

Orosomucoid Like 3 (ORMDL3) Supports Rhinovirus Replication in Human Epithelial Cells¹

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Abstract

Polymorphism at the 17q21 gene locus and wheezing responses to rhinovirus (RV) early in childhood conspire to increase the risk of developing asthma. However, the mechanisms mediating this gene-environment interaction remain unclear. In this study, we investigated the impact of one of the 17q21 encoded genes, *ORMDL3* on RV replication in human epithelial cells. *ORMDL3* knockdown inhibited RV-A16 replication in HeLa, BEAS-2B, A549, NCI-H358 epithelial cell lines, and primary nasal and bronchial epithelial cells. Inhibition varied by RV species, as both minor and major group RV-A subtypes, RV-B52 and RV-C2 were inhibited, but not RV-C15 or RV-C41. *ORMDL3* siRNA did not affect expression of the major group RV-A receptor ICAM-1 or initial internalization of RV-A16. The two major outcomes of *ORMDL3* activity, serine palmitoyl-CoA transferase (SPT) inhibition and endoplasmic reticulum (ER) stress induction were further examined: silencing *ORMDL3* decreased RV-induced ER stress and IFN- β mRNA expression. However, pharmacologic induction of ER stress and concomitant increased IFN- β inhibited RV-A16 replication. Conversely, blockade of ER stress with tauroursodeoxycholic acid augmented replication, pointing to an alternative mechanism for the effect of *ORMDL3* knockdown on RV replication. In comparison, the SPT inhibitor myriocin increased RV-A16 but not RV-C15 replication, and negated the inhibitory effect of *ORMDL3* knockdown. Further, lipidomics analysis revealed opposing regulation of specific sphingolipid species (downstream of SPT) by myriocin and *ORMDL3* siRNA, correlating with the effect of these treatments on RV replication. Together this data revealed a requirement for *ORMDL3* in supporting RV replication in epithelial cells via SPT inhibition.

Keywords: *ORMDL3*, asthma, rhinovirus, epithelial cells, SPT, unfolded protein response

Introduction

Asthma is one of the most prevalent chronic diseases of childhood, affecting more than 7 million children under the age of 18 in the United States (1). The development of asthma involves contributions from multiple genes and environmental factors such as the microbiome and viral infection (2). Aberrant wheezing responses early in life to respiratory viruses, rhinovirus (RV) in particular, predispose to subsequent development of asthma (3-5). Regarding asthma genetics, variation at the 17q21 gene locus remains one of the strongest genetic risk factors for the development of childhood asthma (6). Two genes within this locus, *GSDMB* (gasdermin B) and *ORMDL3* (orosomucoid like-3), are co-regulated by asthma-risk associated polymorphisms (7-9). An increasing body of evidence in murine models has implicated *ORMDL3* in asthma development (10-12). *ORMDL3* over-expressing transgenic mice present compelling evidence for *ORMDL3* as a pathogenic factor (10): these mice manifest spontaneous airway remodeling, increased fibrosis, mucus production, inflammation and methacholine-induced hyper-responsiveness (10). *ORMDL3* has also been implicated in allergen-triggered lung disease in knockout mice and in the transgenic mouse model (10, 13).

ORMDL3 is an endoplasmic reticulum (ER)-resident transmembrane protein that has two identified functions: 1): inhibition of the SPT (serine palmitoyl CoA transferase) enzyme, the rate limiting step in *de novo* sphingolipid synthesis (14) and 2): inhibition of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2b) pump that maintains the calcium gradient between ER and cytoplasm (8, 15). Disruption of the ER-cytosolic calcium gradient induces an ER stress response known as the “Unfolded Protein Response” (UPR) (16, 17). Both of these *ORMDL3* functions appear related to abnormal airway physiology in asthma (15, 18). Treatment of mice with the SPT inhibitor myriocin and SPT heterozygous mice display airway hyper-

responsiveness, but not airway remodeling or inflammation (19). Regarding SERCA inhibition, one study revealed decreased SERCA2b expression in airway smooth muscle cells from subjects with moderate to severe asthma (20). An ER stress pathway induced by *ORMDL3* overexpression, involving the transcription factor activating transcription factor 6 alpha ($ATF6\alpha$), regulates allergen-induced smooth muscle proliferation and airway hyper-responsiveness (10, 21, 22).

Investigation of the high-risk COAST (Childhood Origins of ASThma) cohort identified an intriguing interplay between 17q21 genotype, wheezing responses to RV and asthma development. Ninety percent of the cohort children with the asthma risk genotype at 17q21, conferring increased expression of *ORMDL3*, who wheezed in response to RV before the age of 3 years, developed asthma in childhood. RV stimulation of peripheral blood mononuclear cells from these subjects induced further upregulation of *ORMDL3* expression (3). A follow up study in multiple blood leukocyte cell types revealed an association between 17q21 asthma-risk genotype, increased *ORMDL3* expression, and greater RV-induced UPR target gene and IFN- β induction (23). However, it was unclear if there were any functional interactions responsible for the noted associations. In addition, RV replicates well in epithelial cells, but not in peripheral blood leukocytes.

Currently, the role of *ORMDL3* in regulating RV replication in epithelial cells is unclear. One report in the *ORMDL3* transgenic mice suggested *ORMDL3* over-expression limits RV infection (11). In this study, we sought to understand how *ORMDL3* and its primary cellular activities modulate RV infection in human epithelial cells (Supplemental Figure E1). *ORMDL3* gene knockdown limited RV replication, more so in RV-A and RV-B than in RV-C species. Further exploration of ER stress and sphingolipid synthesis inhibition suggested the latter

property is responsible for the effect of *ORMDL3* knockdown on RV replication, as myriocin, a SPT inhibitor and *ORMDL3* mimic, enhanced RV-A replication and negated the effects of *ORMDL3* knockdown. Together these results identify a requirement for *ORMDL3* and implicate modulation of the sphingolipid pool in supporting RV replication in human epithelial cells.

Materials and Methods

Cells and stimulations:

Cell lines, primary epithelial cells and RV strains are described in the supplemental methods. For primary cells, written consent was obtained under an Internal Review Board-approved protocol. Epithelial cells were expanded as submerged monolayers in collagen type IV-coated flasks (Sigma #C7521). The tauroursodeoxycholic acid (TUDCA) sodium salt (EMD Millipore) stock solution was 100mg/ml in DMEM, tunicamycin (Sigma) stock solution was 1mg/ml in methanol, and myriocin (Sigma) 1mg/ml in methanol. Cell viability using the CellTiter-Glo® kit from (Promega) was >93.47% for all doses of tunicamycin. For RV infections, sucrose-purified RV was added at 2 pfu per cell for 24-72h, except for internalization studies (cells infected with 10pfu/cell for 2h). Following infection, the media was removed and cells resuspended in TRIzol (Invitrogen).

Gene knockdown:

Cells were transfected with 200 pM scrambled control or *ORMDL3* siRNA (Thermofisher) using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. *ORMDL3* western blot: *ORMDL3* siRNA-transfected HeLa cells were lysed with RIPA buffer containing protease inhibitors cocktails (Sigma). Whole-cell lysates were resolved by 12% SDS-PAGE, transferred to LFP polyvinylidene difluoride membrane (PVDF) (GE Healthcare Amersham

Biosciences) and immunoblotted with rabbit anti-ORMDL3 (Millipore) and anti- β -Actin (Santa Cruz), followed by IRDye a-800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG antibodies (Licor). Proteins were visualized and quantitated with the Odyssey system (Licor). See on-line supplement for more detail.

Quantitative PCR:

Cells were lysed with TRIzol (Invitrogen) and processed according to the manufacturer's instructions. RNA was treated with DNase I (Invitrogen) prior to reverse transcription using a Super Script kit (Thermofisher). Gene expression was quantitated by Power UP SYBR Green (Thermofisher) fluorescence detected on a StepOne real time PCR machine (Thermofisher). Reaction efficiency and specificity were assessed using serially diluted standard curves and product melting curve analysis. Relative mRNA expression was normalized 18S rRNA using the standard $\Delta\Delta C_t$ method. For primers, see on-line supplement.

RV plaque assay: see on-line supplemental methods.

Immunofluorescence microscopy:

Infected cells on coverslips were fixed in 4% paraformaldehyde, washed with PBS, and blocked with 10% goat serum in 0.1 M, pH 7.6 Tris, 0.1% Triton X-100, and 0.2% BSA. Antibodies were used at 1:100 dilution: rabbit anti-ORMDL3 (Millipore), mouse anti-RV-A antibody (24) and β -actin rabbit monoclonal antibody labelled with Alexa Fluor[®] 647 (Cell signaling technology). Secondary antibodies were goat anti rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (Thermofisher). Images were acquired on a Nikon Eclipse 50i fluorescence microscope.

Flow cytometry:

Cells were incubated with CD54/ICAM-PE (Miltenyi Biotec). Flow cytometry was performed on an LSRII (BD Bioscience) using FlowJo analysis software (version 9.9.5; Tree Star Inc).

Lipidomics: See on-line supplemental methods.

Statistical analysis:

Comparisons between sample groups were conducted using a generalized linear model (see on-line methods) and two-sample t-test for normally distributed outcomes or non-parametric Wilcoxon rank sum test with SAS software (SAS institute, version 9.4). Bar graphs denote means across independent experiments and error bars denote corresponding standard errors of the means. In all figures, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.005$, **** $P \leq 0.001$.

Results

RV infection increases expression of ORMDL3, type I interferon and UPR indicators in HeLa cells.

We previously reported a correlation between RV infection and increased *ORMDL3* and UPR target gene (*HSPA5*) induction in peripheral blood mononuclear cells (PBMC) (23). To determine if RV also increased expression of these genes in epithelial cells permissive for RV replication, we infected HeLa cells with RV-A16 (Figure 1A) (25). RV-A16 infection significantly increased *ORMDL3*, *DDIT3* (26), spliced *XBPI* mRNA (*XBPI(s)*), and *IFNBI*, and showed a trend for increased *HSPA5* (BiP) ($p=0.08$), suggesting a wider involvement of UPR pathways in these epithelial cells than previously noted in Bjab and THP-1 cell lines, and greater degree of target gene induction than in PBMCs (23).

ORMDL3 knockdown significantly inhibits RV replication

RV replicates in unique lipid organelles near the Golgi and ER (27). *ORMDL3* is a transmembrane ER-resident protein (28). Immunofluorescence microscopy of RV-infected HeLa cells suggested a close spatial proximity between *ORMDL3* and intracellular RV 48hr post infection (Figure 1B). To investigate the functional relationship between *ORMDL3* and RV-A16 infection, HeLa cells were knocked down for *ORMDL3*, and then infected with RV-A16.

ORMDL3 siRNA decreased *ORMDL3* mRNA by ~75% and total *ORMDL* protein by >2-fold (Figure 2A). *ORMDL3* siRNA did not significantly affect *ORMDL1* or *ORMDL2* expression compared to control siRNA (Supplemental Figure E2), though the control siRNA transfection exerted a modest non-specific effect on all three *ORMDL* mRNAs compared to non-transfected untreated cells. RV-A16 infection significantly increased *ORMDL3* expression in untreated, and control siRNA transfected HeLa cells (Figure 2A, left). *ORMDL3* siRNA significantly decreased RV RNA levels (~6-fold) compared to no siRNA or control siRNA treatment. (Figure 2B). The RV-A16 replication decrease observed by quantitative PCR was confirmed by plaque forming unit (PFU) assay (Figure 2C). *ORMDL3* siRNA suppression of RV-A replication was tested in a time course as well (Supplemental Figure E3). *ORMDL3* siRNA significantly and comparably inhibited RV-A16 replication (fold change vs. control siRNA) at 24h, 48h, and 72h post infection, thus 48h post-infection was selected for further studies. To investigate the effect of *ORMDL3* knockdown in airway epithelial cells, BEAS-2B cells, originally isolated from the normal human bronchial epithelium (29) were transfected with control siRNA or *ORMDL3* siRNA and then infected with RV-A16 (Figure 2D). *ORMDL3* siRNA dramatically inhibited RV-A16 replication in BEAS-2B cells. To determine if these observations applied to primary human epithelial cells, nasal epithelial cell (NEC) (26) brushings were collected from patients

and cultured. Bronchial epithelial cells (BEC-53) were obtained during lung transplant (30). The NEC and BEC were knocked down for *ORMDL3*, infected with RV-A16, and analyzed by qPCR for *ORMDL3* mRNA and RV (Figure 2E and F). In contrast to the HeLa and BEC-53 cells, RV-A infection did not upregulate *ORMDL3* expression in primary NEC. However, *ORMDL3* knockdown again significantly decreased RV-A16 RNA in both NEC and BEC. We further confirmed the suppression of RV-A16 by *ORMDL3* knockdown in more human lung epithelial cell lines (A549 and NCI-H358) and another primary bronchial epithelial donor cell culture (BEC-05, supplemental Figure E4). Together these results suggest *ORMDL3* expression is required for optimal RV-A16 replication in multiple epithelial cell types.

ORMDL3 knockdown exerts varied effects on different strains of RV

The three RV species (RV-A, RV-B and RV-C) include over 160 genotypes (31). RV uses three cellular membrane glycoproteins to gain entry into the host cell: intercellular adhesion molecule-1 (ICAM-1) (the majority of RV-A and all RV-B), low-density lipoprotein receptor (LDLR) family members (RV-A “minor group” types), and cadherin-related family member 3 (CDHR3) (RV-C) (32). RV-A and RV-C viruses have primarily been implicated as triggers of asthma exacerbations (32). In order to directly compare RV with different receptor requirements in the same population of cells, we utilized HeLa-E8 cells expressing ICAM-1 and engineered to express CDHR3 (33). HeLa-E8 cells were transfected with *ORMDL3* or control siRNA, then assessed for replication of RV-A1 (minor group, binds LDLR), RV-A16, RV-A36 (both major group), RV-B52, RV-C2, RV-C15 and RV-C41. Interestingly, *ORMDL3* siRNA significantly inhibited all three RV-A strains and a representative RV-B strain. In contrast to RV-A and RV-B, only the RV-C2 showed a trend for inhibition (>60% inhibition) by *ORMDL3* siRNA, whereas replication of the other two strains, RV-C15 and RV-C41 were not significantly

impaired by *ORMDL3* knockdown (Figure 3). The failure of *ORMDL3* siRNA to impact specific RV-C strains was not related to inefficient *ORMDL3* knockdown during RV-C infection or overwhelming replication by RV-C, as the species-specific effect of *ORMDL3* siRNA was observed even for viruses with similar levels of replication (e.g. RV-A36 and RV-C15, Supplemental Figure E5). These results suggest that *ORMDL3* plays a different role in supporting RV-A vs. specific RV-C infections, with more consistent inhibition of RV-A species by *ORMDL3* knockdown.

A recent report showed that *ORMDL3* knockdown inhibited ICAM-1 upregulation in IL-1 β stimulated A549 respiratory epithelial cells (34). Down-regulation of the surface receptor for RV-A could explain differences in effects on RV-A major group and RV-B vs. RV-C replication. However, flow cytometry of siRNA-treated uninfected and RV infected HeLa cells did not reveal any differences in surface ICAM-1 expression (Figure 4A). Therefore, *ORMDL3* siRNA did not appear to be acting via alterations in RV surface receptor levels. Furthermore, *ORMDL3* knockdown did not impair RV-A16 internalization 2h following infection (Figure 4B). Indeed, *ORMDL3* knockdown slightly increased RV-A16 uptake. Together these data suggest *ORMDL3* knockdown impacts rhinovirus replication later in the viral life cycle, after cellular entry.

ER stress inhibits RV replication

To determine the mechanism underlying the inhibition of RV-A replication, we investigated two of the major outcomes of *ORMDL3* activity, ER stress (via SERCA pump inhibition and ER calcium perturbation) and SPT inhibition (Figure E1). The effect of UPR induction on RV replication is not well described, though there is one report of suppression in the setting of cystic fibrosis epithelial cells (35). To directly assess the effects of ER stress on RV replication, HeLa cells were pre-treated with the UPR inducer tunicamycin (Tm, Figure 5A) or the UPR-inhibitor

tauroursodeoxycholic acid (TUDCA, Figure 5B) prior to infection with RV-A16 and RV-C15 strains. Increased ER stress significantly inhibited RV-A, but less so RV-C replication ($p=0.11$ for $1\mu\text{g/mL}$). The converse, UPR inhibition with TUDCA augmented RV-A replication greater than 2-fold, but increased RV-C by less than 30%. We confirmed the suppressive effects of Tm and enhancement by TUDCA of RV-A16 replication in primary bronchial epithelial cells (Supplemental Figure E6). We have previously linked ER stress with augmented IFN- β production. Indeed, Tm was sufficient to induce abundant *IFNB1* mRNA expression in HeLa cells. Tm pre-treatment augmented RV-induced *IFNB1*, whereas TUDCA had the opposite effect (Figure 5C). These data raised the possibility that the process of knocking down *ORMDL3* was paradoxically increasing ER stress. To test this idea, we first confirmed that the transfection reagent alone did not upregulate BiP/*HSPA5* or CHOP/*DDIT3* mRNA (not shown). To determine how *ORMDL3* siRNA affected type I IFN and ER stress, IFN- β and UPR target genes were evaluated in knockdown cells infected with RV (Figure 5D). In the *ORMDL3* siRNA knockdown cells, RV-A16 infection resulted in lower levels of IFN- β and UPR target gene induction, correlating with the decreased detectable RV. Together these data suggested that *ORMDL3* knockdown was not inhibiting RV-A replication by increasing type I IFN or ER stress.

SPT inhibition enhances RV-A16 replication

In light of these results, we next focused on the other major activity of *ORMDL3*, SPT inhibition. Overexpressing *ORMDL3* induces ER stress (23, 36); therefore, it would be difficult to tease apart conflicting effects of ER stress and SPT inhibition using *ORMDL3* transfection. Myriocin is a highly “specific” SPT inhibitor (19) and hence inhibits *de novo* sphingolipid synthesis (37). In this way, it acts as an *ORMDL3* mimic without inducing ER stress (data not shown). HeLa-E8 cells were treated with different concentrations (5, 10, and 20 $\mu\text{g/mL}$) of

myriocin for 3 h, and then infected with RV-A16 or RV-C15 (Figure 6). Myriocin at 5 $\mu\text{g/mL}$, and even more so at 10 $\mu\text{g/mL}$ significantly increased RV-A16 replication, but not RV-C15 replication (Figure 6A). Myriocin also increased RV-A16 replication in primary bronchial epithelial cells (Figure 6B, Supplemental Figure E6). Furthermore, myriocin was able to restore RV-A replication suppressed by *ORMDL3* siRNA (Figure 6C). Together this data suggested the effect of *ORMDL3* knockdown on RV-A replication is mediated through increased SPT activity.

In the murine *ORMDL3* transgenic model, *ORMDL3* over-expression exerted specific effects on individual circulating ceramide species (42). We hypothesized that certain *ORMDL3*-regulated sphingolipid species might be detrimental to RV replication. To define the effect of myriocin and *ORMDL3* siRNA on the sphingolipid milieu in our experimental system, we took a discovery lipidomics approach (Figure 7, Supplemental Figure E7). HeLa cells were untreated (NT), treated with myriocin or transfected with control, or *ORMDL3* siRNA. We identified 453 distinct lipid species. Average inter-replicate coefficient of variation was 22.6%. Perhaps not unexpectedly, transfection of control siRNA with the cationic lipid-based transfection reagent profoundly affected the lipid status of the cell, making this an essential comparator for the specific effects of *ORMDL3* siRNA. The diverse lipid perturbations from these treatments are evident in the principal components analysis (PCA), Figure 7A. In assessing overall effects of the treatments on sphingolipids (Supplemental Figure E7), about one third were downregulated by myriocin, and one-third upregulated by all three treatments compared to no treatment. However, myriocin and *ORMDL3* siRNA disparately affected specific species of sphingolipids, particularly among the hexosylceramides (bar in Figure 7B) and one of the ceramide species (arrows in 7B and E7). The sphingolipid species with the greatest increase during *ORMDL3*

knockdown was Cer(4) d18:0_24:1 (~3-fold, $p=0.037$, Figure 7B). Spingosine-1 phosphate was not identified in this unbiased lipidomics analysis.

Discussion

In this study, we sought to gain a greater understanding of the interaction between *ORMDL3* expression and RV infection in human epithelial cells. *ORMDL3* knockdown reproducibly suppressed RV-A replication in HeLa cells, primary nasal and bronchial epithelium and airway epithelial cell lines. RV-B, RV-A major and minor group strains were comparably affected, despite having different surface receptors for attachment (ICAM-1 and LDLR). Indeed, surface staining of the ICAM-1 receptor and internalization studies suggest *ORMDL3* expression is important later on in the viral life cycle. In comparison to the RV-A viruses, *ORMDL3* siRNA decreased only one of the three RV-C strains tested (RV-C2). C15 and C41 strains are closer phylogenetically, with C2 belonging to a different clade (33, 38, 39), however, the reason for the difference in effect of *ORMDL3* knockdown is not clear. The results from pharmacologic manipulation of ER stress and use of the SPT inhibitor myriocin suggest the SPT inhibition activity of *ORMDL3* is more likely to play a role in supporting RV replication than its effects on UPR induction.

The decrease in UPR and IFN genes observed with *ORMDL3* siRNA may reflect both direct contributions from decreased *ORMDL3* expression, as well as indirect effects related to reduced RV-A replication. Selectively inhibiting the UPR with TUDCA enhanced RV-A replication. Interestingly UPR inhibition did not significantly impact RV-C15, suggesting RV-A may be more sensitive to infection-associated ER stress. Pharmacologically-induced ER stress negatively affected replication of both RV-A and less so RV-C viruses. This effect may be mediated by the increased type I IFN observed in these treatments. Our results are consistent with a report in

cystic fibrosis epithelial cells, in which pharmacologically enhanced UPR suppressed RV replication (35). Our results with exogenous tunicamycin are also consistent with the implications of a previous report examining the interaction between ORMDL3 and RV infection in a mouse model. Song et al reported decreased RV viral load in the ORMDL3 overexpressing transgenic mouse model (11). ORMDL3 overexpression has also been shown by this group to cause induction of a UPR signaling pathway (ATF6) and increased expression of CXCL10 *in vitro* and *in vivo* (36). Infection of these ORMDL3 transgenic mice resulted in much greater type I IFN production and IFN-response genes, likely causing the decrease in RV load. It should be noted however that RV does not replicate well in any wild-type or transgenic mice. What our study highlights is that the converse does not necessarily hold: in our study, decreased ORMDL3 did not augment RV replication.

The results of UPR manipulation in this study suggested *ORMDL3* knockdown must influence RV through a mechanism other than ER stress modulation, namely altered SPT activity. In support of this idea, the greater impact of *ORMDL3* knockdown on RV-A vs RV-C viruses was consistent with the observation that the SPT inhibitor myriocin augmented RV-A16, but not RV-C15 replication. The capacity of myriocin to counteract *ORMDL3* siRNA also supports the idea that the RV-suppressing effect of *ORMDL3* knockdown is related to decreased SPT inhibition (myriocin achieves enzymatic “epistasis”). The role of ORMDL3 in regulating SPT has been contentious in the literature. There have been reports that *ORMDL3* knockdown alone is insufficient to impact SPT (40). However, other studies have shown an effect of ORMDL3 on specific sphingolipid entities: *ORMDL3*^{-/-} epithelial cells had an increase in sphingosine-1-phosphate, a product of SPT activity, and ORMDL3 transgenic mice had decreased sphinganine and ceramide 24:0 in serum (41, 42). In a study by Oeniyran et al.(12), *ORMDL3* siRNA

increased C:24 Ceramide species. In liver cells, *ORMDL3* siRNA increased dihydrosphingosine and multiple dihydroceramide species. Thus, the effect of decreased *ORMDL3* may be specific to different cell types and degree of gene expression change (knockdown vs knockout, timing, and compensatory mechanisms). In our experiments in HeLa cells, *ORMDL3* knockdown exerted a limited effect on the sphingolipid pool, significantly increasing Cer(5) d18:0_24:1 (~3 fold, Figure 7) and less significantly Cer[NS](5) d 18:0_16:0 (2-fold, uncorrected $p < 0.05$). Decreases in multiple ceramide and hexosylceramide species upon myriocin treatment is consistent with expectations for an inhibitor of de novo sphingolipid synthesis. However, one sphingomyelin species paradoxically increased with myriocin treatment (SM 33:1) perhaps reflecting compensatory salvage pathways.

Sphingolipids regulate multiple steps in viral replication, from receptor binding and endocytosis, to replication and budding of new particles (43). Rhinoviruses enter cells via ceramide and GM1-rich lipid rafts (44). Acid sphingomyelinase favors rhinovirus replication by generating ceramides (45). However, too much ceramide may adversely affect membrane fluidity. One way to interpret our results is that a decrease in overall ceramide or hexosylceramide levels are beneficial to RV-A replication, and elevation of particular ceramides, those possessing 18 or 24 carbon acyl chains could be problematic for replication. This idea is also consistent with a report on host lipid changes resulting from RV infection, as assessed via lipidomics (46). In that study, Ceranib 1, a ceramidase inhibitor (decreases sphingosine and increases ceramides) markedly suppressed RV-1A replication. This report also identified a marked decrease in sphingosine-1-phosphate synthesis during RV infection, again suggesting decreased SPT activity may favor RV replication.

One would not expect the consequences of SPT modulation to be isolated to immediate lipid metabolites in the sphingolipid pathway, since sphingolipid synthesis is part of a greater interacting network of lipid regulation. Therefore, some of the effects of myriocin vs *ORMDL3* siRNA treatment may reflect changes in other lipid species. Formation of RV replication domains at the ER-Golgi interface requires lipid remodeling through phosphatidylinositol 4-phosphate (PI4P) – cholesterol “counter currents” (27). Thus, generation of specific phosphatidylinositol substrates could aid or hamper this process. The lipidomic study from Nguyen et al. identified increases in shorter chain phosphoinositols (C32 and C34) and myriocin treatment also increased these species (Supplemental Figure E8)(46). *ORMDL3* siRNA also increased 16 chain species of phosphoglycerides, but the significance of these findings is not yet clear. Finally, RV-A but not RV-C bind short 12-carbon fatty acid-like molecules known as “pocket factor” (47). Although the role of pocket factor in replication is somewhat contentious, different effects of *ORMDL3* vs. myriocin on the generation of pocket factor could affect RV-A over RV-C. Future studies will investigate which aspect of the rhinovirus life cycle is impacted by these opposing manipulations.

The goal of the present study was to determine how differences in *ORMDL3* expression regulate RV infection. The data presented here identify a previously unknown role for *ORMDL3* and its target SPT in regulating RV-A replication in human epithelial cells. Putting these data into context with previous reports (Supplemental Figure E9), optimal RV replication may require a balance between helpful sphingolipid pool modulation by *ORMDL3* and the predilection of *ORMDL3* to cause ER stress. When *ORMDL3* expression is low, the negative impact of specific ceramide generation may outweigh the benefit of decreased ER stress. In the setting of high *ORMDL3* expression, increased ER stress and type I IFN production may predominate (11). In

regards to asthma susceptibility, our results suggest decreased ORMDL3 expression (seen in children less likely to develop asthma (3)) may be protective by limiting the extent of RV replication in epithelial cells. On the other hand, increased ORMDL3 may exacerbate illness through UPR augmented cytokine responses to viral infection (23). It will be interesting to see how these opposing roles for ORMDL3 in RV infection play out in human subjects.

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Figure Legends:

Figure 1: RV-A16 co-localizes with ORMDL3 and increases mRNA expression of *ORMDL3*, UPR target genes and type I interferon. A) HeLa-H1 cells were infected with RV-A16 for 48 h, and then lysed in Trizol. RNA was reversed transcribed and gene expression analyzed by qPCR with normalization to 18S rRNA. Bars depict averages of independent experiments with error bars denoting SEM. (N=3-6 independent experiments for individual genes). NI= No Infection. B) HeLa-H1 cells were infected with RV-A16 for 48h and then fixed. The cells were co-stained with anti-ORMDL3 (green), anti-RV-A (red), anti- β -actin (purple) antibodies, and nuclei were stained with DAPI. Bar is 10 μ m.

Figure 2: ORMDL3 supports RV-A replication in human epithelial cells. Open bars: uninfected. Black bars: RV-A16 infected. A). *ORMDL3* expression and protein: HeLa cells were transfected with control (C-Si) or *ORMDL3* siRNA (ORMDL3i) 24h prior to infection for 48h. Cells were processed for qPCR and data normalized to 18S rRNA (left, N=5). Right: cell lysates from siRNA-transfected cells were analyzed for ORMDL protein by western blot. ORMDL optical density (O.D.) was normalized to β -actin O.D. (right, N=3, $p < 0.05$ vs. NT and control siRNA). B) Samples from (A) were analyzed by qPCR for RV-A16 RNA with normalization to 18S rRNA. Results are from 5 independent experiments. C) A PFU assay from siRNA transfected cells following 48h of infection. N=3 independent experiments. $P < 0.001$ compared with NT or C-Si cells. D) BEAS-2B cells were knocked down for *ORMDL3*, infected with RV-A16 for 48h and processed as above. QPCR results were normalized to 18S rRNA. N=4 independent experiments. E) Primary nasal epithelial cells (NEC) were knocked down for *ORMDL3* and then infected with RV-A16 for 48h. RNA was processed for qPCR. RV-A RNA was normalized to C-Si samples (set=1). The experiment was repeated with 4 individual donors.

F) Primary bronchial epithelial cells from 1 donor (BEC-53) were knocked down for *ORMDL3*, infected with RV-A16 for 48h, processed and normalized as in (E). N=3 independent experiments using cells from this donor.

Figure 3: Different effects of *ORMDL3* knockdown on replication of various RV strains.

HeLa-E8 cells were transfected with *ORMDL3* or control siRNA, and infected with 3 strains of RV-A (1, 16 and 36), RV-B52, and 3 strains of RV-C (2, 15, and 41). 48h later, RNA was harvested and quantitated for RV by qPCR. Levels of RV in the *ORMDL3* knockdown cells was normalized to control siRNA transfected cells (control SiRNA set =1, N=4 experiments).

Figure 4: *ORMDL3* knockdown does not affect ICAM-1 expression or decrease initial RV-

A16 internalization. A) HeLa-E8 cells were uninfected or infected with RV-A16 in triplicate for 48 hours and then stained with anti-ICAM-1 or no antibody for analysis by flow cytometry. Top panel shows representative histograms (lines: uninfected; gray: RV infected). Bottom panel: Open bars are uninfected cells and black bars are RV-16A-infected cells. gMFI: geometric mean fluorescence intensity. Histograms are representative of N=3. B) HeLa-E8 cells were infected with RV-A16 for 2h, washed, lysed, and RNA processed for qPCR detection of RV RNA. Bars are means +/- SEM from N = 3 experiments.

Figure 5: UPR induced by low dose tunicamycin inhibits RV replication. A) HeLa-E8 cells were pre-treated with vehicle, 0.5 μ g/mL or 1 μ g/mL tunicamycin (Tm) 1h prior to 48h infection with RV-A16 (black) or RV-C15 (hatched). qPCR results were normalized to vehicle treated controls. N=5 for RV-A infections and N=3 for RV-C. B) HeLa-E8 cells were pretreated with 0.5 mg/mL or 1mg/mL tauroursodeoxycholic acid (TUDCA) 1h prior to infection and analyzed as in (A), N=4. C) Samples from (A) and (B) were assessed for IFN- β (*IFNB1*) by qPCR, N=3.

D) HeLa-E8 cells transfected with control (C-Si) or *ORMDL3* siRNA (*ORMDL3i*) were infected with RV-A16 for 48h prior to RNA harvest. IFN- β (*IFNB1*) and UPR target genes (*HSPA5*/BiP, *DDIT3*/CHOP) were assessed by qPCR with normalization to 18S rRNA. N=3 experiments.

Figure 6: Myriocin, an SPT inhibitor enhances RV-A replication and counteracts *ORMDL3*

knockdown. HeLa-E8 cells were treated with different concentrations of myriocin for 3h, and then infected with RV-A16 (black) or RV-C15 (hatched) for 48h. RV RNA levels were quantified by qPCR, with normalization to vehicle control (N=5). B) Primary bronchial epithelial cells (BEC-53) were treated with 10 μ g/mL myriocin prior to infection with RV-A16 for 48h. RV levels quantified by qPCR (N=3). C) HeLa-E8 cells were not transfected, or transfected with control or *ORMDL3* siRNA the day before myriocin treatment (10 μ g/mL) and RV infection. Black bars are RV only, and striped myriocin+RV. Samples were processed for RNA, which was quantitated by qPCR with normalization to 18S rRNA (N=7).

Figure 7: Lipidomics analysis of HeLa myriocin treatments and siRNA transfections.

HeLa cells were treated in triplicate with 10 μ g/mL myriocin or transfected with control or *ORMDL3* siRNA for 72 h. Cells were pelleted and processed for analysis by lipid mass spectrometry. A) Principal components analysis (PCA) of the means for the different treatment groups. B) Sphingolipids displaying at least one significant difference between a treatment group (myriocin or *ORMDL3* siRNA) and the untreated controls when adjusted for FDR (q-values designated with asterisks) and at least an 0.7-fold change. The 8 ceramide species significantly impacted by control siRNA alone are not shown. Cer[AS] and Cer[NS] refer to ceramide species (alpha-hydroxy fatty acid sphingosine and non-hydroxy fatty acid sphingosine respectively), HexCer[NS] to hexosylceramide non-hydroxy fatty acid sphingosine species and SM to

sphingomyelin. Note the log scale. Arrow: ceramide specie significantly increased by *ORMDL3* siRNA.

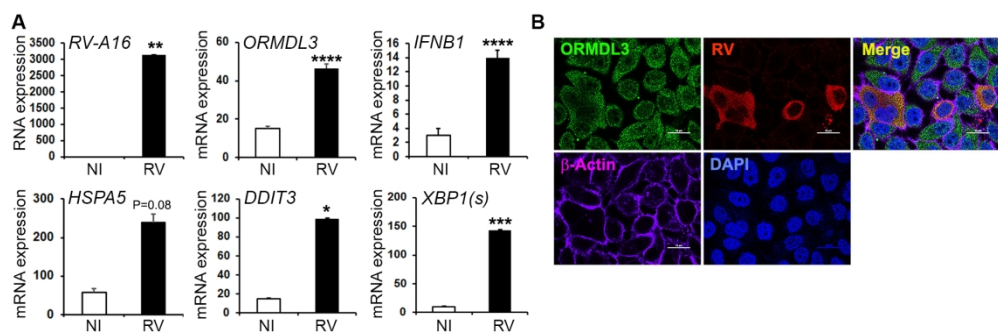


Figure 1

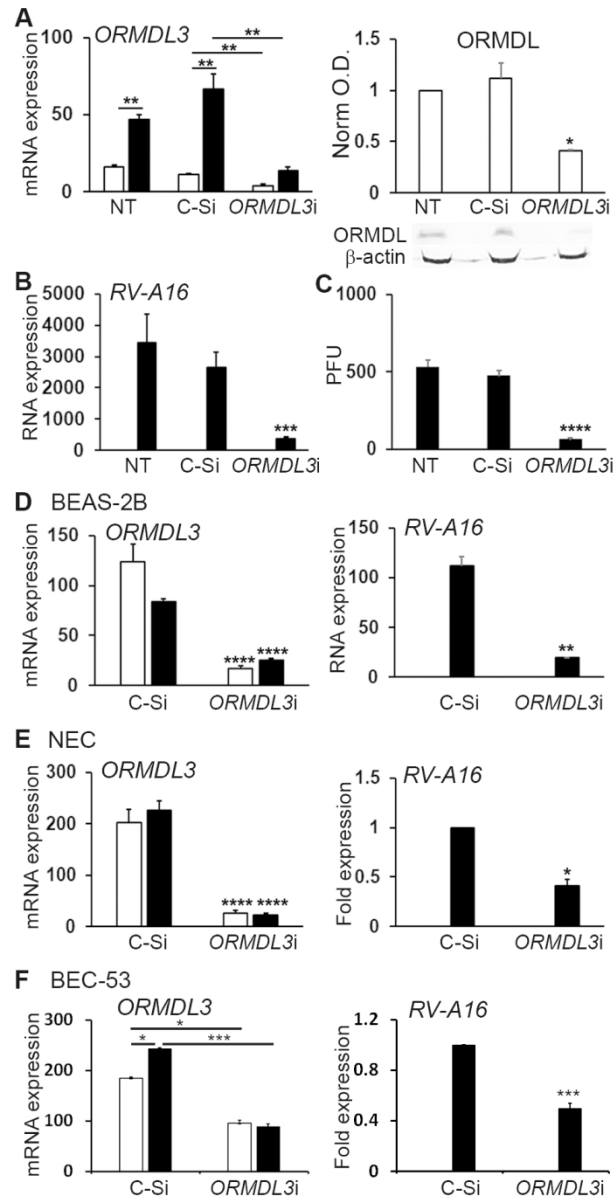


Figure 2

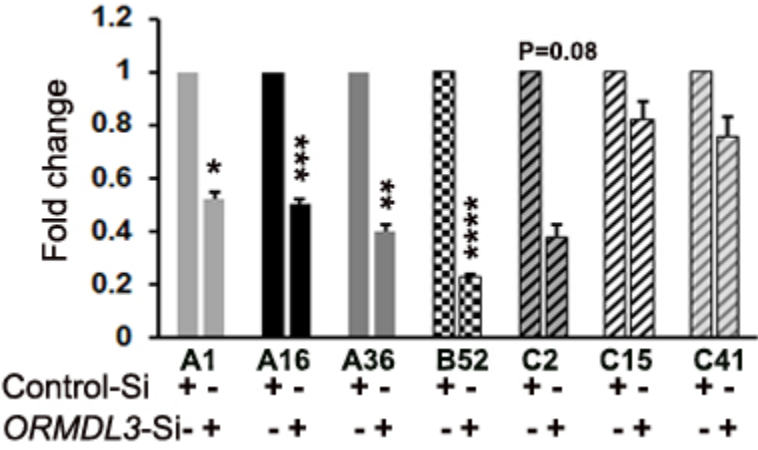


Figure 3

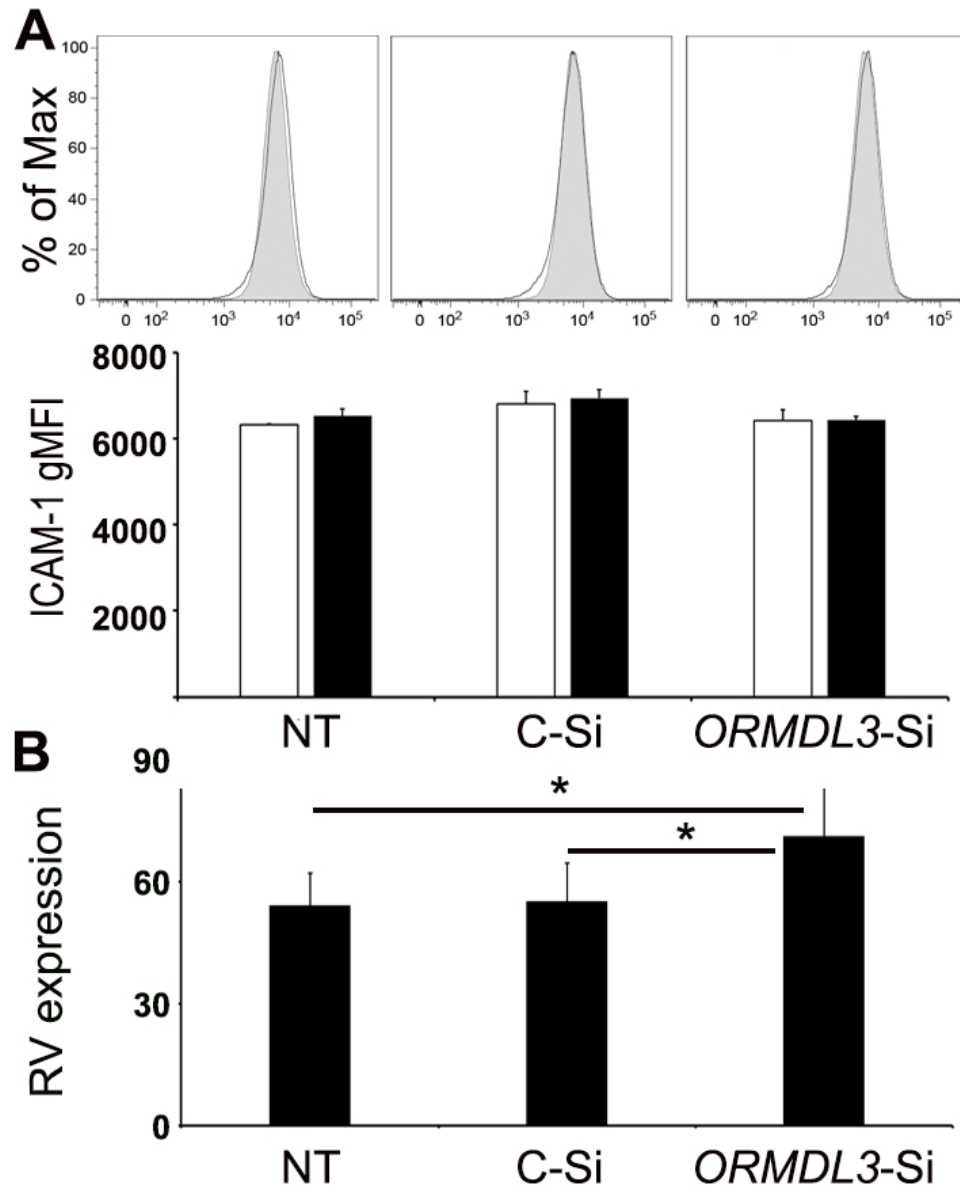


Figure 4

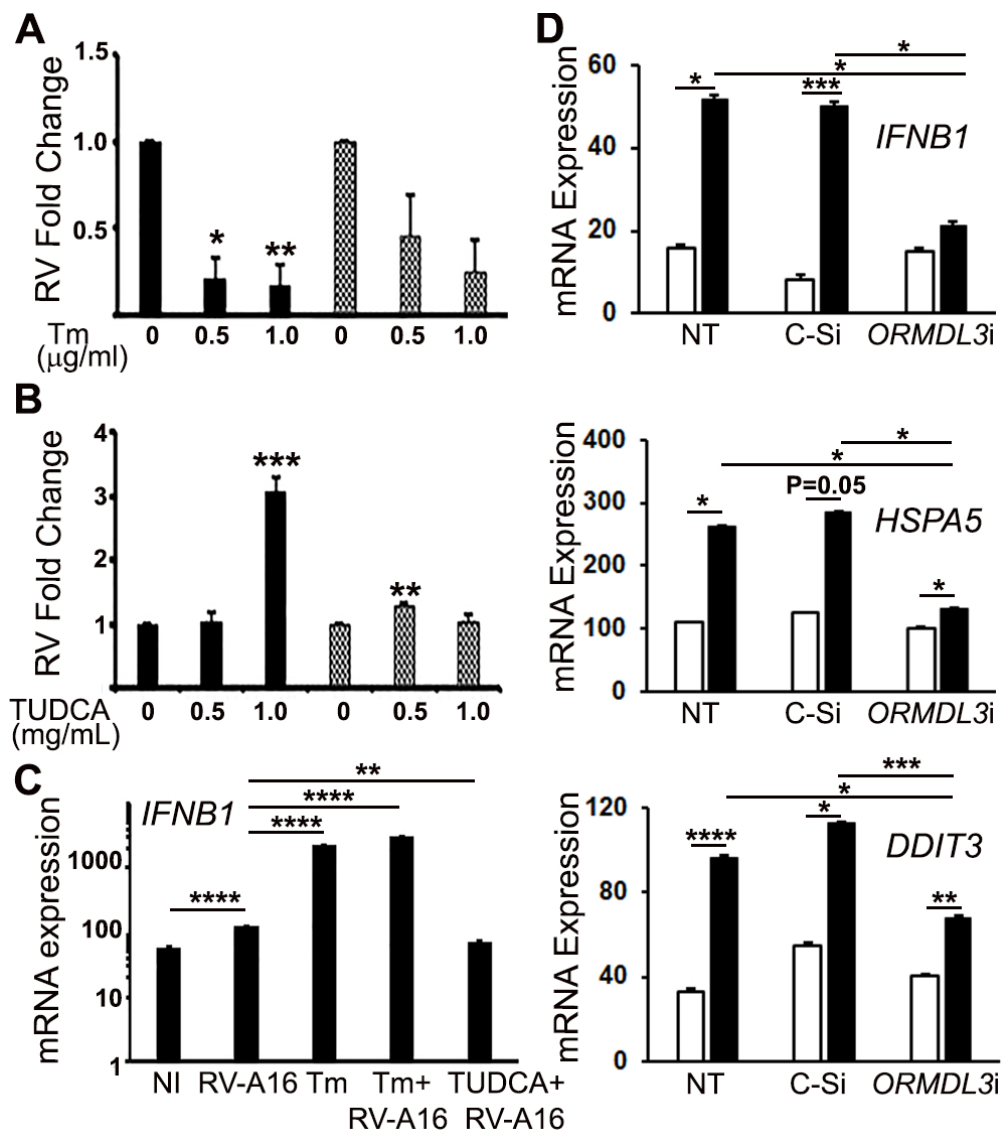


Figure 5

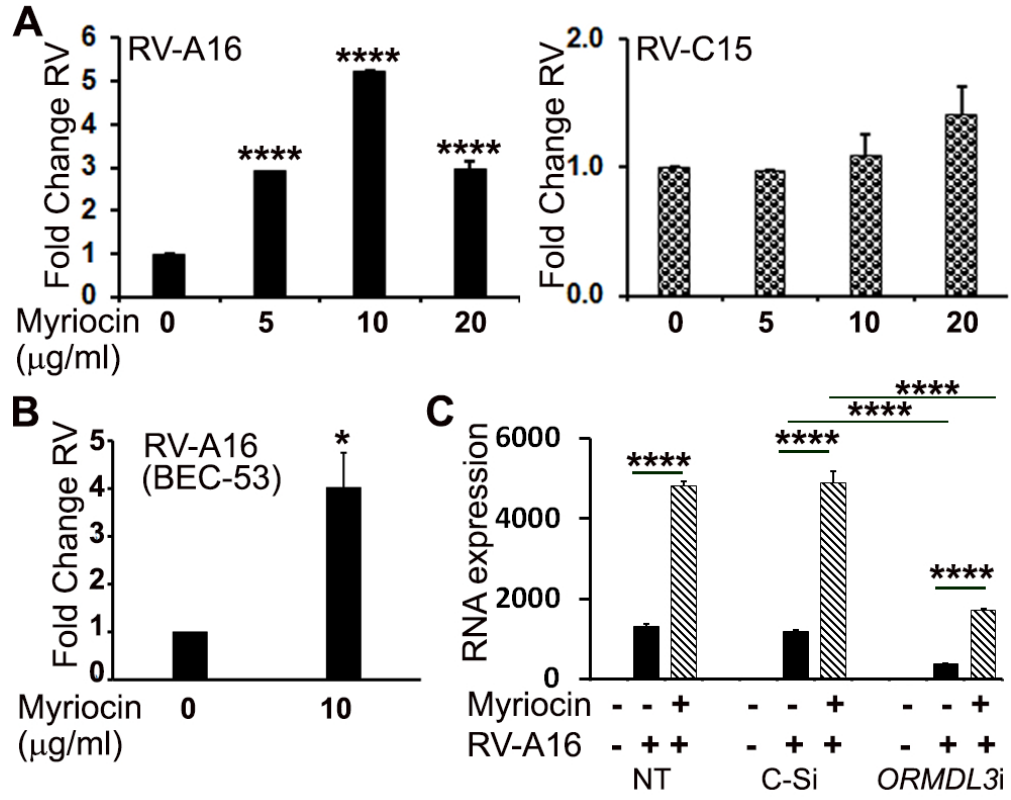


Figure 6

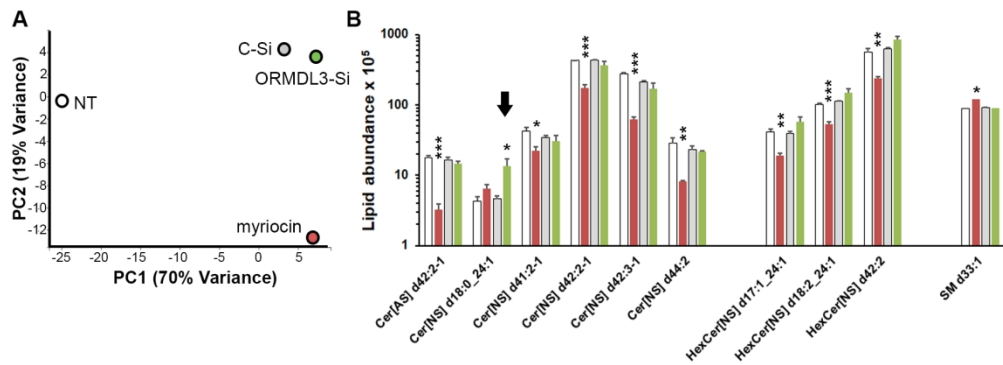


Figure 7

Online Data Supplement

Cells and Rhinoviruses:

HeLa-H1 (American Type Culture Collection (ATCC) #CRL-1958) and HeLa-E8 cell lines were maintained in DMEM/high glucose (Mediatech), 10% fetal bovine serum (FBS) (Hyclone) and antibiotic–antimycotic solution (Mediatech). HeLa-E8 cells have been transduced to stably express the RV-C receptor Cadherin Related Family Member 3 (CDHR3) (1, 2). In general, HeLa-H1 cells, which do not naturally express CDHR3, were used for RV-A16 infections and HeLa-E8 cells, which express both ICAM-1 and CDHR3, for experiments directly comparing infections with RV-A, RV-B and RV-C species. BEAS-2B bronchial epithelial cells, A549 human alveolar basal epithelial cells and NCI-H358 non-small cell lung cancer cells (ATCC) were cultured in 5% FBS BEGM media (Lonza). Primary human nasal epithelial cells (NEC) were collected from the inferior turbinate following the protocol described (3). Primary human bronchial epithelial cells (BEC-05 and BEC-53) were obtained by enzymatic digestion of residual bronchial tissue from lung transplants as previously described, cultured in collagen-coated flasks and used at passage 2-3 (4). Human rhinovirus (RV) type A1a was obtained from ATCC (#VR-1559). RV-A16 is a previously described laboratory strain (5, 6). RV-C15, RV-A36, RV-B52, RV-C2 and RV-C41 are clinical isolates that were cloned in plasmid vectors and produced by reverse genetic methods (7, 8).

Western blot experimental details:

PVDF membranes were incubated with rabbit anti-ORMDL3 antibody, used at a concentration of 1 µg/mL and mouse anti-β-actin at 0.5 µg/mL overnight at 4° C. Blots were subsequently

incubated with IRDye a800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit secondary antibodies, both at 0.1 µg/mL at room temperature for 30 minutes.

Primer sequences:

Primers were designed using Beacon design software (Premier Biosoft).

18S rRNA, F: GGACACGGACAGGATTGACAG3; R: ATCGCTCCACCAACTAAGAACG;

IFNB1, F: TGGCTAATGTCTATCATCA; R: CTCAGTTTCGGAGGTAA;

HSPA5, F: GTGGAATGACCCGTCTGTG; R: TTGGTTGCTTGGCGTTGG;

DDIT3, F: CTAGTGCCAATGATGTGA; R: ATATACAAGCTGAGACCTT;

ORMDL3, F: ATCCCGTTTGTGAGTGTCC; R: CTTAGTGTAGAAGCTG GTGAGG;

ORMDL1, F: AATGGCTGGTCCTTCAAGTGCT; R: ACCCTCACTGTGATGCCCTTTA;

ORMDL2, F: ACACACTGGGAGCAAATGGACT; R: AGTGCGCAGCATCATACTTGGT;

spliced *XBPI*, F: TGCTGAGTCCGCAGCAG GTG, R: GCTGGCAGGCTCTGGGGAAG;

RV: F-CCTCCGGCCCCCTGAAT, R-AAACACGGACACCCAAAGTAGT.

RV plaque assay:

The RV plaque assay was performed as previously described (9). HeLa cells were transfected with control siRNA, ORMDL3 siRNA or mock-transfected. 24h later, cells were infected with RV-A16 at the concentration of 2pfu/cell for 48h. The infected HeLa cells were trypsinized and lysed with 3 rounds of -80°C freeze and thaw. The cell solution was centrifuged at 12000g for 30 min. at 4°C. The RV-containing supernatant was serially diluted with DMEM to 10x, 100x and 1000x dilutions. These dilutions were used to infect confluent HeLa cells in 6-well plates. After

3 h of incubation at 34°C, the liquid medium was removed and replaced with 1.5 mL of EMEM (Mediatech) containing 0.04 M magnesium chloride, 0.1% BSA, and 0.8% DIFCO noble agar (Fisher Scientific Cat# BD 214230). When this layer had solidified, an additional 1.5 mL of the above medium, lacking agar was added. The plates were incubated at 34°C for 2 days. The cells were then fixed by the addition of 3.4% formaldehyde and stained with 0.1% crystal violet in 20% ethanol. Plaques were counted to enumerate infected cells.

Lipid extraction and mass spectrometry:

In triplicate, HeLa-E8 cells were untreated, or treated with 10 µg/mL myriocin, or transfected with control siRNA or ORMDL3 siRNA for 72h. Cell pellets (1 million cells) were stored at -80°C until analysis. Lipids were extracted using previously published techniques (10) Briefly, 100 µL glass beads (425-600µM, 30-40 US sieve and ice-cold 900 µL CH₃Cl:MeOH (2:1 v/v) were added to samples. The samples were vortexed and chilled on ice for 30s 4 times and transferred to a fresh tube. To the extract, 200 µL 1M HCl was added, samples were vortexed and chilled on ice for 30s 4 times. For phase separation, samples were centrifuged 2 min at 5000g at 4°C. The aqueous layer was discarded and 450 µL of organic layer dried by vacuum concentrator for 1.5h. Dried samples were reconstituted with 100 µL ACN:IPA:H₂O (65:30:5 v/v/v). Samples were analyzed as previously described (11) 10 µL of sample was injected onto an Acquity CSH C18 column held at 50 °C (100 mm x 2.1 mm x 1.7 µm particle size; Waters) using a Vanquish Binary Pump (400 µL/min flow rate; Thermo Scientific). Mobile phase A consisted 10 mM ammonium acetate in ACN:H₂O (70:30, v/v) with 250 µL/L acetic acid and Mobile phase B consisted of 10 mM ammonium acetate in IPA:CAN (90:10 v/v), also with 250 µL/L acetic acid. The following gradient was used: 0-2 min at 2% Mobile phase B, 2-5 min

increasing to 30% B, 5-6 min increasing to 50% B, 6-20% increasing to 85%, 20-21 min increasing to 99%, then 28-30 min at 2% B. The LC system was coupled to a Q Exactive Orbitrap mass spectrometer (MS) through a heated electrospray ionization (HESI II) source (Thermo Scientific) with polarity switching. Source and capillary temperature was set at 300 °C, sheath gas flow rate at 25 units, auxiliary gas flow rate at 10 units, sweep flow gas rate at 0 units, spray voltage at 5.0 kV (positive mode) and 3.5 kV (negative mode) and S-lens RF at 90.0 units. Full MS and MS2 spectra (Top2) were collected for each polarity. MS scan resolution was set to 17,500, with 1×10^6 automatic gain control (AGC) target, 100 ms ion accumulation time (max IT) and 200-1600 m/z scan range. MS2 scans were collected with a resolution of 17,500, 1×10^5 AGC target, 50 ms max IT, 1.0 m/z isolation window, stepped normalized collision energy (NCE) at 20, 30, 40, and a 10.0 s dynamic exclusion. Data were processed using Compound Discoverer 2.1 (Thermo Scientific) and Lipidex (11). Chromatographic peaks within 0.4 min to 23 min retention time and 100 Da to 5000 Da precursor mass were aggregated into distinct compound groups using a 10-ppm mass and 0.2 min retention time tolerance, and minimum peak intensity of 5×10^6 , maximum peak-width of 0.75, a signal to noise ratio of 1.5 and 5-fold intensity increase over solvent blanks. Spectra were searched against the LipiDex HCD Acetate library. Only matches with a dot product score >500 and reverse dot product score >700 were assigned. Lipid MS/MS spectra which contained no significant interference (<75%) from co-eluting isobaric lipids, eluted within a 3.5 median absolute retention time deviation (M.A.D. RT) of each other, and found within at least 2 processed files were then identified at the individual fatty acid substituent level of structural resolution. If individual fatty acid substituents were unresolved, then identifications were made with the sum of the fatty acid substituents. Peak areas were normalized to sum of identified lipids within a sample.

To generate the z-score lipidomics heatmap, data were transformed using R function scale (R Core Team (2017). R: a language and environment for statistical computing, Vienna, Austria, R Foundation for Statistical Computing) into z-scores, and then visualized with the pheatmap (Kolde, R. (2015). Pheatmap: Pretty Heatmaps) function of the pheatmap package in R.

Statistical generalized linear model:

A generalized linear model with an identify link function was used to compare normally distributed outcomes between experimental condition. In this analysis, experimental condition was included as a predictor variable in the model. Estimation was conducted using the least squares estimation approach.

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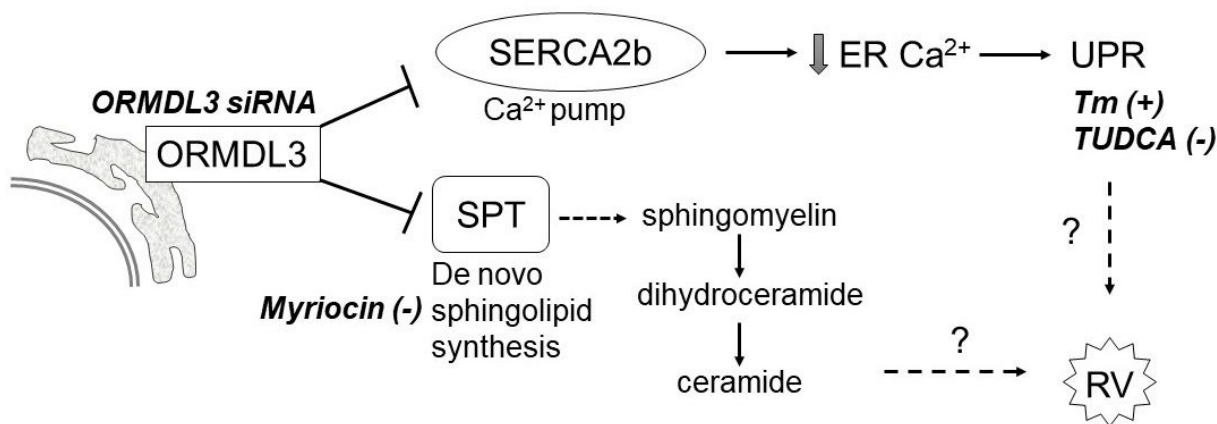
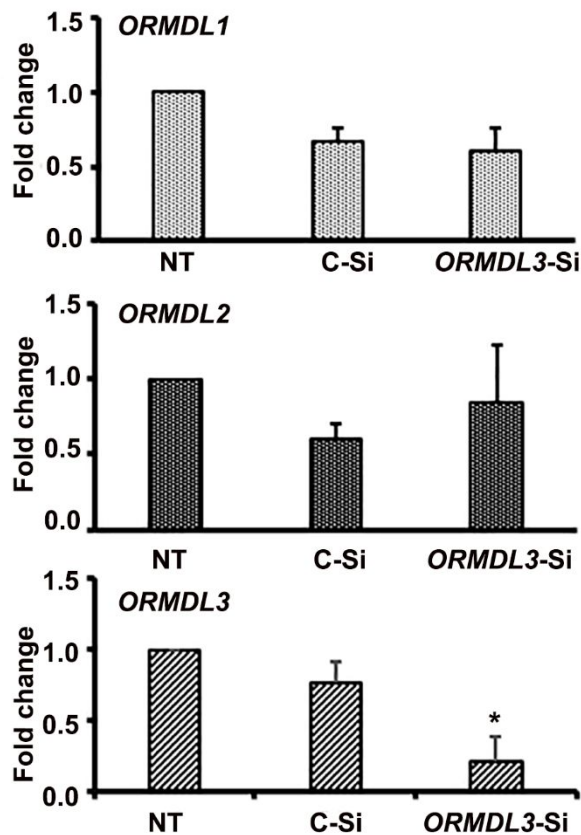
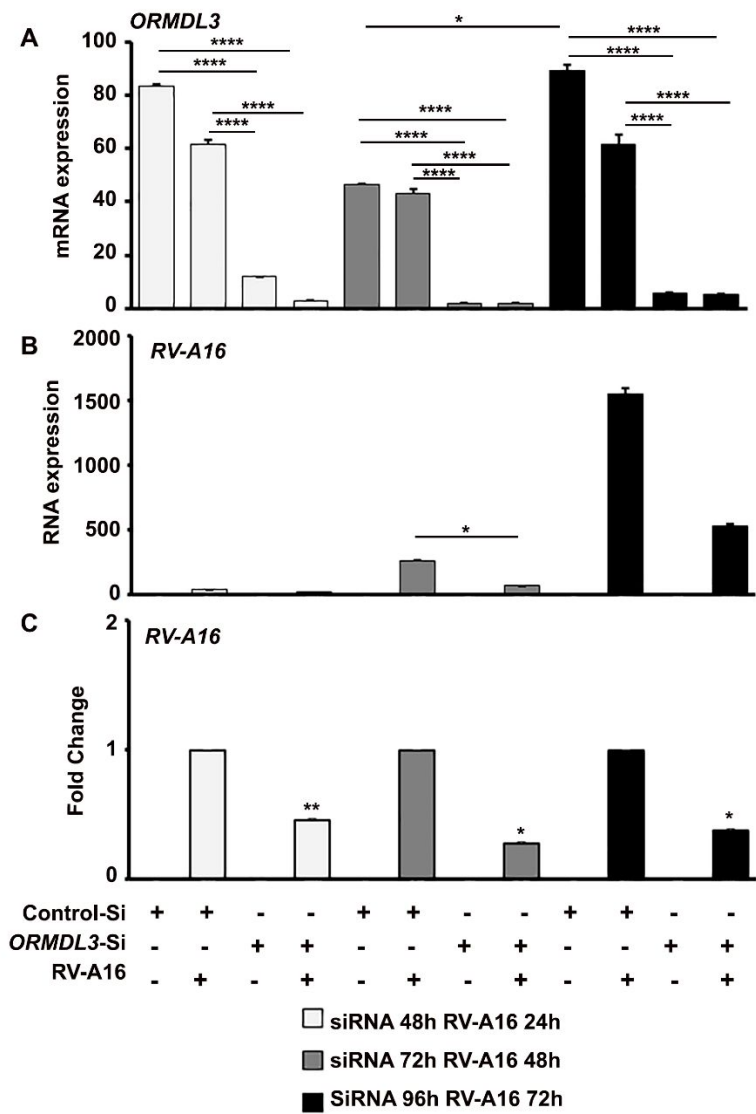


Figure E1: Study approach. To determine the role of ORMDL3 in RV replication in epithelial cells, the primary approach was gene knockdown via siRNA. *ORMDL3* knockdown would be expected to lead to decreased UPR induction and increased SPT activity. These two major outcomes of ORMDL3 activity were further investigated using pharmacologic manipulation via myriocin (SPT inhibitor), TUDCA (UPR inhibitor) and tunicamycin (Tm, UPR trigger).

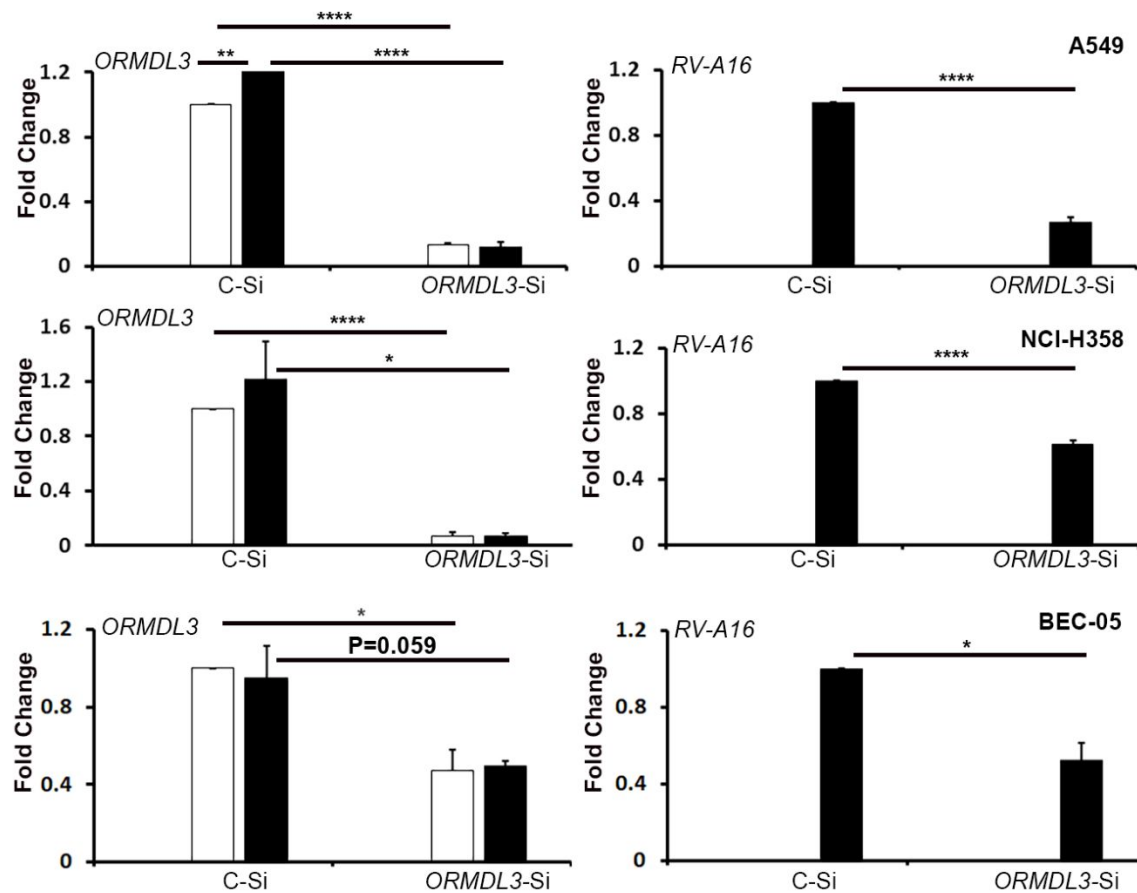


Supplemental Figure E2: *ORMDL3* siRNA did not alter *ORMDL1* or *ORMDL2* expression compared to control siRNA. H1 HeLa cells were untransfected (NT), transfected with control siRNA or *ORMDL3* siRNA for 72h. Expression of *ORMDL1*, *ORMDL2* and *ORMDL3* were determined by qPCR. (N=5).

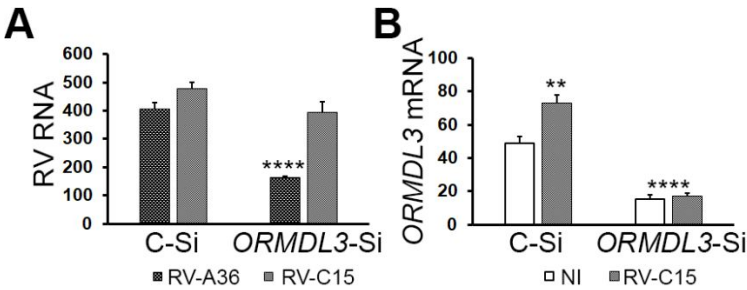


Supplemental Figure E3: Time course of ORMDL3 siRNA inhibition of RV-A16

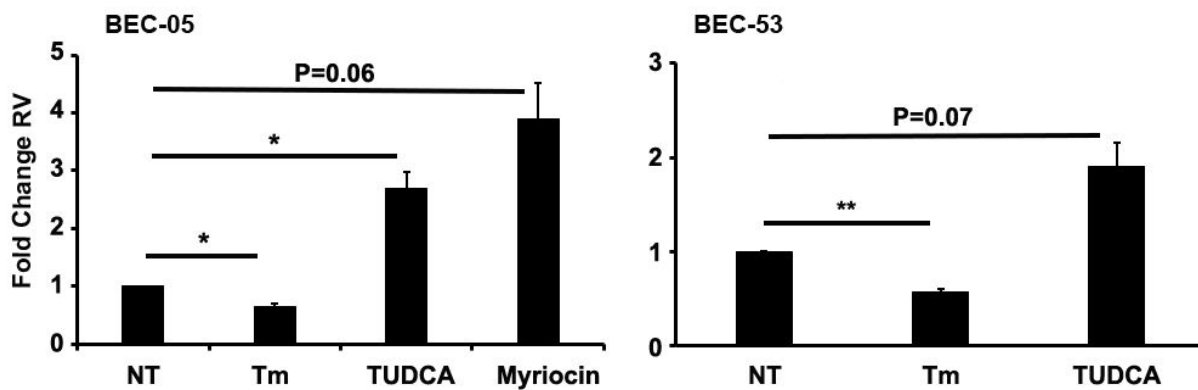
replication. HeLa-H1 cells were transfected with control siRNA or *ORMDL3* siRNA for 24 h, then infected with RV-A16. 24, 48 and 72 hours post infection, *ORMDL3* mRNA (A) and RV RNA levels (B, C) were quantitated by qPCR. B) Bars depict 18S rRNA normalized data, showing increase in RV levels over time. C) Bars represent fold change for *ORMDL3* siRNA vs. control siRNA in RV-A16 infected cells at each time point. N=3



Supplemental Figure E4: *ORMDL3* knockdown decreases RV-A16 replication in lung cell lines and primary bronchial epithelial cells. A549, NCI-H358, or primary bronchial epithelial cultures (BEC-05) were transfected with control siRNA or *ORMDL3* siRNA. The following day, the transfected cells were infected with RVA-16. After 48h, the cells were lysed and analyzed for expression of *ORMDL3* (left panels) or RV RNA (right panels) by qPCR with normalization to 18S rRNA and control siRNA uninfected cells (left) or control siRNA RV-infected cells (right). Open bars: uninfected. Black bars: RV infected. Results are from 4 independent experiments in cell lines and 3 in primary cells.



Supplemental Figure E5: Different impact of *ORMDL3* knockdown for RV viruses with similar levels of replication in HeLa-E8 cells. A) HeLa-E8 were transfected with control siRNA or *ORMDL3* siRNA, then infected side by side with RV-A36 or RV-C15 for 48h prior to RNA-harvest. RV levels were quantitated using qPCR, with normalization to 18S rRNA (N=4 independent experiments). B) HeLa-E8 were transfected with control or *ORMDL3* siRNA 24h prior to infection with RV-C15. *ORMDL3* mRNA levels at 48h of infection were assessed by qPCR (N=8)



Supplemental Figure E6: Effects of UPR and SPT modulation on RV-A16 replication in

primary bronchial epithelial cells. Primary bronchial epithelial cell cultures (BEC-05 and

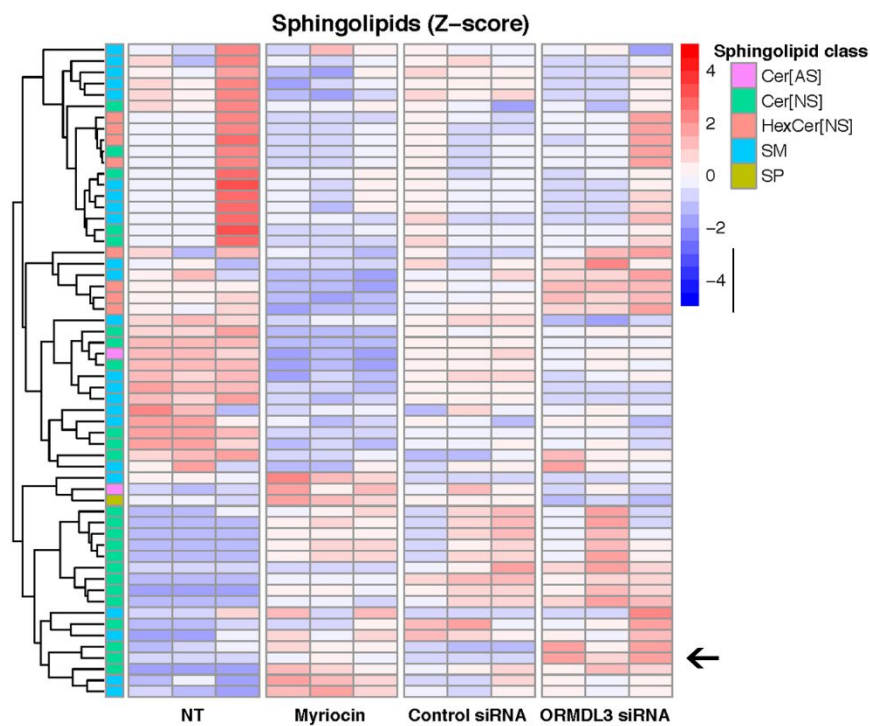
BEC-53) were untreated (NT), pre-treated with 1 µg/mL tunicamycin (Tm), 1 mg/mL

tauroursodeoxycholic acid (TUDCA), or 10 µg/mL myriocin prior to infection with RV-A16.

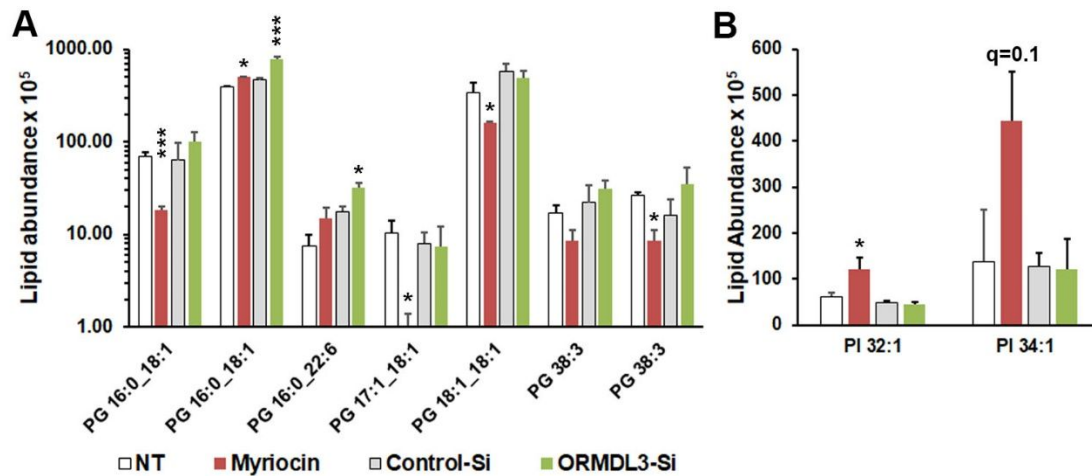
After 48h, cells were lysed, processed for RNA and RV levels determined by qPCR with

normalization to 18S rRNA and non-pre-treated controls (NT). N=3 independent experiments.

Myriocin results for BEC-53 are in Figure 6B of the main text.



Supplemental Figure E7: Sphingolipids from lipidomics analysis. Triplicate cultures of HeLa cells were untreated (NT), treated with 10 $\mu\text{g/mL}$ Myriocin or transfected with control or ORMDL3 siRNA for 72 h. Cells were pelleted and processed for analysis by lipid mass spectrometry. Graph is the heat map of sphingolipid z-scores. Vertical bar: hexosylceramides downregulated by myriocin but upregulated by *ORMDL3* siRNA. Arrow: ceramide upregulated by *ORMDL3* siRNA only.



Supplemental Figure E8. Impact of myriocin and *ORMDL3* siRNA on phosphoglycerides

and phosphoinositol. Triplicate cultures of HeLa cells were untreated, treated with 10 μ g/mL

myriocin or transfected with control or *ORMDL3* siRNA for 72h. Cells were pelleted and

processed for analysis by lipid mass spectrometry. A) Phosphoglycerides (PG) displaying at least

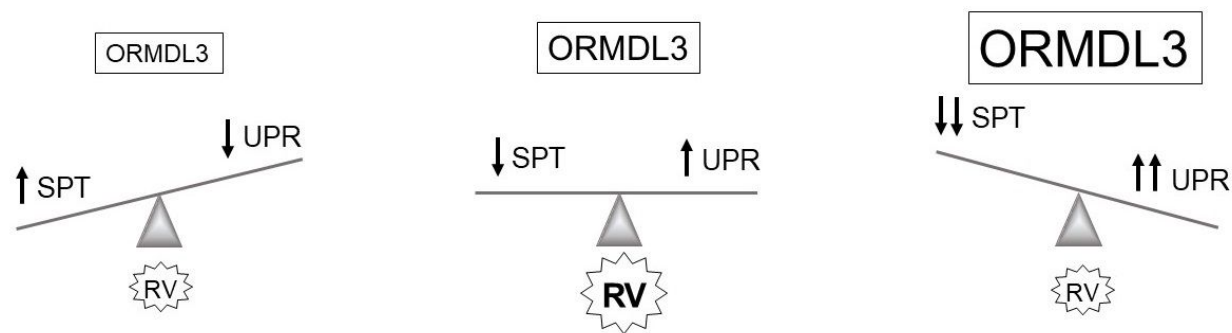
one significant difference between a treatment group (myriocin or siRNA) and the untreated

controls (NT) when adjusted for FDR (q-values designated with asterisks). Phosphoglycerides

significantly impacted by control siRNA alone are not shown. Note the log scale. B) Levels of

phosphoinositol 32:1 and 34:1. For PI 32:1, q-value=0.036 vs. NT. PI 34:1 increased >3-fold, but

only the uncorrected p-value was significant at 0.042.



Supplemental Figure E9: The balance between opposing functions of ORMDL3 and their impact on RV replication. Whereas SPT inhibition is helpful for RV replication, UPR induction and concomitant type I IFN appears to limit RV replication. When ORMDL3 levels are optimal, these two opposing effects balance and RV replication is supported. When ORMDL3 levels are high, the UPR-inducing function dominates and inhibits RV replication. In the setting of low ORMDL3 expression, the altered sphingolipid milieu, with increases in specific ceramides, becomes problematic for RV replication.