Si Materials and methods

Solution composition

HBSS: composition in mM: 5.3 KCl, 0.44 KH_2PO_4, 137.9 NaCl, 0.336 Na_2PO_4, 2.33 $CaCl_2$, 0.79 $MgSO_4$, 10 glucose, 10 HEPES buffer, pH adjusted to 7.4 with NaOH)

Krebs: (composition in mM: 110 NaCl, 0.82 $MgSO_4$, 1.2 KH_2PO_4, 3.4 KCl, 25.7 $NaHCO_3$, 2.4mM $CaCl_2$, and 5.6 glucose, pH at 7.4, bubbled with 95/5% O_2/CO_2 gas mixture)

Ca2+ free Krebs, as normal Krebs without $CaCl_2$

Preparation, transport, and dissection

Lungs were recovered within 2 h after disconnection from the ventilator. Whole lung blood vessels were flushed with histidine-tryptophan-ketoglutarate (HTK) or University of Wisconsin (UW) solution after which the lungs were shipped in HTK or UW solution and arrived at our facility within 24 h of cross-clamp time.

Airway trees were dissected free from the parenchyma, taking care to minimize stress on the airways. Airway segments between the 3rd and 5th branching generations (2-6mm internal diameter) were dissected out. Segments were placed under a dissection microscope in Ca^{2+} free Krebs solution on ice for further dissection. Airway segments were cut open longitudinally, pinned down and epithelium was removed by stripping or cutting. ASM strips were carefully lifted out of the airway by cutting through the connective tissue layer. Muscle tissue strips were subsequently cleaned from any visible remaining connective tissue and aluminum foil clips were attached on either end of the tissue. ASM area of these dissected tissue strips, as determined from cross-sectional histology slides, averaged 0.0665 ± 0.0123mm^2.

Rejection criteria

As the availability of human lungs is unpredictable and certain measures were rejected because of technical difficulties, the actual sample size varied. To reduce variability, we tested two intrapulmonary and two trachealis tissues for each lung. Tissues that did not contract in response to EFS or MCh, or tissues that showed a >10% reduction in contractile force with repeated contractions, were rejected. Some tissues could not be tested for all protocols because of equipment malfunction, or because of post-hoc determined flaws in control parameter settings (inability to achieve a force control without overshoot). For details see Table S1.

Equilibration

Tissues were subsequently equilibrated using electrical field stimulations (EFS, 25 V/cm at 50Hz, 2ms pulse width for 10s) every 5min for 30min or until the resulting contractile force stabilized. This was followed by at least 5 contractions with MCh 10^{-5} M or 10^{-6} M MCh until a stable baseline and contractile force was achieved. 10^{-6} M MCh was used in earlier experiments where only trachealis muscle was tested, while 10^{-5} M MCh was found to be required for more recent experiments in which intrapulmonary muscle was also tested, as these tissues did not equilibrate well with 10^{-6} M MCh.

Dose response

Tissues were exposed to increasing concentrations of MCh from 10^{-8} M to 10^{-4} M every 100s (Fig. S1A). The peak force reached before each subsequent dose was taken as the force representative of that dose. To calculate stress, the force values were divided by the ASM cross-sectional area which was calculated as follows. Tissues were pinned down at L_{ref} on silicone strips and placed in 10% formalin for >24h, and fixed upright in paraffin to allow for cross-sectional cuts for histological analysis. 5µm thick slices were stained with alpha actin or Masson's Trichrome staining, each providing good contrast between ASM and non-muscle tissue. The ASM cross-section was traced and the area calculated. Both maximal stress and EC_{50}, the dose at which 50% of contractile stress was achieved, were calculated by fitting the data with a sigmoidal dose response curve.

After the tissue was exposed to the last MCh dose, it was exposed every 100s to increasing concentrations of isoproterenol (10^{-8} to 10^{-4}M) to measure its ability to relax (Fig. S1A). The minimum force achieved during each 100s interval was taken as the force representative of that dose. Both maximal relaxation and EC_{50}, the dose at which 50% of relaxation was achieved, were calculated by fitting the data with a sigmoidal dose response curve.

Shortening velocity

Shortening velocity measurements were performed at multiple time points to indirectly assess potential differences in cross-bridge cycling rates and their development during a contraction. Tissues were first shortened briefly by 25% of L_{ref} after which the reference zero force was measured (F_{zero}) to compensate for force drift in the force transducer. Subsequently the tissues were contracted with EFS for 5, 8 or 10s, immediately followed by a rapid 120ms force clamp (Fig. S1B, inset) to a force of 5, 7, 10, 20, 40 or 80% of the force just prior to the force clamp relative to F_{zero}, for a total of 21 individual EFS contractions. The slope of the length signal between 80 and 120ms of the force clamp duration was taken as the shortening velocity at this force clamp (Fig. S1B, inset). Force velocity plots were generated using these shortening velocity data and the post-hoc measured actual force clamp value between 80 and 120 ms. Force-Velocity curves were calculated by performing a perpendicular least squares fitting method of the classic hill curve ($V=b(F_0-F)/(a+F)$ (1).

Viscoelastic properties

To probe the viscoelastic properties of the muscle, a continuous 30Hz, 0.5% L_{ref} peak-to-peak length oscillation was applied to the tissue while it was contracted for 5 min with 10^{-5} M MCh (Fig. S1C). The normalized stiffness was calculated from the peak-to-peak change in stress ($\Delta\sigma$) divided by the strain, or fractional length change. Hysteresivity, a dimensionless quantity that expresses hysteresis as a fraction of the elastic potential energy during an oscillation, was calculated as in (2). In short, when applied to sinusoidal oscillations, η can be calculated as $\eta = \tan(\sin{-1}\ (4A/\pi\Delta F\Delta L))$, with A the area of the force-length loop, ΔF the peak-to-peak force amplitude and ΔL the peak-to-peak length amplitude.

Mass Spectrometry

Ultra-high Liquid Chromatography tandem Mass Spectrometry (uHPLC-MS/MS) was used with the following parameters: After running a single SDS PAGE stacking gel band containing all proteins for each sample, the gel band was reduced, alkylated and digested using trypsin (mass spec sequencing grade, ProMega). Peptides were loaded onto a Thermo Acclaim Pepmap (Thermo, 75uM ID X 2cm C18 3uM beads) precolumn and then onto an Acclaim Pepmap Easyspray analytical column (Thermo, 75uM X

15cm with 2uM C18 beads) separation using a Dionex Ultimate 3000 uHPLC at 220 nl/min with a gradient of 2-35% organic (0.1% formic acid in acetonitrile) over 3 h. Peptides were analyzed using a Thermo Orbitrap Fusion mass spectrometer operating at 120,000 resolution (FWHM in MS1, 15,000 for MS/MS) with HCD sequencing all peptides with a charge of 2+ or greater. The raw data were converted into *.mgf format (Mascot generic format) and searched using Mascot 2.6.2 against Uniprot human sequences. The database search results were loaded onto Scaffold Q+ Scaffold_4.8.9 (Proteome Sciences) for spectral counting. Proteins with an average exclusive peptide count across subjects below 2 were excluded. To isolate differences in the smooth muscle of the whole airway samples, each protein was checked against its prevalence in smooth muscle and non smooth muscle tissues of the airways (cartilage tissue, epithelium, adipocytes, fibroblasts and pneumocytes) as listed in The Protein Atlas (3). Only those proteins which were listed as “not detected” in the non smooth muscle cells and tissues, or were listed as “low” in non smooth muscle tissues and “high” in smooth muscle tissues, were used. To correct for smooth muscle content differences between subjects, we calculated the relative total exclusive spectrum counts (i.e. the mean per protein normalized to 1) for each smooth muscle protein and normalized these by the sum of all the relative spectrum counts of smooth muscle proteins.

Supplementary Figures and Tables

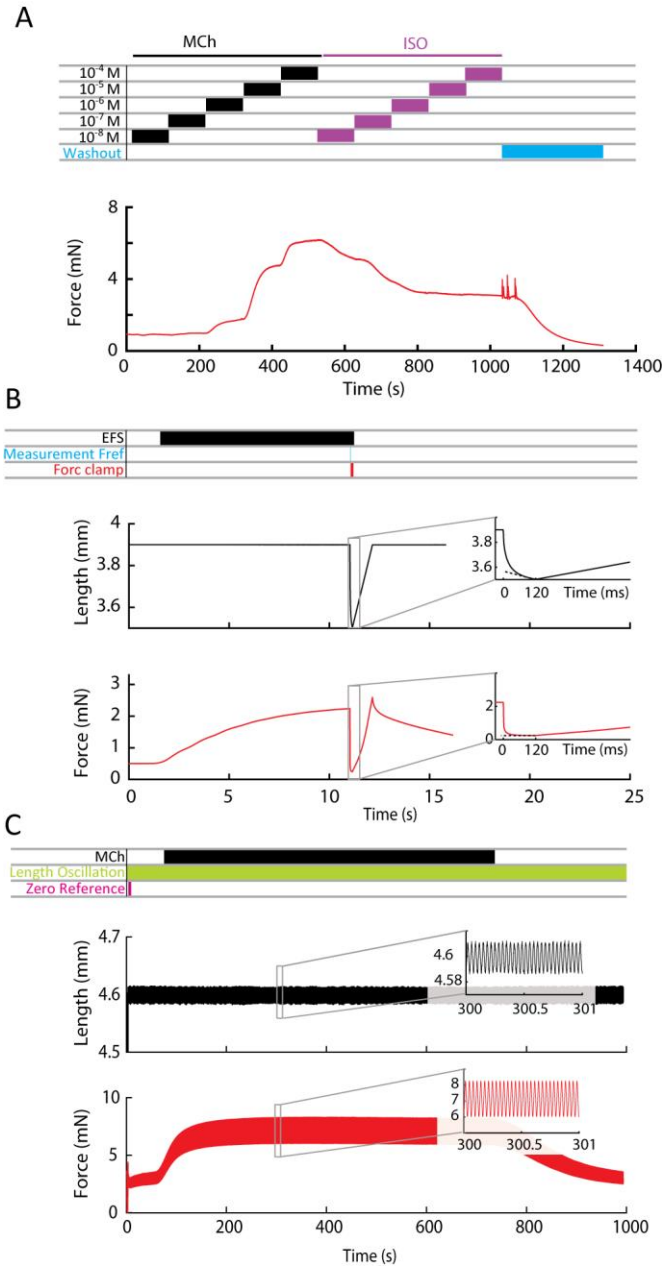

Figure S1: Traces of airway smooth muscle (ASM) mechanics experiments. (A) Sample trace of methacholine (MCh) and isoproterenol (Iso) dose responses. (B) Sample trace of a single EFS force clamp protocol. Top inset shows detail of length during a force clamp, with the slope of the dashed line indicating the shortening velocity. Bottom inset shows detail of force during a force clamp, with force maintained at 5% of the reference force (F_{ref}) for 120ms. (C) Sample trace of length and force oscillations for stiffness and hysteresivity estimation. Insets show detail of length (top) and force (bottom) oscillations.

Table S1: Definition of Abbreviations: A=African American; W=White; H=Hispanic; CVA=Cerebrovascular Accident; HT=Head Trauma; ICH=Intracerebral Hemorrhage; SIGSW=Self Inflicted Gunshot Wound; YA=Years Ago; P.Y.=Per Year. Mechanics Data indicate which experiments were performed on tissue from subject: T=Trachea ASM, IB=Intrapulmonary bronchi ASM, MCh=Methacholine Dose response, Iso=Isoproterenol Dose Response, EFS10=EFS force velocity at 10s, EFS5, 8, 10= EFS force velocity at 5, 8 and 10s, Prot=Proteomics data. *=maximum stress was determined.

Subj.	Sex	Age	BMI	Ethn.	Cause of Death	Asthma history	Other	Medication(s)	Medication in hospital	Data
Asthmatic patients										
1	M	72	44	W	CVA	Age of diagnosis unknown	Smoking 16 P.Y., quit 50 Y.A.			MCh-T*; EFS10-T
2	F	34	32	W	Anoxia from drug intoxication	Diagnosed 20 Y.A., hospitalised twice with exacerbations		Inhaler-Prednisone	Methylprednisolone; Norepinephrine;	MCh-T*
3	M	29	31	W	Anoxia cardiovascular event	Diagnosed 7 years ago	Chewing tobacco for 1 year	Albuterol-Inhaler	Esmolol; Norepinephrine	MCh-T*; EFS10-T
4	M	35	29	W	Anoxia, cardiovascular event	Asthma since childhood.		Albuterol, Budesonide	Norepinephrine; Methylprednisolone; Albuterol	MCh-T*; EFS10-T-IB
5	M	40	27	A	Anoxia, cardiovascular event	Asthma Diagnosed 5 Y.A.		Mometasone and formoterol	Norepinephrine; Methylprednisolone	MCh-T; EFS10-T
6	F	38	36	W	Anoxia cardiovascular event	Asthma diagnosed at 8		Montelukast	Norepinephrine; Epinephrine; Dopamine;	MCh-T-IB; EFS10-T-IB
7	M	23	17	H	CVA	Asthma, unknown when diagnosed	Smoking 7 P.Y. Oxycodone-paracetamol abuse, marijuana & cocaine 1 year	Albuterol	Phenylephrine; Methylprednisolone	MCh-T-IB*; EFS5810-T-IB; Prot
8	F	53	20	W	CVA	Asthma, unknown when diagnosed	Seizure 15 Y.A. , illicit drug use (non IV) 25 Y.A.	Albuterol	Norepinephrine; Epinephrine; Dopamine; Methylprednisolone	MCh-T*-IB*; Iso-T-IB; EFS5810-T-IB; Prot
9	M	23	24	A	Anoxia (Asthma)	Asthma diagnosed age 10, seasonal allergies	Smoking 6 P.Y.	Asthma inhaler (unspecified)	Methylprednisolone, Albuterol	MCh-T*-IB*; Iso-T-IB; EFS5810-T-IB; Prot
10	F	41	24	H	CVA	Asthma, unknown when diagnosed	Hypothyroidism	Asthma medication unknown	Phenylephrine, Vasopressin, Epinephrine, Norepinephrine	MCh-T*-IB*; Iso-T-IB, EFS5810-IB; Prot
11	M	27	26	H	Anoxia, cardiovascular event	Asthma, unknown when diagnosed		Steroid	Norepinephrine, Methylprednisolone, Albuterol	MCh-T*-IB*; Iso-T-IB; EFS5810-T-IB; Prot
12	F	52	33	A	Anoxia, cardiovascular event	History of asthma, resolved but recently	Multiple allergies, diabetes,	Metformin, HTN medications, hemodialysis	Lorezapam; norepinephrine, furosemide;	Prot

						return of symptoms after pneumonia	hypertension, congenital heart disease and heart failure		mannitol	

Control subjects

12	M	22	23	W	Head Trauma 2nd to SIGSW	Asthma as child, not taken medication in 7 years	Smoked Hookah past year	Albuterol as child	Norepinephrine; Phenylephrine; Ipratropium bromide; Albuterol; Methylprednisolone;	MCh-T
13	M	47	26	W	CVA				Phenylephrine	MCh-T*; EFS10-T
14	F	35	22	W	Anoxia from intracranial hemorrhage		Remote marijuana use		Norepinephrine;	MCh-T*; EFS10-T
15	M	30	22	W	Anoxia from Asphyxiation		Smoking: 10 P.Y., Marijuana, Cocaine		Methylprednisolone	MCh-T-IB; EFS10-T-IB; Prot
16	F	54	36	W	CVA		Smoking: 10 P.Y., Quit 8 Y.A.		Epinephrine;	MCh T; EFS10-T
17	F	62	31	W	CVA		Hypertension, Basal cell carcinoma in nose 3 Y.A.		Dopamine	MCh-T*; EFS10-T
18	M	55	27	W	Head trauma		Marijuana occasionally, alcohol abuse		Phenylephrine; Methylprednisolone	MCh-T*-IB*; EFS10-T-IB
19	M	31	24	W	Anoxia, electrocution		Smoking, quit 10 Y.A.		Esmolol, Albuterol	MCh-IB; EFS10-IB; Prot
20	F	58	34	W	CVA				Phenylephrine; Methylprednisolone	EFS10-T
21	F	54	25	W	Head trauma		Smoking 30 P.Y., quit 16 Y.A.; Hypertension		Norepinephrine; Albuterol	MCh-T*; EFS10-T
22	M	49	25	W	HT 2nd GSW		Alcoholism			MCh-T*-IB*; EFS5810-T-IB
23	M	28	22	W	Anoxia 2nd Asphyxiation		Smoking 5 P.Y., Marijuana		Norepinephrine	EFS5810-T-IB
24	F	33	27	A	Anoxia 2nd Drug Intoxication		Bipolar-Depression, Smoking 5 P.Y. Methadone	Depression medication	Norepinephrine, Methylprednisolone	MCh-T*-IB*; Iso-T-IB, EFS5810-T-IB
25	M	38	31	W	Head trauma				Methylprednisolone	MCh-T*-IB*; Iso-T-IB; EFS5810-T-IB
26	F	63	27	W	CVA		Hyperlipidemia, osteopenia	Zocor, methotrexate, Bovina	N/A	MCh-T*-IB*; Iso-T; EFS5810-T-IB
27	F	53	33	W	CVA		Hypertension, anti-phospholipid syndrome, multiple CVA	N/A	Norepinephrine	MCh-T*-IB*; Iso-T-IB; EFS5810-IB; Prot

28	M	18	37	W	Anoxia, cardiovascular event		Autism, Penicillin Allergy	Fluoxetine, Alprazolam, Lisdexamphetamine, Depakote, lithium, Guanfacine, Topiramate, Buspirone, N-Acetyl Cysteine, Prilosec, Clozapine, Clonazepam	Epinephrine, Norepinephrine	MCh-T*-IB*; Iso-T-IB; EFS5810-T-IB; Prot
29	M	55	24	A	CVA		Diabetes, HTN, smoking 40 P.Y., Alcohol 2 per day	Metformin	Methylprednisolone, Epinephrine, Norepinephrine, Heparin, Mannitol, Furosemide, Vasopressin	Prot
30	M	37	37	C	Anoxia, electrocution			none	N/A	Prot

Table S2: Smooth muscle specific proteins

ZYX	Zyxin
PECAM1	Platelet endothelial cell adhesion molecule
SLMAP	Sarcolemmal membrane-associated protein
MYL12A	Myosin regulatory light chain 12A
PTX3	Pentraxin-related protein PTX3
TINAGL1	Tubulointerstitial nephritis antigen-like
SMTN	Smoothelin
VCAN	Versican core protein
SYNPO2	Synaptopodin-2
ACTC1	Actin, alpha cardiac muscle 1
ITGB1	Integrin beta-1
LAMA4	Laminin subunit alpha-4
TPM1	Tropomyosin alpha-1 chain
CNN1	Calponin-1
PALLD	Palladin
MYLK	Myosin light chain kinase, smooth muscle
PGM5	Phosphoglucomutase-like protein 5
TAGLN	Transgelin
DES	Desmin
ACTA2	Actin, aortic smooth muscle
MYH11	Myosin-11o
AEBP1	Adipocyte enhancer-binding protein 1
SRPRB	Signal recognition particle receptor subunit beta
THBS1	Thrombospondin-1
CALD1	Caldesmon
ACTB	Actin, cytoplasmic 1
FLNA	Filamin-A

Table S3: Significantly altered proteins in total airway extract

KANK2	KN motif and ankyrin repeat domain-containing protein 2
SYNM	Synemin
SORBS1	Sorbin and SH3 domain-containing protein 1
SMTN	Smoothelin
LPP	Lipoma-preferred partner
MCAM	Cell surface glycoprotein MUC18
FLNC	Filamin-C
BASP1	Brain acid soluble protein 1
GPX3	Glutathione peroxidase 3
COL4A2	Collagen alpha-2(IV) chain
SYNPO2	Synaptopodin-2
PGM5	Phosphoglucomutase-like protein 5
PALLD	Palladin
POSTN	Periostin
NID1	Nidogen-1
MYL9	Myosin regulatory light polypeptide 9
NDUFA9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial
FERMT2	Fermitin family homolog 2
CORO1C	Coronin-1C
SUN2	SUN domain-containing protein 2
MYH11	Myosin-11o
MYLK	Myosin light chain kinase, smooth muscle
CYC1	Cytochrome c1, heme protein, mitochondrial
ITGB1	Integrin beta-1
CRIP2	Cysteine-rich protein 2
EMILIN1	EMILIN-1
CALD1	Caldesmon
PFKL	ATP-dependent 6-phosphofructokinase, liver type
CAVIN3	Caveolae-associated protein 3
DES	Desmin
CSRP1	Cysteine and glycine-rich protein 1
PGM2	Phosphoglucomutase-2
ATP2B4	Plasma membrane calcium-transporting ATPase 4
LGALS3	Galectin-3
ILK	Integrin-linked protein kinase
LAMA5	Laminin subunit alpha-5
LAMB2	Laminin subunit beta-2
TNS1	Tensin-1
FLNA	Filamin-A
RAB10	Ras-related protein Rab-10
DSTN	Destrin
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein
CLIC4	Chloride intracellular channel protein 4
CHCHD3	MICOS complex subunit MIC19
RSU1	Ras suppressor protein 1
VCL	Vinculin
AOC3	Membrane primary amine oxidase
AIFM1	Apoptosis-inducing factor 1, mitochondrial
TLN1	Talin-1

CAVIN1	Caveolae-associated protein 1
H1F0	Histone H1.0
RAP1B	Ras-related protein Rap-1b
WDR1	WD repeat-containing protein 1
EHD2	EH domain-containing protein 2
CAND1	Cullin-associated NEDD8-dissociated protein 1
ACTA2	Actin, aortic smooth muscle
MYO1C	Unconventional myosin-Ic
DPYSL3	Dihydropyrimidinase-related protein 3
RPS27A	Ubiquitin-40S ribosomal protein S27a
SRI	Sorcin
H2AFY	Core histone macro-H2A.1
SERPINB6	Serpin B6
DLD	Dihydrolipoyl dehydrogenase, mitochondrial
UQCRC2	Cytochrome b-c1 complex subunit 2, mitochondrial
DLST	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial
HMGB1	High mobility group protein B1

Table S4: Database for Annotation, Visualization and Integrated Discovery (DAVID) gene ontology cell component enrichment.

Gene Ontology Term	Count	Bonferroni
GO:0005856 cytoskeleton	26	1.61E-04
→ GO:000015629 actin cytoskeleton	17	7.59E-05
GO:0030054 cell junction	10	0.015271
→ GO:0070161 anchoring junction	10	0.001082
→ GO:0005912 adherens junction	10	0.001082
→ GO:0005924 cell-substrate adherens junction	9	6.60E-04
→ GO:0005925 focal adhesion	8	0.005607
→ GO:0030055 cell-substrate junction	9	9.97E-04
→ GO:0005924 cell-substrate adherens junction	9	6.60E-04
→ GO:0005925 focal adhesion	8	0.005607
GO:0043292 contractile fiber	10	0.005995
→ GO:0044449 contractile fiber part	10	0.004638
GO:0016323 basolateral plasma membrane	10	0.009736

Count column shows the number of proteins that were enriched within this term, out of 66 proteins that were enriched in subjects with asthma. The Bonferroni column shows the Bonferroni corrected p-value of the enrichment. Grey lines indicate duplicate gene ontology terms that appear in multiple hierarchies.

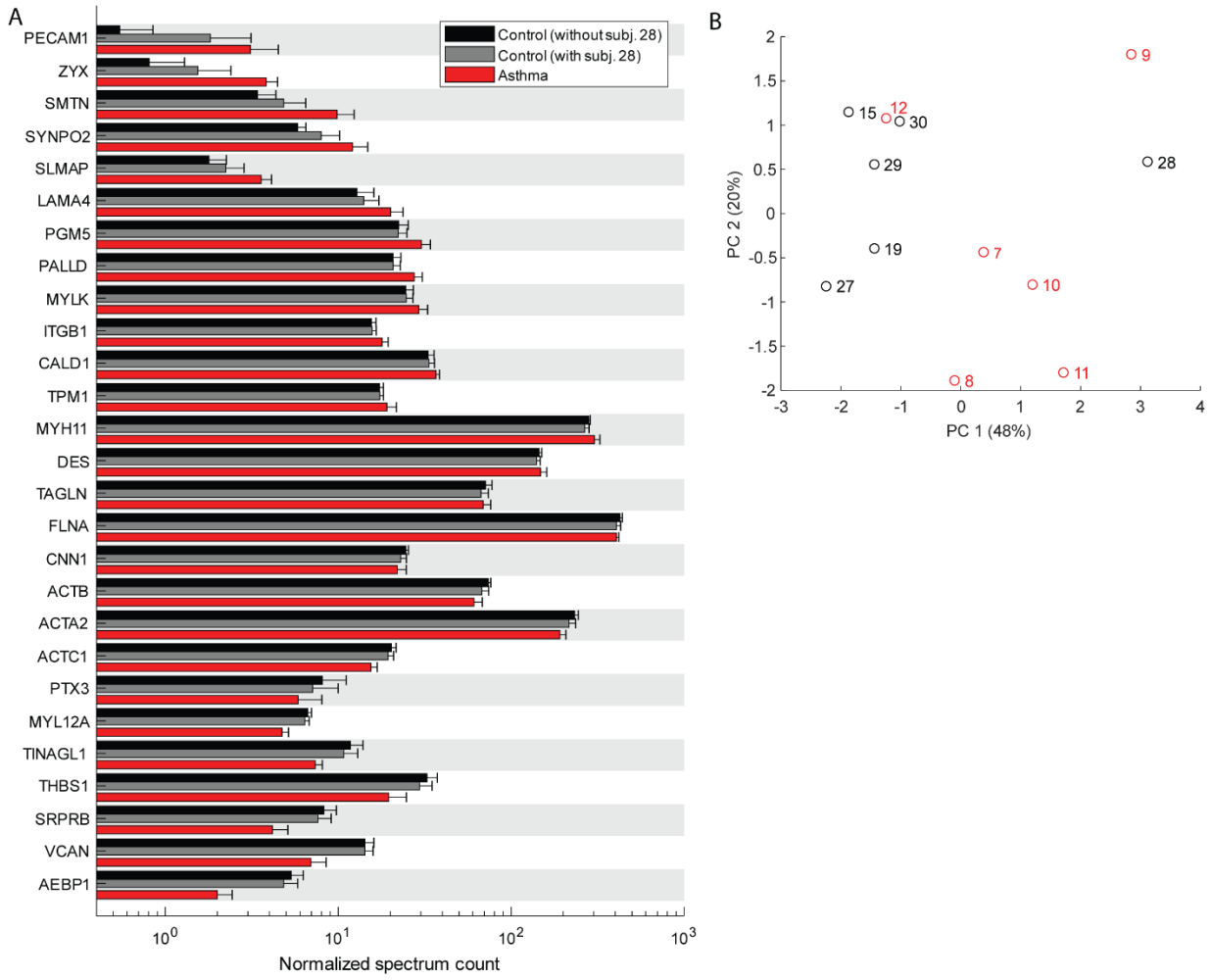

Figure S2 Proteomics data from airway smooth muscle (ASM) specific proteins. (A) Normalized spectrum counts for all smooth muscle specific proteins with a minimum average spectrum count of 2. Black bars are all control subjects excluding clinical outlier subject 28. Grey bars are all control subjects. Red bars are all subjects with asthma. (B) PCA for smooth muscle specific proteins. Black circles are control (subject numbers between brackets) and red circles are asthmatics.

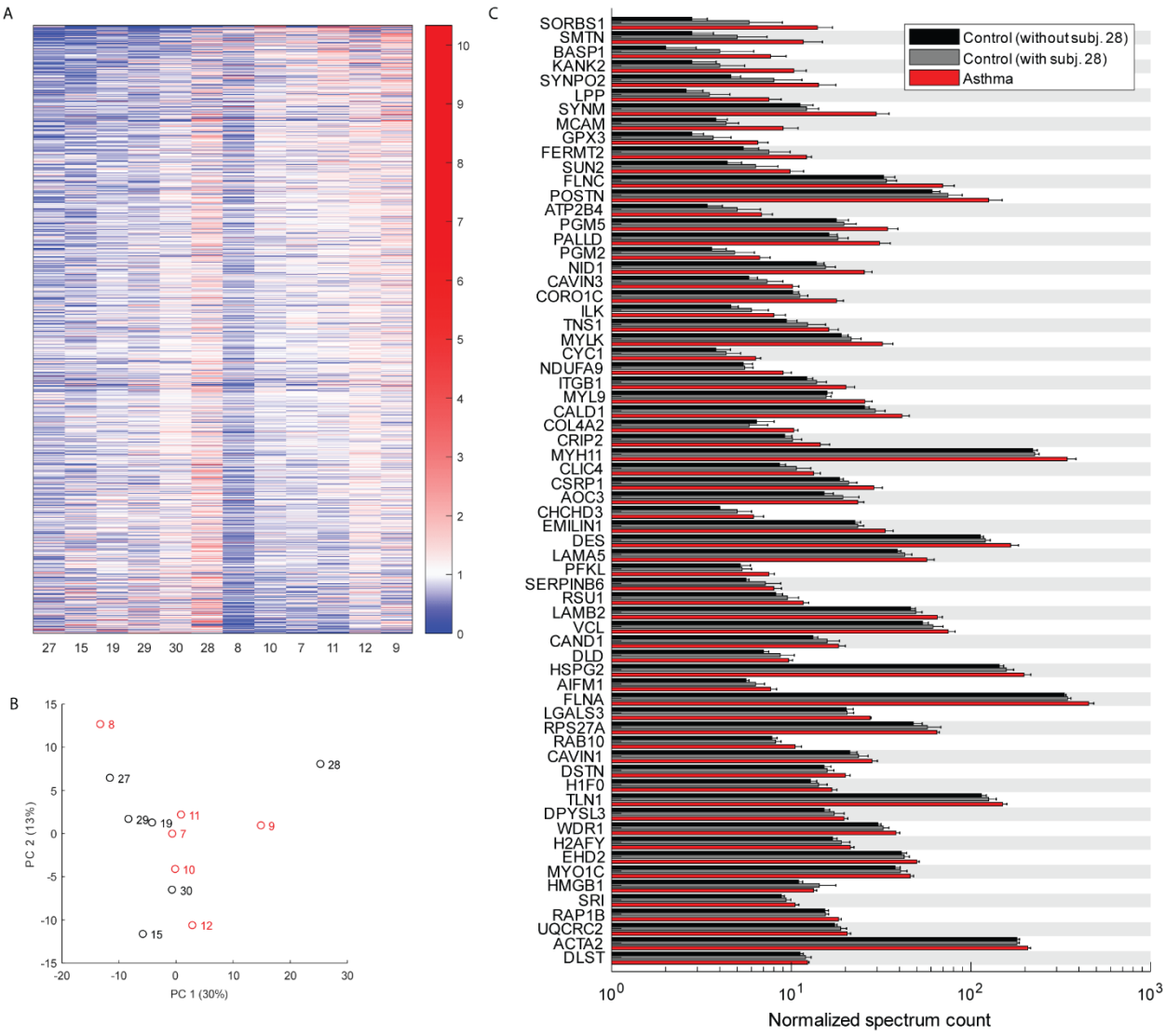

Figure S3: Proteomics data for whole airway extracts. (A) Heatmap of all proteins detected. Subject numbers as in Table 1 (B) PCA for all proteins and all subjects. Black circles are control (subject numbers between brackets) and red circles are asthmatics. (C) Normalized spectrum counts for the 66 proteins that are significantly different in subjects with asthma ($p<0.05$ without correction for multiple comparison). Black bars are all control subjects excluding clinical outlier subject 28. Grey bars are all control subjects. Red bars are all subjects with asthma.

References

1. Bullimore SR, Saunders TJ, Herzog W, & MacIntosh BR (2010) Calculation of muscle maximal shortening velocity by extrapolation of the force–velocity relationship: afterloaded versus isotonic release contractions. *Can. J. Physiol. Pharmacol.* 88(10):937-948.
2. Fredberg JJ*, et al.* (1997) Airway smooth muscle, tidal stretches, and dynamically determined contractile states. *Am J Respir Crit Care Med* 156(6):1752-1759.
3. Uhlén M*, et al.* (2015) Tissue-based map of the human proteome. *Science* 347(6220):1260419.
4. Ammit AJ, Armour CL, & Black JL (2000) Smooth-Muscle Myosin Light-Chain Kinase Content Is Increased in Human Sensitized Airways. *Am J Respir Crit Care Med* 161(1):257-263.
5. Walsh MP*, et al.* (2011) Rho-associated kinase plays a role in rabbit urethral smooth muscle contraction, but not via enhanced myosin light chain phosphorylation. *American Journal of Physiology-Renal Physiology* 300(1):F73-F85.