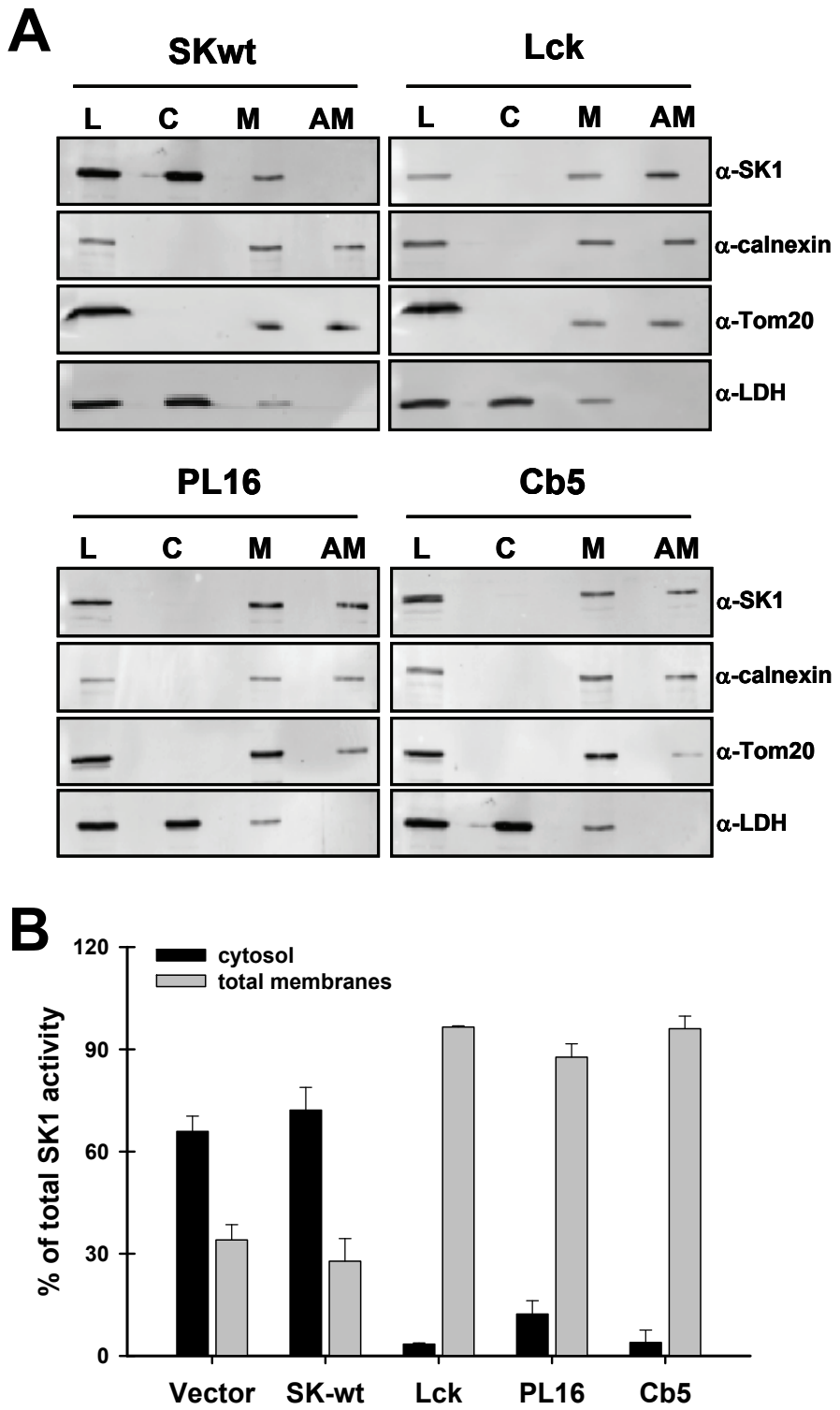
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



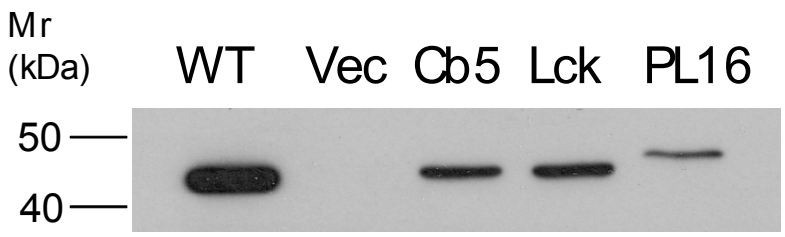
Supplemental Figure 4

A

Construct	Activity with Sphingosine*	Activity with Dihydrosphingosine*
Endogenous	0.037 ± 0.004	0.035 ± 0.004
Wild-type	73.30 ± 2.7	72.3 ± 2.1
Lck-SK	7.03 ± 0.72	7.26 ± 0.36
Cb5-SK	11.6 ± 0.93	13.1 ± 0.53
PL16-SK	11.0 ± 0.51	9.14 ± 0.90

*pmol S1P or DHS1P/ min/μg protein

B



Supplemental Figure 5