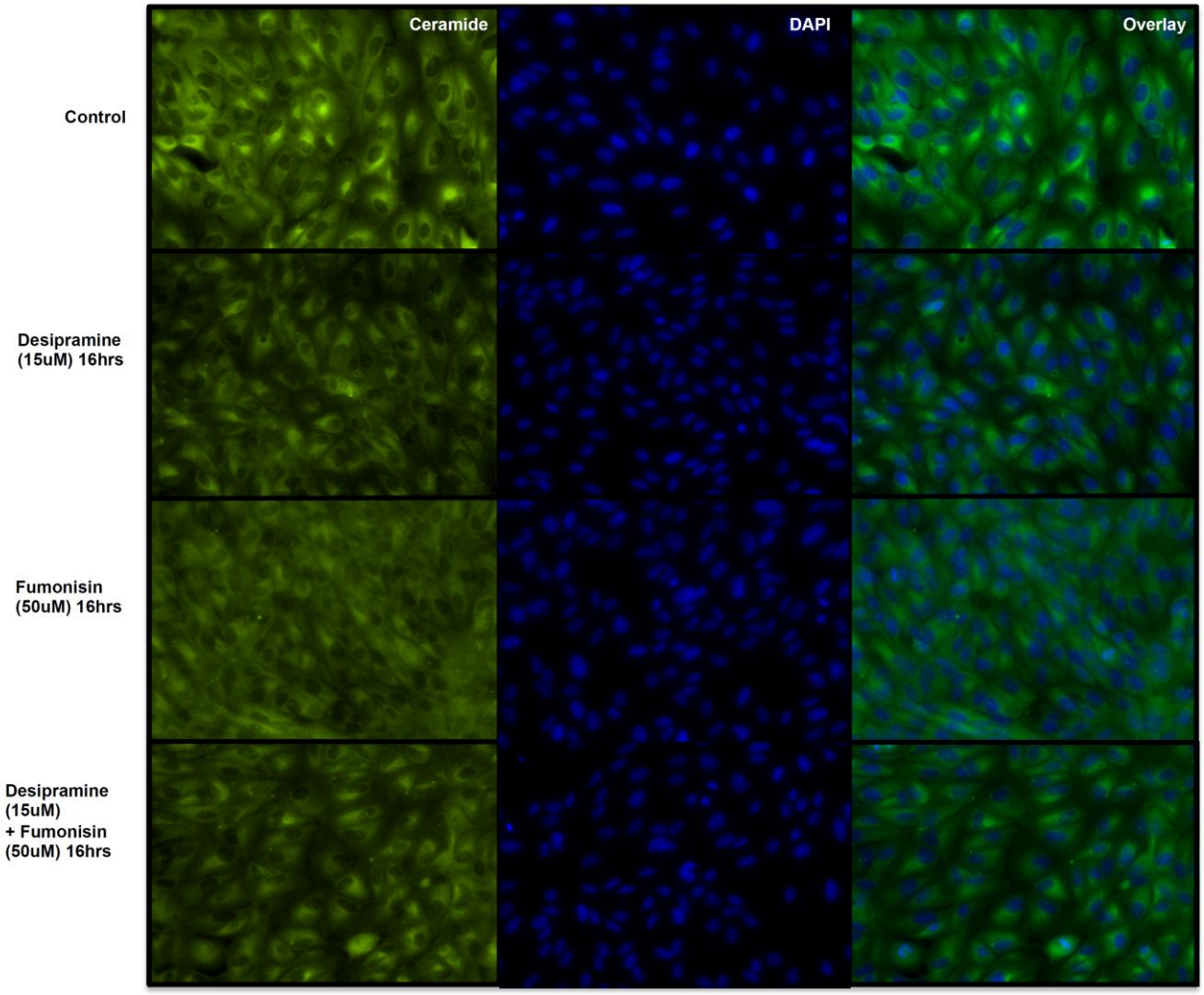
SUPPLEMENTARY DATA

Supplementary Figure 1. ARPE19 cells treated with either 15 uM Desipramine (ASMase inhibitor), 50 uM Fumonisin (ceramide synthase inhibitor) or both, were immunolabeled for ceramide (green) and DAPI (blue). Ceramide immunofluorescent staining demonstrates that inhibition of either ASMase or ceramide synthase significantly reduce ceramide immunostaining compared to the control.



SUPPLEMENTARY DATA

Supplementary Figure 2. High resolution tandem mass spectrometry of epidermal and tight junction putative acyl ceramide. Positive ion mode Orbitrap Higher Energy Collisional Dissociation (HCD) MS/MS at 100,000 resolving power of m/z 942.8562, corresponding to putative protonated AcylCer (d18:1/h24:0) in lipid extracts of mouse epidermis (top panel) and ARPE-19 tight junctions (bottom panel). * Matrix ion. The top panel shows the epidermis tandem mass spectrum from HCD-MS/MS of m/z 942.8562, initially identified as a putative omega-linked acyl-ceramide h42:1 (total acyl carbons: total double bonds). This ion corresponds to the $[M+H]^+$ ion of the $[M+HCO_2\text{-}H]^-$ molecular ion observed at m/z 986.8393 in Figure 4A and Figure 4B. Structural analysis was performed by positive ion MS/MS due to the preferential formation of dehydrated sphingosine derivatives upon fragmentation of ceramide $[M+H]^+$ ions, compared to the dominant loss of formate upon HCD-MS/MS of ceramide $[M+HCO_2\text{-}H]^-$ ions. The product ion at m/z 264.2682 in the top spectrum is indicative of the dehydrated d18:1 sphingosine backbone. The bottom panel shows a similar HCD-MS/MS analysis of ARPE19 TJs purified by anti-PKC zeta IP with a lipid of m/z 942.8562. A product ion at m/z 264.2682 was also observed in the TJ MS/MS spectrum, although at lower abundance than that observed for the epidermis lipid extract. Additional criteria used for assignment of putative acyl-ceramide species included absence of these ions in sample blanks and Protein-A bead negative controls. A search of the LIPID MAPS lipid database (http://www.lipidmaps.org) revealed that 1-O-linked acyl ceramides, including 1-O-26:1 (d18:1/18:0), are present within 0.1 m/z of the theoretical exact masses of the putative omega-linked h(42:1) acylceramide $M+H^+$ ions. This mass difference is sufficient to enable differentiation and quantitation of these two different forms of acylceramide molecular species based on MS measurements, and no putative 1-O-26:1 (d18:1/18:0) was detected above the level of noise in either the plasma membrane or the TJ lipid mass spectra. However, it should be noted that the 1.5 m/z mass window used for isolation of precursor ions for HCD-MS/MS does overlap with the m/z of potential O-linked acylceramides. Therefore, the potential exists for confounding presence of O-linked 1-O-26:1 (d18:1/18:0) acylceramide present below the limit of detection for MS measurements, to contribute to the formation of the sphingosine derivative ion at m/z 264.2682 upon HCD-MS/MS of m/z 942.8562.