

RHINOVIRUS INDUCES MUC5AC IN A HUMAN INFECTION MODEL, & *IN VITRO* VIA NF- κ B & EGFR PATHWAYS

Christopher A. Hewson¹, Jennifer J. Haas¹, Nathan W. Bartlett¹, Simon D. Message^{1,2}, Vasile Laza-Stanca¹, Tatiana Keadze¹, Gaetano Caramori³, Jie Zhu⁴, Mark R. Edbrooke⁵, Luminita A. Stanciu¹, Onn M. Kon², Alberto Papi³, Peter K. Jeffery⁴, Michael R. Edwards¹ and Sebastian L. Johnston^{1,2}.

¹ Department of Respiratory Medicine, National Heart and Lung Institute, Centre for Respiratory Infection & MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, Imperial College London, London, UK

² Imperial College Healthcare NHS Trust, London, UK

³ Research Centre on Asthma and COPD, University of Ferrara, Ferrara, Italy

⁴ Department of Gene Therapy, National Heart and Lung Institute, Imperial College London at the Brompton Hospital, London, UK

⁵ Gene Interference, Functional Genomics, GlaxoSmithKline R&D, Stevenage, UK

Address correspondence to:

Prof. Sebastian L. Johnston, Department of Respiratory Medicine, National Heart and Lung Institute, Imperial College London, Norfolk Place, London, W2 1PG, UK.

Tel: +44 (0) 20 7594 3764

Fax: +44 (0) 20 7262 8913

Email: s.johnston@imperial.ac.uk

ABSTRACT

Rhinovirus (RV) infections are the major cause of asthma exacerbations - the major cause of morbidity and mortality in asthma. MUC5AC is the major mucin produced by bronchial epithelial cells. Whether RV infection up-regulates MUC5AC *in vivo* is unknown, and the molecular mechanisms involved incompletely understood.

We investigated RV induction of MUC5AC *in vivo* and *in vitro* to identify targets for development of new therapies for asthma exacerbations.

RV infection increased MUC5AC release in normal and asthmatic volunteers experimentally infected with RV-16; and in asthmatic, but not normal subjects, this was related to virus load. Bronchial epithelial cells were confirmed a source of MUC5AC *in vivo*. RV induction of MUC5AC in bronchial epithelial cells *in vitro* occurred via nuclear factor- κ B dependent induction of matrix metalloproteinase mediated transforming growth factor- α release, thereby activating an epidermal growth factor receptor-dependent cascade culminating, via mitogen-activated protein kinase activation, in specificity protein-1 trans-activation of the MUC5AC promoter.

RV induction of MUC5AC may be an important mechanism in RV-induced asthma exacerbations *in vivo*. Revealing the complex serial signalling cascade involved identifies targets for development of pharmacologic intervention to treat mucus hyper secretion in RV induced illness.

KEYWORDS:

Asthma, chronic obstructive pulmonary disease, epidermal growth factor receptor, MUC5AC, nuclear factor κ B, rhinovirus

INTRODUCTION

Acute exacerbations are the major cause of morbidity, mortality and health care costs in asthma. Exacerbations continue to occur despite the availability of prophylactic medication [1]. Therapies better able to reduce the impact of acute exacerbations are needed.

Increased production and secretion of mucus is associated with acute exacerbations, accelerated decline in lung function and with fatal asthma [2-4]. Twenty-one different mucin genes have been identified. Of these, MUC5AC and MUC5B are the major respiratory mucins produced by bronchial epithelium and sub mucosal glands respectively [5, 6]. MUC5AC is the pre-dominant mucin in mild to moderate asthma [7] and increases in both MUC5AC and MUC5B proteins have been observed in fatal asthma [4]. MUC5AC is induced in respiratory epithelial cells by a wide variety of stimuli implicated in the pathogenesis of asthma, including cytokines (interleukin (IL) -9 and IL-13), neutrophil elastase, epidermal growth factor receptor (EGFR) ligands and air-pollutants [5], however the importance and mechanisms of mucin induction in asthma exacerbations has not been extensively investigated.

Rhinoviruses (RV) are associated with the majority of asthma exacerbations [8]. Human experimental RV infections have been used to investigate exacerbation pathogenesis, with infection increasing markers of eosinophil activation, IL-8 and neutrophilia [9]. Only one study has investigated RV induction of mucus secretion in humans [10], however this study did not evaluate specific mucin gene expression or protein release, did not relate virus load to mucin secretion and did not evaluate the molecular signalling pathways involved.

The primary target of RV is the bronchial epithelial cell [11]. One recent report investigated RV induction of MUC5AC in tracheal epithelial cells and reported nuclear factor (NF) κ B, mitogen-activated protein kinase kinase (MEK) and Src to be involved [12]. In contrast most other reports investigating induction of MUC5AC by other stimuli in respiratory epithelial

cells implicate the EGFR and downstream pathways [13-15]. The full mechanistic pathways involved in RV induction of MUC5AC therefore remain unclear.

We hypothesised that RV infection would induce MUC5AC secretion *in vivo* and having confirmed this, investigated the detailed molecular regulation of RV induction of MUC5AC *in vitro*. Our findings provide targets for the development of new therapies for mucus hypersecretion in RV induced illness.

MATERIALS AND METHODS

An experimental model for RV induced acute exacerbations of asthma

The clinical model, sampling and analysis are described in detail elsewhere [16]. Briefly, experimental infections were induced in RV-16 seronegative atopic asthmatic and normal nonatopic adult subjects by inoculating a 10,000 tissue culture ID₅₀/ml (TCID₅₀) dose on day 0 by nasal spray. The study was approved by St Mary's NHS Trust ethics committee and all subjects gave informed consent. Broncho-alveolar lavage (BAL) and bronchial biopsies were taken at baseline ~2 weeks prior to infection, on day 4 after virus inoculation during acute infection and at convalescence (6 weeks after infection). BAL was assayed by ELISA for MUC5AC protein as described below. Peak virus load during acute infection was determined in daily nasal lavage samples taken for 8 days following inoculation by qPCR as described [16].

Immunostaining of MUC5AC in bronchial biopsies

Biopsies were fixed and set in paraffin wax. Sections were stained with mouse anti-human MUC5AC (1:2000v/v – Santa Cruz Biotechnology) and biotinylated horse anti-mouse Ab and counterstained with haematoxylin.

Cell and viral culture

NCI-H292 cells were cultured in RPMI-1640 supplemented with 10%v/v FCS (Invitrogen). RV stocks were grown in HeLa cells [17]. Viruses were titrated on HeLa cells to ascertain their TCID₅₀ [18]. The identities of all RVs were confirmed by neutralisation using serotype-specific Ab (ATCC). UV inactivation was performed and filtered virus produced by passing RV stocks through a 30 KDa membrane (Millipore) [17].

Infection of cells with RV

NCI-H292 cells were cultured for 24h before being serum-starved for 24h. Cells were infected at a multiplicity of infection (MOI) of 1 (unless otherwise stated) for 1h.

For inhibition studies, actinomycin D, AG1478, PD98059, U0126, mithramycin A, CAPE, cycloheximide and GM6001 were purchased from Calbiochem. AS602868 was kindly provided by Prof. Ian Adcock (Imperial College, London, UK). Cells were pre-treated for 1-2h before infection.

For the MUC5AC promoter studies, NCI-H292 cells were transfected with MUC5AC promoter constructs [15], including 0.4µg MUC5AC-construct, 0.2µg pCMVSPORT-βgal (Invitrogen) and 0.4µg poly(rI:C), or poly(dI:C) as control (Sigma Aldrich). Luciferase levels were assessed and normalised to β-gal levels (Promega) [15].

ELISA to evaluate MUC5AC protein

Supernatants from NCI-H292 cells and BAL diluted 1:100v/v in carbonate-bicarbonate buffer were assayed for MUC5AC protein by ELISA [15].

RNA extraction, reverse transcription and real-time qPCR

RNA extraction, reverse transcription and qPCR analysis of MUC5AC gene expression was performed as previously described [15].

Site-directed mutagenesis of MUC5AC -324 promoter construct

Site-directed mutagenesis of the NF-κB and IRF transcription factor binding sites was carried out as previously described [15]; NF-κB GGGGAGGACCCCT to TTTTAGGACCCCT and IRF TCACTTCTGG to TCACGGGACC (mutated bases underscored).

Western blotting for EGFR phosphorylation

Cells were infected with RV-16, or transfected with 0.4µg poly(rI:C) or poly(dI:C). Cells were harvested, and proteins resolved and analysed by immunoblotting with mouse anti-phospho-EGFR or sheep anti-EGFR (1:1000v/v – Upstate) and HRP-conjugated secondary antibodies.

ELISA to quantify ERK1/2 phosphorylation

At 3, 6 and 24h post-infection, proteins were extracted using Cell Extraction Buffer (Biosource) including protease inhibitors (Pierce Biotechnology). Total and phospho-specific ERK1/2 were quantified using specific ELISAs (Biosource).

ELISA to quantify TGF- α release

At 8h post-infection, supernatants were harvested and assayed for TGF- α release using a human TGF- α Quantikine ELISA (R&D Systems).

Confocal microscopy for RV infection and p65 translocation

At 6h post-infection, cells were fixed, permeabilised and incubated with a rabbit anti-RV 3C protease Ab (1:200v/v – provided by Dr. James Gern, University of Wisconsin, Madison, USA [19]) and mouse anti-p65 Ab (1:500v/v – Santa Cruz Biotechnology). These were detected using goat anti-mouse AlexaFlour546 (1:200v/v) and anti-rabbit AlexaFlour488 (1:200v/v) Ab (Invitrogen). Slides were counter-stained with DAPI.

Statistical analysis

Data are presented as mean \pm the standard error of the mean. All data were analysed using ANOVA and Bonferroni's multiple comparison *post hoc* test. Correlations between MUC5AC concentrations and virus load were examined using Spearman's rank correlation. Data were accepted as significantly different when $P < 0.05$.

RESULTS

RV induction of MUC5AC protein secretion in vivo & relation to virus load.

Nine atopic asthmatic and 15 non-atopic normal volunteers were experimentally infected with RV-16 as described [16]. MUC5AC protein was quantified in BAL harvested prior to (baseline), 4-days after and 6-weeks after infection. In addition, nasal lavage samples were taken to determine peak virus load during the infection [16].

RV-infection led to a significant increase in BAL MUC5AC protein between the baseline and acute infection phases ($P=0.033$), which was resolved by 6-weeks ($P=0.002$, Fig 1A). Asthmatic patients had greater mean levels of MUC5AC BAL protein at each time point compared with normal subjects, however, these differences were not statistically significant (Fig 1B).

In asthmatic patients alone, MUC5AC levels in BAL were highly correlated with virus load ($r=0.750$, $P=0.02$, Fig 1C), indicating induction was related to severity of infection. No significant relationship was observed for normal subjects ($P=NS$).

Bronchial epithelial cells are a source of MUC5AC in vivo.

Bronchial biopsies taken at baseline and 4-days post-infection [16] were stained for MUC5AC protein expression. In both normal (Fig 1D, upper panels) and asthmatic (Fig 1D, lower panels) subjects, epithelial cells were identified as MUC5AC producing cells. Quantitative analysis could not be performed due to loss of epithelium in many of the asthmatic subjects and therefore while we were able to identify RV-induced release of MUC5AC (Fig 1A & B) we were unable to definitively demonstrate an increase in production. However, these data do demonstrate that bronchial epithelial cells are a source of MUC5AC protein in the human lung *in vivo*.

RV stimulates de novo MUC5AC synthesis and secretion in respiratory epithelial cells in vitro.

Having found RV induction of MUC5AC *in vivo*, we next investigated mechanisms *in vitro*. We infected the bronchial epithelial cell-line NCI-H292 with RV-16 and observed a time-dependent increase in MUC5AC protein compared to uninfected control cells (Fig 2A). This induction was dose-dependent, with significant induction of MUC5AC protein by RV-16 at MOI of 1 and 0.5, but not 0.1 (Fig 2B).

To determine whether RV increased MUC5AC transcription, we next assessed MUC5AC mRNA expression - this was significantly increased between 8 and 48h post-infection, peaking at 24h, but had returned to baseline by 72h (Fig 2C). Induction of mRNA expression was dose-dependent (Fig 2D).

As the RV family contains both major and minor serotypes (which use different receptors to infect), we demonstrated that RV-16, RV-9 (both major group) and RV-1B (minor group) significantly increased MUC5AC expression (Fig 2E), indicating that induction was neither serotype nor receptor restricted. In addition, we confirmed induction was virus specific as virus-free inocula and UV-inactivated RV-16 failed to significantly induce MUC5AC expression (Fig 2E).

We next investigated whether the increased MUC5AC expression was due to increased *de novo* transcription, using actinomycin D to inhibit new mRNA synthesis and suppress induction of MUC5AC mRNA expression (Fig 2F), confirming increased expression was due to increased transcription. Additionally, we monitored MUC5AC mRNA over time and observed no difference in degradation rates in the presence of RV-16 (Fig 2G), confirming that RV-16 infection did not alter MUC5AC mRNA degradation.

RV induces MUC5AC expression via an EGFR, MEK/ERK, Sp1 signalling cascade.

Previous studies report EGFR activation is required for induction of MUC5AC expression by a wide range of mediators [13-15]. We therefore investigated this for RV-induced MUC5AC expression.

AG1478, a specific inhibitor of EGFR phosphorylation and activation, resulted in dose-dependent and complete inhibition of MUC5AC expression (Fig 3A). We next confirmed that RV infection activated EGFR using phospho-specific Western blotting (Fig 3B) and also confirmed that poly(rI:C) - a frequently used model of virus infection - activated EGFR in the same manner (Fig 3C).

We then investigated the signalling pathways downstream of EGFR. PD98059 an inhibitor of MEK activation led to a dose-responsive and complete inhibition of MUC5AC mRNA expression (Fig 4A), as did a second MEK-specific inhibitor, U0126 (Fig 4B). We confirmed that RV infection induced extracellular-regulated kinase (ERK) phosphorylation from 3-24h (Fig 4C) and that mithramycin A, an inhibitor of specificity protein (Sp) 1 binding, resulted in a dose-responsive inhibition of MUC5AC expression (Fig 4D). To confirm the requirement for Sp1, we transfected NCI-H292 cells with MUC5AC promoter-luciferase constructs (Fig 4E) [15] and observed that serial truncation of the promoter from -1330 to -192bp did not alter induction of the MUC5AC promoter by poly(rI:C) (Fig 4F); however when the promoter was further truncated to the -63bp fragment, thus removing the Sp1 sites, induction was abolished. Finally, we mutated the 3 Sp1 sites within the -192bp promoter fragment, identifying that all 3 sites were active, and that on deletion of all Sp1 sites there was no longer significant induction (Fig 4G).

These data demonstrate that RV induction of MUC5AC expression requires activation of the EGFR, followed by activation of MEK and ERK, culminating in trans-activation of the MUC5AC promoter by Sp1 binding to 3 specific sites within the proximal promoter.

RV induction of MUC5AC requires NF- κ B mediated induction of TGF- α release upstream of EGFR activation.

With the identification that EGFR was required and knowing that EGFR ligands are cleaved from precursors by matrix metalloproteinases (MMP) that may be induced by NF- κ B, which is implicated in RV-induced inflammation [17], we next investigated the role NF- κ B in RV induction of MUC5AC.

Two pharmacologically distinct inhibitors of NF- κ B activation, caffeic acid phenethyl ester (CAPE) - an inhibitor of p65 nuclear translocation (Fig 5A) and AS602868 - an inhibitor of inhibitor of κ B kinase (IKK) β (Fig 5B) resulted in a dose-responsive inhibition of MUC5AC induction. To confirm that RV infection caused activation of NF- κ B, using confocal microscopy we stained cells for RV 3C protease, a non-structural protein only expressed in actively infected cells [19], the p65 subunit of NF- κ B and DAPI. We observed NF- κ B nuclear translocation (red) only in cells infected with RV (green), while uninfected cells retained p65 in their cytosol (Fig 5C).

Virus infections induce both NF- κ B and interferon regulatory-factors and the MUC5AC promoter contained binding sites for both (Fig 4E). Site-directed mutagenesis of these sites did not alter promoter activity of the -324 construct (Fig 6A).

Having demonstrated that NF- κ B did not transactivate the MUC5AC promoter directly, we investigated the role of NF- κ B in activation of EGFR ligands. Pro-transforming growth-factor (TGF) α cleavage, activation and release from the cell surface has previously been demonstrated upstream of MUC5AC expression [20]. We therefore investigated whether RV infection induced the release of TGF- α , and whether this was NF- κ B dependent using AS602868. Infection of NCI-H292 cells with RV-16 resulted in significant induction of TGF- α release into supernatants, which was significantly blocked by NF- κ B inhibition (Fig 6B).

We next sought to confirm that RV induction of MUC5AC expression required the synthesis of an intermediate protein. Treatment of cells with cycloheximide to prevent *de novo* protein synthesis significantly inhibited MUC5AC gene expression (Fig 6C), confirming requirement of a newly synthesised intermediate protein in this process.

As MMPs are known to be induced by NF- κ B and to cleave pro-TGF- α to active TGF- α [21], we investigated the involvement of MMPs using the inhibitor GM6001 (Fig 6D) and observed that GM6001 significantly inhibited RV-induction of MUC5AC mRNA.

These data demonstrate that RV-induction of MUC5AC expression requires NF- κ B activation in addition to the EGFR-MEK/ERK-Sp1 pathway. However, NF- κ B does not directly transactivate the MUC5AC promoter, but rather activates expression and translation of intermediate MMPs that induce TGF- α release upstream of EGFR activation.

RV induction of MUC5AC protein requires the same key signalling events

Finally, having defined in detail the mechanisms involved in RV-induced MUC5AC gene expression, we wished to confirm our key findings for MUC5AC protein release.

We repeated our experiments using the inhibitor of NF- κ B (CAPE), the inhibitor of MMP (GM6001), the inhibitor of EGFR (AG1478), and the inhibitors of MEK activation (PD98059 and U0126) and demonstrated a statistically significant reduction in RV-induced MUC5AC protein release with all inhibitors (Fig 7).

These data confirmed the signalling pathway of RV induction of NF- κ B to activate MMPs and a subsequent EGFR-MEK/ERK pathway leading to MUC5AC synthesis and secretion.

DISCUSSION

RVs are the major cause of asthma exacerbations and mucus secretion is important in exacerbation pathogenesis, however RV induction of MUC5AC has not been demonstrated in humans and the molecular mechanisms regulating RV induction of MUC5AC are poorly understood.

We have demonstrated that MUC5AC release into BAL is increased by RV infection *in vivo* and, in asthmatic subjects only, BAL MUC5AC levels correlated with peak virus load. It is likely that this correlation is related to the impaired innate anti-viral immune response to RV infection recently reported in bronchial epithelial cells in asthma [11].

We next confirmed bronchial epithelial cells as a source of MUC5AC *in vivo* and observed that RV infection stimulated *de novo* MUC5AC gene expression and protein secretion from respiratory epithelial cells *in vitro*. This was RV serotype and receptor independent and replication dependent. Next we identified activation of the EGFR/MEK-ERK signalling pathway, culminating in Sp1 binding the proximal MUC5AC promoter. In addition, we identified a second signalling cascade upstream of EGFR activation: RV-induction of MUC5AC was NF- κ B dependent, however NF- κ B did not directly transactivate the promoter. Instead, NF- κ B was required for MMP-dependent release of the EGFR ligand TGF- α [14] thus completing definition of a complex signalling cascade (Fig 8).

A recent publication has described RV-14 mediated up-regulation of MUC5AC via Src/MEK/NF- κ B [12]. However, these authors overlooked the role of EGFR and implied that NF- κ B directly induced MUC5AC release. We have demonstrated that NF- κ B is involved indirectly via increasing transcription of an intermediate MMP. Another recent publication also identified involvement of the EGFR/ERK pathway in RV/dsRNA induction of MUC5AC – in this case initiated by TLR-3 recognition of the poly(rI:C) used [22]. While the paper did not fully characterise the complete pathway described here and did not identify the

induction of an intermediate MMP, their identification of upstream TLR-3 signalling increases the range of potential therapeutic targets for development of new approaches to therapy.

This combination of two serially linked signalling cascades both necessary for induction of MUC5AC is a novel pathway for mucin induction, so far unique for RV induction. However, it is possible that studies investigating other stimuli may have overlooked this pathway. We have previously reported that for PMA induced MUC5AC, an EGFR pathway culminating in Sp1 transactivation of the promoter was required [15] and others have highlighted Sp1 transactivation of MUC2 and MUC5AC promoters [23]. We noted at that time that PMA induction of MUC2 in colonic epithelium was also reported to involve EGFR-mediated signalling pathways, but in contrast culminated in NF- κ B activation of the promoter [24]. However, these authors did not demonstrate any effect on MUC2 promoter activity when the putative NF- κ B site was removed. With our demonstration that *both* NF- κ B and Sp1 are required in the complex RV-induction of MUC5AC, it is possible that both are also required for induction of MUC2 and MUC5AC by other stimuli. There are several other reports demonstrating Sp1 mediated activation of MUC2 in intestinal [25] and respiratory epithelium [26]; and there are, equally, several reports of requirement for NF- κ B in MUC2 [27] as well as MUC5AC expression [28]. With the extensive literature establishing the importance of the EGFR pathway (which culminates in Sp1 transactivation of MUC2 and MUC5AC promoters) [13, 14], it is likely that these studies identifying a requirement for NF- κ B may be highlighting this pre-EGFR signalling cascade we have identified with RV. This possibility should therefore be considered for other secretagogues.

Our findings also have important implications for acute exacerbations of other illnesses, as RV are reported to be a major cause of acute exacerbations of COPD [29] and other respiratory illnesses [30] in which mucus hyper-secretion is likely to play an important role.

In conclusion, this study has identified that RV infection induces MUC5AC protein release *in vivo* and the key molecules involved in a complex serial induction pathway. These data provide targets for the development of novel interventions against MUC5AC hyper secretion in RV induced exacerbations of airway diseases.

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FIGURE LEGENDS

Fig 1. MUC5AC is induced by RV *in vivo*; correlating with virus load and produced by bronchial epithelial cells. BAL and nasal lavage were taken from 15 normal and 9 asthmatic subjects at baseline, 4-days and 6-weeks post-infection. MUC5AC was quantified in the BAL by ELISA. RV load was quantified by qPCR in nasal lavage. Closed symbols indicate asthmatic subjects (AA); open symbols indicate normal subjects (N). The solid line indicates correlation for AA subjects. (A) Quantification of MUC5AC protein secreted into BAL for all patients. (B) Quantification of MUC5AC protein secreted into BAL for normal and asthmatic patients. (C) Correlation between peak virus load and BAL MUC5AC at day 4. *P* and *r*-values are indicated as appropriate for each correlation. (D) Bronchial biopsies were taken at baseline and 4-days post-infection. Biopsies were stained for MUC5AC (brown) and counter-stained with haematoxylin (blue). All images 200x magnification. (1) Representative immunohistochemistry images from baseline normal subject (2) and at day 4 and from baseline asthmatic patient (3) and at day 4 (4).

Fig 1

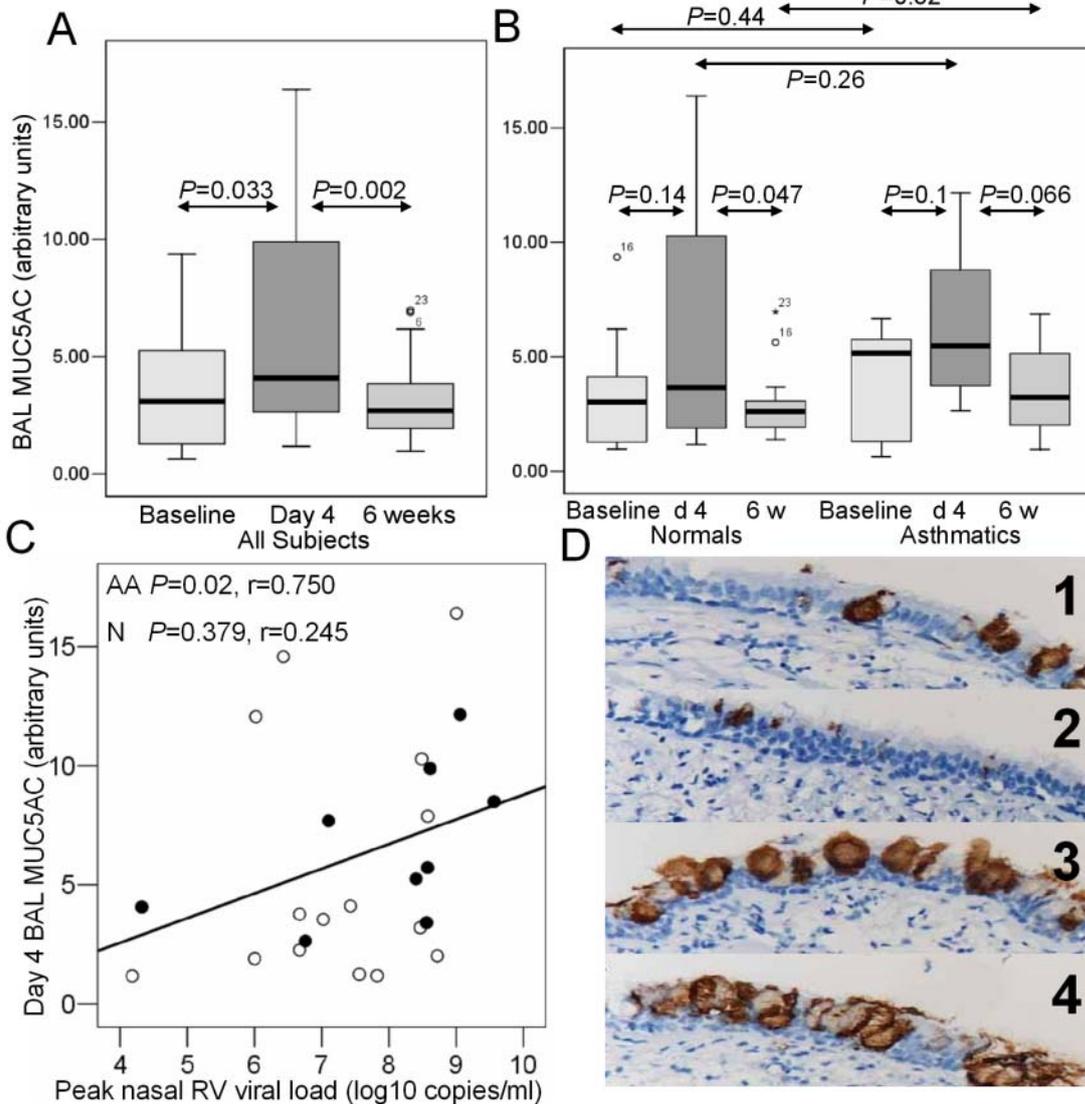


Fig 2. RV infection leads to time-, dose- and replication-dependent increases in *de novo* MUC5AC expression. (A) Cells were infected with RV-16 and MUC5AC protein quantified by ELISA at 8, 24, 48 and 72h post-infection (n=5). (B) Cells were infected with RV-16 at MOIs of 0.1, 0.5 and 1 or equivalent volume of filtered RV-16 and MUC5AC protein quantified by ELISA at 24h post-infection (n=3). (C) Cells were infected as (A) and MUC5AC mRNA harvested and quantified by qPCR (n=4). (D) Cells were infected as (B) and MUC5AC mRNA harvested and quantified by qPCR 8h post-infection (n=4). (E) Cells

were infected with RV-16, RV-1B, RV-9, UV-inactivated RV-16 or equivalent volume of filtered RV-16 and MUC5AC mRNA quantified by qPCR 8h post-infection (n=5). *, ** and *** indicate $P<0.05$, $P<0.01$ and $P<0.001$ respectively compared to uninfected control cells. # indicates $P<0.001$ compared to RV-16 infected cells. (F) Cells were pre-treated with actinomycin D at doses indicated and infected with RV-16. MUC5AC mRNA was quantified by qPCR 8h post-infection (n=4). (G) Cells were infected with RV-16 and treated with 100nM actinomycin D 16h later. MUC5AC mRNA was harvested and quantified by qPCR at 0, 4, 8 and 24h post-actinomycin D (n=4). ** and *** indicate $P<0.01$ and $P<0.001$ respectively compared to time 0.

Fig 2

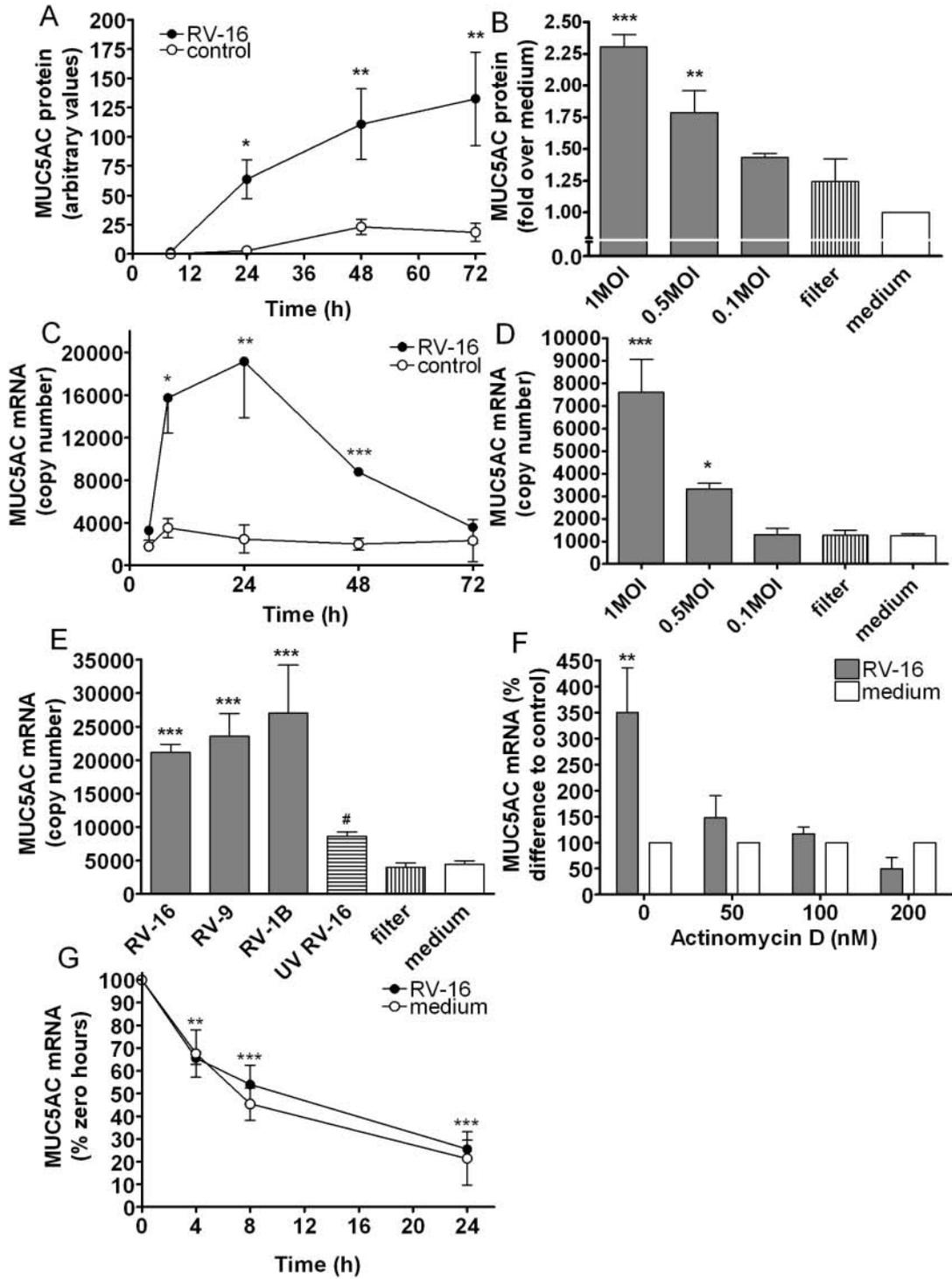


Fig 3. RV induction of MUC5AC requires activation of EGFR. (A) Cells were pre-treated with AG1478 at doses indicated and infected with RV-16. MUC5AC mRNA was harvested and quantified by qPCR 8h post-infection (n=5). *, ** and *** indicate $P<0.05$, $P<0.01$ and $P<0.001$ respectively compared to control infected cells. (B) Cells were pre-treated with anti-EGFR Ab and infected with RV-16. Ab was replaced and MUC5AC mRNA quantified by qPCR 8h post-infection (n=4). *** and N/S indicates $P<0.001$ and $P>0.05$ as indicated. (C) Cells were infected with RV-16 and proteins harvested 4, 6, 8 and 12h post-infection. Total and phospho-specific EGFR was detected by Western blotting. (D) Cells were stimulated with poly(rI:C), or poly(dI:C) as control, and proteins harvested 1.5, 2 and 3h later. Total and phospho-specific EGFR was detected by Western blotting. Western blots are representative of 3 experiments.

Fig 3

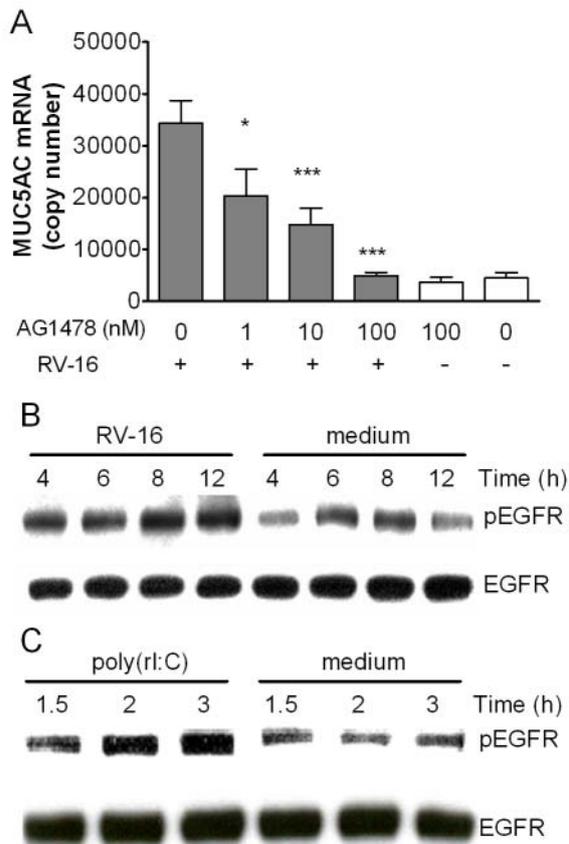


Fig 4. RV induction of MUC5AC requires MEK-ERK and Sp1 transactivation of the MUC5AC promoter. (A) Cells were pre-treated with PD98059 at doses indicated and infected with RV-16. MUC5AC mRNA was quantified by qPCR 8h post-infection (n=4). (B) As (A), but cells pre-treated with U0126 as indicated (n=4). (C) Cells were infected with RV-16 and total- or phospho-ERK quantified by ELISA at 3, 6 and 24h post-infection (n=4). (D) As (A) and (B), but cells pre-treated with mithramycin A as indicated (n=5). For (A), (B) and (D) *, ** and *** indicate $P<0.05$, $P<0.01$ and $P<0.001$ respectively compared to uninhibited infected cells. For (C), * and *** indicates $P<0.05$ and $P<0.001$ respectively compared to uninfected control cells. (E) Map of cloned MUC5AC promoter, indicating the five truncations and putative transcription factor binding sites. (F) Cells were transfected with the

five constructs, alongside poly(rI:C), or poly(dI:C) as control. Luciferase expression was quantified 48h post-infection (n=5). (G) Cells were transfected with -192 construct containing mutated Sp1 binding sites alongside poly(rI:C), or poly(dI:C) as control. Luciferase expression was quantified 48h post-infection (n=5). * and *** indicates $P < 0.05$ and $P < 0.001$ respectively, compared to -1330 (F) or -192 (G) constructs. N/S indicates $P > 0.05$ as indicated.

Fig 4

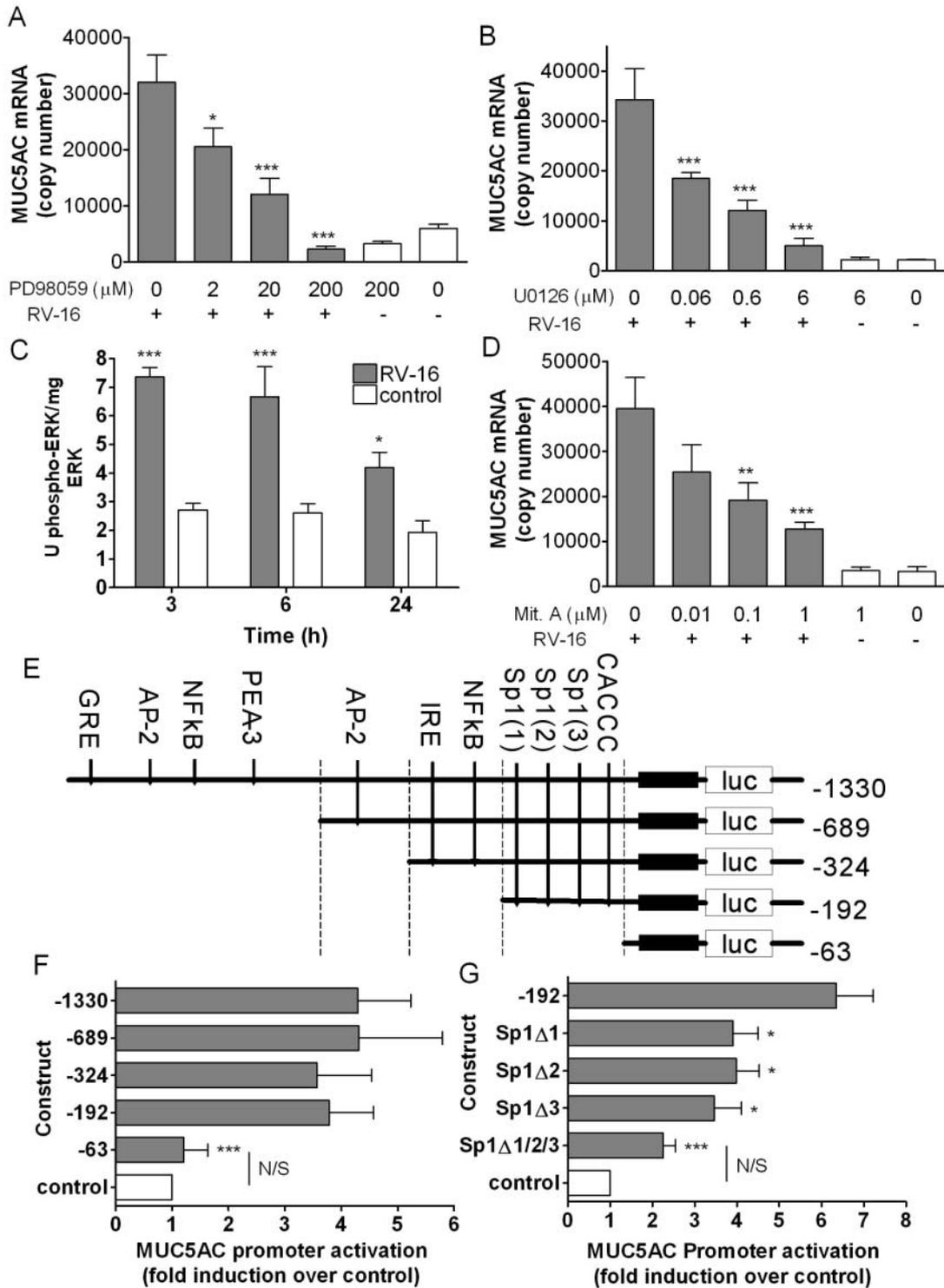


Fig 5. RV induction of MUC5AC requires NF- κ B activation. (A) Cells were pre-treated with CAPE at doses indicated and infected with RV-16. MUC5AC mRNA was quantified by

qPCR 8h post-infection (n=4). (B) As (A), but cells pre-treated with AS602868 as indicated. * and *** indicates $P < 0.05$ and $P < 0.001$ respectively, compared to control infected cells (n=4). (C) Cells were infected with RV-16 before RV 3C protease and p65 was detected and visualised by confocal microscopy 6h post-infection. Arrows indicate co-localised 3C protease and p65 nuclear translocation. Confocal image is representative of 3 experiments.

Fig 5

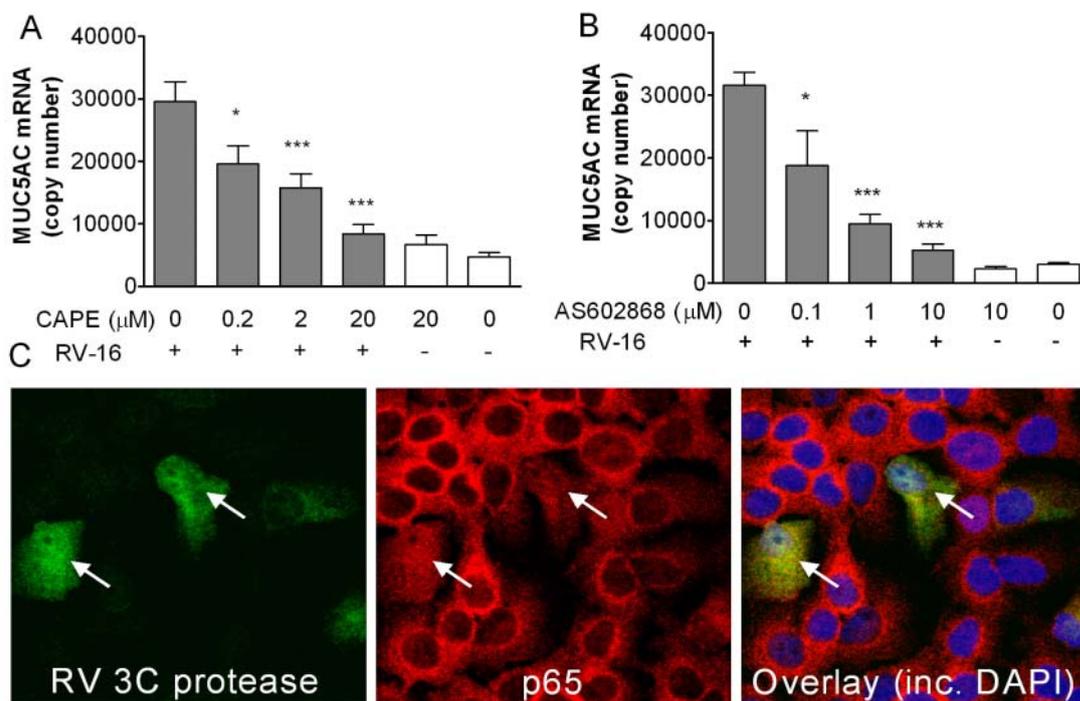


Fig 6. RV induction of MUC5AC requires an NF- κ B dependent, MMP-mediated release of TGF- α . (A) Cells were transfected with wild type or -324 constructs containing mutated NF- κ B and IRF binding sites alongside poly(rI:C), or poly(dI:C) as control. Luciferase expression was quantified 48h post-infection (n=4). (B) Cells were pre-treated with 1×10^{-6} M AS602868 and infected with RV-16. TGF- α release was quantified 6h post-infection (n=3). ** indicates $P < 0.01$ compared to control cells. # and N/S indicate $P < 0.05$

and $P > 0.05$ respectively. (C) Cells were pre-treated with cycloheximide at doses indicated and infected with RV-16. MUC5AC mRNA was quantified 8h post-infection (n=3). *** indicates $P < 0.001$ compared to control infected cells. ##, ### and N/S indicates $P < 0.01$, $P < 0.001$ and $P > 0.05$ respectively comparing infected and control cells at each concentration of cycloheximide. (D) Cells were pre-treated with 4×10^{-5} M GM6001 and infected with RV-16. MUC5AC mRNA was quantified 8h post-infection (n=3). *** indicates $P < 0.001$ and N/S indicates $P > 0.05$ compared to control cells or as indicated.

Fig 6

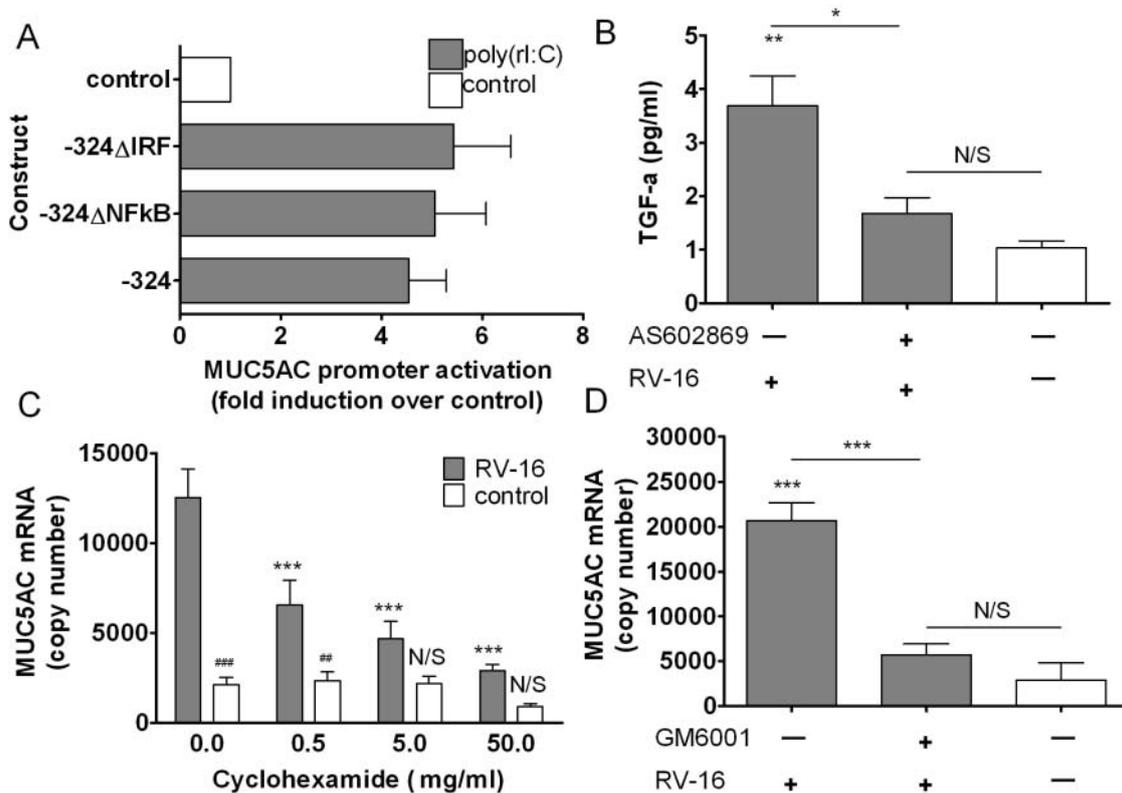


Fig 7. RV induction of MUC5AC protein release requires activation of NF- κ B, MMP, EGFR and MEK/ERK pathways (A) Cells were pre-treated with CAPE at doses indicated and infected with RV-16. MUC5AC protein was quantified by ELISA 24h post-infection (n=7). (B) As (A), but cells pre-treated with GM6001 as indicated (n=3). (C) As (A), but

cells pre-treated with AG1478 as indicated (n=4). (D) As (A), but cells pre-treated with PD98059 as indicated (n=4). (E) As (A), but cells pre-treated with U0126 as indicated (n=3). *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively compared to uninhibited infected cells.

Fig 7

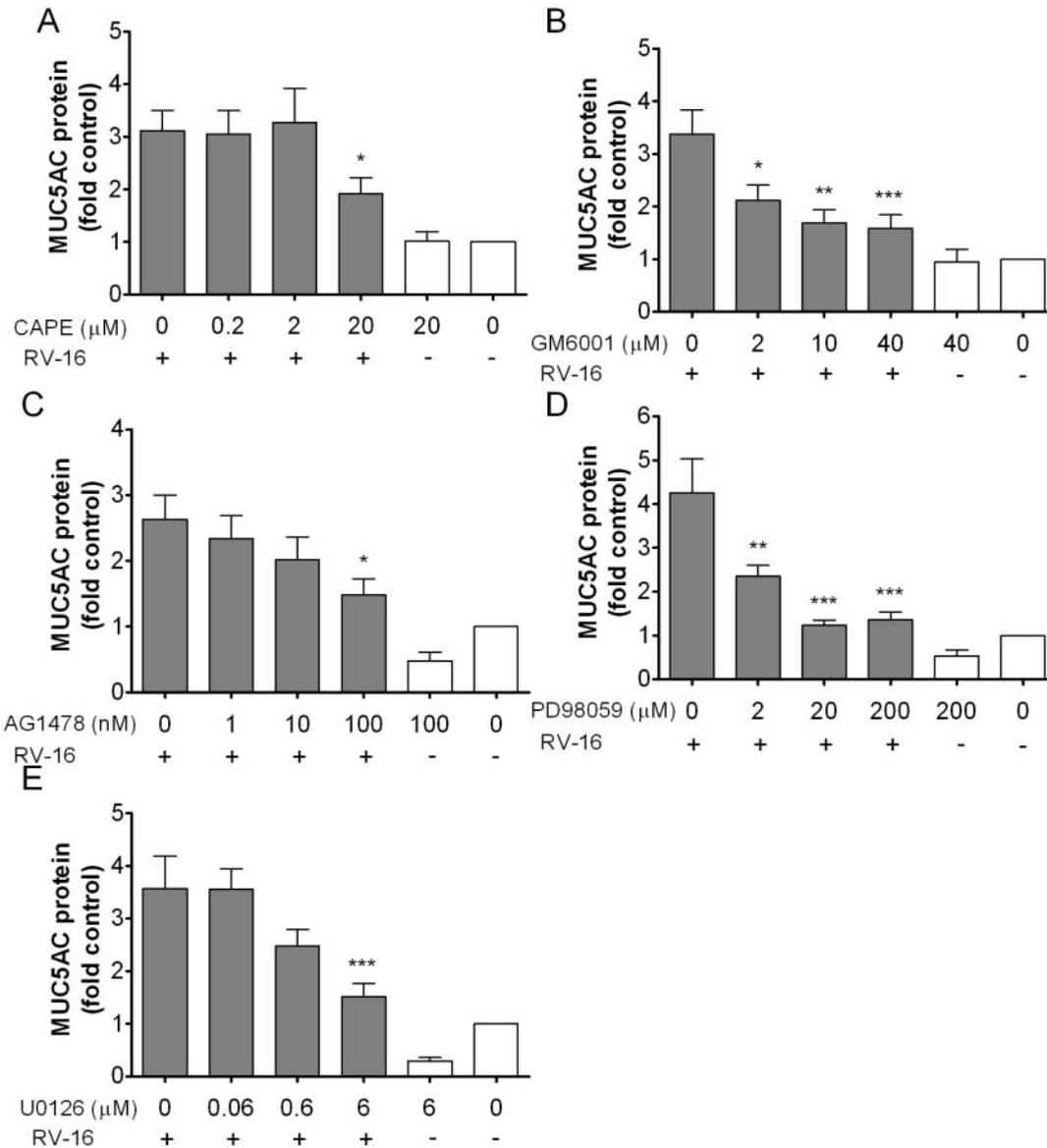


Fig 8. Molecular mechanisms of RV induction of MUC5AC synthesis and secretion. RV infects respiratory epithelial cells causing activation and nuclear translocation of NF- κ B. This

causes transcription and translation of MMPs that cleaves pro-TGF- α and active TGF- α is released from the cell surface. The active TGF- α then binds to and activates EGFR present on the surface epithelium in a para/autocrine mechanism. Phosphorylation of the intracellular domain of EGFR activates a cellular signalling cascade including MEK and ERK activation and culminates in Sp1 transactivation of the MUC5AC promoter. This drives *de novo* transcription of the MUC5AC gene, increasing MUC5AC mRNA quantities and ultimately resulting in secretion of MUC5AC protein.

