

Accepted Manuscript

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PII: S0091-6749(17)31427-6

DOI: [10.1016/j.jaci.2017.08.015](https://doi.org/10.1016/j.jaci.2017.08.015)

Reference: YMAI 12994

To appear in: *Journal of Allergy and Clinical Immunology*

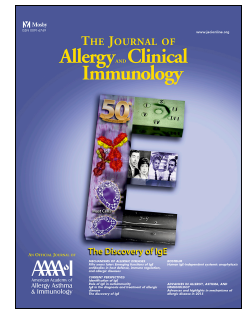
Received Date: 8 March 2017

Revised Date: 25 July 2017

Accepted Date: 24 August 2017

Please cite this article as: Chen J, Miller M, Unno H, Rosenthal P, Sanderson M, Broide DH, Ormdl3 Upregulates Airway Smooth Muscle Proliferation, Contraction, and Ca^{2+} Oscillations in Asthma, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.08.015.

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**ORMDL3 UPREGULATES AIRWAY SMOOTH MUSCLE PROLIFERATION,
CONTRACTION, AND Ca^{2+} OSCILLATIONS IN ASTHMA**

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Funding: This work was supported by NIH grants AI 107779, AI 38425, AI 070535, AI
72115, AI 242236 to D.H.B

ABSTRACT

Background: Airway hyperresponsiveness (AHR) is a major feature of asthma attributed predominantly to an extrinsic immune/inflammatory response increasing airway smooth muscle (ASM) contractility.

Objective: We investigated whether increased ASM expression of ORMDL3, a gene on chromosome 17q21 highly linked to asthma, induced increased ASM proliferation and contractility *in vitro*, as well as influenced airway contractility and calcium flux in ASM in precision cut lung slices from WT and hORMDL3^{Zp3-Cre} mice (which express increased levels of human ORMDL3).

Methods: Levels of ASM proliferation and contraction were assessed in ASM cells transfected with ORMDL3 *in vitro*. In addition, airway contractility and calcium oscillations were quantitated in ASM cells in precision cut lung slices derived from naïve WT and naïve hORMDL3^{Zp3-Cre} mice which do not have a blood supply.

Results: Increased ASM expression of ORMDL3 *in vitro* resulted in increased ASM proliferation and contractility. Precision cut lung slices derived from naïve hORMDL3^{Zp3-Cre} mice which do not have airway inflammation exhibit increased airway contractility with increased calcium oscillations in ASM cells. Increased ASM ORMDL3 increases ASM sarcoplasmic reticulum Ca²⁺ ATPase 2b (SERCA2b) which increases ASM proliferation and contractility.

Conclusion: Overall, these studies provide evidence that an intrinsic increase in ORMDL3 in ASM can induce increased ASM proliferation and contractility which may contribute to increased AHR in the absence of airway inflammation in asthma.

KEY MESSAGES

- Increased ASM expression of ORMDL3 induces increased ASM proliferation and contractility in vitro.
- Precision cut lung slices derived from naïve hORMDL3^{Zp3-Cre} mice (which express increased levels of human ORMDL3) and do not have airway inflammation exhibit increased airway contractility with increased calcium oscillations in ASM cells.
- In addition to extrinsic inflammation, intrinsic ASM expression of ORMDL3, can increase ASM contractility and proliferation and might contribute to increased AHR in asthma.

CAPSULE SUMMARY

This study demonstrates that an intrinsic increase in ORMDL3 in ASM can induce increased ASM proliferation and contractility which might contribute to increased AHR in the absence of airway inflammation in asthma.

KEY WORDS

- ORMDL3
- Asthma
- Airway smooth muscle
- SERCA2b
- Airway hyperresponsiveness

ABBREVIATIONS

AHR,	airway hyperresponsiveness
ASM,	airway smooth muscle
$[Ca^{2+}]_i$,	intracellular Ca^{2+} concentration
CPA,	cyclopiazonic acid
5-HT,	5-hydroxytryptamine
GWAS,	genome wide association study
MCh,	methacholine
MLC,	myosin light-chain
OG,	Oregon Green
ORMDL3,	oroscomucoid like 3
PCLS,	precision-cut lung slices
SERCA,	sarcoplasmic reticulum Ca^{2+} transport ATPases
SNP,	single nucleotide polymorphism
SR,	sarcoplasmic reticulum
TG,	transgenic
WT,	wild type

INTRODUCTION

Airway hyperresponsiveness (AHR) is a major feature of asthma¹. Conceptually AHR may either be due to an intrinsic abnormality in airway smooth muscle (ASM) resulting in increased contractility, or to an extrinsic effect of inflammatory cells and their mediators on ASM to increase contractility. The effects of extrinsic airway inflammation on ASM is considered to be the primary driving force for increased AHR mediated by ASM in asthma. Thus, the presence of airway inflammation in asthma forms the basis for NIH expert panel recommendations regarding early institution of anti-inflammatory therapy to reduce persistent asthma symptoms, exacerbations, and improve lung function and AHR¹. The episodes of airway inflammation in asthma are commonly triggered by either viruses or allergen and are associated with release of inflammatory mediators from a variety of lung resident (mast cell, epithelium, macrophage) and recruited cells (eosinophils, T cell, neutrophil) that may contribute to increased ASM contractility, AHR, airway remodelling, and decline in lung function¹.

In contrast to this well established role of extrinsic inflammation contributing to increased ASM contractility and AHR, the role of an intrinsic abnormality in ASM contributing to increased ASM contractility and AHR are less well established. Theoretically the contribution of an intrinsic abnormality in ASM to AHR may be mediated by either increased amounts of ASM, increased expression of contractile proteins in ASM cells, and/or increased activation of the contractile apparatus²⁻⁴. ASM contraction is initiated by the binding of calcium and calmodulin to activate myosin light chain (MLC) kinase, which

phosphorylates MLC to form cross-bridge cycling of MLC with actin filaments⁴. Thus, the regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) in ASM is essential for airway contraction. Mathematical models suggest that an increased amount of ASM in the airway submucosa is sufficient to cause AHR through mechanical effects alone⁵⁻⁷. The presence of increased airway smooth muscle mass in large and small airways in asthma is well described^{3,8-13} and is considered to be a major contributor to bronchoconstriction of airways, and persistent airflow obstruction^{6,11}. The increase in ASM in asthma can be due to hyperplasia³ and/or hypertrophy¹¹. There are studies demonstrating ASM hypertrophy in severe asthma^{10,11} but not in mild asthma³. There are several studies demonstrating that *ex vivo* asthmatic ASM proliferation (i.e. hyperplasia) is increased compared to non-asthmatics^{12,13}. Studies of ASM obtained by laser capture from mild asthmatics and controls have shown that ASM cell number was nearly twofold higher in subjects with asthma, and the amount of ASM in the submucosa was increased 50-83%³. As with studies of hypertrophy of ASM in asthma, there are some studies that have not demonstrated increased proliferation of asthmatic ASM *ex vivo*¹⁴. Thus, while there is extensive evidence from multiple laboratories that there is increased ASM in asthma^{3,8-13}, the mechanism mediating the increased ASM is incompletely understood. Current paradigms consider ASM as a target of mediators released from inflammatory cells in the airway that result in ASM changes (hypertrophy, hyperplasia) and increased AHR. Thus, the mechanisms behind these changes in ASM are considered to be mediated by repeated episodes of airway inflammation in asthma, rather than an intrinsic abnormality in ASM¹.

Our previous studies of ORMDL3 (oroscomucoid 3), a gene on chromosome 17q21 which is highly linked to asthma in genome wide association studies (GWAS)¹⁵, demonstrated that these mice generated to express universal increased levels of human ORMDL3 (hORMDL3^{Zp3-Cre} mice) spontaneously developed increased ASM, and increased AHR in the absence of airway inflammation¹⁶. These results suggest that ORMDL3 may be a key gene regulating ASM hypertrophy and hyperplasia, and more importantly, that lung structural cells (such as ASM), rather than inflammatory cells, expressing increased ORMDL3, may be the cells contributing to the increased AHR as observed in hORMDL3^{Zp3-Cre} mice. In this study, we make the novel observation that ORMDL3 upregulates cardinal features of asthma including ASM proliferation, contractility, and Ca²⁺ oscillations in ASM cells. We also provide evidence that ORMDL3 (localized to the endoplasmic reticulum)¹⁷ upregulates sarcoendoplasmic reticulum calcium ATPase 2b (SERCA2b) (localized in proximity to ORMDL3 in the sarcoplasmic endoplasmic reticulum)¹⁸ which regulates Ca²⁺ flux and contractility in ASM cells. Therefore, this ORMDL3, SERCA2b, Ca²⁺ oscillation, and ASM contractility pathway in ASM may be an important genetic pathway intrinsic to ASM leading to increased AHR in the 62% of asthmatics harboring the SNP on chromosome 17q21 linked to increased levels of ORMDL3 expression¹⁵. These studies support the paradigm that ASM changes and AHR in asthma are not only driven by extrinsic inflammation, but can also be mediated by an intrinsic abnormality in lung structural cells such as ASM.

METHODS

Generation of hORMDL3^{zp3-Cre} chimeric mice

hORMDL3^{zp3-Cre} chimeric mice were generated as described in the online supplement¹⁶.

ASM studies in vitro.

The methods for mASM or hASM culture, transfection with ORMDL3 or SERCA2b, siRNA knockdown with SERCA2b, quantitation of ASM proliferation and contraction in vitro, and qRT-PCR have been described in the online supplement^{16,19}.

Mouse precision-cut lung slices (PCLSs)

The methods for generation of PCLSs, measurement of mouse airway contraction and relaxation, and measurement of Ca²⁺ signaling have been described in the online supplement²⁰⁻²³.

Statistical Analysis

All data are reported as the mean ± SEM. A P<0.05 was considered significant. Detailed methods for statistical analysis have been described in the online supplement.

RESULTS

Chimeric hORMDL3^{Zp3-Cre} mice have increased ASM without airway inflammation

As our previous studies had demonstrated that naïve hORMDL3^{Zp3-Cre} mice have increased ASM without airway inflammation¹⁶, we generated bone marrow chimeric mice in which the hORMDL3^{Zp3-Cre} chimeric mouse had wild type (WT) bone marrow (with no expression of hORMDL3) and the mouse lung structural cells expressed increased levels of hORMDL3. Establishment of chimerism (WT bone marrow into irradiated hORMDL3^{Zp3-Cre} recipient) was confirmed by RT-qPCR demonstrating hORMDL3 expressed in the lungs but not the blood or bone marrow leukocytes of hORMDL3^{Zp3-Cre} chimeric mouse (**Fig 1A**). These studies demonstrated that 12 week old naïve chimeric hORMDL3^{Zp3-Cre} mice had increased levels of peribronchial smooth muscle ($p < 0.05$ vs WT control) as assessed by image analysis quantitation of the area of α -smooth muscle actin immunostaining (**Fig 1 B-E**). The naïve chimeric hORMDL3^{Zp3-Cre} mice with increased ASM did not have evidence of airway inflammation (**Fig 1F**). These studies provide evidence that lung structural cell expression of hORMDL3 is sufficient to induce increased ASM independent of airway inflammation. However, these bone marrow chimera studies are unable to identify which lung structural cell expressing ORMDL3 (ASM, fibroblast, epithelium) is able to induce increased ASM mass independent of airway inflammation.

Human ASM cells expressing increased levels of ORMDL3 have increased proliferation, contraction, and expression of SERCA2b

To determine whether ASM expression of ORMDL3 itself could contribute to the increased ASM observed in naïve chimeric hORMDL3^{Zp3-Cre} mice, we transfected normal human ASM cells (hASM) with hORMDL3 (**Fig 2A, 2B**). These studies demonstrated that Ad-ORMDL3-GFP transfected hASM cells had significantly increased proliferation assessed by Ki67 mRNA (**Fig 2C**) and hASM cell counts (**Fig 2D**) as compared to empty vector control. In addition, Ad-ORMDL3-GFP transfected hASM had significantly increased contractility in response to acetylcholine (ACh) (**Fig 2E, Fig 2F**). These studies demonstrate that increased ORMDL3 in hASM can induce increased proliferation and increased hASM contractility a major feature of asthma.

We have previously demonstrated that hORMDL3^{Zp3-Cre} mice have increased expression of the calcium pump SERCA2b in their lungs¹⁶, but have not previously investigated whether ORMDL3 regulates SERCA2b in ASM, and whether this influences ASM contractility. Thus, we transfected Ad-ORMDL3-GFP into hASM cells which resulted in significantly increased levels of expression of SERCA2b in hASM (**Fig 2G**). The importance of SERCA2b induction by ORMDL3 is suggested from subsequent studies in which we transfected normal hASM with Ad-SERCA2b. These studies demonstrated that Ad-SERCA2b transfected hASM cells had significantly increased proliferation as assessed by Ki67 mRNA (**Fig 2H**) as compared to empty vector control. In addition, Ad-SERCA2b transfected hASM cells had significantly increased contractility compared to control transfected hASM ($p < 0.05$) (**Fig 2I, Fig 2J**), consistent with in vivo studies demonstrating increased cardiac smooth muscle contractility in mice expressing increased levels of

SERCA2b in the heart^{24,25}. Incubation of Ad-SERCA2b transfected hASM cells with Ach in vitro resulted in a trend for increased ASM contractility which was not statistically significant (Ad-SERCA2b vs Ad-SERCA2b + Ach) ($p=0.13$) (**Fig 2J**). Thus, the majority of the increased ASM contractility in Ad-SERCA2b transfected ASM incubated with Ach was due to Ad-SERCA2b transfection rather than Ach. These studies demonstrate that in hASM cells, increased SERCA2b (a downstream pathway from ORMDL3)^{16,17} can induce increased proliferation and increased hASM contractility providing evidence for an intrinsic pathway in hASM in which ORMDL3 upregulates SERCA2b resulting in increased hASM and increased contractility.

Increased airway contraction in hORMDL3^{Zp3-Cre} mouse precision cut lung slices (PCLSs)

To determine whether ORMDL3 expression in the lung influences airway contraction, we visualized (**Figure 3A, and Supplemental Video 1**) and quantitated (**Figure 3B-E**) airway contractility in naïve hORMDL3^{Zp3-Cre} mouse precision cut lung slices (PCLSs) which reflect the *in situ* physiology of mASM of relatively smaller airways devoid of a blood supply and thus unable to recruit inflammatory or immune cells from the circulation. To quantitate airway contraction in PCLSs, we used both MCh (a cholinergic agonist used to assess AHR in asthma) and 5-hydroxytryptamine (5-HT) which each trigger Ca^{2+} oscillation and contraction in ASM²⁶ by binding to their respective ASM cell surface receptors, which as a consequence activate different downstream G-protein coupled pathways each generating inositol triphosphate (IP_3)^{4,27-28}. IP_3 then binds to intracellular IP_3 receptors on the SR to release Ca^{2+} from the SR into the cytoplasm^{4,27-28}.

There was no significant difference in the baseline size of airways chosen for testing between hORMDL3^{zp3-Cre} and WT group (**supplemental Figure S1A-D**). In addition, no pre-contraction of the airways was observed in either hORMDL3^{zp3-Cre} or WT mice, as none of the airways showed relaxation to (R,R)-formoterol, a long-acting β_2 -receptor agonist that relaxes ASM by inactivating MLC kinase and activating MLC phosphatase (**supplemental Figure S1E and S1F**). Both MCh (**Figure 3B**) and 5-HT (**Figure 3C**) induced a dose-dependent increase in airway contraction in WT mouse PCLSs at 37°C. As compared to WT mice, the extent of airway contraction in hORMDL3^{zp3-Cre} mice were significantly increased for both MCh (**Figure 3B, 3D, and Supplemental Video 1**) and 5-HT (**Figure 3C, 3E**) at all concentrations tested (100 to 2000 nM) suggesting an overall increased airway contractility in hORMDL3^{zp3-Cre} mice. A similar pattern of increased airway contraction in hORMDL3^{zp3-Cre} mice were noted at room temperature (**supplemental Figure S2**). These studies demonstrate that increased ORMDL3 expression in naïve lung PCLSs without a blood supply results in increased airway reactivity to agonists including MCh, a major feature of asthma.

Increased Ca^{2+} oscillation frequency in ASM cells in hORMDL3^{zp3-Cre} mouse PCLSs

As ORMDL3 induced increased ASM expression of SERCA2b (**Fig 2G**), which regulates $[\text{Ca}^{2+}]_i$ that control ASM contraction, we further examined whether ASM cells with increased ORMDL3 expression in PCLSs from hORMDL3^{zp3-Cre} mice could exhibit

increased frequency of Ca^{2+} oscillations in response to either MCh or 5-HT, as Ca^{2+} oscillation frequency has been implicated in ASM contractile responses *in vivo*²⁶⁻²⁸. No spontaneous Ca^{2+} signaling was observed in mASM cells derived from either WT or hORMDL3^{zp3-Cre} mice. In response to both MCh and 5-HT at 37°C, mASM cells in WT PCLSs showed a rapid onset within 15 seconds of oscillatory increases of $[\text{Ca}^{2+}]_i$, which occurred in the form of Ca^{2+} waves propagating along the length of the mASM cells (**Figure 4A-4E, and Supplemental Video 2**). These Ca^{2+} oscillations were accompanied by contraction and shortening in mASM cells resulting in airway contraction (**Figure 3A**). In the mASM cell in PCLSs of WT mice (**Figure 4F**), MCh induced a concentration dependent increase in Ca^{2+} oscillation frequency in a similar way as observed in airway contraction. As compared to WT mice, the Ca^{2+} oscillation frequency in mASM cells in PCLSs of hORMDL3^{zp3-Cre} mice were significantly increased for both MCh (**Figure 4G and 4H**) and 5-HT (**Figure 4I**) at all concentrations tested (100 to 2000 nM). A similar pattern of increased Ca^{2+} oscillations in mASM cells of hORMDL3^{zp3-Cre} mice were noted at room temperature (**supplemental Figure S3**). These results demonstrate that in the absence of airway inflammation, mASM cells with increased ORMDL3 expression exhibit increased Ca^{2+} oscillation frequency response to agonists. Thus, mASM with increased Ca^{2+} oscillations is associated with increased airway contractility in hORMDL3^{zp3-Cre} mice.

Knockdown or inhibition of SERCA2b inhibits ASM contractility and Ca^{2+} oscillations

To determine the role of SERCA2b expression induced by ORMDL3 in mediating increased contractility and Ca^{2+} oscillations in hASM, we initially demonstrated that the levels of

SERCA2b in mASM cells derived from hORMDL3^{zp3-Cre} mice were increased compared to WT mice (**Fig 5A**) as quantitated by RT-qPCR. In hASM, SERCA2b but not SERCA2a was detected (**Fig 5B**). We then used siRNA to knockdown SERCA2b expression in hASM cells. siRNA knockdown of SERCA2b was efficient in hASM cells (**Fig 5C**). siRNA knockdown of SERCA2b significantly inhibited hASM contraction *in vitro* in response to histamine starting within 15 minutes of exposure to histamine (**Fig 5D**). Control scrambled siRNA did not inhibit histamine induced hASM contraction. These results demonstrated an important role of SERCA2b in ASM contractility *in vitro*.

To further confirm the role of SERCA2b in airway contraction and underlied Ca^{2+} oscillations in ASM cells, we investigate the effect of cyclopiazonic acid (CPA), a specific SERCA pump inhibitor²⁹, on the MCh-induced airway contraction, as well as the Ca^{2+} oscillations in ASM cells in mouse PCLSs (**Fig 5E-H; supplemental Figure S4**). Treatment of MCh-contracted airways with CPA (from 500 nM to 5 μM) relaxed airway contraction in a concentration-dependent manner (**Fig 5E and 5F**), 5 μM CPA could induce nearly 73% relaxation as normalized to the control (**Fig 5F**). Consistently, CPA also inhibited MCh-induced Ca^{2+} oscillations in a concentration-dependent manner (**Fig. 5G and 5H**), while 5 μM CPA gradually stopped Ca^{2+} oscillations in 15 min (**Fig. 5G**). Taken together, these results suggest that SERCA2b expression or activity is important to sustain ASM contractility via regulating Ca^{2+} oscillation frequency in ASM cells. Thus, increased SERCA2b expression induced by ORMDL3 in ASM cells could contribute to the increased

305 reactivity of ASM in response to agonists such as MCh and 5-HT as observed in the PCLSs
306 of hORMDL3^{Zp3-Cre} mice.

309 DISCUSSION

310 This study makes the novel observation that ORMDL3 expression in ASM may explain the
311 strong genetic linkage of ORMDL3 on chromosome 17q21 to asthma¹⁵. In addition, this
312 study provides evidence that intrinsic expression of a gene highly linked to asthma in ASM
313 can lead to increased ASM proliferation and contractility independent of extrinsic
314 inflammation. Thus, asthma may be a disease of both airway inflammation as well as an
315 intrinsic change in ASM. In support of this, we demonstrate that ORMDL3 expression in
316 ASM cells *in vitro* induces increased ASM proliferation and increased ASM contractility to
317 Ach. In addition, *in vivo* studies demonstrate that naïve hORMDL3^{Zp3-Cre} chimeric mice with
318 ORMDL3 expressed in lung structural cells (but not in bone marrow derived inflammatory
319 and immune cells) have increased ASM in the absence of lung inflammation. Importantly,
320 *in vivo* studies demonstrate that ASM in naïve hORMDL3^{Zp3-Cre} mouse PCLSs without a
321 blood supply have increased contractility and increased Ca²⁺ oscillation frequency, a
322 fundamental signaling mechanism mediating ASM contraction, in response to MCh and
323 5-HT. Taken together, these studies underscore the important role that ORMDL3 expressed
324 in ASM plays in inducing increased ASM proliferation and increased ASM contractility to
325 MCh, a major feature of asthma. However, this study is unable to determine whether
326 ORMDL3 induces increased AHR by effects on increasing ASM proliferation and/or by

increasing ASM contractility. As ORMDL3 is highly linked in genetic epidemiology studies to childhood asthma¹⁵, ORMDL3 expression in ASM may help to explain why individuals with the SNP linked to chromosome 17q21 and increased ORMDL3 expression have increased AHR, a major feature of asthma.

The concept that Th2 inflammation is important to the development of allergic disease in the upper airway and lower airway is well accepted. However, there is limited insight into why certain individuals with the same level of Th2 inflammation will either only have allergic rhinitis (with no asthma), while others will have allergic rhinitis and asthma. In this study we provide evidence that ORMDL3, a gene most highly linked to asthma¹⁵ (but not linked to allergic rhinitis)³⁰, when expressed in a lung structural ASM cells in vitro or in vivo spontaneously induced increased airway contractility to MCh without an additional inflammatory stimulus. This may help to explain how Th2 inflammation when combined with an ASM expressed asthma associated gene such as ORMDL3 can result in allergic rhinitis and asthma, whereas Th2 inflammation alone in the absence of an abnormality in ASM may result in allergic rhinitis but not asthma. ORMDL3 has been linked asthma¹⁵ but not to allergic rhinitis³⁰, underscoring differences between asthma and allergic rhinitis. In addition, the SNP linked to increased ORMDL3 expression in asthma is common affecting approximately 62% of childhood asthmatics¹⁵. Another example of a structural gene being important to allergic disease is the loss of function of the filaggrin gene in epithelial cells in the skin in atopic dermatitis which influences barrier function in Th2 inflammation in the skin³¹.

Previous studies have also provided evidence that AHR can be independent from airway inflammation. For example, a study of allergic asthmatics did not find a correlation between AHR to MCh and levels of inflammatory cells in sputum, BAL, or bronchial biopsy³². In addition, inhaled corticosteroids can effectively reduce levels of airway inflammation, but only attenuate AHR³³. Patients with eosinophilic bronchitis can also have severe inflammation but no AHR³³. Population studies have also demonstrated that AHR is a heritable trait, with an estimated heritability of 30 to 66%^{34,35}. Thus, while airway inflammation plays an important role in the pathogenesis of asthma, there are likely genetic, as well as airway components (airway inflammation; airway remodeling) that all contribute to levels of AHR in asthma³⁶.

Our study also provides evidence that SERCA2b may be the downstream pathway by which ORMDL3 influences ASM contractility. Our prior studies have demonstrated that in human lung epithelial cells increased expression of hORMDL3 activates several downstream pathways including ATF6 α , SERCA2b, sphingolipids, chemokines, and remodeling pathways^{16,17}. As ORMDL3 regulates several downstream pathways, some downstream pathways may be more important in ASM compared to other cell types expressing ORMDL3. For example, the importance of increased SERCA2b to ASM hypertrophy and increased contractility is suggested from studies of the heart, in which mice expressing increased levels of SERCA2b in the heart had increased cardiac contractility with increased Ca²⁺ flux in cardiomyocytes, and increased cardiac muscle hypertrophy^{24,25}. SERCA are intracellular ATP driven Ca²⁺ pumps that transport Ca²⁺ from the cytoplasm into the

371 sarco-endoplasmic reticulum^{18,37} (the domain of the endoplasmic reticulum that lacks
 372 ribosomes). SERCA proteins are encoded by three genes *Serca1*, *Serca2*, and *Serca3*, of
 373 which SERCA2 is the only SERCA expressed in ASM^{18,37}. Alternative splicing of the
 374 *Serca2* gene encodes two major protein isoforms, SERCA2a which is expressed
 375 predominantly in cardiomyocytes and slow twitch skeletal muscle^{18,37}, while SERCA2b, the
 376 focus of this study is expressed in ASM and most tissues^{18,37}. Much of the Ca^{2+} entering the
 377 ASM cell and released from stores may be sequestered by the superficial SR through
 378 SERCA. Cycling between SR Ca^{2+} uptake and release mechanisms leads to Ca^{2+}
 379 oscillations^{27,28}. Force generation by ASM is dependent on actin–myosin cross-bridge
 380 formation^{4,28}. When cytosolic Ca^{2+} levels are increased, the combination of Ca^{2+} and
 381 calmodulin activate myosin light chain (MLC) kinase, which phosphorylates MLC, leading
 382 to enhanced contraction^{4,38}. Therefore, ASM cell contraction is regulated by the frequency
 383 of oscillations in $[\text{Ca}^{2+}]_i$ ^{27,28}, which correspond to the speed of cyclic release of Ca^{2+} from
 384 the SR and re-uptake of Ca^{2+} from the SR by SERCA^{38,39}. In this study we demonstrate that
 385 ORMDL3 increases levels of SERCA2b in ASM which is associated with increased
 386 frequency of Ca^{2+} oscillations in ASM. In addition, increased levels of SERCA2b in ASM
 387 increases ASM proliferation, while siRNA knockdown of SERCA2b reduces ASM
 388 contractility. Furthermore, inhibition of SERCA by giving CPA in PCLSs reduced Ca^{2+}
 389 oscillation frequency in ASM cells and thus airway contraction in a dose-dependent way.
 390 These results together suggest that SERCA2b, regulated by ORMDL3 in ASM, plays an
 391 important role in sustaining agonists-induced Ca^{2+} oscillations in ASM cells and is
 392 associated with increased airway contractility as shown in PCLSs of hORMDL3^{Zp3-Cre} mice.

Transfection of SERCA2b into ASM resulted in greater enhancement of contraction than that noted with transfection of ORMDL3. One possible explanation for these results is that transfection of ORMDL3 not only increases SERCA2b (which increases ASM contractility), but also inhibits the function of the enzyme serine palmitoyltransferase or SPT⁴⁰ (which could reduce ASM contractility). For example, increased levels of ORMDL3 inhibit the enzyme SPT, the first and rate limiting step in sphingolipid synthesis resulting in lower levels of sphingosine 1 phosphate (S1P)⁴⁰. As S1P induces ASM contraction⁴¹, lower levels of S1P induced by increased levels of ORMDL3 could reduce ASM contractility. Thus, ORMDL3 induction of SERCA2b could increase ASM contractility, while its concomitant induction of the enzyme SPT could reduce S1P and reduce ASM contractility. Thus, the net effect of ORMDL3 on ASM contractility may be less than that of SERCA2b, because of the induction not only of SERCA2b, but also inhibition of S1P by ORMDL3. Interestingly, therapeutically targeting Ca^{2+} signaling pathways in smooth muscle in asthma to influence smooth muscle contractility and AHR is currently an active area of investigation⁴². However, there are also studies which question whether Ca^{2+} signals change in asthmatic ASM while stimulated with agonists⁴³⁻⁴⁵.

Interestingly, some studies have reported that decreased expression of SERCA in ASM from asthmatics contributes to airway remodeling⁴³. It should be noted that our study differs from the prior study⁴³ in examining the effect of increased ORMDL3 (simulating asthmatics with the SNP on chromosome 17q21 linked to increased ORMDL3 expression) on SERCA2b in ASM, whereas the prior study did not genotype asthmatics to determine whether they had

the SNP associated with increased ORMDL3 expression which we demonstrate is associated with increased SERCA2b expression. Thus, we hypothesize that based on genotype not all asthmatics will have increased SERCA2b expression. In addition, prior studies have demonstrated that suppressed SERCA by cytokines might slow $[Ca^{2+}]_i$ response to agonists, and thus maintain a relatively high $[Ca^{2+}]_i$ leading to increased ASM contractility⁴⁶. In agreement with this, our results showed that treatment of PCLSs with high dose (10 μ M) of CPA stopped MCh-induced Ca^{2+} oscillations and lead to a rise of $[Ca^{2+}]_i$ baseline in ASM cells with partially contracted airway (**supplemental Fig S4**). However, lower dose of CPA (from 500 to 5000 nM) reduced Ca^{2+} oscillations and airway contraction in a dose-dependent manner, suggesting that under a certain range, increased/decreased SERCA is proportional to increased/decreased Ca^{2+} oscillations and therefore increased/decreased airway contraction, while fully block or knockdown of SERCA would result in an $[Ca^{2+}]_i$ plateau that mediates only impaired ASM contraction response to agonists⁴⁷.

ORMDL3 is expressed in multiple cell types including lung structural cells (epithelium, smooth muscle) and immune and inflammatory cells (T cells, eosinophils, and macrophages)^{16,17}. As the SNP linking chromosome 17q21 to asthma is associated with increased ORMDL3 expression¹⁵ we have previously used cre-lox techniques to generate mice selectively deficient in ORMDL3 in airway epithelium (*Ormdl3*^{*Δ2-3/Δ2-3*}*CC10*)⁴¹ to simulate an inhaled therapy that effectively inhibited ORMDL3 expression in the airway. In contrast to the anticipated reduction in AHR, allergen challenged *Ormdl3*^{*Δ2-3/Δ2-3*}*CC10* mice

had a significant increase in AHR compared to WT mice⁴¹. Levels of sphingosine-1-phosphate (S1P) were significantly increased in *Ormdl3*^{Δ2-3/Δ2-3}*CC10* mice as well as in airway epithelial cells in which ORMDL3 was inhibited with siRNA and incubation of S1P with ASM cells significantly increased contractility⁴¹. Overall, *Ormdl3*^{Δ2-3/Δ2-3}*CC10* mice exhibit increased allergen induced AHR independent of inflammation and associated with increased S1P generation⁴¹. These studies suggest that therapies that selectively and effectively inhibit ORMDL3 in airway epithelium in asthma, may not be the best cell to target. Further studies are needed to determine whether targeting reduction of ORMDL3 in airway ASM would reduce AHR.

In summary, in this study we provide evidence that an intrinsic gain of function in ORMDL3 in ASM can influence ASM contractility in asthma, by demonstrating that increased ORMDL3 expression in ASM cells induces increased expression of SERCA2b with consequent increased Ca^{2+} oscillation in ASM cells, and increased ASM contractility. Overall, these studies provide evidence that ORMDL3 (a gene on chromosome 17q21 highly linked to asthma in GWAS)¹⁵ can induce increased ASM contractility in the absence of inflammation. As AHR may conceptually be driven by either chronic inflammation inducing increased ASM contractility, and/or an intrinsic change in ASM inducing increased ASM hyperplasia and contractility, this study provides evidence that targeting a gene inducing a fundamental abnormality in ASM such as ORMDL3, may result in a new therapeutic approach to the treatment of asthma.

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463 **ACKNOWLEDGEMENTS**

464 Research reported in this publication was supported by NIH grants AI 107779, AI 38425,
465 AI 070535, AI 72115, AI 242236 to D.H.B

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FIGURE LEGENDS

Figure 1. hORMDL3^{zp3-Cre} chimeric mice and increased airway smooth muscle

Wild type (WT) and hORMDL3^{zp3-Cre} mice (n= 8 mice/group) were irradiated and each injected i.v. with WT bone marrow and sacrificed 12 weeks later. (A) Efficiency of chimerism was assessed by RT-qPCR to detect expression of human ORM DL3 in blood and lungs. (B-E). Lungs of chimeric mice (n= 8 mice/group) were immunostained with an anti- α smooth muscle actin antibody and the area of peribronchial anti- α smooth muscle actin immunostaining quantitated by image analysis. (F). Lungs of chimeric mice (n= 8 mice/group) were immunostained with either an anti-CD4, anti-F4/80, anti-MBP, or anti-neutrophil elastase antibody and the number of peribronchial cells immunostaining positive quantitated by image analysis.

Figure 2: ORMDL3, SERCA2b, and airway smooth muscle proliferation and contraction

Primary human airway smooth muscle cells (hASM) were transfected with Ad-ORMDL3-GFP or as a control the empty vector Ad-GFP. Efficiency of transfection was assessed by immunofluorescence microscopy (**A**) and FACS (**B**). hASM proliferation was assessed 72 hours later either by (**C**) RT-qPCR of Ki67 mRNA levels (n= 3/group), or (**D**) by ASM cell counts by light microscopy (n= 6/group). (**E-F**). hASM contraction (n= 3/group) was assessed in a gel contraction assay in hASM cells transfected with either Ad-ORMDL3-GFP or Ad-GFP in the presence or absence of acetylcholine (Ach). Ad-ORMDL3 vs Control, $p < 0.05$; Ad-ORMDL3 vs Ad-ORMDL3+Ach, $p < 0.05$; Ad-ORMDL3+Ach vs Control +Ach, $p < 0.05$; Ad-ORMDL3 vs Control + Ach, $p = 0.20$; Control vs Control + Ach, $p < 0.05$. (**G**). Levels of SERCA2b (n= 3/group) were quantitated by RT-qPCR in hASM cells transfected with either Ad-ORMDL3-GFP or Ad-GFP. (**H**) Levels of hASM proliferation (n= 3/group) were assessed by RT-qPCR of Ki67 mRNA levels in hASM cells transfected with either Ad-SERCA2b or control. (**I-J**). hASM contraction (n= 3/group) was assessed in a gel contraction assay in hASM cells transfected with either Ad-SERCA2b or Ad empty vector control in the presence or absence of Ach. Ad-SERCA2b vs Control, $p < 0.05$; Ad-SERCA2b vs Ad-SERCA2b+Ach, $p = 0.13$; Ad-SERCA2b+Ach vs Control +Ach, $p < 0.05$; Ad-SERCA2b + vs Control + Ach, $P < 0.05$.

Figure 3. hORMDL3^{zp3-Cre} mice have increased airway contraction compared to WT mice

(A) Representative phase-contrast images of two airways in a precision cut lung slices (PCLSs) derived from WT and hORMDL3^{zp3-Cre} mouse, respectively, under resting condition (left panel, scale bar = 100 μ m), exposed to 2000 nM MCh (right panel) at 37°C. The response of airways in PCLSs derived from WT (blue line) and hORMDL3^{zp3-Cre} (red line) mouse, respectively, to increased dose of MCh (**B**) and 5-HT (**C**) at 37°C, followed by a comparison of dose response curve to MCh (**D**) and 5-HT (**E**) between WT (blue line) and hORMDL3^{zp3-Cre} (red line) mouse. hORMDL3^{zp3-Cre} vs WT, *P<0.05. Each line in (B) and (C) represents the mean and each point represents the mean $\pm$ SEM at selected time points of the airway lumen area normalized to the initial size at t = 0 s. Data in (B) and (D) “WT” represent n = 9 airways, “hORMDL3^{zp3-Cre}” represent n = 11 airways from 5 mice, respectively; data in (C) and (E) “WT” represent n = 9 airways, “hORMDL3^{zp3-Cre}” represent n = 13 airways from 5 mice, respectively.

Figure 4. hORMDL3^{zp3-Cre} mice have increased Ca²⁺ oscillations in airway smooth muscle cells compared to WT mice

(A-D) Representative 2-photon images of MCh-induced Ca²⁺ oscillations in the same airway smooth muscle cell (labeled ASMC with white arrow) in mouse precision cut lung slices (PCLSs). A cycle of Ca²⁺ oscillations in the same ASMC over time can be visualized in **A-D** as a change in fluorescence intensity of Oregon Green (white color change in ASMC in figure **A-C**, and normalized intensity change in figure **D**) recorded at baseline pre-MCh at 0

s (A), and at two time points after MCh, 1.07 s (B), and 1.97 s (C) (scale bar = 20 μ m). (E) Representative trace shows that 400 nM MCh induced Ca²⁺ oscillations in an ASMC at 37°C, which sustained until MCh was washed off by sHBSS, adding back MCh would induce Ca²⁺ oscillations again. Representative traces show Ca²⁺ oscillations of ASMC in PCLSs derived from WT (F) and hORMDL3^{zp3-Cre} mouse (G), respectively, response to increased dose of MCh at 37°C, followed by a comparison of dose response curve to MCh (H) and 5-HT (I) between WT (blue line) and hORMDL3^{zp3-Cre} (red line) mouse. hORMDL3^{zp3-Cre} vs WT, *P<0.05. Representative traces are expressed as intensity (F_t) normalized to the initial intensity at t = 0 s (F₀), measured from a region of interest with size of 8 × 10 pixel within a single ASMC. Data in (H) “WT” represent n = 26 ASMCs, “hORMDL3^{zp3-Cre}” represent n = 31 ASMCs from 5 mice, respectively; data in (I) “WT” represent n = 17 ASMCs, “hORMDL3^{zp3-Cre}” represent n = 26 ASMCs from 5 mice, respectively.

Figure 5. Knockdown or inhibition of SERCA2b inhibits airway smooth muscle contractility and Ca²⁺ oscillations

(A). RT-qPCR was used to detect levels of SERCA2b mRNA in mouse airway smooth muscle (mASM) derived from either hORMDL3^{zp3-Cre} mice or WT control mice (n= 6/group). (B) Levels of SERCA2a and SERCA2a and 2b mRNA were quantitated by RT-qPCR in human ASM (hASM) cells (n= 6/group). (C). Levels of SERCA2b mRNA were quantitated in hASM cells in which SERCA2 siRNA was used to knockdown SERCA. Scrambled siRNA was used as a control (n= 4/group). (D). hASM contraction was assessed

in a gel contraction assay for 0-60 minutes in the presence of histamine in hASM cells in which siRNA (or scrambled control siRNA) was used to knockdown SERCA2 (n=8/group). SERCA2 siRNA vs Scrambled siRNA. **P<0.01. (E) The effect of cyclopiazonic acid (CPA) (500 to 5000 nM) on airways contracted with 400 nM MCh in precision cut lung slices (PCLSs) derived from WT mice, followed by (F) a summary of relaxation effect normalized to control. Controls were treated with vehicle of 0.5% DMSO in sHBSS, P<0.01. Each line in (E) represents the mean and each point represents the mean $\pm$ SEM at selected time points of the airway lumen area normalized to the initial size at t = 0 s. Data in (F) represent n = 8 - 10 airways for each bar from 5 mice. (G) Representative trace shows 5 μ M CPA gradually stopped Ca^{2+} oscillations induced by 400 nM MCh in a mASM cell in mouse PCLSs. (H) A summary for the effect of CPA on Ca^{2+} oscillation frequency induced by 400 nM MCh in mASM cells in PCLSs, P <0.05. Data in (H) represent n = 17 - 19 mASM cells for each bar from 5 mice.

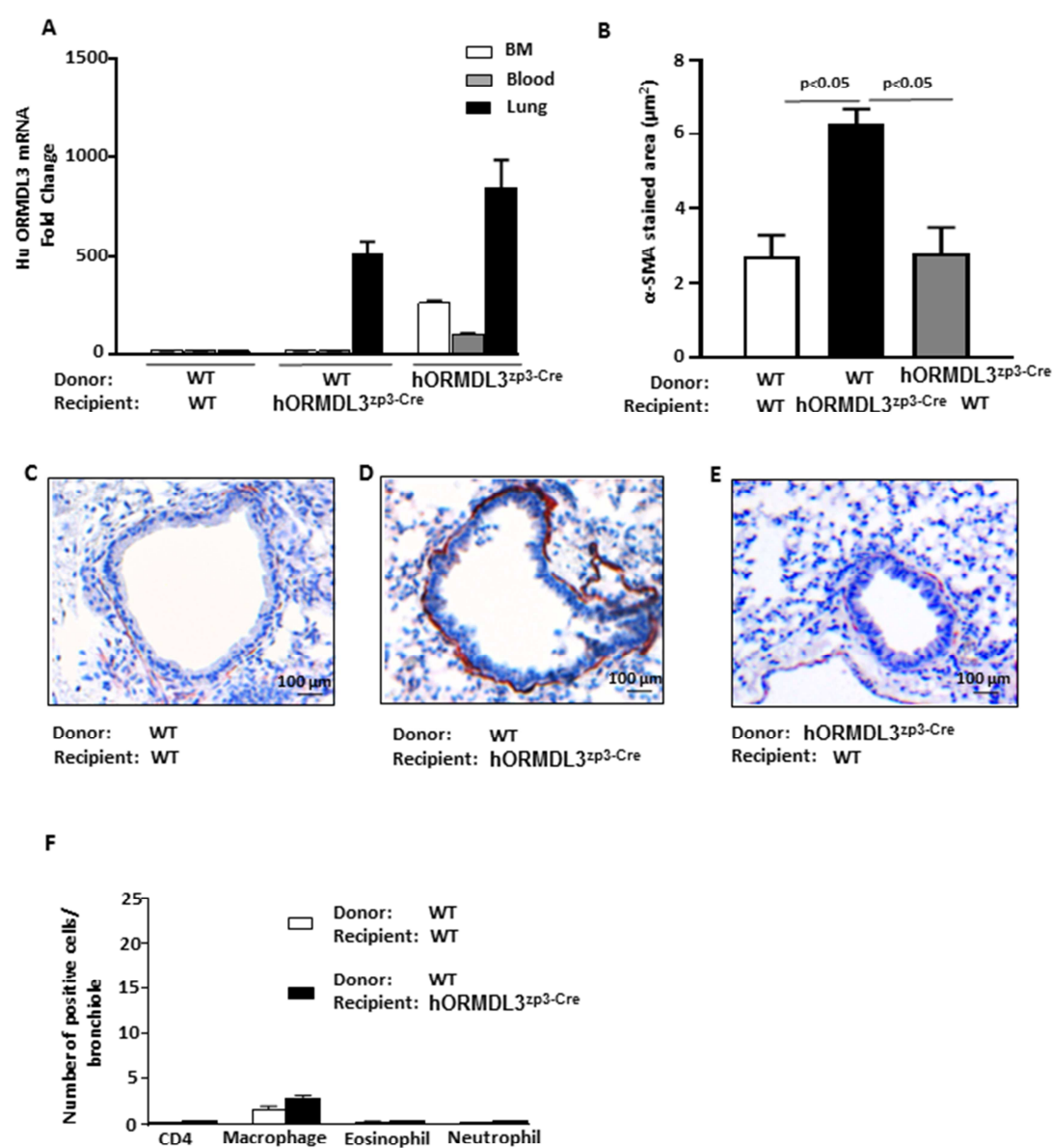


Figure 1

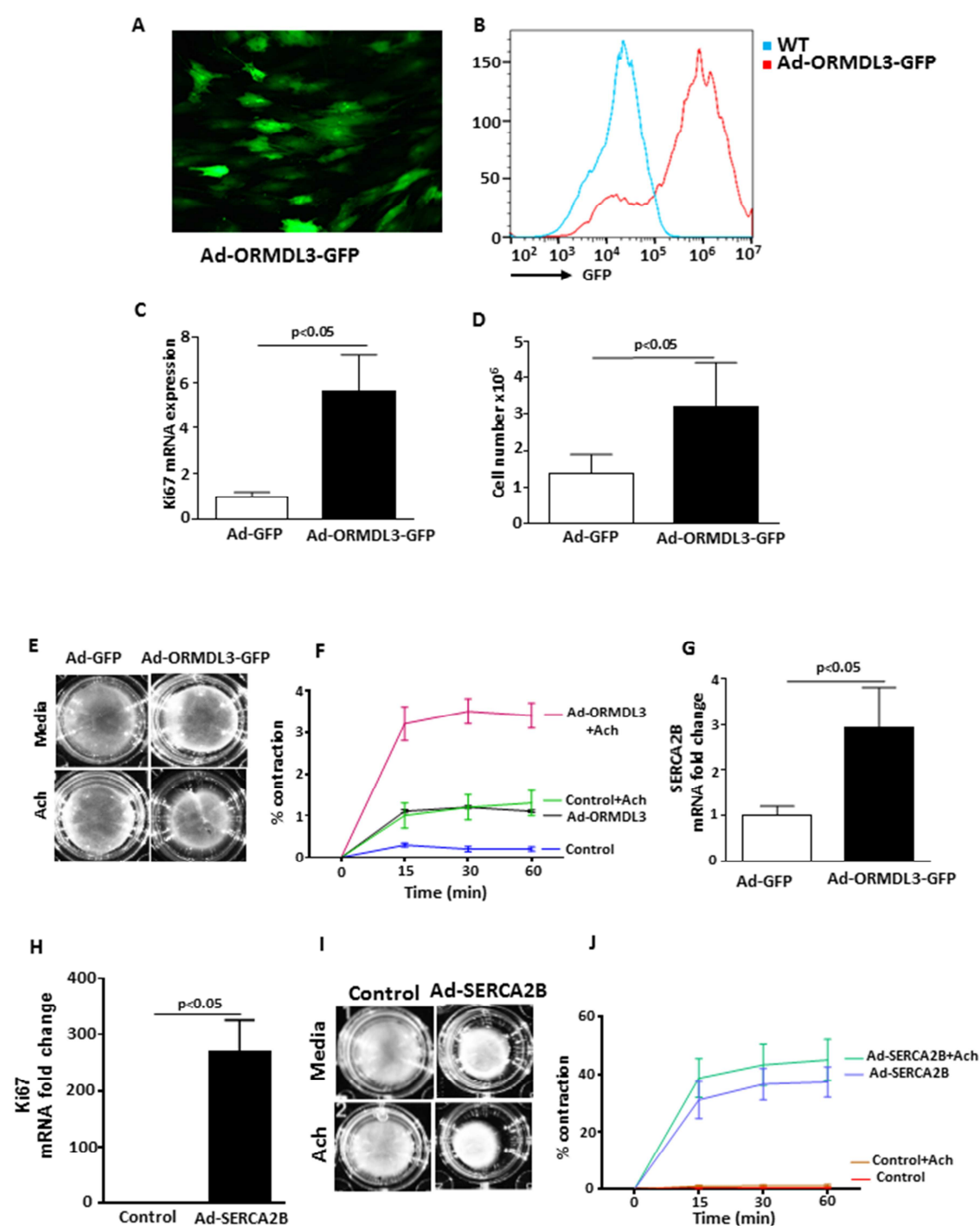


Figure 2

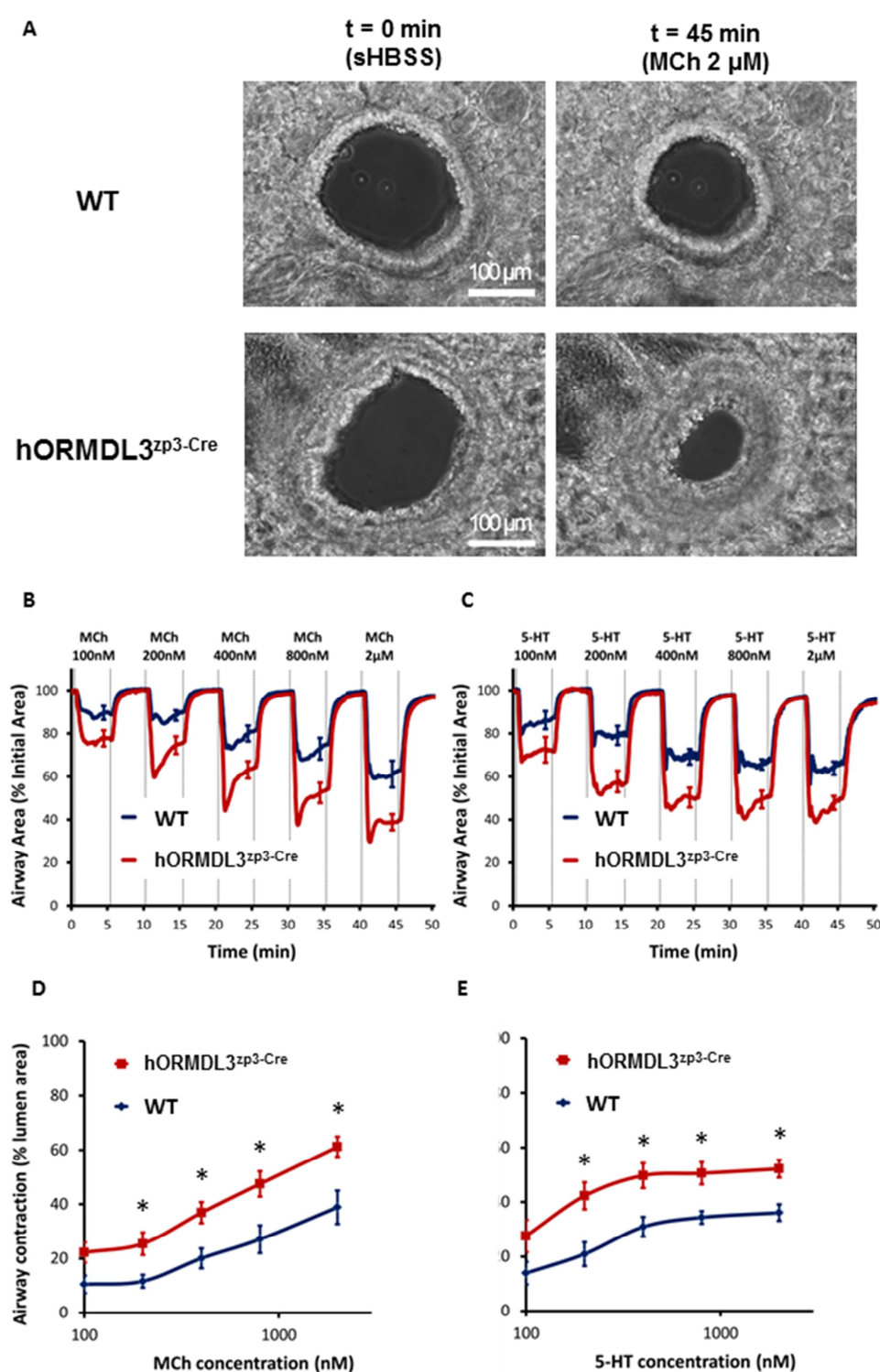


Figure 3

● $t = 1.97 \text{ s}$

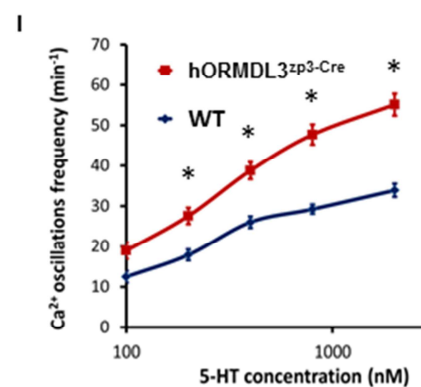
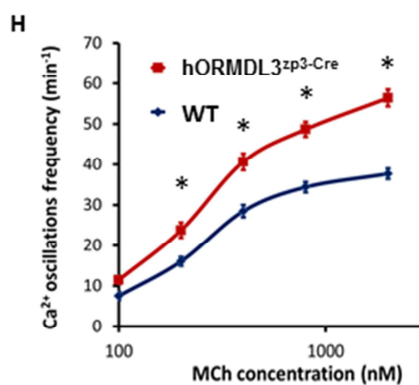
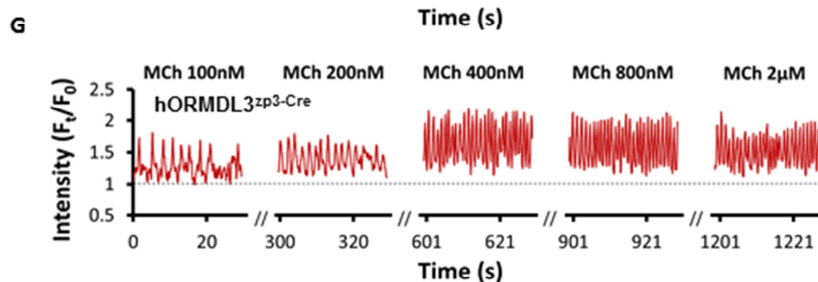
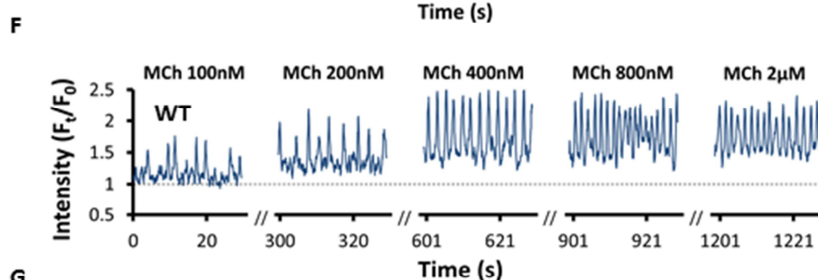
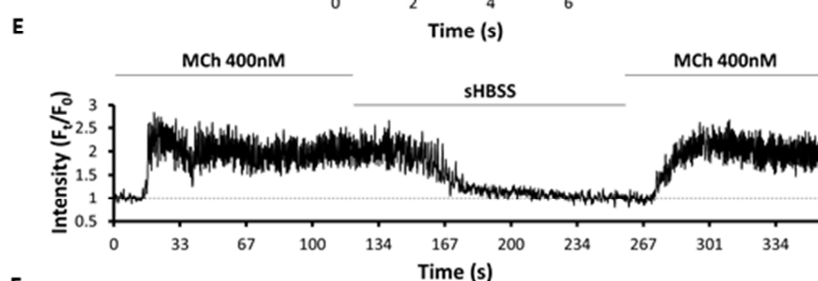
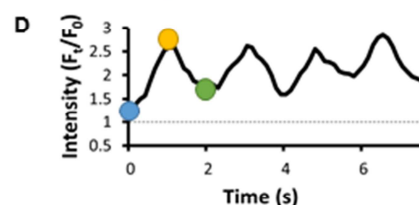


Figure 4

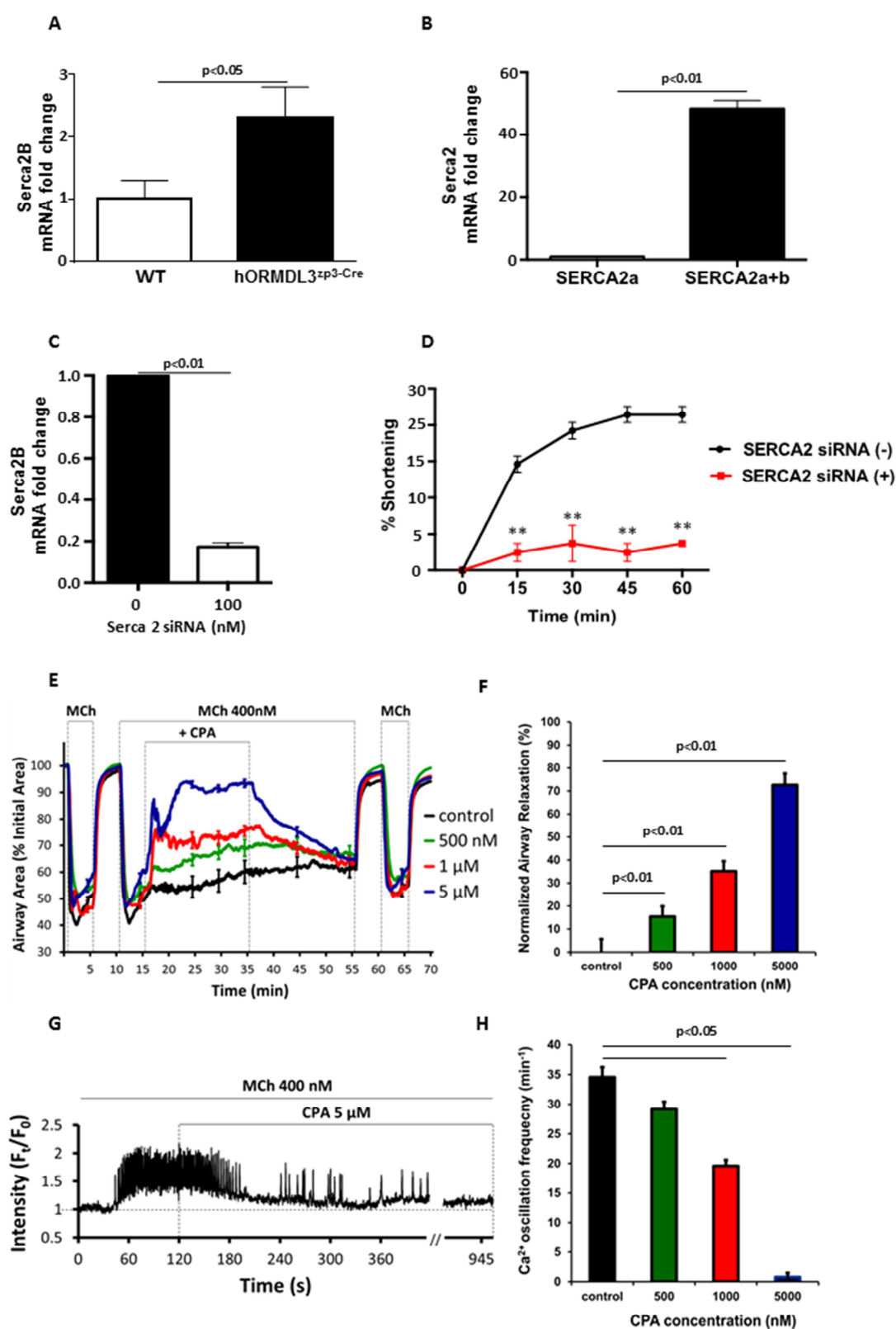


Figure 5

ONLINE SUPPLEMENTAL MATERIAL**ORMDL3 UPREGULATES AIRWAY SMOOTH MUSCLE PROLIFERATION,
CONTRACTION, AND Ca^{2+} OSCILLATIONS IN ASTHMA**

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MATERIALS AND METHODS

Animal care and use.

All the mouse experimental protocols described in the online methods were approved by the UCSD and University of Massachusetts Medical School Institutional Animal Care and Use Committees.

Generation of hORMDL3^{zp3-Cre} chimeric mice

hORMDL3^{zp3-Cre} mice on a C57Bl/6 background which universally express the human ORM DL3 gene were genotyped to confirm expression of the human ORM DL3 gene and utilized in these studies as previously described in this laboratory^{E1}. Littermate controls are referred to as wild type (WT) mice. Recipient mice (WT and hORMDL3^{zp3-Cre} mice) were irradiated once with 450 rad. Donor bone marrow from WT or hORMDL3^{zp3-Cre} mice was isolated from both tibias and fibulas, and approximately 10 million cells were injected 1 hour after irradiation to recipient mice via tail veins. Mice were rested for 30 days before sacrifice. Efficiency of chimerism was assessed by RT-qPCR to detect human ORM DL3 expression in peripheral blood and lung.

Lung processing

Lungs were processed for immunohistology (paraffin-embedded lung sections) as previously described in this laboratory^{E2}. For paraffin-embedded sections, lungs were equivalently inflated with an intratracheal injection of the same volume of 4%

paraformaldehyde solution (Sigma Chemicals, St. Louis, MO) to preserve the pulmonary architecture.

Peribronchial smooth muscle layer.

The thickness of the mouse airway smooth muscle layer (mASM) was measured by α -smooth muscle actin immunohistochemistry as previously described^{E3}. Lung sections were immunostained with an anti- α -smooth muscle actin primary Ab (Sigma-Aldrich) to detect peribronchial smooth muscle. Species- and isotype-matched Abs were used as controls in place of the primary Ab. The area of peribronchial α -smooth muscle actin staining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS, Leica Microsystems) attached to an image analysis system (Image-Pro Plus, Media Cybernetics, Bethesda, MD) as previously described^{E1}. Results are expressed as the area of peribronchial α -smooth muscle actin staining per micrometer length of basement membrane of bronchioles 150–200 μ m internal diameter.

Lung inflammation: eosinophils, CD4+ lymphocytes, neutrophils, and F4/80 positive macrophages

Levels of lung eosinophils, CD4+ lymphocytes, neutrophils, and F4/80 positive macrophages were quantitated in the peribronchial space in mouse lung sections processed for immunohistochemistry using an anti-mouse MBP Ab (kindly provided by James Lee PhD, Mayo Clinic, Scottsdale, Arizona), an anti-mouse CD4 Ab (GeneTex), an anti-neutrophil elastase antibody (Santa Cruz), and an anti-mouse F4/80 Ab (AbD Serotec)

as previously described^{E1,E2}. The number of individual cells staining positive in the peribronchial space was counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive per bronchiole with 150–200 μ m of internal diameter. At least five bronchioles were counted in each slide.

Human airway smooth muscle transfection with ORMDL3 or SERCA2b in vitro.

Human airway smooth muscle cells (hASM) were transfected with 1.5 MOI of either Ad-ORMDL3-GFP or Ad-GFP without the ORMDL3 insert as a control (OriGene), and Ad-Serca2b (custom made, ViGene, Rockville, MD), or Ad without the SERCA2b as the control. hASM proliferation and contraction were determined 72 h after transfection.

Human airway smooth muscle proliferation and contraction in vitro.

hASM cell counts were performed by either RT-qPCR detection of levels of Ki67 mRNA, or quantitation of ASM cell number by light microscopy. hASM cells were used in an *in vitro* smooth muscle gel contraction assay, previously described in our laboratory^{E3}. Briefly, transfected ASM cells were seeded in collagen gels free of LPS (Advanced BioMatrix, San Diego, Calif). After 3 day incubation in collagen gels containing smooth muscle complete media (ScenCell), hASM cells were cultured in the presence or absence of an agonist (50 μ l acetylcholine or histamine) or media control for 15, 30, and 60 minutes. With agonist-induced hASM contraction, the area of the gel decreases significantly^{E3}. The area of the gels was quantitated by using a Bio-Rad ImageDR transilluminator and Versadoc

scanner (Bio-Rad Laboratories, Hercules, Calif) with an accompanying image-capture and analysis program to generate area in square millimeters.

qRT-PCR

Quantitative RT-PCR (qRT-PCR) was performed as previously described in this laboratory^{E3}. In brief, total RNA was extracted with RNA-STAT-60 (Tel-Test) and reverse transcribed with Oligo-dT and SuperScript II kit (Life Technologies). qRT-PCR was performed with TaqMan PCR Master Mix and Ki67, or SERCA2b primers (all from Life Technology). The relative amounts of transcripts were normalized to those of housekeeping gene (GAPDH) mRNA and compared between the different genes by the $\Delta\Delta$ cycle threshold method as previously described in this laboratory^{E3}.

Preparation of mouse precision-cut lung slices (PCLSs)

Either wide type (WT) or hORMDL3^{zp3-Cre} mouse (5-7 weeks old, male) was killed by cervical dislocation at UCSD. After opening the thoracic cavity, the lungs were inflated with ~1 mL air via an intratracheal catheter, tighten by a thread on the top end of the trachea, and shipped in DMEM supplemented with 20 mM HEPES at 4°C overnight to University of Massachusetts Medical School. The lung was then warmed up to ~37°C, deflated by inserting an intratracheal catheter, and further inflated with ~1 mL of 1.8% agarose in sHBSS, followed by ~0.2 mL air at ~37°C. The agarose was then gelled with ~4°C sHBSS. The lung lobes were removed and sectioned into ~180 μ m thick PCLSs by a vibratome (VF-300, Precisionary Instruments, Greenville, NC, USA) as previously

described^{E4}. The PCLSs were maintained in DMEM with antibiotics at either 37°C or 25°C and 10% CO₂ for up to 2 days. The PCLSs experiments were performed at 37°C with constant perfusion using a custom-made, temperature-controlled microscope enclosure as previously described^{E5}.

Measurement of mouse airway contraction and relaxation

Briefly, mouse PCLS was mounted on a perfusion chamber created by sandwiching the PCLS between two cover-glasses with the edges sealed by silicone grease. A 200 µm nylon mesh with a hole aligned over a selected airway was used to hold down the PCLS. Phase-contrast images of airway lumen in response to different compounds was collected at a rate of 1 image per 2 seconds with a CCD camera mounted on an inverted microscope (Nikon Diaphot or Olympus IX71) with a 10X objective, and controlled by a custom-programed software based on Video Savant 4 (IO Industries, Montreal, Canada) as previously described^{E5}. The change in lumen area of a selected airway was analyzed by ImageJ, then normalized by the formulae: % initial area = the lumen area at a certain time / initial lumen area to quantify the airway contractility as described^{E6}. For the airway contraction assay, the normalized contraction at specific time point (5, 15, 25, 35, 45 min, respectively) when responding to a different concentration of agonists were used for dose-response curve drawing and statistical analysis.

To record cyclopiazonic acid (CPA) induced airway relaxation (%), we initially normalized the contractile state of an airway (measured at the mid-time point at 30 minutes

in figure 5E) to its own initial contraction (measured at 15 minutes) by the formulae: % Relaxation (R) = $(Area_{30min} - Area_{15min}) / (100 - Area_{15min})$. We then normalized the CPA induced relaxation to the amount of relaxation that occurred in the control response by the formulae: % Normalized Relaxation = $(R_{cpa} - R_{control}) / (100 - R_{control})$. These normalized relaxation responses to different concentrations of CPA were used for comparison and statistical analysis.

Measurement of intracellular Ca^{2+} signaling

Briefly, mouse PCLSs were incubated with sHBSS containing 20 μ M Oregon Green (OG) 488 BAPTA-1-AM, 0.1% Pluronic F-127 and 200 μ M sulfobromophthalein (SB) in the dark at $\sim 30^{\circ}\text{C}$ for ~ 1 h, followed by sHBSS containing 200 μ M SB for an additional 30 min at room temperature to allow the de-esterification of OG. The mouse PCLS was mounted in the perfusion chamber as described above. The intracellular Ca^{2+} signaling as reflected by the fluorescence change of OG in ASM cells of PCLSs was monitored with a custom-built, video-rate scanning 2-photon laser microscope^{E4,E7} at a rate of 15 images per second, and controlled by a custom-written image acquisition software based on Video Savant. The fluorescence intensity in a region of interest (ROI, 8×10 pixels) within an ASM cell, with respect to time, was determined using custom-written software. Relative fluorescence intensity was expressed as F_t/F_0 , a ratio of the fluorescence intensity at a particular time (F_t) normalized to the initial time (F_0).

Mouse airway smooth muscle (mASM) cell culture

Mouse airway smooth muscle (mASM) was obtained from WT and hORMDL3^{zp3-Cre} mice as previously described^{E1}. In brief, after mouse sacrifice, the tracheas were excised, cut longitudinally with a scalpel to expose the lumen and then transferred to 0.14% Pronase (Roche Applied Science) in Ham's F-12 nutrient medium and incubated at 4°C overnight. The tracheas were then brushed with a cotton swab to remove the remaining adherent epithelial cells, cut into small pieces (~30 per trachea), and cultured in DMEM/F-12 and 10% FBS for attachment and outgrowth. Four days later, mASM cell outgrowth was apparent, and the cells were left to multiply for 2 more days, after which they were trypsinized and plated into a 175-cm² flask. Confluency was reached within about 3-5 days. Experiments were done on the second passage, which was split 1:2 from the first passage.

Human airway smooth muscle culture and transfection with SERCA2b siRNA

Primary hASM were obtained from ScienCell (Carlsbad, CA)^{E3}. hASM were maintained and cultured in flasks or plates (6, 24 and 96 well) using smooth muscle cell media with smooth muscle growth supplement (ScienCell) and 2% fetal bovine serum. hASM were transfected with either control siRNA or SERCA2 siRNA (100nM) by using transfection reagents siTran1.0 (OROGENE) and Opti-MEM (Thermo Fisher Scientific) according the manufacturer's instructions. The transfected hASM cells were used 48 hrs after the transfection in all experiments. We assessed the efficiency of SERCA2a and SERCA2a and 2b gene knockdown by qRT-PCR as previously described in this laboratory^{E3}. There are no

primers specific for SERCA2b. In brief, total RNA was extracted with RNA-STAT-60 (Tel-Test) and reverse transcribed with Oligo-dT and SuperScript II kit (Life Technologies). qRT-PCR was performed with TaqMan PCR Master Mix and SERCA primers (all from Life Technology). The relative amounts of transcripts were normalized to those of housekeeping gene (GAPDH) mRNA and compared between control siRNA transfected samples and ATF6 α siRNA transfected samples by the $\Delta\Delta$ cycle threshold method as previously described in this laboratory^{E3}.

Chemicals and reagents

Oregon Green 488 BAPTA-1 AM was obtained from Life Technologies (Grand Island, NY, USA); Ryanodine was obtained from Abcam Inc. (Cambridge, MA, USA); Other reagents were obtained from either Sigma-Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Pittsburgh, PA, USA). Hanks' Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES buffer (sHBSS) was adjusted to pH 7.4. Hanks' 0-Ca²⁺ solution (0-Ca²⁺ sHBSS) was prepared by supplementing HBSS without Ca²⁺ with 20 mM HEPES, 0.9 mM MgSO₄ and 1 mM Na₂H₂-EGTA. The stock solutions for CPA were prepared in DMSO with final working solutions containing 0.01% (or less) DMSO. Corresponding concentrations of DMSO were included in all control solutions.

Statistical Analysis

UCSD: Results of experiments performed at UCSD (Fig 1, Fig 2, and Fig 5 A-D) were analysed using GraphPad Prism (San Diego, CA). All data are reported as the mean $\pm$ SEM.

A $P < 0.05$ was considered significant. A t test was used for analysis of two groups.

ANOVA analysis was use when more than two groups were compared.

University Massachusetts: Results of experiments performed at the University Massachusetts (Fig 3, Fig 4, Fig 5E-H, Supplement Fig S1, S2, and S3) were analyzed using Origin 2016 software (OriginLab Corporation, Northampton, MA). All data are reported as the mean $\pm$ SEM. A $P < 0.05$ was considered significant. For multiple comparisons, one-way (Figure 5F, 5H) or two-way (Figure 3D, 3E, 4E, 4F and supplemental Figures S2C, S2D, S3C, S3D) ANOVA analysis of variance followed Tukey's honestly significant difference analysis (Tukey's HSD). For between group comparison of supplemental Figure S1, a paired/unpaired samples t test was used.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. No size difference or pre-contraction was observed on tested airways derived from either hORMDL3^{zp3-Cre} or WT mice

There was no difference in the initial airway lumen area recorded for analysis in hORMDL3^{zp3-Cre} compared to WT mice at room temperature (**A**) or body temperature (**B**).

There was no difference in the initial airway size recorded for analysis in hORMDL3^{zp3-Cre} compared to WT mice at room temperature (**C**) or body temperature (**D**). A representative trace shows that 400 nM (R,R)-formoterol can relax MCh-induced airway contraction (**E**), but not airways (without stimulation) derived from either hORMDL3^{zp3-Cre} (red line) or WT mice (black line) (**F**). Each line in (**F**) represents the mean and each point represents the mean $\pm$ SEM at selected time points of the airway lumen area normalized to the initial size at $t = 0$ s. Data in (**A**) and (**C**) “WT” represent $n = 13$ airways, “hORMDL3^{zp3-Cre}” represent $n = 13$ airways from at least 4 mice, respectively; data in (**B**) and (**D**) “WT” represent $n = 17$ airways, “hORMDL3^{zp3-Cre}” represent $n = 20$ airways from at least 5 mice, respectively; data in (**F**) “WT” represent $n = 3$ airways, “hORMDL3^{zp3-Cre}” represent $n = 4$ airways from 3 mice, respectively.

Figure S2. hORMDL3^{zp3-Cre} mice have increased airway contraction compared to WT mice

The response of airways in PCLSs derived from wild type (WT, blue line) and hORMDL3^{zp3-Cre} (red line) mouse, respectively, to increased dose of MCh (A) and 5-HT (B) under room temperature, followed by a comparison of dose response curve to MCh (C) and 5-HT (D) between WT (blue line) and hORMDL3^{zp3-Cre} (red line) mouse. hORMDL3^{zp3-Cre} vs WT, *P<0.05. Each line in (A) and (B) represents the mean and each point represents the mean $\pm$ SEM at selected time points of the airway lumen area normalized to the initial size at t = 0 s. Data in (C) and (D) “WT” represent n = 6 airways, “hORMDL3^{zp3-Cre}” represent n = 9 airways from 4 mice, respectively; data in (C) and (D) “WT” represent n = 8 airways, “hORMDL3^{zp3-Cre}” represent n = 6 airways from 4 mice, respectively.

Figure S3. hORMDL3^{zp3-Cre} mice have increased Ca²⁺ oscillations in airway smooth muscle cells compared to WT mice

Representative traces show Ca²⁺ oscillations of ASM cells in PCLSs derived from wild type (WT) (A) and hORMDL3^{zp3-Cre} mouse (B), respectively, response to increased dose of MCh under room temperature, followed by a comparison of dose response curve to MCh (C) and 5-HT (D) between WT (blue line) and hORMDL3^{zp3-Cre} (red line) mouse. hORMDL3^{zp3-Cre} vs WT, *P<0.05. Representative traces are expressed as intensity (F_t) normalized to the initial intensity at t = 0 s (F₀), measured from a region of interest with

size of 8×10 pixel within a single ASM cells. Data in (C) “WT” represent $n = 12$ ASM cells, “hORMDL3^{zp3-Cre}” represent $n = 13$ ASM cells from 4 mice, respectively; data in (D) “WT” represent $n = 10$ ASM cells, “hORMDL3^{zp3-Cre}” represent $n = 13$ ASM cells from 4 mice, respectively.

Figure S4. The effect of 10 μ M CPA on airway contraction and Ca^{2+} oscillations in ASM cells induced by 400 nM MCh in mouse PCLSs

(A) The effect of 10 μ M CPA on airways contracted with 400 nM MCh in mouse PCLSs, followed by (B) a summary of relaxation effect normalized to control. Controls were treated with vehicle of 0.5% DMSO in sHBSS, $P < 0.05$. (C) Representative trace shows 10 μ M CPA gradually stopped 400 nM MCh-induced Ca^{2+} oscillations and lead to an increased Ca^{2+} baseline in an ASM cell of mouse PCLSs. Each line in (A) represents the mean and each point represents the mean $\pm$ SEM at selected time points of the airway lumen area normalized to the initial size at $t = 0$ s. Representative traces (C) are expressed as intensity (F_t) normalized to the initial intensity at $t = 0$ s (F_0), measured from a region of interest with size of 8×10 pixel within a single ASM cells. Data in (A) and (B) represent $n = 8 - 10$ airways for each line from 5 mice. Data in (C) represent $n = 7$ ASM cells from 5 mice.

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330 **SUPPLEMENTAL VIDEO LEGENDS**

331

332 **Video S1. Airway contraction in mouse precision cut lung slices**

333 A representative comparison of airway contraction in mouse precision-cut lung slices
334 (PCLS) induced by increasing doses of MCh in hORMDL3^{zp3-Cre} and WT mouse.

335

336 **Video S2. Ca²⁺ oscillations in airway smooth muscle cells in mouse precision cut**
337 **lung slices**

338 Representative Ca²⁺ oscillations in airway smooth muscle cells (ASMCs) induced by
339 400 nM MCh in precision-cut lung slices (PCLS) derived from WT mouse. Note that
340 Ca²⁺ oscillations occurred in the form of Ca²⁺ waves propagating along the length of
341 ASMCs.

342

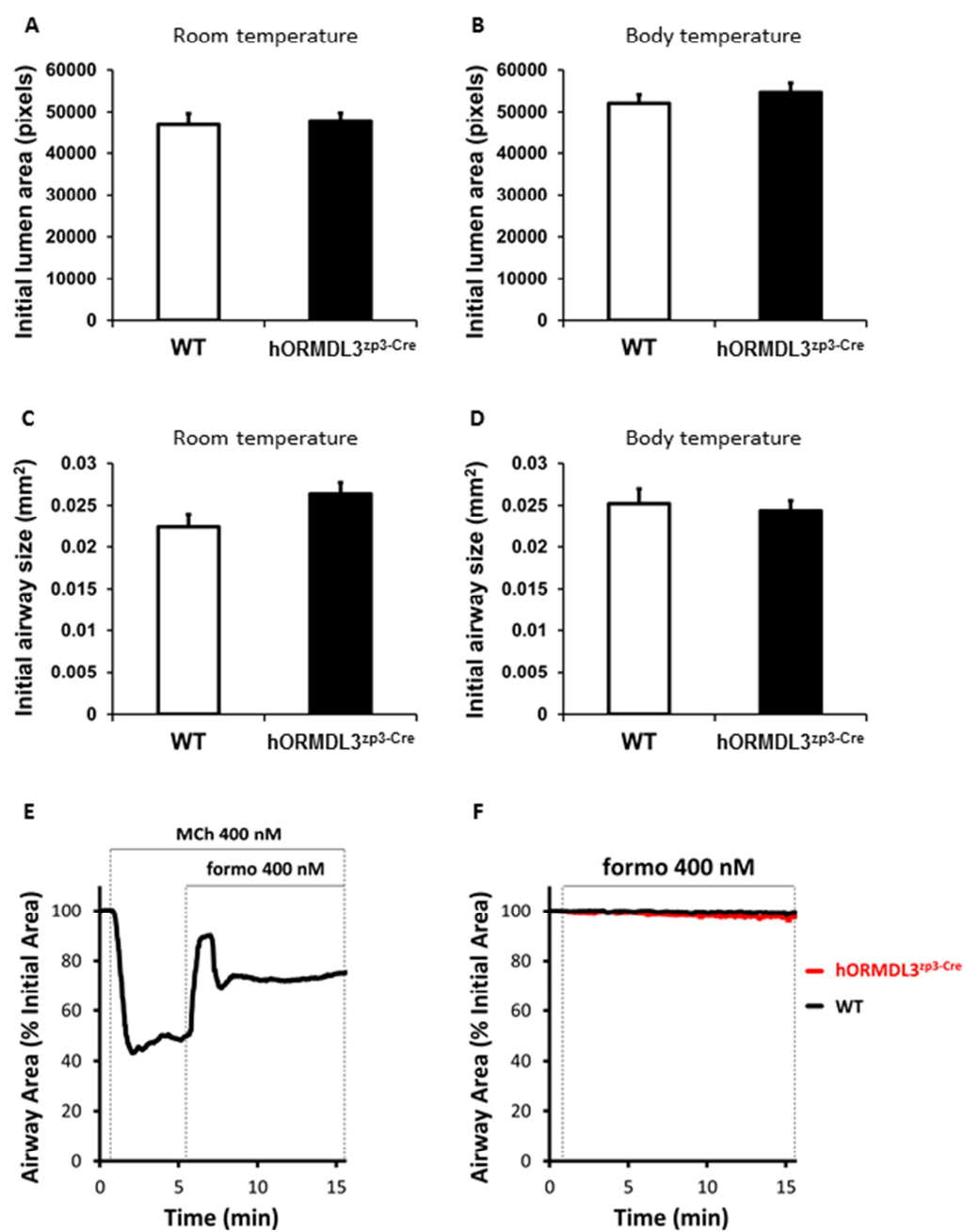


Figure S1

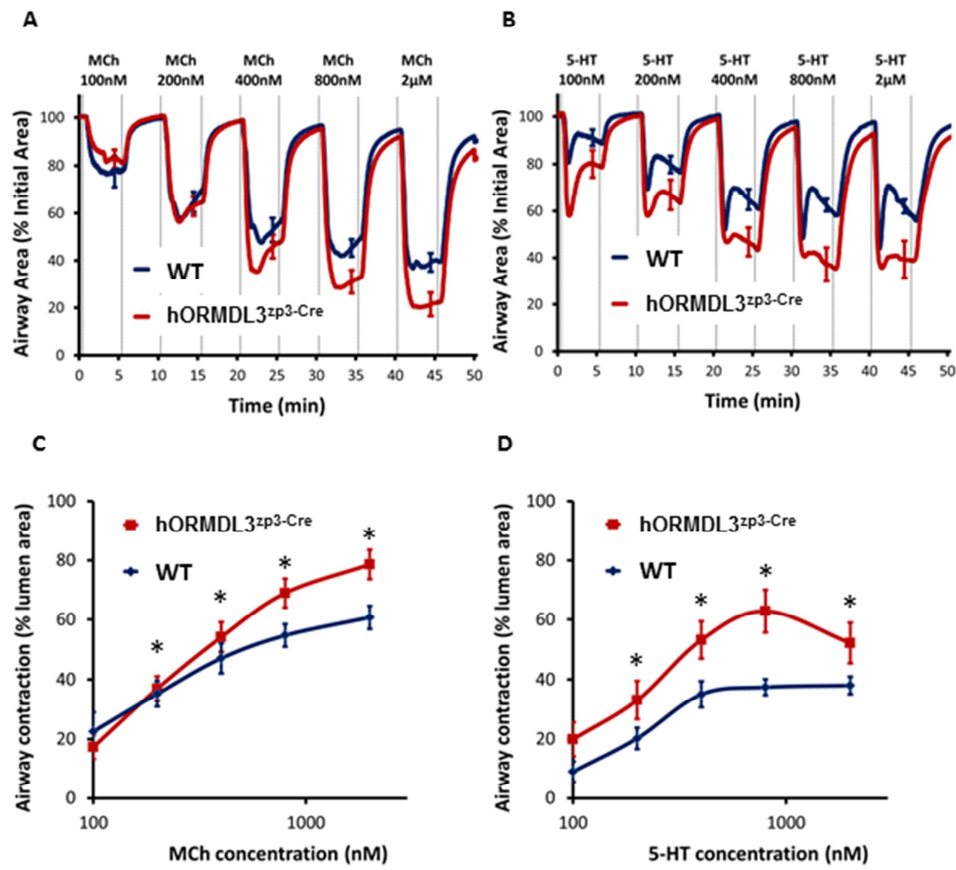


Figure S2

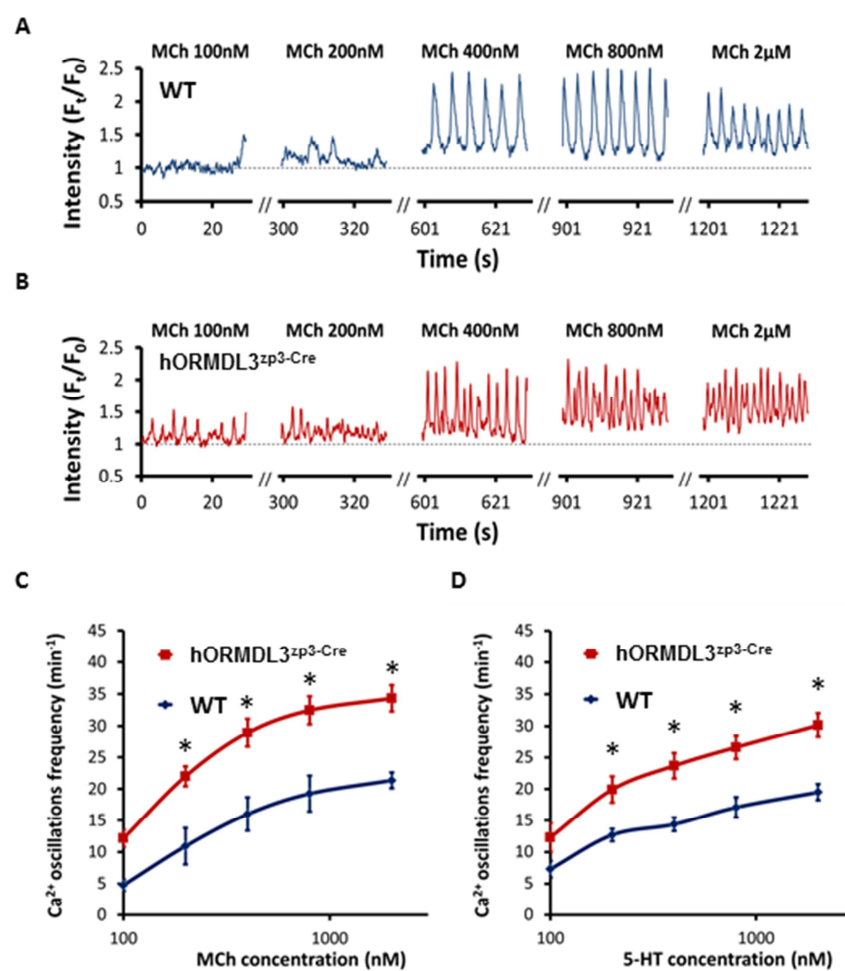


Figure S3

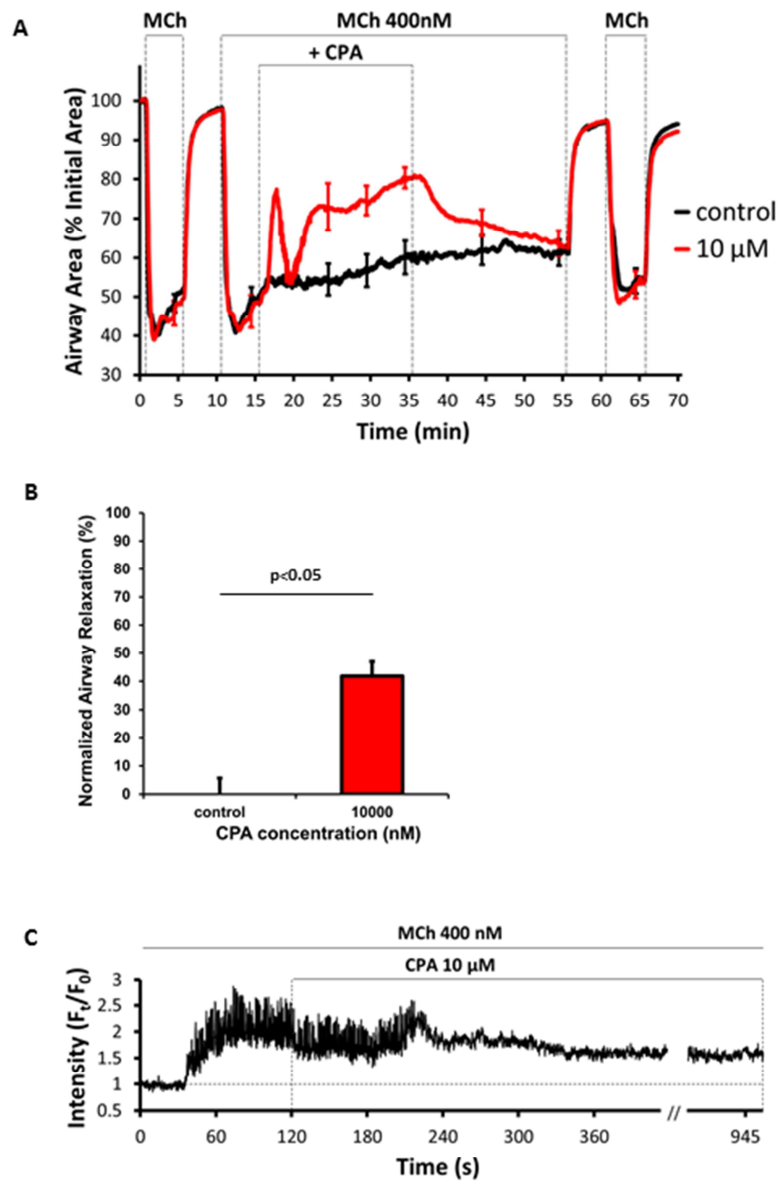


Figure S4