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Munc18b is an essential gene in mice whose expression is limiting for secretion by airway epithelial and mast cells

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Airway mucin secretion and MC (mast cell) degranulation must be tightly controlled for homoeostasis of the lungs and immune system respectively. We found the exocytic protein Munc18b to be highly expressed in mouse airway epithelial cells and MCs, and localized to the apical pole of airway secretory cells. To address its functions, we created a mouse with a severely hypomorphic Munc18b allele such that protein expression in heterozygotes was reduced by ~50 %. Homozygous mutant mice were not viable, but heterozygotes showed a ~50 % reduction in stimulated release of mucin from epithelial cells and granule contents from MCs. The defect in MCs affected only regulated secretion and not constitutive or transporter-mediated secretion. The severity of passive cutaneous anaphylaxis was also reduced by ~50 %, showing that reduction of Munc18b expression results in an attenuation of physiological responses dependent on MC degranulation. The Munc18b promoter is controlled by INR (initiator), Sp1 (specificity protein 1), Ets, CRE (cAMP-response element), GRE (glucocorticoid-response element), GATA and E-box elements in airway epithelial cells; however, protein levels did not change during mucous metaplasia induced by allergic inflammation. Taken together, the results of the present study identify Munc18b as an essential gene that is a limiting component of the exocytic machinery of epithelial cells and MCs.

Key words: exocytosis, mast cell, mucin, mucus, Munc18, secretion.

INTRODUCTION

SNARE [SNAP (soluble *N*-ethylmaleimide-sensitive factor-attachment protein) receptor] proteins and SM (Sec1/Munc18) proteins collaborate to mediate vesicular transport at every step of the exocytic and endocytic pathways of eukaryotic cells [1–5]. SNARE proteins form a four-helix bundle that is responsible for the specific pairing of donor and target membranes and for inducing membrane fusion. SM proteins provide a scaffold for the assembly of SNARE bundles, and in regulated exocytosis they can also hold the SNARE protein Syntaxin in a closed conformation to prevent its unregulated interaction with other SNARE proteins. Together, SM and SNARE proteins comprise the core vesicle trafficking machinery, whereas other trafficking proteins help to position the SM and SNARE proteins for productive interactions or regulate the timing of their function.

There are seven SM proteins in mammals, and three of these function in regulated exocytosis: Munc18a, b and c. Munc18a is expressed in neurons [6–8], and its deletion in mice causes a complete failure of neurotransmitter release, death at birth and a gradual cell-autonomous loss of neuronal viability [9]. Munc18c is expressed ubiquitously [10], and in polarized epithelial cells is localized to the basolateral membrane [11,12]. Munc18c knockout mice die either *in utero* or shortly after birth, and heterozygous mice have impaired glucose tolerance due to partial defects in both insulin release from pancreatic islet cells and translocation of glucose transporters to the plasma membrane of adipocytes and skeletal muscle cells [13–15]. Munc18b is expressed in epithelial cells and leucocytes [10,16–20], but Munc18b-knockout mice have not been reported. In epithelial cells, Munc18b is localized to the apical membrane [11,21] and appears to participate in apical exocytosis based upon *in vitro* overexpression experiments [22–24]. In MCs (mast cells), Munc18b overexpression or knockdown results in impaired degranulation [25,26]. We targeted the murine Munc18b gene to study its role in airway epithelial and leucocyte secretion.

Secretory epithelial cells of the conducting airways synthesize mucin glycoprotein polymers and secrete them into the airway lumen [27,28]. After secretion, mucins become hydrated to form a viscoelastic mucus gel that is swept from distal to proximal airways by the beating of ciliated epithelial cells intercalated among the secretory cells. Mucociliary clearance removes inhaled particles and pathogens from the lungs by transporting them to the pharynx to be swallowed. Insufficient mucus leaves the lungs vulnerable to injury, but excessive mucus obstructs the airway lumen and contributes to the pathophysiology of common lung

Abbreviations used: AB-PAS, Alcian Blue/periodic acid/Schiff reagent; bHLH, basic helix–loop–helix; CCSP, Clara cell secretory protein; Clca3, chloride channel, calcium-activated, family member 3; CRE, cAMP-response element; DNP, 2,4-dinitrophenol; FBS, fetal bovine serum; FcεRIα, high-affinity IgE receptor, α subunit; FRT, flippase recognition target; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid-response element; HA, haemagglutinin; HSA, human serum albumin; HRP, horseradish peroxidase; IL-3, interleukin-3; INR, initiator; ISH, *in situ* hybridization; MC, mast cell; mBMMC, mouse bone-marrow-derived MC; mClca3, mouse Clca3; MFI, mean fluorescent intensity; mtCC, mouse transformed Clara cell; NK, natural killer; OCT, optimal cutting temperature compound; PAFS, periodic acid/fluorescent Schiff reagent; PBST, PBS containing 0.05 % Tween 20; PGD_2, prostaglandin D_2; PGK, phosphoglucokinase; SCF, stem cell factor; SM, Sec1/Munc18; SNAP, soluble *N*-ethylmaleimide-sensitive factor-attachment protein; SNARE, SNAP receptor; Stxbp2, syntaxin-binding protein 2; TK, thymidine kinase; TNFα, tumour necrosis factor α; WT, wild-type; YFP, yellow fluorescent protein.

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diseases such as asthma and cystic fibrosis. Thus tight control of mucin synthesis and secretion is essential for lung health.

Similarly, regulated exocytosis is a key effector function of leucocytes. For example, MCs store preformed inflammatory mediators in secretory granules whose contents can be rapidly released upon activation of receptors for IgE, complement fragments or ATP [29]. This response helps to initiate an effective defence against pathogens, but can lead to anaphylaxis or allergies if dysregulated [30]. As another example, cytotoxic T-cells and NK (natural killer) cells release the contents of secretory granules to kill target host cells infected with viruses and to reduce leucocyte numbers after their expansion during an infection. Mutations in genes encoding the exocytic proteins Munc13-4, Syntaxin 11 and Munc18b or the lytic granule protein perforin cause familial haemophagocytic lymphohistiocytosis [31–33]. These examples indicate the importance of leucocyte secretory function for homoeostasis.

EXPERIMENTAL

WT (wild-type) and transgenic mice

All mice were handled in accordance with the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. WT C57BL/6J mice were purchased from the Jackson Laboratory. To generate HA (haemagglutinin)–YFP (yellow fluorescent protein)–Munc18b transgenic mice, the HA epitope tag and Venus YFP were cloned by PCR in tandem with rat Munc18b and inserted into a mammalian expression vector containing the mouse CCSP (Clara cell secretory protein) promoter, a rabbit β-globin intron, and the bovine growth hormone polyadenylation signal [34,35]. The DNA was purified and microinjected into the pronucleus of fertilized C57BL/6 oocytes, which were then implanted into pseudopregnant C57BL/6 females. Transgenic offspring were identified by PCR analysis of genomic DNA from tail biopsies using a 5′ primer specific to the CCSP promoter (5′-CTGTGTCTTTGTCCTTCCCTGTG-3′) and 3′ primer specific to Munc18b (5′-TTACAACAGGATGACAAGATTCG-3′). Three transgenic lines were established.

Munc18b hypomorphic mice

Homology arms and insertion fragments were generated by PCR using the BAC (bacterial artificial chromosome) clone RP24-321J16 as a template. The backbone vector (a gift from Professor Thomas Südhof, Stanford Institute for Neuro-Innovation and Translational Neurosciences, Stanford University School of Medicine, Stanford, CA, U.S.A.) contains the neomycin-resistance gene under the control of the PGK (phosphoglucokinase) promoter (PGK-Neo) flanked by two FRT (flippase recognition target) sites situated between the two cloning sites for the recombination arms. The herpes virus TK (thymidine kinase) gene under the control of the same promoter (PGK-TK) is situated outside the homology region. The 5′ homology arm was obtained with the primers 5′-GTCGACGGCTTTCCTCCTCACAATT-3′ and 5′-GTCGACATGTTGATCACATTCACCCT-3′, and included 2927 bp of the region upstream to exon 1 of the *Stxbp2* (Syntaxin-binding protein 2) gene (encoding Munc18b; NCBI Gene ID 20911). This was cloned into the SalI site of the vector. The 3′ homology arm was obtained with primers 5′-CTCGAGGGCTGACGAGTTTCTGAA-3′ and 5′-CTCGAGGGTAACTGAGGAAGACA-3′, included 3292 bp between introns 1 and 7, and was cloned into the XhoI site. The replacement insert was obtained with primers 5′-GGTACCCATTATATGCATGTATGA-3′ and 5′-ATCGATCAGGGTTCAGAAACTCGT-3′, included 1068 bp surrounding exon 1, and was cloned between the ClaI and KpnI sites. This targeting vector was electroporated into B6129SF1 ES cells that were then subjected to positive selection of recombination events with neomycin and to negative selection of non-homologous recombination events with gancyclovir. ES cell clones were assessed by PCR with primers inside the construct and outside the homology arms to confirm homologous recombination: 5′-TTCCATTTGTCACGTCCTGCAC-3′ and 5′-TTCCAGCACCCACATCCAGAG-3′ on the 5′ side, and 5′-CCCTGGAACTCCTGCTGTCAT-3′ and 5′-CCGCTTCCTCGTGCTTTACG-3′ on the 3′ side. Confirmed clones were expanded and ES cells were injected into C57BL/6J-*Tyr*$^{c-2J}$/J blastocysts, which were then implanted into pseudopregnant females of the same strain. Chimaeric males were crossed with C57BL/6J-*Tyr*$^{c-2J}$/J females, and non-albino pups were screened for the desired mutation by PCR with three primers: 5′-AAGGCGGTGGTAGGGGAAAGT-3′, 5′-CCGCTTCCTCGTGCTTTACG-3′, and 5′-CTCGGGTGATCCATGATGAGG-3′. The lengths of the PCR products were 1337 bp from the WT allele and 997 bp from the mutant allele, and the same strategy was used for all subsequent genotyping. The expected homologous recombination event would flank exon 1 with two loxP sites and insert FRT-PGK-Neo-FRT into intron 1. The original goal was to remove exon 1 with Cre recombinase, eliminating the start codon for translation of Munc18b, and any alternative ATG codon in exons 2 and 3 would induce frameshift mutations with early stop codons. Sequencing the genomic region surrounding exon 1 in heterozygous mice revealed that the loxP site 5′ of exon 1 was not introduced during recombination. Nonetheless, we analysed whether the presence of PGK-Neo in intron 1 disrupted expression of the Munc18b gene. The hypomorphic mice used in these studies were on a 75–93 % C57BL6 background. Experimental and control animals were obtained by crossings of heterozygous mutants with C57BL/6 mice.

Cell harvesting and culture

Peritoneal MCs were isolated as described previously [36]. In brief, after killing, the peritoneal cavity was lavaged with 10 ml of PBS. The resulting lavage fluid was centrifuged at 450 ***g*** for 5 min at room temperature (22 °C), and the obtained cell pellet was resuspended in 0.5 ml of PBS. Cells were counted using a Neubauer chamber, and differential cell counts were performed in cytospin samples stained with Wright–Giemsa. mBMMCs (mouse bone-marrow-derived MCs) were obtained as described previously [29]. In brief, harvested bone marrow cells from mouse femora and tibiae were cultured for ∼6 weeks in medium supplemented with 5 ng/ml recombinant mouse IL-3 (interleukin-3) and 100 ng/ml SCF (stem cell factor) (both from R&D Systems). To follow their development, aliquots (100 μl) of 5×10^4 non-adherent cells in the culture were analysed at different time intervals by flow cytometry as described below.

Antibodies

Three different rabbit polyclonal antibodies to Munc18b were used, designated M18b-O, M18b-A and M18b-D. M18b-O was raised against a recombinant polypeptide and supplied by Vesa Olkkonen [18]; M18b-A was raised against a synthetic peptide and purchased from Abcam (catalogue number ab3451); M18b-D was raised by us against the isoform-specific peptide QSYSSLIRNLEQLGGTVTNSAGSGTSS (amino acids 432–458). The performance of M18b-O has been described previously [18]. The selectivity of M18b-A and M18b-D was tested with

immunoblots using Munc18 proteins expressed in bacteria and purified as described previously [37,38]. M18b-A reacted with Munc18a ~3-fold more intensely than Munc18b, but did not detect Munc18c (results not shown); M18b-D detected Munc18b, but not Munc18a or Munc18c in 100-fold greater amounts (Supplementary Figure S1 at http://www.BiochemJ.org/bj/446/bj4460383add.htm). Rabbit polyclonal antibodies against Syntaxin 1 (#S1172) and Syntaxin 4 (#S9924) were purchased from Sigma–Aldrich, to Syntaxin 2 were purchased from Synaptic Systems (#110-022), and to Syntaxin 3 were supplied by Vesa Olkkonen [21]. Rabbit monoclonal antibodies against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were purchased from Cell Signaling Technology (#14C10), and rabbit polyclonal antibodies against mClca3 [mouse Clca3 (chloride channel, calcium-activated, family member 3)] [39] were a gift from Dr Karen Affleck (GlaxoSmithKline, Stevenage, U.K.).

Immunoblots

Tissues from mice were placed in iced 0.5 % PBS containing a protease inhibitor cocktail (Sigma, #P8340, diluted 1:100), and manually homogenized using a ground glass tissue grinder (Kontes). The human lung cancer cell lines A549, Calu-3, Mes-1, H-2170 and H-292 were acquired from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured according to the supplier's recommendations. mtCCs (mouse transformed Clara cells) [40] were provided by Professor Francesco DeMayo (Program in Developmental Biology, Baylor College of Medicine, Houston, TX, U.S.A.) and cultured in high-glucose Dulbecco's modified Eagle's medium with 5 % (v/v) FBS (fetal bovine serum) (Gibco). Cell pellets from a confluent 10 cm plate were washed and resuspended in 10 ml of PBS containing 1 % Triton X-100 and protease inhibitor cocktail as above, then homogenized by sonication at the maximal setting for 30 s (Sonic Dismembrator F50, Fisher Scientific). Tissue and cell homogenates were centrifuged to remove particulates, protein concentrations in the supernatants were measured by a bicinchoninic acid assay (Pierce), and homogenates were diluted in PBS to 2 mg/ml, then boiled in SDS sample buffer [0.1 M Tris, 24 % (w/v) glycerol, 8 % (w/v) SDS, 0.2 M DTT (dithiothreitol) and 0.02 % Coomassie Blue G-250]. Samples were resolved by SDS/PAGE using 11 % Tris-glycine or 4–15 % Tris/HCl linear gradient gels, transferred to PVDF membranes (Millipore) and blocked with 5 % (w/v) non-fat milk in PBST (PBS containing 0.05 % Tween 20). Blots were incubated with primary antibodies in 5 % (w/v) non-fat milk in PBST for 1 h, washed sequentially with PBST and PBS, then detected using HRP (horseradish peroxidase)-conjugated secondary antibodies (Jackson ImmunoResearch) and chemiluminescence reagents (Pierce). GAPDH, detected with a specific antibody (Abcam), was used as a loading control.

ISH (*in situ* hybridization)

ISH was performed in collaboration with the Gene Expression Core Service of Baylor College of Medicine [41,42], but the published protocols using frozen tissue were modified for fixation and embedding to obtain better resolution as follows. The lungs of an adult C57BL/6 mouse were inflated intratracheally with 3.7 % formaldehyde in 0.1 M phosphate buffer (pH 7.0) at 15 cmH_2O of pressure (1 $cmH_2O = 98.0665$ Pa), fixed *in situ* for 10 min, then removed from the thoracic cavity, immersed in the same solution overnight at 4 °C, embedded in paraffin and sliced into 3 μm sections. After deparaffination, acetylation and rehydration, slides were assembled in flow-through hybridization chambers and placed into a Tecan Genesis 200 liquid-handling robot, which executed a script for non-radioactive ISH using 120 nM digoxygenin-labelled RNA antisense probes purchased from Exiqon. The probe sequences were 'Munc18b-antisense' 5′-AGCCCCAAGTTTGCTGTATTTT-3′, and 'DD-scrambled' 5′-GTGTAACACGTCTATACGCCCA-3′ that has no homology to known RNAs. Hybridized probes were detected by catalysed reporter deposition using biotinylated tyramide followed by avidin-coupled alkaline phosphatase, resulting in a dark blue precipitate.

Immunohistochemistry

Lungs were inflated, fixed and embedded as described previously [43]. Deparaffinated sections were washed with PBS, permeabilized with 0.05 % Triton X-100 for 15 min, washed with PBST, exposed for 30 min to 6 M guanidinium chloride in 50 mM Tris buffer (pH 7.5) for antigen retrieval, washed with PBST, exposed to 3 % H_2O_2 in 90 % methanol for 30 min to quench endogenous peroxidases, then washed with PBST. Specimens were blocked with 5 % (v/v) goat serum and 5 % (v/v) FBS in PBS for 2 h, washed with PBST, labelled overnight at 4 °C with M18b-O antibody diluted 1:500 in 5 % (v/v) FBS and 0.5 % saponin in PBS, then incubated for 1 h at 21 °C with HRP-labelled goat anti-(rabbit Fab) (Jackson ImmunoResearch) diluted 1:1000 in 5 % (v/v) FBS and 0.5 % saponin in PBS, washed in PBST, and developed with 3-3′ diaminobenzidene (Vector Laboratories). Nuclei were counterstained with Methyl Green.

Fluorescence microscopy

For fluorescence microscopy of YFP–Munc18b, the lungs of transgenic mice were inflated and fixed with 4 % (w/v) paraformaldehyde as above, then serially dehydrated at 4 °C in 20 % sucrose for 4 h, 50 % sucrose for 2 h, 50 % sucrose and 50 % OCT (optimal cutting temperature compound) overnight, then instilled with OCT intratracheally and frozen. Sections 5 μm thick were washed twice for 10 min with PBS at 21 °C, then viewed through an upright Olympus BX60 microscope with a Chroma Technology 41017 filter set.

For fluorescence microscopy of MCs, ears were excised, fixed overnight at 4 °C in 4 % (w/v) paraformaldehyde (pH 7.0), dehydrated and embedded in paraffin. Avidin binds strongly to the heparin-containing serglycin proteoglycans in the secretory granules of MCs. Thus 5 μm cross-sections were deparaffinized, rehydrated, incubated with FITC–avidin and Hoechst dye 33342 (Molecular Probes®, Life Technologies) for 1 h at 25 °C, and then mounted with Fluoromount (Diagnostic BioSystems). Images were acquired using a fluorescent microscope with a triple fluorescent filter [DAPI (4′,6-diamidino-2-phenylindole), GFP (green fluorescent protein) and Texas Red]. Taking advantage of the autofluorescence of the cartilage and muscle observed in the red channel, we used the Image Pro Plus software package to delineate the dermis of the ears as the tissue between the epidermal layers, excluding all muscle and cartilage. All dermal FITC–avidin-positive cells with a Hoechst-positive nucleus were counted. Results were expressed as the number of mature MCs/mm^2 of dermis [44].

Electron microscopy

Cells were processed as described previously [29,44]. In brief, 5×10^4 peritoneal cells or mBMMCs were fixed in 2.5 % (v/v) glutaraldehyde/0.1 M sodium cacodylate (pH 7.2) for 2 h and then incubated in 1 % (w/v) OsO_4 for 1 h. After washing with double-distilled water, the cells were pelleted and embedded in Araldite

(Huntsman Advanced Materials), sectioned at 100 nm with an ultramicrotome, stained with uranyl acetate and lead citrate, and then examined with a JEOL 200CX electron microscope. Cell profiles from each section were photographed using an unbiased random sampling technique. The fraction of cell profiles identified morphologically as MCs (e.g. by the presence of their characteristic electron-dense granules, non-segmented nucleus and surface microplicae) was assessed by unbiased stereology. The volume fraction (which represents the fraction of the total volume of the cell occupied by granules) and the surface density (which is directly proportional to the membrane surface of the granules per unit of volume) were calculated with the point-counting method using a cycloid grid. The area of the cell profiles was measured using a point grid.

Flow cytometry

Aliquots of 5×10^4 mBMMCs were incubated with 200 ng of anti-mouse Kit/CD117 PE-Cy7 and 200 ng of anti-mouse FcεRIα (high-affinity IgE receptor, α subunit) Alexa Fluor® 647 (eBioscience) in 100 μl of PBS for 30 min at 4 °C. The labelled cells were washed twice with PBS, resuspended in 0.5 ml of PBS and analysed in a four-laser LSRII flow cytometer (Becton-Dickinson). The number of CD117$^+$/FcεRIα^+ cells and their MFI (mean fluorescent intensity) at the emission ranges of the fluorophores were recorded, since the obtained data should be proportional to the amount of the two receptors expressed on the surfaces of the MCs [36].

Mucin secretion

Mucous metaplasia was induced in the airways of mice by intraperitoneal immunization and aerosol challenge with ovalbumin [45,46]. At 3 days after ovalbumin aerosol exposure, half of the mice were exposed for 5 min to an aerosol of 100 mM ATP in 0.9 % NaCl solution to induce mucin secretion, then killed after 20 min. Lungs were inflated and fixed with paraformaldehyde as above, then fixed, embedded, sectioned and stained with AB-PAS (Alcian Blue/periodic acid/Schiff reagent) or PAFS (periodic acid/fluorescent Schiff reagent) as described previously [45]. Littermates were used as controls for the secretion experiments because of the mixed genetic background. For the quantification of intracellular mucin, data are presented as the epithelial mucin volume density, derived from analysis of images of airways stained with PAFS as described previously [45,46]. Images were acquired and analysed by investigators blinded to mouse genotype and treatment.

MC secretion assays

To test secretory responses, 10^6 mBMMCs were resuspended in 1 ml of culture medium and sensitized with anti-DNP (2,4-dinitrophenol) IgE (5 μg/ml) for 3 h. After repeated washes, the cells were stimulated with 100 ng/ml DNP-HSA (human serum albumin). Secreted products were measured in supernatants before and after stimulation. Histamine was also measured in cell lysates (10^6 mBMMCs treated with 1 ml of 0.2 % Triton X-100). After stimulation, PGD_2 (prostaglandin D_2) was measured at 30 min, histamine at 1 h and TNFα (tumour necrosis factor α) at 6 h. All products were measured using ELISA (Oxford Biomedical Research) [29]. Other samples of mBMMCs were collected for electron microscopy before and after stimulation. To stop degranulation at specified times, the cells were placed on ice and 1 ml of 5 % (v/v) glutaraldehyde/0.2 M sodium cacodylate (pH 7.2) was added to the aliquots. To test secretory responses from peritoneal MCs, peritoneal lavages obtained as described above were enriched for MCs as follows. Cells suspended in 1 ml of modified Tyrode's buffer (10 mM Hepes, pH 7.3, 130 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose and 1 mg/ml BSA) were layered over a 2 ml solution of 22.5 % metrizamide (Nycomed/Accurate Chemical), then centrifuged at 400 *g* for 15 min at 4 °C, and MCs were recovered at the bottom. Purification was confirmed by Toluidine Blue staining or by flow cytometry [44], then 10^5 peritoneal MCs were sensitized and stimulated, and histamine in cell lysates and supernatants was determined as described for mBMMCs above.

Passive cutaneous anaphylaxis

Under anaesthesia with isoflurane, 100 ng of both anti-DNP IgE and anti-dansyl IgE (Pharmingen) in 20 μl of PBS were injected intradermally into the right and left ear pinnae respectively. Two days later, the treated mice were challenged intravenously with 100 μg of DNP-HSA in 200 μl of PBS containing 0.5 % Evans Blue. The mice were killed 30 min later, and their ears were excised and incubated in 150 μl of formamide at 55 °C for 24 h. The absorbance of Evans Blue in each supernatant was measured at 610 nm using a μQuant universal microplate spectrophotometer (Bio-Tek Instruments). MC-deficient Kit^{W-sh}/Kit^{W-sh} mice were a gift from Professor Stephen Galli (Stanford University School of Medicine, Stanford, CA, U.S.A.) and used as a negative control as described previously [29].

Statistical analysis

Quantitative data are presented as means $\pm$ S.E.M., and statistical analysis was performed using Student's *t* test (SPSS Statistics, version 16.0). $P < 0.05$ was considered significant.

RESULTS

Munc18b is strongly expressed in airway epithelial cells and leucocytes

The expression of Munc18b protein was analysed in mouse tissues by immunoblotting with isoform-specific M18b-D antibodies. Bands were observed in multiple tissues at 72 kDa (Figure 1A), consistent with the mobility of post-translationally modified Munc18b (the unmodified protein is 67 kDa; Supplementary Figure S1). Munc18b was most strongly expressed in lung and spleen, followed by kidney and colon, and weakly in liver. Munc18b was not detected in brain, heart or duodenum. Similar results were obtained using M18b-O and M18b-A antibodies, along with strong expression in MCs (Supplementary Figure S2 at http://www.BiochemJ.org/bj/446/bj4460383add.htm). Taken together, these results suggest that Munc18b protein is most strongly expressed in secretory epithelia and leucocytes.

To assess Munc18b expression in mouse conducting airways that contain mucin-secreting goblet cells separately from lung alveoli that contain surfactant-secreting Type 2 cells, tracheal tissue was analysed and also showed staining (Figure 1A, lane 1). To assess Munc18b expression in isolated lung epithelial secretory cells, human and mouse lung adenocarcinoma cell lines were analysed, and bands of the appropriate size were observed in all of these (shown in Figure 1A for A549 and in Supplementary Figure S3 at http://www.BiochemJ.org/bj/446/bj4460383add.htm for Calu-3 and mtCC).

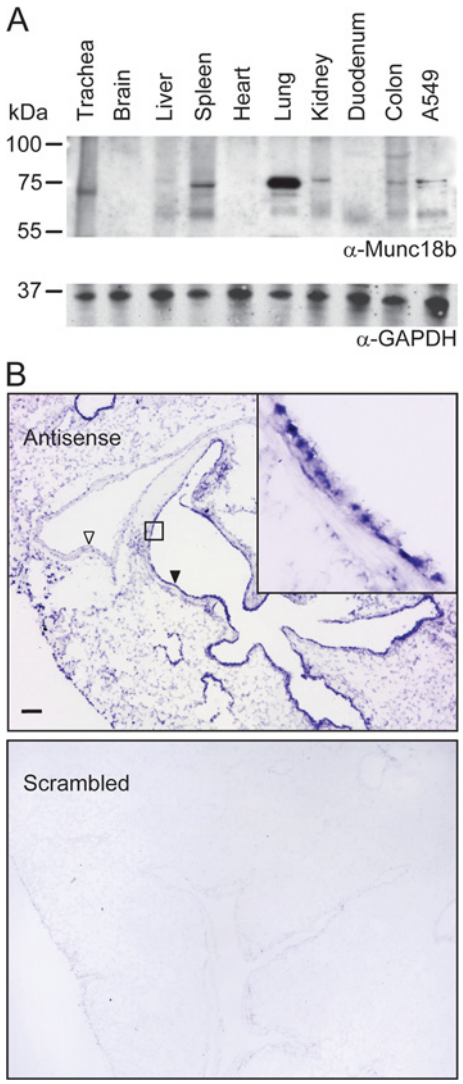

Figure 1 Munc18b is strongly expressed in lung epithelial cells and spleen

(**A**) Immunoblots of C57BL/6 mouse tissues and the A549 human lung adenocarcinoma cell line were probed with an isoform-specific polyclonal anti-Munc18b antibody (M18b-D). Dark bands consistent with post-translationally modified Munc18b are observed at 72 kDa. (**B**) ISH. An antisense RNA probe specific for Munc18b was used to analyse mRNA distribution in a section of mouse lung (upper panel). There is strong linear staining in airway epithelium (solid arrowhead), but not in vascular endothelium (open arrowhead), and scattered staining on the lung surface (lower left) and in alveoli. Scale bar = 200 μm for the low-magnification image and 20 μm for the inset. As a negative control, a section of mouse lung was incubated with a scrambled sequence probe (lower panel).

ISH using a short synthetic isoform-specific probe showed strongly expression of Munc18b in airway epithelial cells (Figure 1B, upper panel, solid arrowhead). In contrast, there was little or no staining in endothelial cells of neighbouring blood vessels within the bronchovascular bundle (Figure 1B, upper panel, open arrowhead), and no staining anywhere with a control probe (Figure 1B, lower panel). In addition to airway epithelial cells, there was linear staining of mesothelial cells on the lung surface (Figure 1B, upper panel, lower left), and scattered punctate staining of alveolar epithelial Type 2 cells and alveolar macrophages (Figure 1B, upper panel). At higher magnification of the airway epithelium (Figure 1B, inset), staining appeared to be present in alternating cells, consistent with the alternating ciliated and secretory phenotypes of airway epithelium [28,45], although cilia or other distinguishing features of either cell type could not be identified with certainty.

Munc18b localizes to the apical pole of airway secretory cells

Cell-specific localization of Munc18b protein in airway epithelium was determined by immunohistochemical analysis using M18b-O antibodies. This revealed intense staining at the apical (lumenal) pole of secretory (Clara) cells, but not ciliated cells (Figure 2A, upper panel). Irrelevant antibodies used as a control did not result in comparable staining (Figure 2A, lower panel).

To more precisely determine the subcellular localization of Munc18b, we generated three transgenic mouse lines expressing an N-terminal HA–YFP tag on the full-length Munc18b protein, targeted to airway secretory cells using the CCSP promoter. The size of the immunoblotted transgenic protein was ~100 kDa, consistent with the combined masses of Munc18b (67 kDa) and YFP (36 kDa), and transgene expression in the highest-expressing line was approximately 0.5-fold that of endogenous protein (results not shown). The airways of transgenic mice viewed by fluorescence microscopy showed linear staining of the apical plasma membrane of domed cells (Figure 2B, upper panel) protruding into the airway lumen by bright-field microscopy (Figure 2B, lower panel), consistent with the known morphology of airway secretory cells [28,45].

Munc18b is an essential gene

To study Munc18b function, we generated mutant mice by homologous recombination in embryonic stem cells (Figure 3A). The loxP site 5′ of exon 1 was repeatedly lost during recombination, resulting in a genomic sequence that is not susceptible to Cre recombinase-mediated removal of the first exon and translational start site. However, these mice were tested for a potential hypomorphic phenotype resulting from the ~1.9 kb insertion of PGK-Neo into intron 1 that might disrupt transcription. To determine Munc18b protein expression from the mutant allele, immunoblotting was performed on lung tissue of heterozygous mutant mice using M18b-A antibodies (Figure 3B). This showed ~50 % of the protein level of WT mice (Figure 3C), indicating that this is a severely hypomorphic or null allele. No truncated proteins were detected using Munc18b-D antibodies on immunoblots (results not shown). We identified Munc18b as an essential gene because screening more than 200 pups from heterozygous crosses never yielded a homozygous mutant mouse. Of the pups from multiple heterozygous parents, 36 % were WT and 64 % were heterozygous for the mutation. The Mendelian percentages of these two genotypes indicate that haploinsufficiency of Munc18b does not induce a significant survival disadvantage under conditions of standard veterinary care prior to 3 weeks of postnatal life when the mice were genotyped. Heterozygous mutant mice appeared healthy beyond 1 year of age, although a precise survival analysis was not performed.

Munc18b protein expression is limiting for stimulated airway mucin secretion

Mucins are secreted in the airways at a low basal rate and a high agonist-stimulated rate uncovered by mutations in different genes [46]. A defect in basal secretion is expressed phenotypically as spontaneous accumulation of intracellular mucin with no increase in mucin gene expression [47], and a defect in stimulated secretion as a failure to release intracellular mucin from mucous

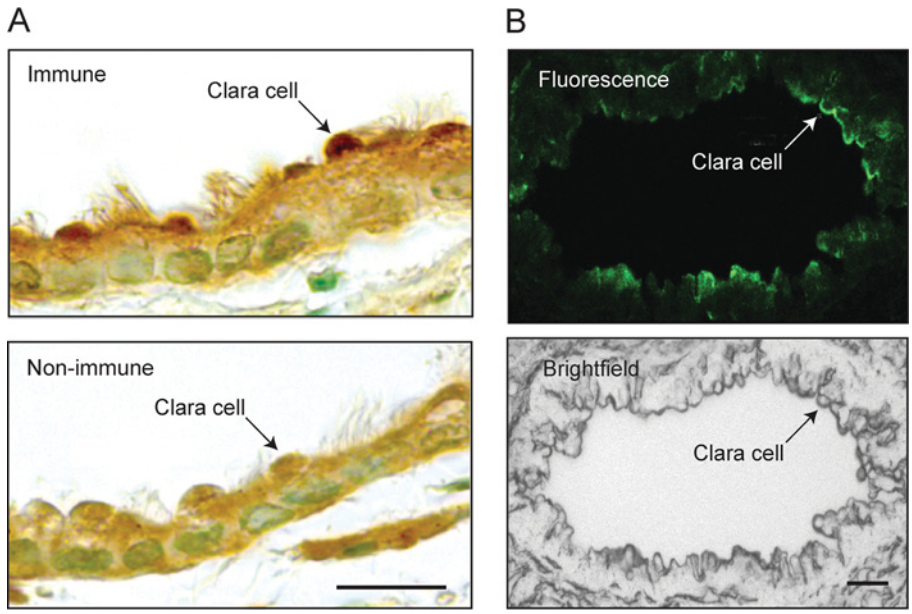

Figure 2 Munc18b localizes to the apical pole of airway secretory cells

(**A**) Immunohistochemistry. Sections of mouse lung were incubated with polyclonal antibodies (M18b-0) against Munc18b (upper panel) or non-immune serum (lower panel). HRP-labelled secondary antibodies showed detection of Munc18b at the apical pole (brown) of domed non-ciliated cells morphologically compatible with secretory (Clara) cells (arrows). Scale bar = 20 μm. (**B**) Immunofluorescence. Sections of frozen lung from a transgenic mouse expressing HA–YFP–Munc18b under the control of a Clara cell promoter were visualized by fluorescence (upper panel) and bright-field (lower panel) microscopy, showing YFP localized at the apical membrane of domed cells (arrows). Scale bar = 20 μm.

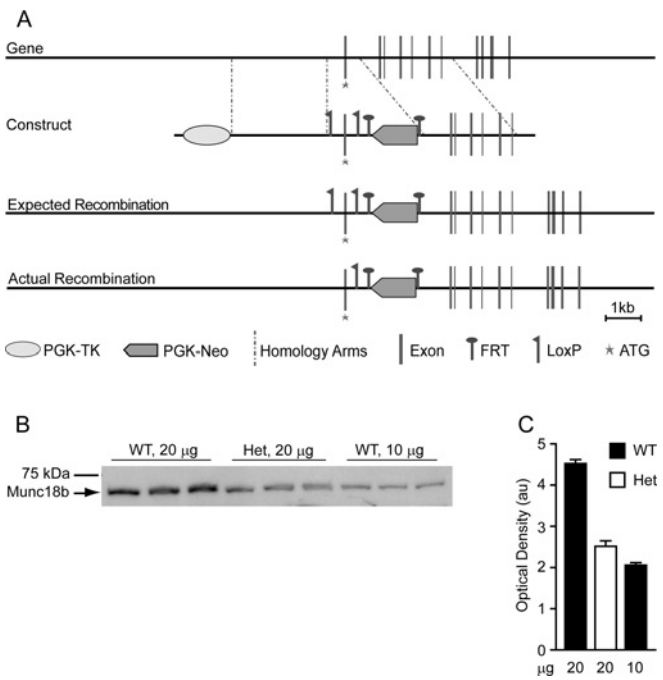

Figure 3 Generation of mice with a hypomorphic Munc18b allele

(**A**) Targeting strategy. Diagram of the exon structure of the mouse Munc18b gene (top row), the construct that was electroporated into embryonic stem cells (second row), the structure of the mutant gene that was intended after homologous recombination (third row) and the actual structure of the recombined gene showing loss of the loxP site 5′ of the first exon (bottom row). (**B**) Immunoblot of lung homogenates from WT and heterozygous mutant (Het) mice probed with a polyclonal anti-Munc18b antibody (M18-A), showing ~50 % decrease in Munc18b protein in heterozygous mice. (**C**) Densitometric quantification of immunoblots such as those in (**B**), confirming ~50 % decrease in Munc18b protein in heterozygous mutant mice. Results are means ± S.E.M. au, arbitrary units.

metaplastic cells after exposure to a high concentration of a secretagogue [46]. The airways of naïve (not exposed to ovalbumin) heterozygous Munc18b mutant mice did not reveal any spontaneous accumulation of intracellular mucin (results not shown). The airways of heterozygous mutant mice sensitized and challenged with ovalbumin to increase mucin production (mucous metaplasia) accumulated levels of intracellular mucin similar to WT mice (Figures 4A and 4B), confirming that there is no impairment of mucin production or change in basal secretory function that might manifest as intracellular levels of mucin lower or higher than the WT. However, heterozygous mutant mice sensitized and challenged with ovalbumin, and then stimulated with an ATP aerosol to induce acute secretion, released only ~50 % as much intracellular mucin as WT mice (Figure 4). Taken together, these results indicate that the WT level of Munc18b protein expression is limiting for stimulated, but not for basal, airway mucin secretion.

Munc18 proteins function through interactions with Syntaxin proteins, and Syntaxins 1, 2, 3 and 4 have been implicated in exocytosis in other cells [1–5]. We found that Munc18b interacted selectively with Syntaxins 2 and 3 compared with Syntaxins 1 and 4 in airway epithelial cell lines (Supplementary Figure S3). However, we did not find a reduction in the level of Syntaxin 2 or 3 protein in the lungs of heterozygous Munc18b mutant mice by immunoblot analysis that might account for the secretory defect in airway epithelial cells (results not shown).

Munc18b expression in airway epithelium does not increase during mucous metaplasia

The production of secreted mucins is markedly increased during airway mucous metaplasia, [43,48], but it is not clear whether expression of the secretory machinery is up-regulated together with the secretory products. To address this, we induced

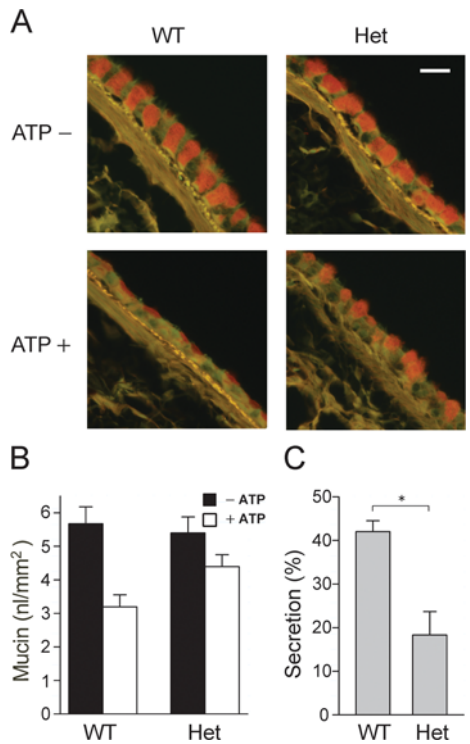

Figure 4 Defect in stimulated mucin secretion in heterozygous mutant Munc18b mice

(**A**) Lung sections stained with PAFS from mice sensitized and challenged with ovalbumin to increase mucin expression, then exposed to aerosolized ATP to induce mucin secretion. Both WT and heterozygous (Het) mutant mice accumulate abundant intracellular mucin (orange) after ovalbumin sensitization and challenge (upper panels). In response to ATP, heterozygous mutant mice release only a small fraction of mucin compared with WT (lower panels). Scale bar = 20 μm. (**B**) The volume density of retained intracellular mucin with or without ATP exposure as in (**A**) was quantified using fluorescence microscopy (n = 17–23 mice per group for four separate experiments). (**C**) The percentage of mucin released for each genotype in response to ATP was calculated from the data in (**B**), and results are weighted means ± S.E.M. for four separate experiments. $^*P < 0.05$.

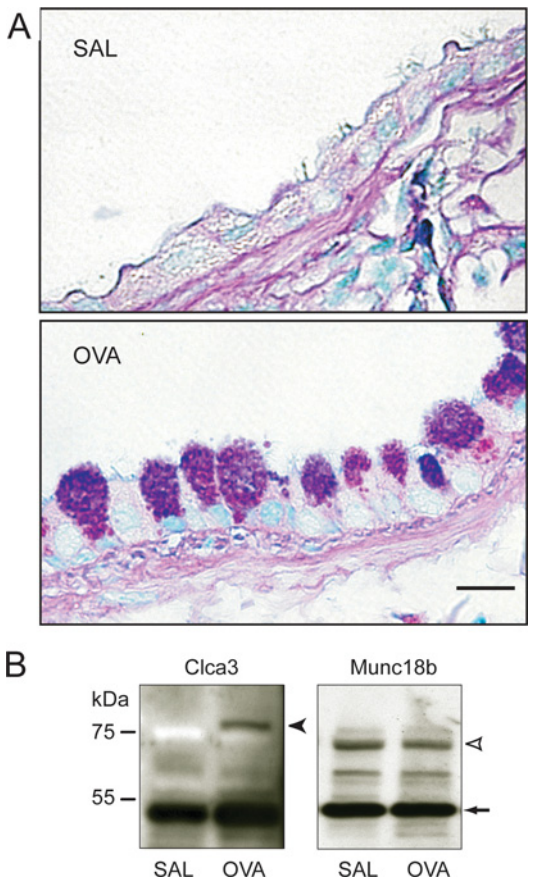

Figure 5 Munc18b expression in airway epithelium is not regulated during inflammatory mucous metaplasia

(**A**) Lung sections stained with AB-PAS from WT mice. Mice were sensitized to ovalbumin by intraperitoneal injection, then exposed to aerosols of 0.9 % saline solution (SAL) as a control or ovalbumin (OVA) in saline solution to induce mucous metaplasia, and then killed 3 days later. Purple staining identifies intracellular mucin. Scale bar = 10 μm. (**B**) Immunoblots of control (SAL) and metaplastic (OVA) C57BL/6 mouse tracheal homogenates probed with antibodies against mClca3 (left-hand panel), a marker of mucous metaplasia, or Munc18b (right-hand panel). Anti-GAPDH antibodies were included as a loading control in both blots (arrow). There is increased expression of mClca3 (solid arrowhead), but not Munc18b (open arrowhead).

mucous metaplasia in WT mice with ovalbumin sensitization and challenge, and then measured Munc18b expression in the trachea, rather than in the whole lung, to enrich for airway epithelium relative to alveolar epithelium, since Munc18b is expressed in both (Figure 1B). The airway epithelium underwent mucous metaplasia evidenced by intracellular accumulation of mucins stained histochemically with AB-PAS (Figure 5A) and up-regulation of Clca3 detected by immunoblotting (Figure 5B, left-hand panel). However, there was no change in the expression of Munc18b protein by immunoblot analysis using M18b-A antibodies (Figure 5B, right-hand panel), suggesting that the level of expression of the exocytic machinery in non-metaplastic airway secretory cells is sufficient for increased apical exocytosis during mucous metaplasia.

Despite the lack of change in expression during inflammatory metaplasia, it is likely that expression of Munc18b is tightly regulated, since it is a limiting component of the airway exocytic machinery (Figure 4). Mutational analysis of a 181 bp core promoter region of the mouse Munc18b gene in A549 and H292 airway epithelial secretory cell lines showed functionally important Sp1 (specificity protein 1), Ets, CRE (cAMP-response element), E-box and GRE (glucocorticoid-response element) sequences upstream of an INR (initiator) sequence 10 bp from the translation initiation site, as well as a GATA site outside the core promoter region at base −598 (Supplementary Figure S4B at http://www.BiochemJ.org/bj/446/bj4460383add.htm). The bHLH (basic helix–loop–helix) transcription factor Math1, which binds E-box motifs and is required for development of the secretory phenotype in gut epithelium [49], was not expressed in metaplastic airway epithelium (Supplementary Figure S4C).

Munc18b protein expression is limiting for MC degranulation and passive cutaneous anaphylaxis

To study Munc18b function in leucocytes, we focused on MCs, which are leucocytes specialized for secretion. We found strong expression of Munc18b in mature peritoneal MCs using M18b-A antibodies in immunoblots (Supplementary Figure S2A) and a ~50 % reduction in expression in mBMMCs derived from heterozygous mutant mice using M18b-D antibodies (Figure 6A). We did not detect any differences in MC numbers, differentiation, morphology, expression of surface receptors or storage of histamine between heterozygous mutant mice and WT littermates (Figures 6B–6F and Table 1).

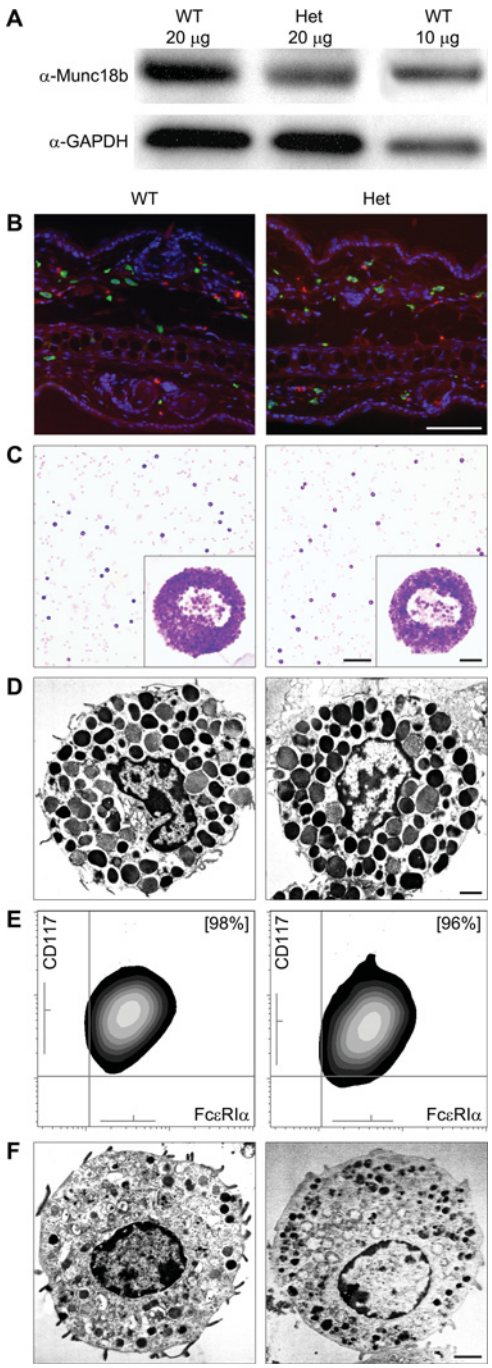

Figure 6 Partial deficiency in Munc18b expression does not affect MC numbers, distribution, development or morphology

Representative images are shown of the multiple experiments analysed in Table 1. (**A**) Immunoblots of lysates of mBMMCs after 6 weeks of culture using anti-Munc18b (M18b-D) and anti-GAPDH (loading control) antibodies. (**B**) Ear sections showing MCs stained with FITC–avidin (green). Hoechst dye (blue) and the autofluorescence of cartilage and muscle (red) were used to delineate the area of dermis to perform the quantification of skin MCs. Scale bar = 100 μm. (**C**) Cytospins of peritoneal lavages stained with Wright–Giemsa showing metachromatic MCs. Scale bar = 100 μm; scale bar in inset = 5 μm. (**D**) Transmission electron microscopy of individual peritoneal MCs used for the unbiased stereology studies. Scale bar = 2 μm. (**E**) Flow cytometry of mBMMCs after 6 weeks of culture in IL-3/SCF-enriched medium. Cells were labelled with fluorescent antibodies against CD117 and FcεRIα; MCs are the double-positive cells. (**F**) Transmission electron microscopy of individual mBMMCs used for the unbiased stereology studies. Scale bar = 5 μm. Het, heterozygous.

Table 1 Quantification, morphometry and cellular contents of MCs

Parameters of MC numbers, morphology and cellular contents compared between WT and heterozygous mutant (Het) mice. Results are means ± S.E.M. for four mice of each genotype.

	WT	Het
Skin MC counts*		
Number/mm^2 of dermis	207 ± 12	201 ± 9
Peritoneal MC counts†		
Percentage	4.3 ± 0.4	4.0 ± 0.4
Number × 10^3/ml	30 ± 4	36 ± 7
Peritoneal MC morphometry‡		
V_v	0.39 ± 0.10	0.44 ± 0.16
S_v (μm^{-1})	33.68 ± 1.71	32.34 ± 1.53
A (μm^2)	45.16 ± 1.26	42.79 ± 0.98
mBMMCs in culture§		
2 weeks (%)	10.3 ± 3.1	11.1 ± 3.3
4 weeks (%)	87.1 ± 4.5	85.9 ± 5.1
6 weeks (%)	98.7 ± 2.1	97.8 ± 1.9
mBMMC receptors and histamine‖		
CD117 MFI (au)	68 ± 3	63 ± 4
FcεRIα MFI (au)	39 ± 4	37 ± 5
Histamine (μg/ml)	1.1 ± 0.3	0.9 ± 0.4
mBMMC morphometry¶		
V_v	0.16 ± 0.06	0.18 ± 0.02
S_v (μm^{-1})	44.64 ± 5.23	43.77 ± 3.64
A (μm^2)	13.34 ± 0.59	14.02 ± 0.64

*FITC–avidin-positive cells with a Hoechst-positive nuclear profile per mm^2 of dermis in random 5 μm sections of ear (8 samples/animal).

†Neubauer chamber counts and Wright–Giemsa staining of cytospins of peritoneal lavages with 10 ml of PBS (3 samples/animal).

‡Stereological analysis of randomly acquired electron microscopy images of peritoneal MCs. V_v, granule volume fraction; S_v, granule surface density; A, cell profile area (10 cell profiles/sample; 5 samples/animal).

§Percentage of CD117$^+$/FcεRIα$^+$ cells in mBMMC cultures assessed by flow cytometry (1 sample/animal).

‖mBMMCs at 6 weeks in culture. Cell surface expression of CD117 and FcεRIα expressed as MFI in arbitrary units (au) of specific labelled primary antibodies bound to the surface of double-positive cells (1 sample/animal). Total histamine concentration in lysates of 10^6 mBMMCs measured by ELISA (1 sample/animal).

¶Stereological analysis of randomly acquired electron microscopy images from mBMMCs at 6 weeks in culture (20 cell profiles/sample; 1 sample/animal).

After stimulating mBMMCs via FcεRI cross-linking, we found that the secretion of histamine, which is released by regulated exocytosis, was reduced by ~50% in cells from heterozygous mutant mice compared with those from WT mice (Figure 7A). In contrast, the secretion of TNFα (Figure 7B), which is released through constitutive exocytosis [29], and of PGD_2 (Figure 7C), which is released by a non-exocytic transporter [29], were unaffected in heterozygous mutant mice. We confirmed the failure of mBMMCs from mutant mice to fully degranulate by electron microscopy, which showed numerous residual electron-dense secretory granules in the cell cytoplasm 60 min after stimulation, whereas mBMMCs from WT mice had very few granules at this time point (Figure 7D). To verify that degranulation is also impaired in mature mutant MCs that developed *in vivo*, we measured histamine secretion from stimulated peritoneal MCs with similar results (Figure 7E).

To test whether the defect in stimulated secretion by mutant MCs resulted in physiological consequences, we compared mutant mice and their WT littermates in a model of passive cutaneous anaphylaxis. The ears of mutant mice showed ~50% reduction in Evans Blue extravasation (Figure 7F), indicating that Munc18b expression is limiting for a physiological response that depends upon MC degranulation.

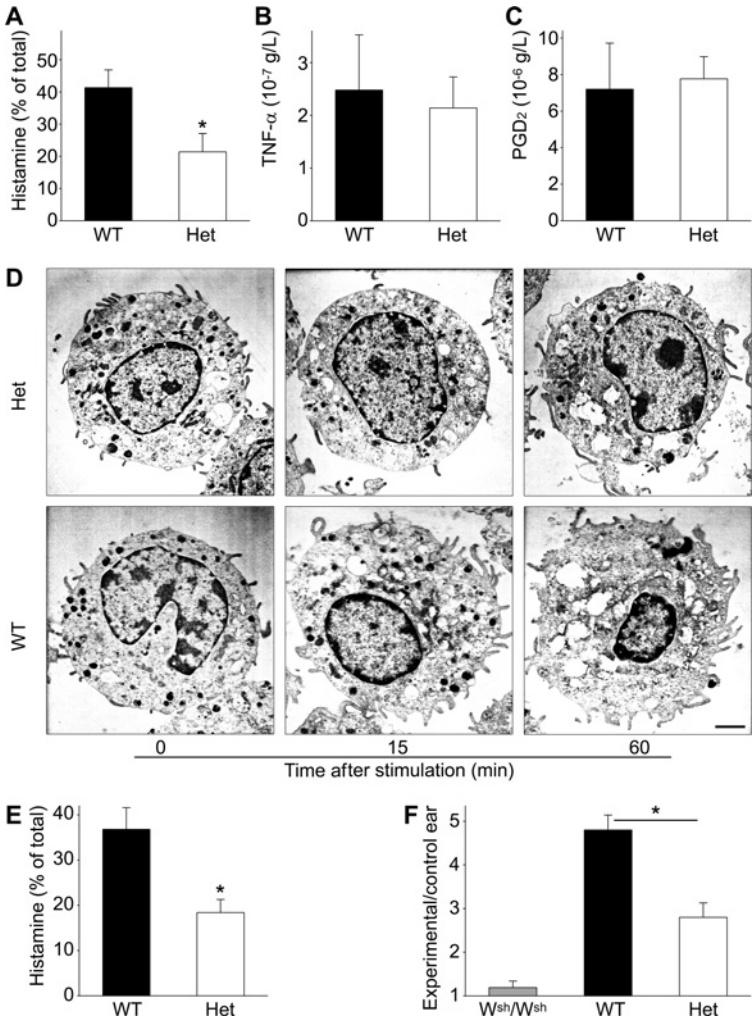

Figure 7 Partial deficiency in Munc18b expression affects MC regulated exocytosis selectively and decreases mouse allergic responsiveness

(**A**–**D**) After 6 weeks in culture, mBMMCs from WT and heterozygous mutant (Het) mice were stimulated by FcεRI cross-linking. There is a ∼50 % reduction in histamine, which is released by regulated exocytic degranulation, in the supernatant of heterozygous mBMMCs (**A**). There are comparable levels of TNFα (**B**), which is released by constitutive exocytosis, and PGD_2 (**C**), which is released by a non-exocytic mechanism, in the supernatants of WT and heterozygous mBMMCs. (**D**) Transmission electron microscopy of individual mBMMCs before (0 min) or after (15 and 60 min) stimulation, showing retention of dense granules in the heterozygous cells compared with the WT cells. Scale bar = 5 μm. (**E**) Peritoneal MCs stimulated by FcεRI cross-linking show a ∼50 % reduction in histamine release, indicating that the defect in mBMMCs is also found in fully mature MCs. (**F**) Passive cutaneous anaphylaxis. MC-deficient W^{sh}/W^{sh} (negative control), WT and mutant mice passively sensitized intradermally in the ears with specific (experimental) or non-specific (control) IgE and then challenged systemically with IgE-specific antigen. The amount of Evans blue extravasated as a consequence of local MC activation was quantified and expressed as the ratio of experimental/control ear, indicating that the MC secretory defect identified *in vitro* has consequences *in vivo*. Results are means ± S.E.M.*$P < 0.05$.

DISCUSSION

Munc18b is an essential gene in mice

To analyse the function of Munc18b, we targeted the gene in mice. Recombination resulted in a severely hypomorphic, functionally null, allele by measurement of protein expression in the lungs and MCs of heterozygous mutant mice (Figures 3 and 6A, and Supplementary Figure S2A). The insertion of PGK-Neo in intron 1 appears to have eliminated expression of the Munc18b gene rather than inducing expression of a dominant-negative truncated protein, since no truncated protein was detected using antibody M18-D generated against amino acids 432–458 in a protein containing 593 amino acids. Similar to Munc18a and Munc18c [9,13], Munc18b is an essential gene with no homozygous mutant mice surviving after birth. The inviability of homozygous mutant Munc18b mice is somewhat surprising in view of the postnatal viability of humans with hypomorphic alleles of Munc18b that result in severe defects of lytic granule exocytosis by cytotoxic T-cells and NK cells causing familial haemophagocytic lymphohistiocytosis [31,32]. This discrepancy suggests that the human alleles are less severely hypomorphic than our mouse allele.

Expression and localization of Munc18b

Previous studies have shown that Munc18b is broadly expressed in secretory epithelia and leucocytes. Whereas Munc18b is most strongly expressed in spleen, liver and kidney as shown by Northern blot analysis [16–18,20], at the level of protein it is more strongly expressed in lung than in any other tissue (Figure 1A). By ISH, Munc18b expression has previously been localized to the lung epithelium of embryonic mice [18,50]. Our higher-resolution analysis in adult mice suggests that Munc18b is most strongly expressed in secretory cells of conducting airways, with

lower levels of expression in alveolar secretory cells, alveolar macrophages and mesothelial cells (Figure 1B). At a subcellular level, Munc18b has been localized to the apical membrane of polarized epithelial cells of the intestine and kidney [11,21]. We similarly find Munc18b localized to the apical membrane of airway secretory cells by immunohistochemistry and by transgenic expression of Munc18b labelled with a fluorescent tag (Figure 2). After lung, Munc18b is most strongly expressed in spleen (Figure 1A), which is rich in leucocytes, and Munc18b is strongly expressed in MCs (Figure 5 and Supplementary Figure S2), which are a leucocyte lineage specialized for secretion.

Function of Munc18b in airway mucin secretion

It is of historic interest that mucin secretion in protozoa was among the earliest exocytic processes studied genetically and by high-resolution electron microscopy [51–53]. To analyse the role of Munc18b in mammalian airway mucin secretion, we targeted the gene in mice. Since homozygous mutant mice were not viable postnatally, function was studied in heterozygous mutant mice. Heterozygous Munc18b mutant mice did not show spontaneous accumulation of intracellular mucin, which would indicate a defect in basal secretion, but did show a $\sim$50 % decrease of stimulated mucin secretion (Figure 4). This suggests that Munc18b is a limiting component of regulated exocytosis at high secretory rates, and a similar dose-dependency of stimulated mucin secretion on synaptotagmin-2 expression has been observed [46]. Fruitflies heterozygous for a null mutation of Rop, the Munc18 homologue in *Drosophila*, also showed a marked reduction in synaptic vesicle release [54], suggesting that Munc18 protein expression is limiting for stimulated secretion broadly across isoforms, cell types and organisms.

Stable post-developmental expression of Munc18b in airway epithelium

In view of its limiting role in stimulated mucin secretion, an important question is whether Munc18b expression is increased along with that of mucins and other secreted gene products during mucous metaplasia. For example, Clca3 transcripts increase more than 1000-fold during mucous metaplasia [48], and Muc5ac mucin transcripts increase more than 40-fold [43]. Despite the marked increase in secreted products, the level of Munc18b protein did not change detectably during mucous metaplasia (Figure 5) in an assay sensitive to a 2-fold change (Figure 3B). The ability of WT epithelial cells to nonetheless acutely release most accumulated mucins in response to a strong stimulus [46] (Figure 4) indicates that airway secretory cells constitutively possess substantial exocytic reserve capacity. The strong and stable expression of Munc18b protein in airway secretory cells should make it a useful marker of the airway epithelial secretory lineage.

Transcriptional control of Munc18b expression

Despite the lack of post-developmental change in Munc18b expression during allergic mucous metaplasia (Figure 5), it is likely that Munc18b expression is tightly regulated during development of the airway epithelium because of its limiting role in secretion (Figure 3). Supporting this inference, the Munc18b promoter contains binding sites for transcription factors known to be important in airway epithelial development (Supplementary Figure S3). These include GRE, CRE and GATA sites, consistent with the well-known role of glucocorticosteroids in lung epithelial secretory cell development [55], the importance of CREB (cAMP-response-element-binding protein) in mucociliary differentiation of airway epithelium in response to retinoids [56,57] and the requirement for GATA-6 in development of the bronchiolar epithelium [55–57]. The promoter also contains an E-box that binds bHLH transcription factors required for development of a secretory phenotype in gut epithelium [49], and an Ets motif that could interact with SPDEF (SAM-pointed-domain-containing Ets factor), which co-activates the expression of multiple lung epithelial genes [55]. Munc18b expression also increases during MC development [58] and the same transcription-factor-binding sites are functional in MCs [20], so comparison of the transcription factors controlling Munc18b expression during development of MCs and airway secretory cells would be informative.

Function of Munc18b in MC secretion

The commensurate reductions in Munc18b expression (Figure 5A) and stimulated degranulation (Figures 6A and 6D) of MCs from heterozygous mutant mice indicates that Munc18b can be a limiting component of the regulated exocytic machinery for leucocytes as well as epithelial cells. The fact that histamine, but not TNF-α, secretion was affected (Figures 6A and 6B) points to a selective role of Munc18b in regulated exocytosis, but not in constituve exocytosis, in MCs. Our control experiments support that the functional differences we found between heterozygote and WT MCs and mice could not be attributed to an intrinsic defect in MC granulation, development, distribution or retention in different tissues (Figures 6B–6F and Table 1). In addition, our findings that the cell surface densities of receptors for IgE and SCF (Figure 6E), and that cytokine and prostaglandin secretion (Figures 7B and 7C), were almost identical between mutant and WT MCs indicate that there was no deficiency in the signal transduction pathways required for MC activation.

The correlation that we found between protein expression and cellular secretory function in MCs was further extended by the finding of a matching reduction in physiological function in heterozygous mice in passive cutaneous anaphylaxis (Figure 6F), a response highly dependent on MC degranulation. Taken together, these results suggest that variation in the expression of Munc18b or other limiting components of the MC exocytic machinery could alter the release of preformed inflammatory mediators by this cell and be a cause of variation in resistance to infection or susceptibility to inflammatory diseases [29,44]. For the same reason, targeting the expression or function of the MC exocytic machinery could be a therapeutic strategy.

Interaction of Munc18b with exocytic Syntaxins

The Syntaxin that interacts with Munc18b to mediate regulated secretion in epithelial cells and MCs is not known with certainty. Munc18b has been found to interact with Syntaxins 1, 2 and 3, but not Syntaxin 4, in other cells [11,16,18,59,60], and we obtained similar results in airway secretory cells (Supplementary Figure S3). Overexpression of Munc18b in epithelial cell lines prevented the association of Syntaxin 3 with SNAP-23 and VAMP (vesicle-associated membrane protein) 3 and 8 [21,61] and inhibited the apical delivery of marker proteins [22–24], suggesting that Munc18b interacts with Syntaxin 3 in apical epithelial exocytosis. However, Syntaxin 3 protein levels were not reduced in our heterozygous mutant mice, indicating that the dependence of stimulated airway mucin secretion on Munc18b is not exclusively mediated by its stoichiometric interactions with Syntaxin 3 or that

Syntaxin 3 does not in fact mediate airway mucin secretion. In MCs, Syntaxin 4 has been implicated in degranulation by over-expression [62], blocking antibodies [63] and small interfering RNA [64,65], even though Munc18b did not physically interact with Syntaxin 4 [25,26]. In cytolytic T-cells and NK cells, mutations in Syntaxin 11 phenocopy mutations in Munc18b in causing defects in lytic granule exocytosis that result in familial haemophagocytic lymphohistiocytosis [31,32], suggesting that Munc18b and Syntaxin 11 collaborate in mediating exocytosis in these cells. We did not assess the expression of Syntaxin 11 or its interactions with Munc18b in airway epithelial cell lines or MCs, but it should also be considered a candidate SNARE in future studies of airway epithelial and MC exocytosis.

AUTHOR CONTRIBUTION

Burton Dickey, Roberto Adachi and Michael Tuvim designed the experiments. Kyubo Kim, Youlia Petrova, Brenton Scott, Rupesh Nigam, Anurag Agrawal, Christopher Evans, Zoulikha Azzegagh, Alejandra Gomez, Elsa Rodarte, Rustam Bagirzadeh, Lucia Piccotti and Binhui Ren performed the experiments. James McNew, Vesa Olkkonen and Joo-Heon Yoon provided key reagents. Burton Dickey, Roberto Adachi and Kyubo Kim wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Munc18b is an essential gene in mice whose expression is limiting for secretion by airway epithelial and mast cells

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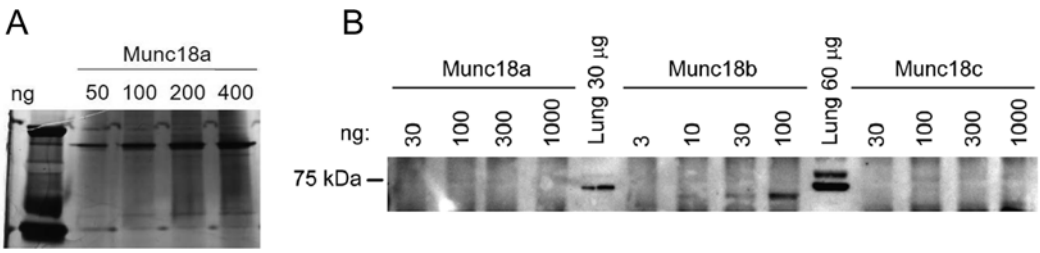

Figure S1 Analysis of the specificity of M18b-D antibodies for Munc18 isoforms

(**A**) Recombinant Munc18 proteins were expressed in bacteria and purified as described previously [1,2]. Protein concentrations were measured by bicinchoninic acid assay, purified proteins were resolved by SDS/PAGE and the purity of recombinant proteins was measured by densitometric analysis of silver-stained gels (shown for Munc18a, 63 % pure). (**B**) Recombinant Munc18 proteins whose concentrations were normalized as in (**A**) were separated by SDS/PAGE, transferred to PVDF membranes, and blots were incubated with M18-D antibodies, then probed with secondary antibodies, all as described in the main text. Staining at the appropriate mobility (68–75 kDa) was visible with 10 ng of Munc18b protein, but not with 1000 ng of Munc18a or Munc18c proteins. Note that recombinant Munc18b runs lower (67 kDa) than Munc18b in lung (72–75 kDa), and that the latter runs as a doublet, suggesting that Munc18b undergoes multiple post-translational modifications in mammalian tissues. This is consistent with the known regulation of Munc18 proteins by phosphorylation [3].

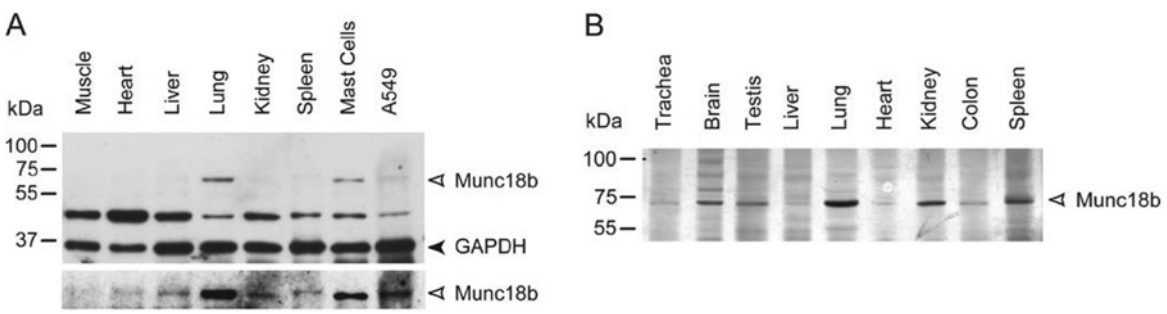

Figure S2 Expression of Munc18b protein in mouse tissues and a human cell line using M18b-A and M18b-O antibodies

Western blots of C57BL/6 mouse tissues and of the human A549 lung adenocarcinoma cell line were probed with M18-A antibodies (**A**) or M18b-O antibodies (**B**) as described in the main text. An overexposed film of the same blot is shown in the lower panel of (**A**) to compare expression in tissues where Munc18b is less strongly expressed. Expression in the Calu-3 human lung adenocarcinoma and mtCC mouse lung adenocarcinoma cell lines was similar to A549 (results not shown, but co-immunoprecipitates from those cells are shown in Figure S4).

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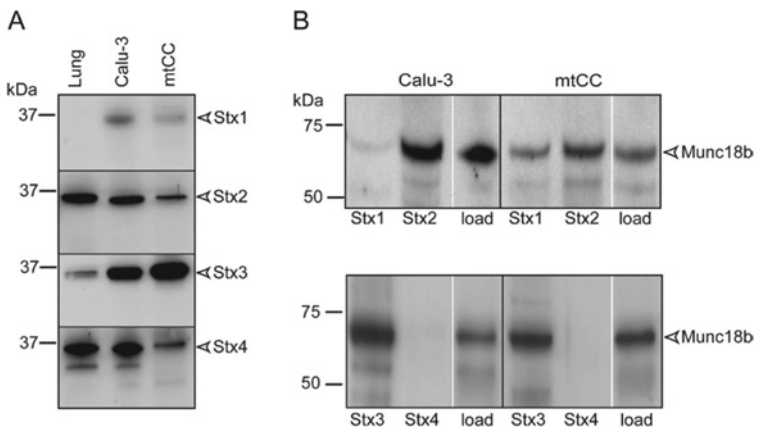

Figure S3 Munc18b interacts selectively with Syntaxins 2 and 3 in airway secretory cell lines

(**A**) Expression of Syntaxin (Stx) exocytic isoforms 1–4 was detected by probing Western blots of homogenates of mouse lung and the Calu-3 human and mtCC mouse lung epithelial cell lines with rabbit polyclonal antibodies. Syntaxins 2, 3 and 4, but not Syntaxin 1, were detectable in whole mouse lung, whereas all four isoforms were detectable in the cell lines. (**B**) Munc18b was immunoprecipitated by incubating Calu-3 and mtCC cell lysates with isoform-specific Syntaxin antibodies and Protein A-coated beads, then probing Western-blotted precipitates with antibodies against Munc18b. Loading controls for the efficiency of Munc18b immunodetection were 1/20 volume of the cellular lysates. Syntaxins 2 and 3 efficiently pulled down Munc18b in both cell lines, whereas Syntaxin 1 pulled down lower amounts of Munc18b, and Syntaxin 4 did not pull down detectable Munc18b. Experimental: in each tube, 50 μl of Protein A agarose bead solution (Invitrogen) was pelleted by centrifugation at 15000 ***g*** for 10 min and rinsed with 1% Triton X-100 in PBS. To the packed beads was added 100 μl of mtCC lysate at 7 μg/μl or Calu-3 cell lysate at 3 μg/μl, and 2 μl of one of the four anti-Syntaxin antibodies. Samples were rotated at 4°C for 2 h, then pelleted, and twice resuspended with 1% Triton X-100 in PBS and pelleted, then finally eluted with 30 μl of SDS sample buffer. Aliquots of the immunoprecipitated samples (25 μl) and the cellular lysates (5 μl) were resolved by SDS/PAGE using 8% Tris-glycine gels, transferred on to PVDF membranes and probed with antibody M18-0.

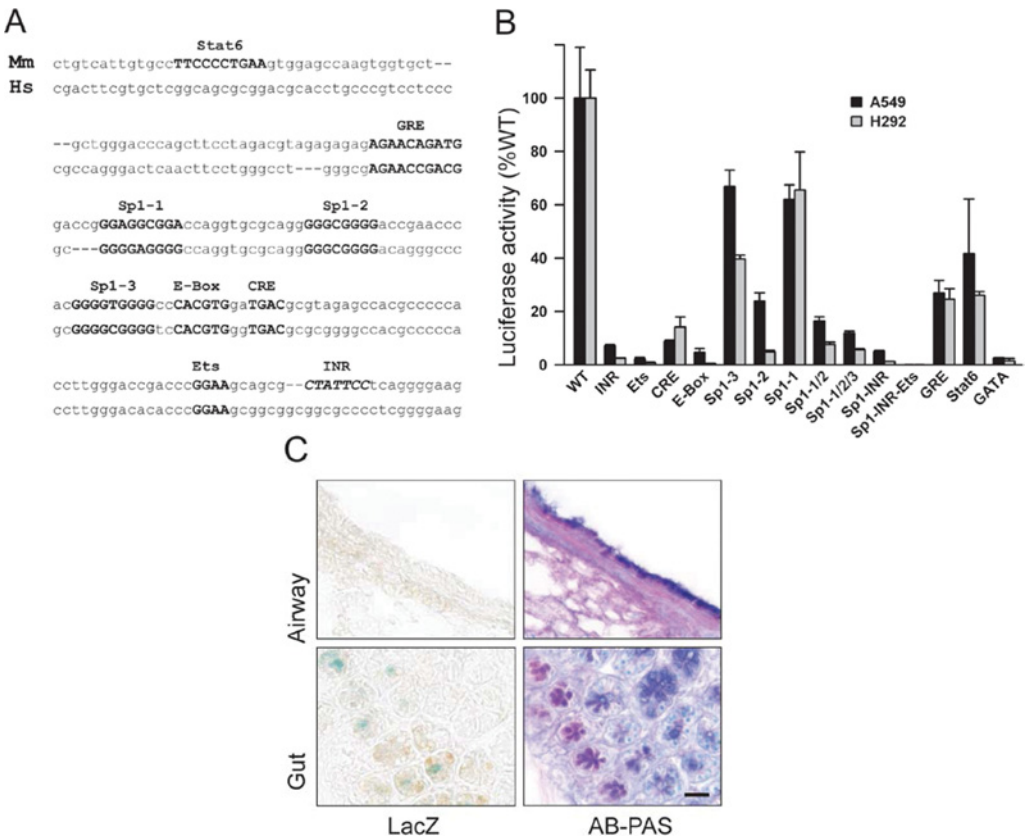

Figure S4 Analysis of transcription factor binding sites in the Munc18b promoter

(**A**) The proximal mouse (Mm) and human (Hs) Munc18b promoters up to the translation start site are aligned to show conserved transcription factor binding motifs in bold upper-case letters, and an INR sequence in the mouse promoter is shown in bold upper-case italic letters. (**B**) Point mutations were introduced into conserved motifs in the mouse Munc18b promoter singly and in combination, and luciferase activity of reporter constructs was measured in A549 and H292 lung epithelial cell lines. In addition, a Stat6 (signal transducer and activator of transcription 6) site poorly conserved between mouse and human promoters was mutated because of the role of Stat6 in mucous metaplasia [4], and a GATA site outside the core promoter region at base -598 was mutated because of the role of GATA6 in lung development [5]. (**C**) No expression of the *LacZ* gene knocked into the *Math1* locus was detected in frozen sections of bronchial airways of heterozygous mutant mice sensitized and challenge with ovalbumin (upper left-hand panel), although expression was detected in intestinal epithelial cells used as a positive control (lower left-hand panel); mucous cells are detected by AB-PAS staining of adjacent sections in airways (upper right-hand panel) and intestine (lower right-hand panel). Scale bar $= 20\ \mu$m. Experimental: analysis of the Munc18b promoter was performed essentially as described previously [6,7]. Briefly, point mutations were introduced into putative transcription factor binding sites identified using TESS (Transcription Element Search System) software (http://www.cbil.upenn.edu/tess) in a 181 bp fragment upstream of the transcription start site that is strongly expressed in MC and lung epithelial cell lines. For analysis of a GATA site at base -598, an 802 bp fragment was used [6,7]. Promoter–luciferase constructs (PGL-3, Promega) were transfected into A549 and NCI-H292 cells using FuGENE6 (Roche) and luciferase activity was measured after 24 h. Since the bHLH transcription factor Math1 that binds to E-box motifs is essential for development of secretory epithelial cells in the mouse intestine [8], we tested whether it is similarly required in the airway using a *LacZ*-knockin/*Math1*-knockout mouse. For analysis of Math1 expression *in situ*, mucous metaplasia was induced in the airways of heterozygous *LacZ* knockin/*Math1* knockout mice with ovalbumin sensitization and challenge as described in the main text, then lungs were inflated with OCT and sections of intestine were immersed in OCT. Frozen tissues were sectioned at 10 μm, fixed with 0.2% glutaraldehyde, stained with X-Gal (Promega), and counterstained with Orange G (Sigma–Aldrich). Adjacent sections were fixed with 3.7% formaldehyde and stained with 1% AB (pH 2.5)-PAS to detect mucins [9]. Sp1, specificity protein 1.

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