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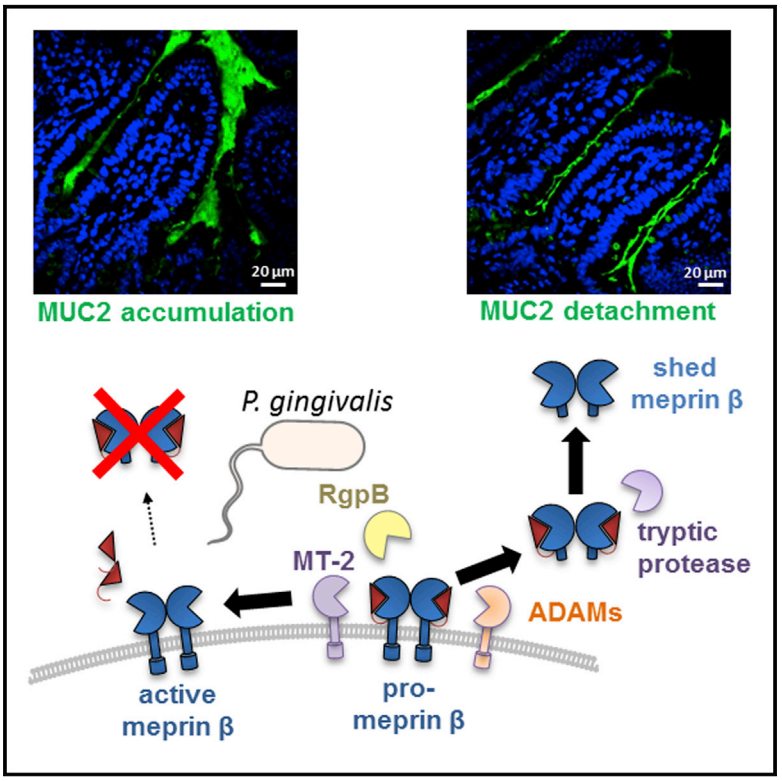
Mucus Detachment by Host Metalloprotease Meprin β Requires Shedding of Its Inactive Pro-form, which Is Abrogated by the Pathogenic Protease RgpB

Graphical Abstract



Highlights

- Meprin β activation and shedding are mutually exclusive events
- ADAM-mediated pro-meprin β shedding is required for proper mucus integrity
- Pathogenic secreted cysteine protease RgpB activates host metalloprotease meprin β
- Activation of membrane-bound meprin β prevents its shedding and mucus detachment

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In Brief

ADAM-mediated meprin β shedding is required for mucus detachment, regulating intestinal integrity. This work by Wichert et al. demonstrates that meprin β is exclusively shed in its pro-form. Activation of meprin β by the serine protease MT-2 or the bacterial virulence factor RgpB abrogates its shedding, resulting in a disturbed mucus barrier.

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Mucus Detachment by Host Metalloprotease Meprin β Requires Shedding of Its Inactive Pro-form, which Is Abrogated by the Pathogenic Protease RgpB

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SUMMARY

The host metalloprotease meprin β is required for mucin 2 (MUC2) cleavage, which drives intestinal mucus detachment and prevents bacterial overgrowth. To gain access to the cleavage site in MUC2, meprin β must be proteolytically shed from epithelial cells. Hence, regulation of meprin β shedding and activation is important for physiological and pathophysiological conditions. Here, we demonstrate that meprin β activation and shedding are mutually exclusive events. Employing *ex vivo* small intestinal organoid and cell culture experiments, we found that ADAM-mediated shedding is restricted to the inactive pro-form of meprin β and is completely inhibited upon its conversion to the active form at the cell surface. This strict regulation of meprin β activity can be overridden by pathogens, as demonstrated for the bacterial protease Arg-gingipain (RgpB). This secreted cysteine protease potently converts membrane-bound meprin β into its active form, impairing meprin β shedding and its function as a mucus-detaching protease.

INTRODUCTION

The metalloprotease meprin β is a type 1 transmembrane protein belonging to the astacin family of zinc endopeptidases (Dumermuth et al., 1991; Sterchi et al., 2008). Meprin β is highly expressed in epithelial cells of the small intestine (Sterchi et al., 1982, 1988), and its role in intestinal homeostasis and associated diseases such as inflammatory bowel disease (IBD) has been extensively studied. In the small intestine, meprin β is responsible for mucin 2 (MUC2) cleavage, which regulates mucus detachment, important for proper barrier function (Schütte et al., 2014). By contrast, meprin β-deficient mice are protected against dextran sulfate sodium (DSS)-induced colitis (Banerjee et al., 2011) because of the loss of the pro-inflammatory cytokine interleukin-18 (IL-18), which is a proposed meprin β substrate (Banerjee and Bond, 2008). Thus, meprin β has important but divergent roles in intestinal homeostasis and in intestinal inflammation, suggesting that meprin β activity must be strictly regulated in the intestine.

Meprin β is expressed as a zymogen and requires activation by serine proteases to remove its inhibitory propeptide (Grünberg et al., 1993; Johnson and Bond, 1997; Bode et al., 1992). Recently, we identified the transmembrane serine protease matriptase-2 (MT-2) as a specific activator of meprin β at the cell surface (Jäckle et al., 2015). In addition to proteolytic removal of the inhibitory propeptide, meprin β undergoes ectodomain shedding from the cell surface by a disintegrin and metalloprotease (ADAM) 10 and 17 (Hahn et al., 2003; Jefferson et al., 2013; Herzog et al., 2014). Importantly, the substrate specificity of meprin β is dependent on whether meprin β is membrane-bound or shed into the extracellular space. The recently identified substrate MUC2 can only be cleaved by soluble meprin β, which requires meprin β shedding from the cell surface of

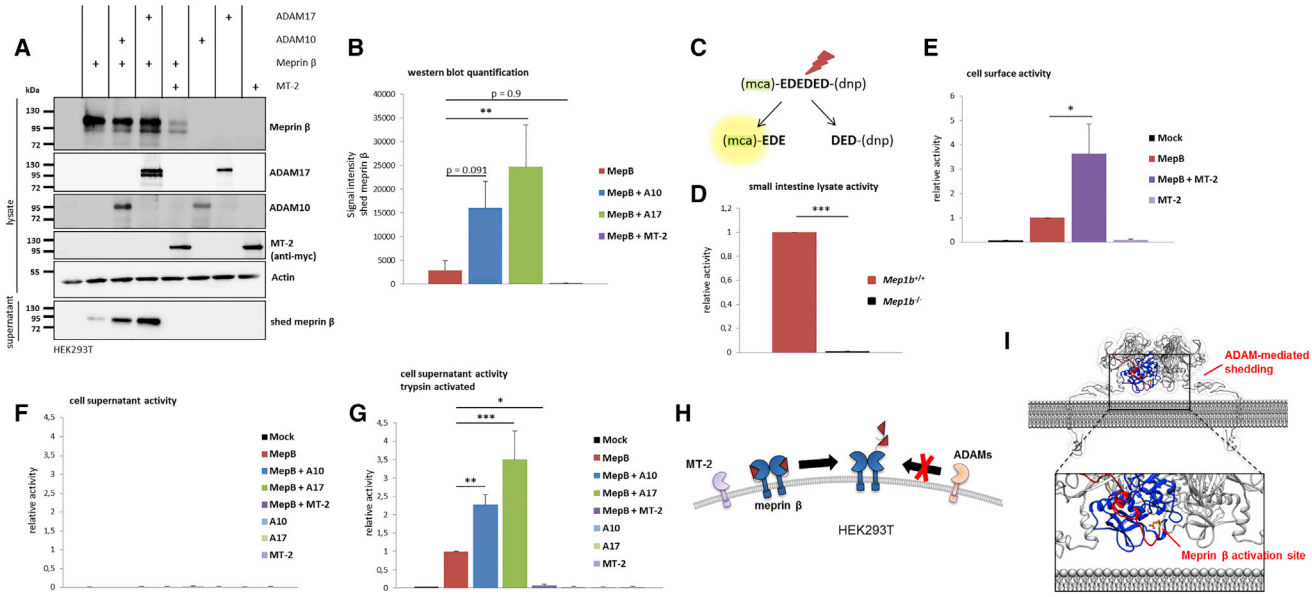


Figure 1. Only Inactive Pro-meprin β Is Shed by ADAM10 and ADAM17

(A) Soluble meprin β levels after co-expression of ADAM10/17 or MT-2 and meprin β in HEK293T cells were detected by immunoblotting.

(B) Densitometric analysis of shed meprin β from three biological replicates as shown in (A).

(C) Meprin β activity was measured using a quenched fluorogenic peptide-based assay. mca, (7-methyloxycoumarin-4-yl) acetyl; dnp, 2,4-dinitrophenyl.

(D) Meprin β activity in small intestinal tissue lysates of WT and *Mep1b*$^{-/-}$ mice (n = 3).

(E) Cell surface activity of meprin β on HEK293T cells upon co-transfection with MT-2.

(F) No meprin β activity was detected in cell supernatants of transfected HEK293T cells.

(G) Meprin β activity was increased in trypsin-activated supernatants. Data are presented as mean ± SD, and statistical analysis was assessed by unpaired Student's t test (D,E) or one-way ANOVA followed by Dunnett's test (B,G) from three biological replicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(H) Model of activation and shedding of meprin β.

(I) Orientation of dimeric meprin β based on the crystal structure (PDB: 4GWM). ADAM-mediated pro-meprin β shedding appears N-terminal of the EGF-like region. Higher magnification of the meprin β activation site shows Arg_{61} in close proximity to the plasma membrane. Ribbon structure of the catalytic domain is displayed in blue and the propeptide is shown in red. Arg_{61} residue at the scissile bond is highlighted as a ball-stick-model in orange.

See also Figure S1.

epithelial cells in the small intestine (Schütte et al., 2014). MUC2 is secreted from goblet cells and represents the main structural component of the intestinal mucus layer required for protecting the host epithelium from bacteria (van Klinken et al., 1999; Johansson et al., 2008). In the presence of meprin β, the mucus layer is only loosely attached in the small intestine, leading to mucus detachment and fast mucus renewal, requiring intestinal peristalsis. In contrast, the mucus layer of meprin β-deficient mice is densely packed and tightly attached to the epithelium (Schütte et al., 2014).

To elucidate the regulation and function of meprin β in the small intestine and its role as a putative therapeutic agent to target intestinal disorders, we aimed to identify the molecular features that determine ADAM-mediated shedding. We found that only inactive pro-meprin β can be shed from the cell surface, whereas MT-2 binding and activation of membrane-bound meprin β completely prevent ADAM-mediated shedding. This unique molecular mechanism of enzyme regulation is exploited by the pathogen *P. gingivalis*, secreting a protease that is capable of activating the membrane-bound host metalloprotease meprin β, preventing its shedding, which would consequently impair its function as a mucus-detaching protease.

RESULTS

ADAM-Mediated Shedding of Meprin β Is Restricted to Its Inactive Pro-form and Is Completely Prevented after Its Activation by MT-2

To analyze shedding and activation of membrane-bound meprin β, HEK293T cells were co-transfected with meprin β and its sheddases ADAM10/17 or its activator MT-2, respectively (Figure 1A). Increased amounts of soluble meprin β were detected in the supernatants of ADAM10/17 co-expressing cells, indicating constitutive shedding of meprin β by those proteases (Figures 1A and 1B). A small amount of shed meprin β was visible in the supernatant of meprin β single-transfected cells, which was probably generated by endogenous ADAM activity (Figure 1A). By contrast, these constitutive levels of soluble meprin β were strongly reduced after MT-2 co-expression (Figures 1A and 1B). To determine the proteolytic activity of shed and membrane-bound meprin β, a highly specific quenched fluorogenic peptide substrate was used (Broder and Becker-Pauly, 2013; Figure 1C). The specificity of the fluorogenic peptide substrate was confirmed using small intestinal tissue lysates of meprin β-deficient mice and corresponding wild-type (WT) animals (Figure 1D). Although co-expression of MT-2 with meprin β resulted

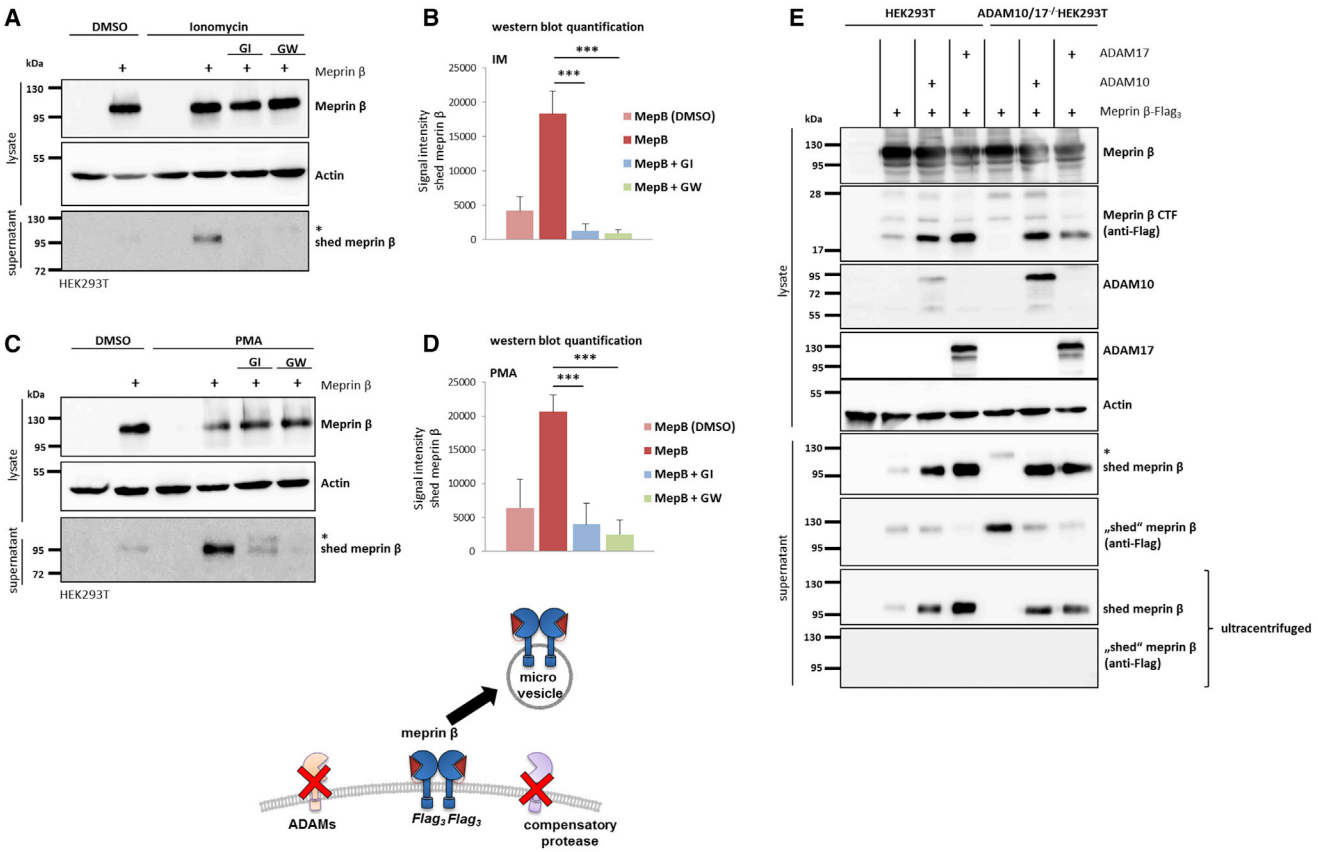


Figure 2. Loss of the Sheddase Activity of ADAM10/17 Leads to Secretion of Meprin β by Microvesicles

(A) Meprin β-transfected HEK293T cells were stimulated with 1 μM IM and additionally treated with 3 μM hydroxamate inhibitors GI or GW. Accumulated shed meprin β was detected in cell supernatants of IM-stimulated cells by immunoblotting. An additional signal for full-length meprin β appeared in supernatants of GI- or GW-treated cells (*).

(B) Densitometric analysis of shed meprin β from three biological replicates as shown in (A).

(C) Meprin β-transfected HEK293T cells were stimulated with 100 nM PMA and additionally treated with GI or GW. Accumulated shed meprin β was detected in cell supernatants of PMA-stimulated cells by immunoblotting. An additional signal for full-length meprin β appeared in supernatants of GI- or GW-treated cells (*).

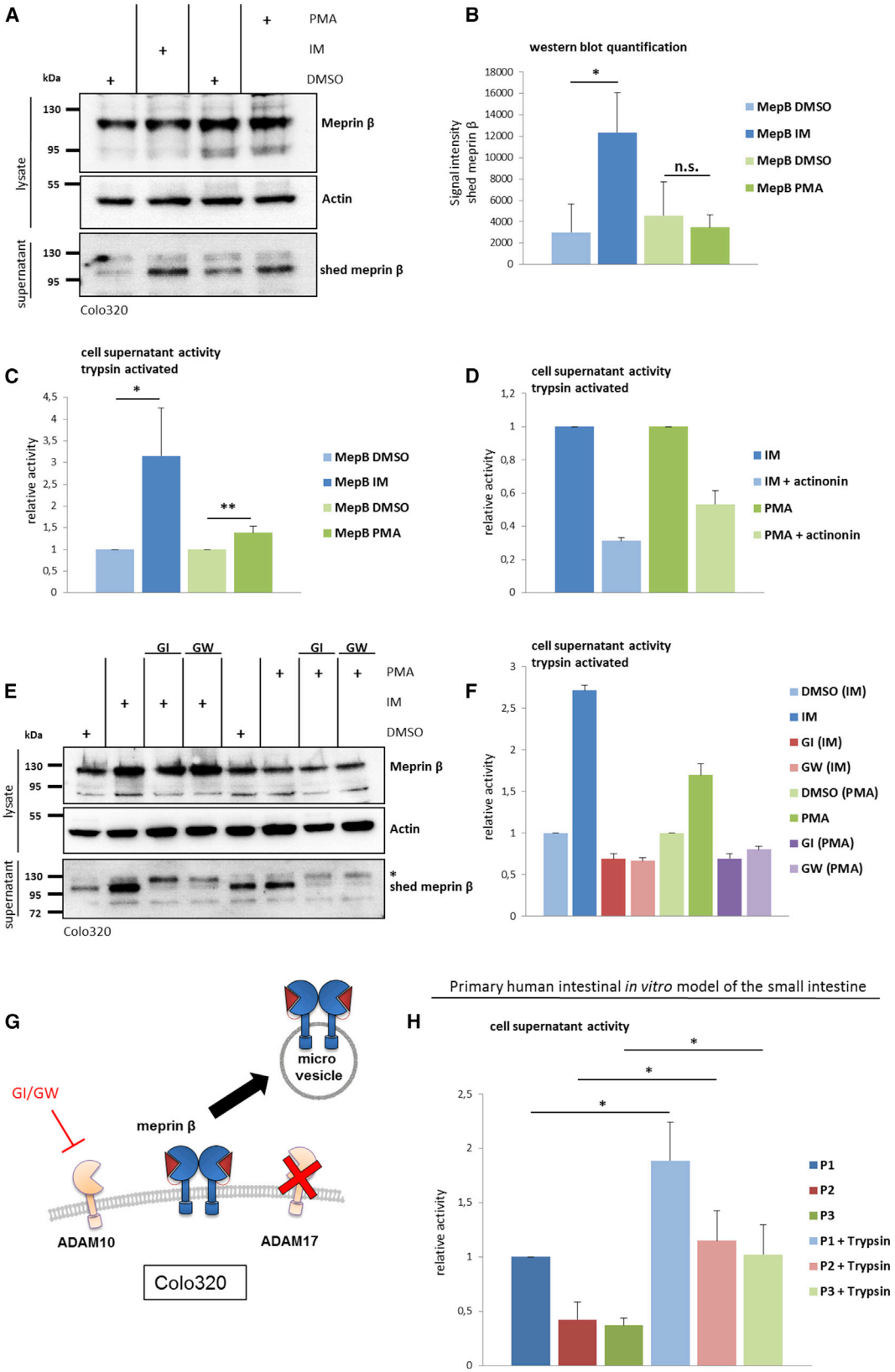
(D) Densitometric analysis of shed meprin β from three biological replicates as shown in (C). Data are presented as mean ± SD, and statistical analysis was assessed by one-way ANOVA followed by Dunnett's test (***$p < 0.001$).

(E) Co-expression of C-terminal FLAG-tagged meprin β and ADAM10/17 in HEK293T and ADAM10/17$^{-/-}$ HEK293T cells (CRISPR/Cas). Full-length meprin β was detected in cell supernatants using a $FLAG_3$ antibody (*). CTF, C-terminal fragment.

See also Figure S2.

in strong activation of meprin β at the cell surface (Figure 1E), no meprin β activity was detected in cell supernatants from any treatment condition (Figure 1F) even though soluble meprin β was readily detected by immunoblotting upon co-transfection with ADAM proteases (Figure 1A). Because MT-2 co-expression completely prevented constitutive meprin β shedding (Figures 1A and 1B), we hypothesized that meprin β is shed solely in its inactive pro-form by ADAM10/17. Indeed, upon pre-incubation of cell supernatants with recombinant trypsin to activate soluble meprin β, we observed increased proteolytic activity in supernatants of ADAM10/17-co-transfected cells (Figure 1G). However, no meprin β activity was detected in the trypsin-activated cell supernatant of MT-2-co-expressing cells (Figure 1G). These results indicate that ADAM10/17-mediated shedding of meprin β is restricted to its inactive pro-form and that meprin β shedding is completely prevented after its activation by MT-2 (Figure 1H), despite the fact that known activation and shedding sites are located differently in the meprin β molecule (Figure 1I). We confirmed these results with either ionomycin (IM) or phorbol 12-myristate 13-acetate (PMA) stimulation to induce endogenous ADAM10 or ADAM17 activity, respectively (Figure S1).

To determine the specificity of pro-meprin β shedding by endogenous ADAMs, hydroxamate inhibitors (GI254023X [GI] for ADAM10 and GW280264X [GW] for ADAM10/17) were used (Ludwig et al., 2005; Hundhausen et al., 2003). IM induced shedding of pro-meprin β by ADAM10 was completely abolished after addition of the inhibitors GI or GW (Figures 2A and 2B), whereas PMA-induced ADAM17-mediated shedding of pro-meprin β was fully inhibited by GW alone (Figures 2C and 2D). Interestingly, GI treatment also decreased meprin β shedding in PMA-stimulated cells, suggesting that ADAM10 plays a crucial role in constitutive pro-meprin β shedding within HEK293T cells. Again, meprin β



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activity in supernatants could only be measured upon trypsin activation and was significantly decreased in supernatants of GI- or GW-treated cells (Figures S2A–S2D). Taken together, these results clearly demonstrate that ADAM10/17 and MT-2 processing of membrane-bound pro-meprin β are mutually exclusive events and that previous activation of membrane-bound meprin β by MT-2 completely prevents its shedding by ADAM10/17.

Inhibition or Knockout of ADAM10/17 Leads to Meprin β Secretion by Microvesicles

During analysis of meprin β shedding from IM- and PMA-stimulated HEK293T cells, an additional band for meprin β migrating at a higher molecular weight (*) appeared after inhibition of ADAMs by GI or GW (Figures 2A and 2C). To investigate the origin of this meprin β isoform, we co-expressed C-terminal FLAG-tagged meprin β and ADAM10/17 in HEK293T cells and in ADAM10/17$^{-/-}$ HEK293T cells (CRISPR/Cas) (Riethmueller et al., 2016). Interestingly, full-length meprin β was detected in the supernatant of ADAM10/17$^{-/-}$ cells using a $FLAG_3$ antibody that recognizes the C terminus of membrane-bound meprin β (Figure 2E). This signal appeared at the same apparent molecular weight as observed upon inhibition of ADAM10/17 by GI or GW (Figures 2A and 2C). Of note, the signal decreased after re-transfection with ADAM10/17, whereas meprin β C-terminal fragments (CTFs) increased again (Figure 2E). However, after ultracentrifugation of cell supernatants, no signal for full-length meprin β was detected in western blots using the $FLAG_3$ antibody, and soluble meprin β was only visible in the presence of its sheddases ADAM10/17 (Figure 2E). Hence, membrane-bound meprin β is released into the supernatant via microvesicles in the absence of its sheddases ADAM10/17. These results indicate that no other constitutive sheddase for pro-meprin β beside ADAM10/17 is present in HEK293T cells.

ADAM10 Is the Constitutive Sheddase of Endogenous Human Pro-meprin β

To identify the constitutive sheddase of pro-meprin β in human intestinal cells, endogenous meprin β shedding was analyzed in human colon carcinoma cells (Colo320) after stimulation with either IM or PMA. Compared with the respective DMSO control levels, IM stimulation induced a significant increase in shed pro-meprin β in cell supernatants, which was not the case for PMA stimulation (Figures 3A and 3B). After trypsin activation, however, a marked increase in the activity of soluble meprin β was detected in the cell supernatants of IM-stimulated and, to a lesser extent, PMA-stimulated cells (Figure 3C), which was blocked using the meprin inhibitor actinonin (Figure 3D). Furthermore, IM as well as PMA stimulation were both sensitive to GI inhibition (Figures 3E and 3F). A slight PMA-stimulating effect on ADAM10 activity has been observed previously (Jefferson et al., 2013; Maretzky et al., 2005). The ability of the ADAM10-selective inhibitor GI to block IM- and PMA-stimulated shedding indicates that ADAM10 is the major sheddase responsible for endogenous pro-meprin β shedding in Colo320 cells (Figure 3E). Moreover, trypsin activated meprin β activity in cell supernatants, and, thus, pro-meprin β shedding was reduced after pre-treatment with GI and GW even below control levels, indicating that constitutive ADAM10 activity was blocked (Figure 3F). In addition, full-length pro-meprin β (*) was released into the supernatant via microvesicles after ADAM10 inhibition, indicating the absence of compensatory meprin β sheddases in Colo320 cells, as previously observed for HEK293T cells (Figures 3E and 3G). These results clearly indicate that ADAM10 is the critical sheddase required for endogenous pro-meprin β shedding in Colo320 cells.

We next analyzed cell supernatants of a primary human *in vitro* transwell-like model of the small intestine (Schweinlin et al., 2016). Here, meprin β activity was measured in cell supernatants, which could be significantly increased after trypsin treatment (Figure 3H), indicating that endogenous levels of shed meprin β were predominantly found in its inactive pro-form.

Shedding and Activation of Membrane-Bound Meprin β Is Dependent on a Motif N-terminal of the Epidermal Growth Factor-like Domain

To elucidate the underlying molecular mechanisms for mutually exclusive proteolytic events of meprin β shedding and activation, we generated a meprin β propeptide variant in which the MT-2 activation site was mutated from arginine residue 61 to a serine residue (R61S) (Jäckle et al., 2015). Because activation of membrane-bound meprin β by MT-2 completely prevents its shedding by ADAM10/17, we hypothesized that Arg_{61} might be a competitive recognition motif for both proteases. However, no differences in ADAM-mediated shedding of the pro-meprin β variant R61S were detected (Figure 4A), even though no

Figure 3. ADAM10 Is the Major Sheddase of Endogenous Pro-meprin β in Colo320 Cells

(A) Colo320 cells were stimulated with 1 μM IM for 30 min or with 100 nM PMA for 2 hr to induce ADAM10/17 activity, respectively. Shed meprin β was detected in cell supernatants by immunoblotting.

(B) Densitometric analysis of shed meprin β from three biological replicates as shown in (A).

(C) Quantification of meprin β activity in cell supernatants (trypsin-activated) from three biological replicates. Data are presented as mean ± SD, and statistical analysis was assessed by unpaired Student's t test (*$p < 0.05$, **$p < 0.01$).

(D) Meprin β activity in trypsin activated cell supernatants was inhibited using the meprin inhibitor actinonin (10 μM). SD was calculated from two technical replicates.

(E) The ADAM inhibitor GI or GW was added to cell supernatants of IM- or PMA-treated Colo320 cells, and pro-meprin β shedding was analyzed by immunoblotting. After inhibition of pro-meprin β shedding, a higher-molecular-weight form of meprin β was released into the supernatant (*).

(F) Pre-incubation with GI or GW reduced meprin β activity in trypsin-activated cell supernatants of stimulated Colo320 cells. SD was calculated from two technical replicates.

(G) Model of pro-meprin β shedding in Colo320 cells.

(H) Quantification of endogenous meprin β activity in supernatants of a primary human small intestinal cell culture model. Data are presented as mean ± SD from three biological replicates, and statistical analysis was assessed by unpaired Student's t test (*$p < 0.05$).

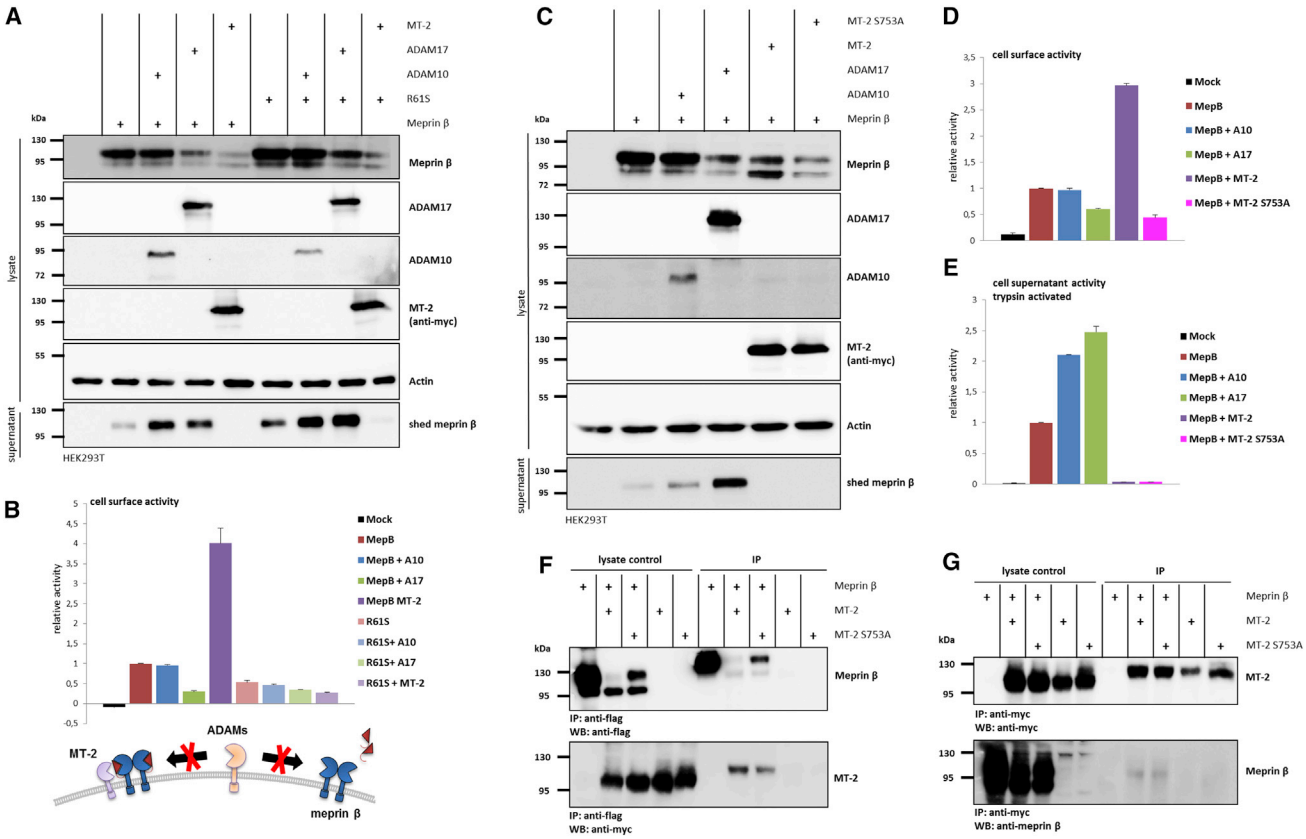

Figure 4. Binding of Proteolytically Inactive MT-2 to Membrane-Bound Meprin β Shields Meprin β from ADAM Interaction

(A) HEK293T cells were co-transfected with the meprin β propeptide variant R61S and ADAM10/17 or MT-2, respectively. Proteins were analyzed by immunoblotting.

(B) No increase in cell surface activity of meprin β could be measured after co-expressing meprin β R61S and MT-2. SD was calculated from two technical replicates.

(C) HEK293T cells were co-transfected with meprin β and ADAM10/17 or the inactive MT-2 variant S753A. Proteins were analyzed by immunoblotting.

(D) No increase in meprin β cell surface activity could be measured in MT-2 S753A co-expressing cells.

(E) No meprin β activity was measured in trypsin-treated cell supernatants of MT-2 S753A co-expressing cells. SD was calculated from two technical replicates.

(F) C-terminal FLAG-tagged meprin β was co-transfected with MT-2 or MT-2 S753A in HEK293T cells and immunoprecipitated for meprin β using a $FLAG_3$-antibody. Co-immunoprecipitation of MT-2 was detected by immunoblotting.

(G) MT-2 or its inactive variant S753A were co-transfected with meprin β in HEK293T cells and immunoprecipitated for MT-2 using a myc antibody. Co-immunoprecipitation of meprin β was detected by immunoblotting.

activation of meprin β R61S by MT-2 was measured in the cell surface activity assay (Figure 4B). Importantly, ADAM-mediated shedding of pro-meprin β R61S in MT-2 co-expressing cells was prevented to a similar extent as observed for WT meprin β, indicating a strong non-proteolytic interaction between MT-2 and membrane-bound meprin β. Upon co-transfection of HEK293T cells with meprin β and the inactive MT-2 variant S753A (Figure 4C), shedding of pro-meprin β by ADAM10/17 was prevented to the same extent as observed for active MT-2 despite no appreciable activation of membrane-bound meprin β (Figure 4D, E). Accordingly, co-immunoprecipitation studies demonstrated strong interactions of meprin β with both active and inactive MT-2 variants (Figures 4F and 4G), which shields meprin β from any ADAM interaction.

To further investigate the binding exclusivity of MT-2 and ADAM10/17 to membrane-bound meprin β, we generated a meprin β chimera in which the previously reported ADAM17 cleavage site in human meprin β (hMeprin β) N-terminal of the epidermal growth factor (EGF)-like domain (Hahn et al., 2003) was exchanged with the corresponding sequence from murine meprin β (mMeprin β) (Figure S3A). Shedding and activation of chimeric meprin β was assessed upon co-transfection with ADAM10/17 or MT-2 in HEK293T cells (Figure S3B). In the supernatant of single transfected HEK293T cells, the same molecular weight shift for chimeric meprin β (*) was detected, as previously observed in ADAM10/17$^{-/-}$ HEK293T cells, indicating impaired shedding of chimeric meprin β and release of the full-length protein via microvesicles. Here, only co-transfection with ADAM17, and not ADAM10, resulted in shedding of chimeric meprin β (Figure S3B). Surprisingly, chimeric meprin β could not be activated by MT-2 even though the identified MT-2 cleavage site at position Arg_{61} was not altered (Figure S3C). However, activation of

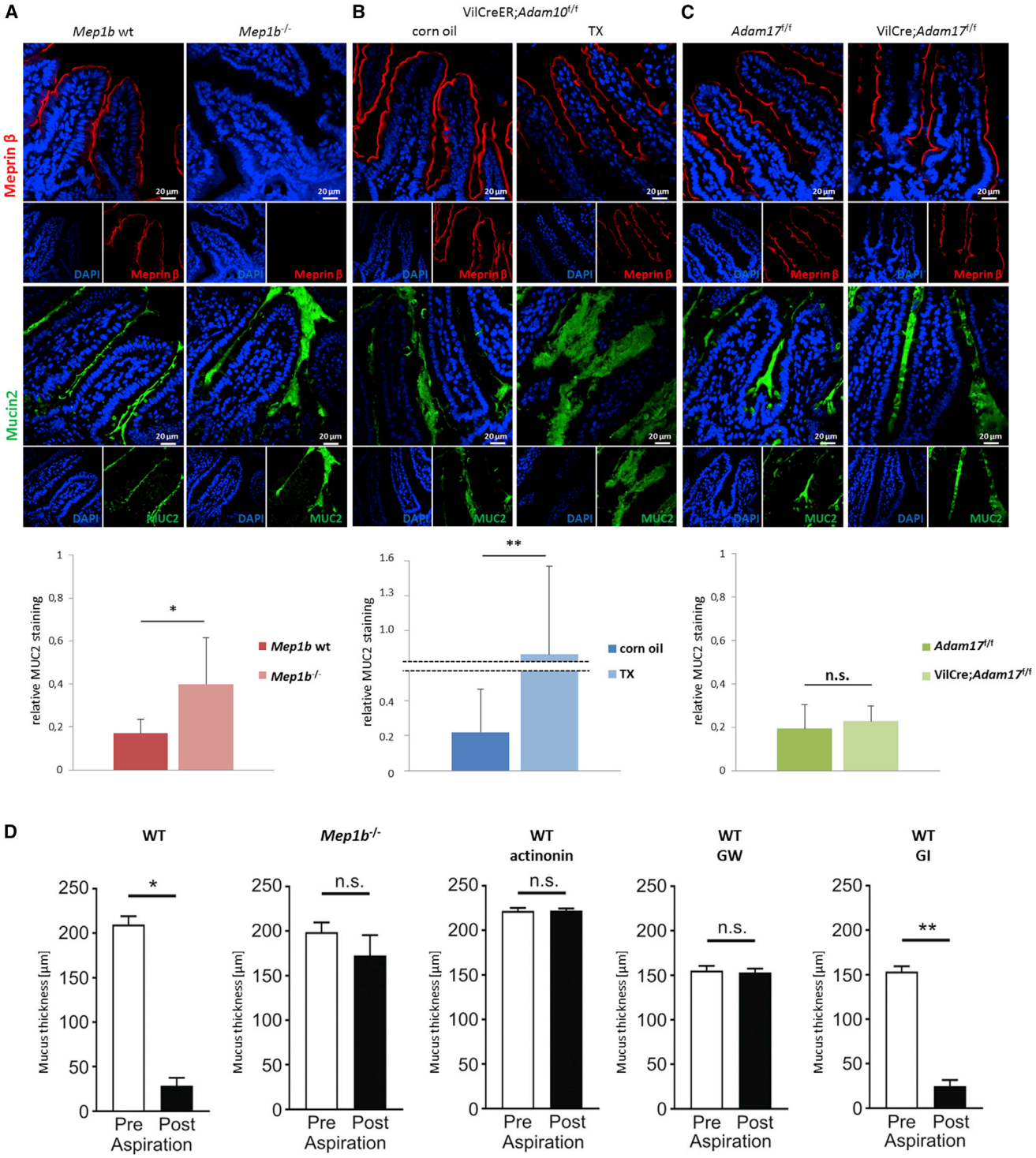

Figure 5. Mucus Attachment in Mouse Ileum Is Dependent on Functional Meprin β

(A–C) Ilea of (A) *Mep1b*$^{-/-}$, (B) tamoxifen (TX)-inducible VilCreER;*Adam*10$^{f/f}$, and (C) VilCre;*Adam17*$^{f/f}$ mice were stained for meprin β (red) and mucin 2 (MUC2, green) and visualized by immunofluorescence microscopy. Scale bars, 20 µm. Meprin β-deficient mice (A, n = 3) and ADAM10-deficient mice (B, n = 4) showed strong accumulation of MUC2, which was not seen in VilCre;*Adam17*$^{f/f}$ animals (C, n = 3). Data are presented as mean ± SD, and statistical analysis was assessed by unpaired Student's t test (*p < 0.05, **p < 0.01).

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chimeric meprin β was not generally abolished because trypsin activated chimeric meprin β as efficiently as WT hMeprin β (Figure S3D). These results suggest that the motif N-terminal of the EGF-like domain of hMeprin β is important for interaction with ADAM10 and MT-2, thus explaining the mutually exclusive binding of both proteins to membrane-bound hMeprin β.

Meprin β Shedding and Activation Are Regulated by Host-Microbiome Interaction

To investigate the role of ADAM-mediated pro-meprin β shedding and its effect on mucus detachment *in vivo*, we studied ADAM10/17-deficient mice. Because meprin β-deficiency as well as the lack of shed meprin β in germ-free mice resulted in impaired mucus detachment in the small intestine (Schütte et al., 2014), we analyzed MUC2 staining and meprin β protein levels in VilCreER;*Adam10*$^{f/f}$ mice (Tsai et al., 2014). These tamoxifen-inducible ADAM10-deficient animals showed a mucus layer that appeared to be similar to that of meprin β-deficient mice (Figures 5A and 5B). However, it should be noted that, in VilCreER;*Adam10*$^{f/f}$ mice, Notch signaling is abolished, leading to goblet cell hyperplasia (Tsai et al., 2014), whereas no differences in goblet cell numbers were observed in meprin β-deficient mice (Figures S4A and S4B). In contrast, VilCre;*Adam17*$^{f/f}$ mice showed no evidence for mucus layer alteration (Figure 5C) nor differences in the amount of specific intestinal cell types (Figure S4C), as shown previously (Feng et al., 2015). To further analyze the influence of ADAM10/17 on meprin β-mediated mucus detachment in the small intestine, we employed a murine *ex vivo* tissue explant model (Gustafsson et al., 2012b). As expected, mucus detachment was abrogated in *Mep1b*$^{-/-}$ explants and could also be inhibited in WT tissue by applying the meprin inhibitor actinonin (Schütte et al., 2014; Gustafsson et al., 2012a; Ermund et al., 2015a, 2015b; Figure 5D). Interestingly, impaired mucus detachment was also observed when ileal explants from WT mice were incubated with the ADAM10/17 inhibitor GW but not upon incubation with the ADAM10-selective inhibitor GI. However, both ADAM10 and ADAM17 are capable of shedding the murine meprin β, as shown by co-expression of mMeprin β with ADAM10/17 in ADAM10/17$^{-/-}$ HEK293T cells, which resulted in increased amounts of meprin β CTFs (Figure S5). Thus, unlike the specific requirement for ADAM10 in human pro-meprin β shedding, both ADAM10 and ADAM17 can contribute to pro-meprin β shedding in the murine small intestine.

In small intestinal organoids derived from meprin β-deficient and WT control mice, no obvious phenotypic differences in growth or morphology were observed (Figure 6A). Remarkably, western blot analysis of organoid lysates from WT mice revealed only a single band for mMeprin β at approximately 130 kDa, corresponding to its membrane-bound full-length form, which was lacking in organoids isolated from *Mep1b*$^{-/-}$ mice (Figure 6B). By contrast, in isolated human small intestinal organoids, although immunoblotting revealed a major band at approximately 120 kDa, corresponding to full-length meprin β, an additional faint faster-migrating band suggestive of shed hMeprin β was detected (Figure 6C). These results provide additional evidence about the differences in shedding observed for murine versus human meprin β and are in line with other studies detecting mMeprin β predominantly in its membrane-bound but not in its soluble form (Marchand et al., 1994; Gorbea et al., 1993; Craig et al., 1987).

Similar to isolated murine organoids, germ-free mice also lack shed meprin β in the small intestine and show a MUC2 phenotype comparable with meprin β-deficient mice (Schütte et al., 2014). These observations raised the possibility that pro-meprin β shedding is triggered by bacterial stimuli *in vivo*. To test this possibility, we stimulated transfected HEK293T cells with the bacterial endotoxins lipopolysaccharide (LPS) or lipoteichoic acid (LTA). LPS from the outer membrane of Gram-negative bacteria is a known stimulator of ADAM17 via activation by polo-like kinase-2 (PLK2) (Schwarz et al., 2014), whereas LTA from the cell wall of Gram-positive bacteria stimulates ADAM10 activity via platelet activation factor receptor (PAFR) (Lemjabbar and Basbaum, 2002). Upon LTA stimulation, increased amounts of shed meprin β, associated with increased proteolytic activity of trypsin-activated soluble meprin β, were detected in the cell supernatant, which was not the case after LPS stimulation (Figures 6D–6F). Consistent with our previous results, ADAM10 was the major sheddase of human pro-meprin β, which could be induced by bacterial LTA in HEK293T cells (Figure 6G). A similar tendency toward LTA-induced pro-meprin β shedding by ADAM10, but not ADAM17, was observed in Colo320 cells (Figure S6).

Membrane-Bound Meprin β Is Activated by the Pathogenic Gingipain Protease RgpB, Impairing Its Shedding

Although interaction of meprin β with MT-2 impairs ADAM-mediated pro-meprin β shedding, and active soluble meprin β was not detected in any experiment, activation of membrane-bound meprin β might abrogate its shedding per se. Because LTA treatment can induce ADAM-mediated pro-meprin β shedding, it raised the possibility that host-microbiome interactions might be involved in this shedding event. To determine whether pathogenic bacteria can interrupt this regulatory mechanism, we analyzed the isolated secreted bacterial protease Arg-gingipain (RgpB) from *P. gingivalis*, which is well studied in periodontitis but is also found to colonize and harm the gastrointestinal tract (Nakajima et al., 2015). Importantly, RgpB can efficiently cleave MUC2, disrupting the MUC2 polymeric network (van der Post et al., 2013). In addition, RgpB exhibits a cleavage preference for arginine in the P1 position (Chen et al., 1992; Potempa et al., 1998), consistent with the already identified activation site of hMeprin β at Arg_{61} (Jäckle et al., 2015). To investigate the effect of RgpB on pro-meprin β shedding, we stimulated

(D) Mucus thickness measurements were performed in WT (n = 4) and *Mep1b*$^{-/-}$ mouse ilea (n = 4). Ileal explants from WT mice were apically incubated with the meprin inhibitor actinonin (n = 4), the dual ADAM10/17 inhibitor GW (n = 7), or the ADAM10 inhibitor GI (n = 5). Data are presented as mean ± SEM, and statistical analysis was assessed by Mann-Whitney test (*p < 0.05, **p < 0.01).
See also Figure S4.

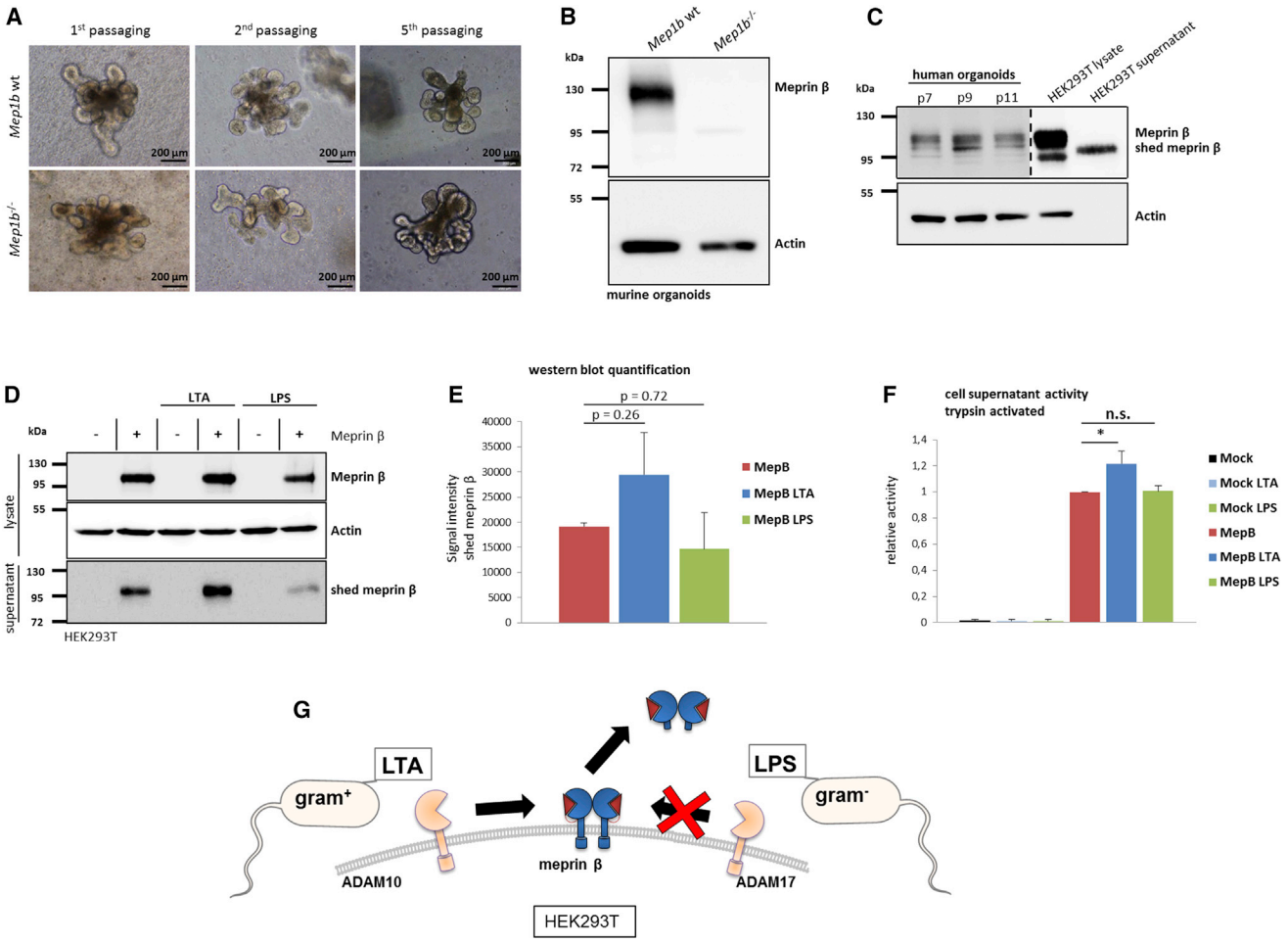

Figure 6. ADAM-Mediated Pro-meprin β Shedding Requires Stimulation by the Host Microbiome

(A) No morphological differences were observed in intestinal organoids isolated from meprin β-deficient mice or control animals. Scale bars, 200 μm.

(B) Immunoblotting of lysates from murine intestinal organoids.

(C) Immunoblotting of lysates from human intestinal organoids. The western blot was exposed in different intensities, marked by the dashed line.

(D) Meprin β-transfected HEK293T cells were stimulated with 50 μg/mL LTA or 1 μg/mL LPS. Proteins were detected by immunoblotting.

(E) Densitometric analysis of shed meprin β from three biological replicates as shown in (D).

(F) Quantification of meprin β activity in cell supernatants (trypsin-activated) from three biological replicates. Data are presented as mean ± SD, and statistical analysis was assessed by one-way ANOVA followed by a Dunnett's test (*$p < 0.05$).

(G) Model of LTA-induced ADAM10-mediated pro-meprin β shedding.

See also Figure S6.

meprin β–expressing ADAM10/17$^{-/-}$ HEK293T cells with RgpB. Interestingly, soluble meprin β levels in supernatants of ADAM10/17 co-expressing cells were decreased after RgpB treatment (Figure 7A). To avoid the contribution of full-length meprin β release via microvesicles, cell supernatants were ultracentrifuged prior to analysis. Indeed, meprin β cell surface activity confirmed the pathogenic protease RgpB to be a specific activator of membrane-bound meprin β (Figure 7B). Here we observed meprin β activity in cell supernatants of RgpB-treated cells without previous trypsin activation, indicating that the gingipain protease RgpB activates soluble pro-meprin β as well (Figure 7C). However, meprin β activity in cell supernatants could be further increased upon trypsin treatment (Figure 7D). Decreased protein levels of soluble meprin β together with reduced meprin β activity in supernatants of RgpB-treated cells (Figures 7A and 7D) demonstrate that only inactive pro-meprin β is shed by ADAM proteases. Furthermore, increasing concentrations of RgpB could completely abolish ADAM-mediated pro-meprin β shedding (Figures 7E and 7F), clearly demonstrating that RgpB-mediated activation of membrane-bound meprin β blocks ADAM-mediated pro-meprin β shedding.

In addition to its crucial role in mucus detachment under physiological conditions, meprin β mRNA is downregulated in biopsies of Crohn's disease (CD) patients, implicating a loss of meprin β function in IBD (Vazeille et al., 2011). To assess changes in meprin β protein levels, we analyzed the amount of membrane-bound meprin β in inflamed and non-inflamed ileal biopsies derived from CD patients using an antibody specific

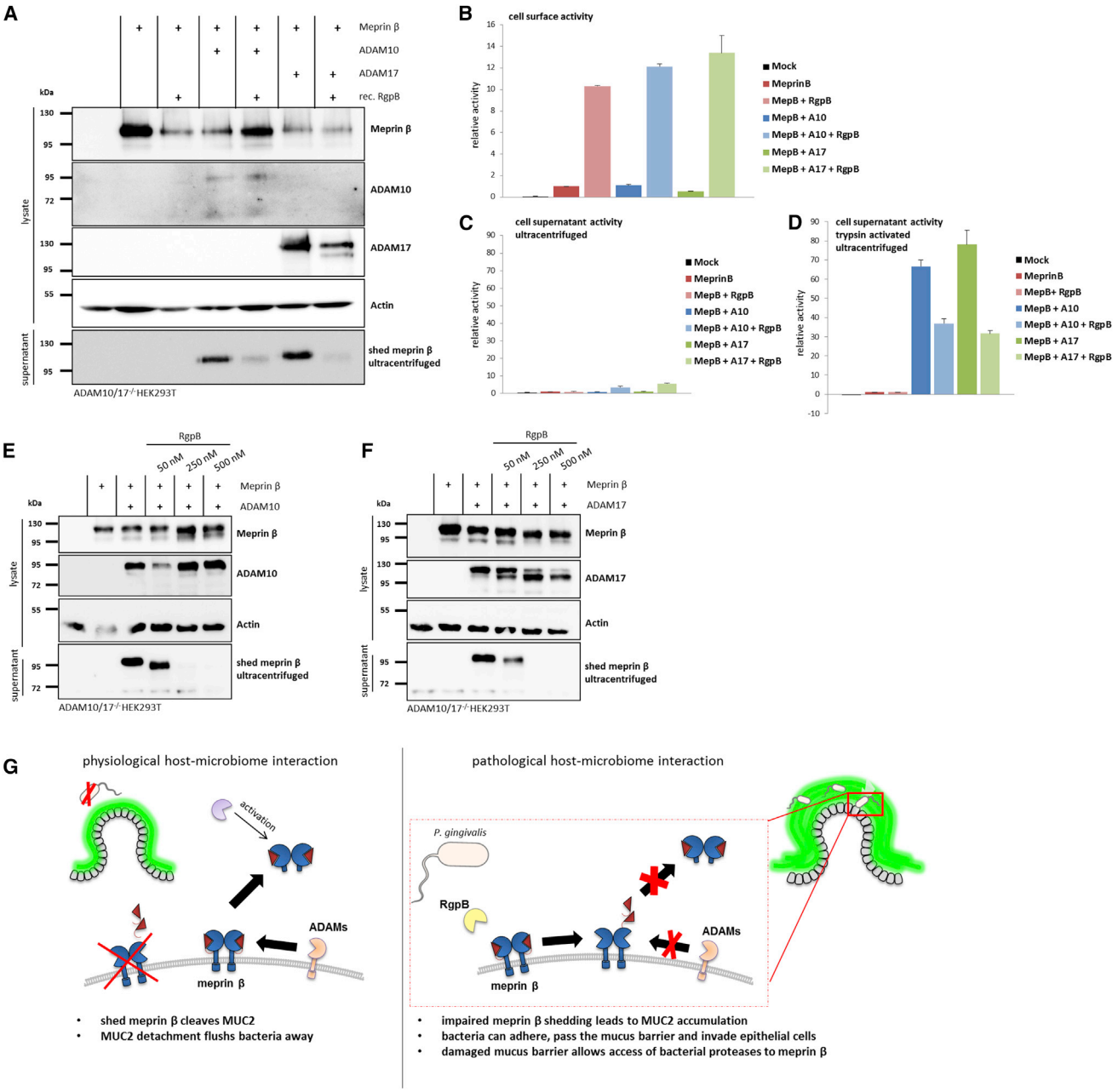

Figure 7. Meprin β Is Activated by the Pathogenic Protease RgpB

(A) ADAM10/17$^{-/-}$ HEK293T cells were transfected with meprin β and incubated with the purified gingipain protease RgpB (100 nM). Proteins were analyzed by immunoblotting.

(B) Cell surface activity of meprin β.

(C) Meprin β activity in ultracentrifuged cell supernatants.

(D) As in (C) but trypsin-activated. SD was calculated from two technical replicates.

(E and F) ADAM10/17$^{-/-}$ HEK293T cells were co-transfected either with (E) ADAM10 and meprin β or (F) ADAM17 and meprin β and stimulated with RgpB in different concentrations.

(G) Model of the regulation of meprin β shedding and activation in health and disease.

for the C terminus of meprin β. Intriguingly, we observed a trend toward increased activated, membrane-bound meprin β protein levels in inflamed tissue samples compared with non-inflamed tissue, suggesting that ADAM-dependent meprin β shedding and mucus detachment are compromised in the inflamed state (Figure S7).

In summary, we have identified a unique regulatory mechanism of meprin β shedding and activation that most likely is

regulated by specific host-microbiome interactions that could directly influence the meprin β substrate repertoire and modulate mucus detachment (Figure 7G).

DISCUSSION

The biological function of meprin β strictly depends on its localization at the cell surface or whether it is released by ectodomain shedding. For example, membrane-bound meprin β sheds APP at the β-secretase cleavage site, leading to the generation of amyloid-β peptides, which tend to aggregate in the brains of Alzheimer's disease patients (Bien et al., 2012). However, this β-secretase activity is completely abrogated for soluble meprin β (Bien et al., 2012). In contrast, MUC2 cleavage in the small intestine is only achieved by soluble meprin β and is important for proper mucus function (Schütte et al., 2014). Disturbed mucus production and detachment lead to pathological conditions such as cystic fibrosis (O'Sullivan and Freedman, 2009).

Here we demonstrate that only inactive pro-meprin β is shed from the cell surface by ADAM proteases. We recently identified MT-2 as a membrane-bound activator of meprin β (Jäckle et al., 2015), and we now show that ADAM-dependent pro-meprin β shedding is completely abolished upon binding and activation by MT-2. Moreover, in ADAM10/17$^{-/-}$ HEK293T cells, meprin β is secreted exclusively via microvesicles, eliminating the possibility that other proteases can compensate for pro-meprin β shedding in this cell line. It is conceivable that microvesicle release is a protective mechanism to avoid an overload of active meprin β at the cell surface. Indeed, in a mouse model of renal injury in which an overload of active membrane-bound meprin β has been reported, meprin β was found to be translocated from the apical to the basolateral surface of renal cells, leading to severe inflammation, increased leukocyte infiltration, and stronger brush border disruption (Bylander et al., 2008). Consistent with the idea that high levels of active membrane-bound meprin β might be detrimental and associated with pathological processes, we detected more active full-length meprin β in inflamed ileal tissues derived from patients suffering from CD than in non-inflamed tissue.

ADAMs and meprin β are highly expressed in epithelial cells of the small intestine (Sterchi et al., 1988; Lottaz et al., 1999; Jones et al., 2016), and soluble pro-meprin β can easily be activated by secreted serine proteases such as pancreatic trypsin or kallikreins (Grünberg et al., 1993). We already excluded the presence of MT-2 in epithelial cells of the murine small intestine (Jäckle et al., 2015), which is a prerequisite for meprin β shedding, because we now show that activation of membrane-bound meprin β by MT-2 completely prevents its shedding. Endogenous meprin β shedding in Colo320 cells provided strong evidence that ADAM10 is the constitutive sheddase of pro-meprin β. In contrast, we observed abolished mucus detachment in *ex vivo* mucus thickness measurements after applying the ADAM10/17 inhibitor GW but not upon incubation with the ADAM10-selective inhibitor GI. However, no increase in MUC2 staining was observed in *Adam17*$^{f/f}$ mice, as shown for meprin β-deficient animals. Increased MUC2 staining in VilCreER;*Adam10*$^{f/f}$ mice can rather be explained by the previously described hyperplasia of goblet cells, which are the main source of MUC2 in the small intestine (Tsai et al., 2014). Thus, in the murine system, ADAM10 and ADAM17 most likely act redundantly on pro-meprin β shedding. Analysis of ADAM10/17 double-deficient mice will show whether there are other, not yet identified proteases involved in pro-meprin β shedding as well.

Generation of an EGF-chimera in which the identified ADAM17 cleavage site for hMeprin β between amino acids Q_{595} and L_{598} (Hahn et al., 2003) was exchanged confirmed involvement of different pro-meprin β sheddases in the murine versus the human system. Although ADAM10 prefers shedding of human pro-meprin β, ADAM17 was still able to shed chimeric meprin β because it prefers cleavage N-terminal of valine residues (Tucher et al., 2014). However, increased levels of meprin β CTFs after co-expressing ADAM10/17 in ADAM10/17$^{-/-}$HEK cells confirmed ADAM-mediated shedding of mMeprin β. Chimeric meprin β could not be activated by MT-2 but by other serine proteases, such as trypsin, using the same activation site at position Arg_{61}. We therefore hypothesize that this exchanged motif in meprin β, which shows the weakest sequence homology between human and murine meprin β (Pischitzis et al., 1999), is essential for the interaction with ADAM10 and MT-2. Competing for the same sequence motif explains the full blockade of ADAM-mediated pro-meprin β shedding upon binding to MT-2.

Germ-free mice lack shed meprin β and exhibit a severe MUC2 phenotype (Schütte et al., 2014). Additionally, only a minimal amount of meprin β was shed from intestinal organoids under sterile culture conditions, suggesting that a bacterial trigger may be required to induce meprin β shedding. We show that the bacterial endotoxin LTA can induce ADAM10-dependent pro-meprin β shedding in HEK293T cells and Colo320 cells. These findings suggest that important host-microbiome interactions of meprin β with commensal microbiota exist that regulate meprin β shedding, which plays a crucial role in the homeostasis of proper mucus function. Additionally, it was shown that soluble shed meprin β diminishes colonization of adherent-invasive *E. coli* in the small intestine by cleaving bacterial type 1 pili (Vazeille et al., 2011). Conclusively, decreased levels of soluble meprin β and increased amounts of active full-length meprin β correlate with the severity of intestinal inflammatory diseases, as we observed for inflamed and control human ileal biopsies from CD patients.

On the other hand, analysis of knockout mice revealed that meprin β expression on leukocytes of the intestinal lamina propria exhibits pro-inflammatory activity in DSS-induced colitis (Crisman et al., 2004; Banerjee et al., 2011). Under inflammatory conditions, the meprin β expression pattern is shifted from epithelial cells to leukocytes of the lamina propria, completely changing its substrate repertoire and further stimulating the pro-inflammatory function (Lottaz et al., 2007). The bivalent function of meprin β regarding pro- and anti-inflammatory properties as well as its changes in localization and subsequent substrate repertoire indicate the importance of regulating meprin β shedding and activation. Most interestingly, we identified RgpB from the pathogen *P. gingivalis* to specifically activate membrane-bound hMeprin β, preventing its shedding.

Different isoforms of gingipain proteases exist in soluble and membrane-bound forms (Potempa et al., 1995), which account

for 85% of proteolytic activity of *P. gingivalis* at the site of infection (de Diego et al., 2014). In a murine periodontitis model, the secreted proteases Kgp and RgpB have been identified as major virulence factors accounting for *P. gingivalis* pathogenicity (Pathirana et al., 2007). Although *P. gingivalis* is rather known from periodontitis, it was also detected in the ileum of mice after oral administration (Arimatsu et al., 2014). Additionally, a positive correlation between gingival and intestinal inflammation has been observed (Figueredo et al., 2017), and increased abundance of *P. gingivalis* has been reported in the feces of colorectal cancer patients (Ahn et al., 2013; Vogtmann et al., 2016). Thus, activation of membrane-bound meprin β by RgpB and consequential loss of pro-meprin β shedding from the cell surface might change the meprin β substrate repertoire in multiple tissues, including the gastrointestinal tract. It is conceivable that the bacterial pathogen *P. gingivalis*, through its secreted protease RgpB, activates membrane-bound meprin β, which leads to a loss of pro-meprin β shedding and perturbations in mucus integrity. Additionally, oral administration of *P. gingivalis* to mice can lead to disturbed gut microbiota in the ileum, systemic inflammation, and increased intestinal permeability (Nakajima et al., 2015; Whiting et al., 2015). Microbial dysbiosis is also characteristic for IBD associated with metabolic effects, mucus disruption, and altered immunological response (Wlodarska et al., 2015). Importantly, the direct effect of RgpB on MUC2 polymer disruption (van der Post et al., 2013), together with its activating properties on membrane-bound meprin β, might provide two distinct mechanisms to disrupt intestinal barrier function and promote bacterial overgrowth. Significantly, RgpB provides a proof of concept that activation of membrane-bound hMeprin β prevents the production of soluble meprin β, which functions as a mucus-detaching protease. In summary, meprin β is a key mediator in the homeostasis of mucus integrity, and its unique posttranslational regulation is required for proper mucus function and protection against bacterial invasion.

EXPERIMENTAL PROCEDURES

Cell Culture Stimulation, Fluorogenic Peptide-based Activity Assay, and Immunoblotting

Meprin β activity was measured in Colo320 cells or in transfected HEK293T cells and ADAM10/17$^{-/-}$ HEK293T cells by applying 50 μM of the highly specific quenched fluorogenic peptide substrate (7-methyloxycoumarin-4-yl) acetyl (mca)-EDEDED-(K-ε-2,4-dinitrophenyl [dnp]). Additionally, cells were stimulated with 1 μM IM (30 min) or 100 nM PMA (2 hr) or with 100 nM of the purified cysteine protease RgpB from *P. gingivalis* (Sztukowska et al., 2012; Veillard et al., 2015) to induce meprin β shedding. Remaining cell lysates and supernatants were used for western blot analysis. Detailed methods and all cDNA constructs or antibodies used can be found in the Supplemental Experimental Procedures.

Mice, Tissue Collection, and Immunofluorescence Staining

All procedures performed in this study involving animals were in accordance with the ethical standards set by the National Animal Care Committee of Germany, approved by the Laboratory Animal Ethics Committee of the University of Gothenburg with certification number 73-2015, and in accordance with the University Committee on the Use and Care of Animals at the University of Colorado (B102614 (01)1E). 8- to 12-week-old male and female mice of the following strains were used for histological sections: *Mepb1b*$^{-/-}$ mice (Norman et al., 2003) and the corresponding C57/BL6N WT mice, VilCre; *Adam17*$^{f/f}$ mice, and *Adam17*$^{f/f}$ control mice (Feng et al., 2015) and tamoxifen-inducible intestinal epithelial cell-specific ADAM10-deficient mice (VilCreER;*Adam10*$^{f/f}$ mice) (Tsai et al., 2014). Preparation and mounting of murine ileal explants for mucus thickness measurements were performed as described previously (Gustafsson et al., 2012b). Murine small intestine was additionally used to isolate primary organoid cultures as described previously (VanDussen et al., 2015). Detailed methods and all antibodies used for immunofluorescence microscopy of ileal tissue can be found in the Supplemental Experimental Procedures.

Human Intestinal Biopsies

Human jejunal tissue biopsies were obtained from different adult donors (n = 3, mean age 53.7 years, 2 males, 1 female) and used to isolate intestinal epithelial cells for a primary human *in vitro* model of the human small intestine (Schweinlin et al., 2016). Informed written consent was obtained beforehand, and the study was approved by the institutional ethics committee on human research of the Julius Maximilians University Würzburg (study approval number 182/10). Human small intestinal enteroid cultures were generated from ileal pinch biopsies obtained from patients undergoing endoscopy in accordance with IRB protocol 14-2012 at the University of Colorado. Non-inflamed (n = 7) and inflamed (n = 5) ileal biopsies from CD patients were analyzed for meprin β protein levels. Patients gave written informed consent prior to colonoscopy for biopsy collection. Approval was granted by the ethics committee of the medical faculty of Kiel University (B2321/98). Detailed methods for tissue preparation and analysis can be found in the Supplemental Experimental Procedures.

Statistical Analysis

Western Blots were quantified with ImageJ, and the protein of interest was normalized to actin levels. For the quantification of MUC2 on immunofluorescence images, a semi-automated method developed in-house was used. Therefore, different tools of ImageMagick were deployed, and the same settings were used for all images analyzed. The MUC2 signal was normalized to nuclear DAPI staining. For activity assays, relative fluorescent units (RFUs) were normalized to the control sample. All statistical analyses were performed with GraphPad Prism for unpaired Student's t test or for one-way ANOVA followed by a Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Values were normalized and are shown as mean ± SD. For mucus attachment assays, data are represented as mean ± SEM. Differences between groups were assessed by Mann-Whitney test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Additional material and methods are available in the Supplemental Experimental Procedures.

DATA AND SOFTWARE AVAILABILITY

The accession number for the raw data reported in this paper is Mendeley Data: 10.17632/3bbcykwgmv.1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.10.087.

AUTHOR CONTRIBUTIONS

Conceptualization, C.B.-P. and R.W.; Methodology, Investigation, and Data Analysis, R.W., A.E., S.S., M. Schweinlin, P.A., F.W., B.P., M.F.-P., P.J.D., and C.B.-P.; Resources, M.K., B.R., K.K., M.M., R.L., J.W.B., S.R.-J., J.P., G.C.H., P.R., S.N., M. Stirnberg, and P.J.D.; Writing, R.W., P.J.D., and C.B.-P.

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