Regulation of Ectodomain Shedding of ADAM33 *In Vitro* and *In Vivo*

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Conflict of Interest Statement

The authors declare the following conflicts of interest:

DED reports personal fees from Synairgen, which is outside the submitted work. All other authors have nothing to declare.

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Capsule summary: TGF-β signaling regulates ectodomain shedding of ADAM33 mainly via an autocatalytic mechanism and is essential for shedding of catalytically-active ADAM33 *in vivo*. These mechanisms have implications for the genetic contribution of *ADAM33* to asthma and BHR.

Key words: ADAM33, TGF-β, ectodomain shedding, asthma, allergy

Abbreviations: ADAM33: a disintegrin and metalloprotease 33; BALF: bronchoalveolar lavage fluid; HDM: house dust mite; IL-33: interleukin-33; TGF-β: transforming growth factor beta

To the Editor:

A disintegrin and metalloprotease (ADAM)33 is a susceptibility gene for asthma and bronchial hyperresponsiveness (BHR). ADAM33 is a transmembrane protein but a soluble protein containing the metalloprotease domain (sADAM33) has previously been identified in bronchoalveolar lavage fluid (BALF) from asthmatic subjects and its levels significantly and negatively correlated with FEV_1, suggesting a role in the development of airflow obstruction (1). We have shown that sADAM33 in asthmatic BALF is enzymatically active and that the sADAM33 metalloprotease causes angiogenesis and myogenesis *in vivo* or *ex vivo* (2, 3). Furthermore, *in vivo* allergen challenge causes shedding of enzymatically-active sADAM33 into BALF of wildtype mice, and in a transgenic mouse model, lung-specific sADAM33 expression causes airway remodeling which enhances eosinophil recruitment with associated BHR following allergen sensitization and challenge (2). Although transforming growth factor beta (TGF-β) is a trigger for sADAM33 release *in vitro* (3), there is no mechanistic information or evidence of *in vivo* relevance. Here we sought to characterize the mechanism(s) of TGF-β-induced ectodomain shedding of murine ADAM33 and to determine its importance for shedding of ADAM33 *in vivo*. As it is not possible to test directly the importance of TGF-β in ectodomain shedding in human asthma, we characterized the mechanism(s) of the TGF-β induced ectodomain shedding of murine ADAM33 and determined its importance for shedding of ADAM33 *in vivo.* Detailed methodology is provided in the Methods section in this article's Online Repository at www.jacionline.org.

Initially, we confirmed that murine ADAM33 was similar to human ADAM33 in its sensitivity to TGF-β induced ectodomain shedding (3). As expected, TGF-β treatment caused a dose-dependent increase in sADAM33 in supernatants of Cos-7 cells expressing murine ADAM33 (Fig E1A, B). The main band had a molecular weight around 102kDa, indicating that the entire

ectodomain had been shed; however further processing was also evident suggesting loss of the inhibitory pro-domain. Consistent with this, there was a significant increase in ADAM33 enzymatic activity in cell-free supernatants following TGF-β treatment (Fig E1C). Pericellular proteolysis is frequently mediated by members of the ADAM or MT-MMP families that are sensitive to the broad-spectrum hydroxamic acid based inhibitor, GM6001; however we found it did not affect the shedding (Fig E2A,B) or activity (Fig E2C) of ADAM33. We also confirmed that GM6001 also had no effect on the activity of purified recombinant ADAM33 (Fig E2D). As ADAM33 has a unique substrate-binding site and its catalytic activity is insensitive to GM6001 (4), we postulated that ADAM33 ectodomain shedding was autocatalytic. Consistent with this, mutation of E347A in the catalytic site suppressed shedding of sADAM33 both at baseline and in response to TGF-β (Fig 1A) and this was accompanied by a significant reduction in enzymatic activity in cell free supernatants (Fig 1B). These data suggest that a substantial component of the shedding of sADAM33 is autocatalytic.

TGF-β activates multiple signals including SMAD and non-SMAD pathways. Inhibition of SMAD signaling using SB341452, significantly suppressed TGF-β_1 induced shedding of immunoreactive and enzymatically active ADAM33 (Fig 1C,D). In contrast, the MAP2K/MEK inhibitor, PD98059 dose-dependently increased shedding of immunoreactive and enzymatically active forms of sADAM33 (Fig 1E,F). These data suggest a complex effect of TGF-β signaling with SMAD activation being stimulatory for ADAM33 shedding, whereas MAPK activation has a negative regulatory effect. The latter effect may be mediated via TIMP-3, a known inhibitor of ADAM33 enzymatic activity (5), which is reported to be induced by TGF-β via MAPK signaling (6). Thus, inhibiting MAPK activation with PD98059 may inhibit TIMP3 expression and release ADAM33 from the effects of this natural inhibitor leading to increased autocatalytic shedding and augmented enzyme activity. While ectodomain shedding

92 can be regulated by natural protein inhibitors, other mechanisms including membrane
93 trafficking, protein maturation and substrate presentation have also been shown to be important
94 for the regulation of other sheddases (7). Whether release of ADAM33 is preceded by its
95 membrane trafficking or maturation and/or by assembly into higher order complexes requiring
96 additional protein interactions to exosites located either on the ADAM33 or adapter proteins
97 remains to be determined.
98
99 Epithelial injury leads to release of TGF-β and we have postulated that this is the source of the
100 TGF-β that drives the increase in sADAM33 which is observed in response to allergen
101 challenge *in vivo* (2). To test this hypothesis, we utilized BALF from mice in which lung
102 epithelial *Tgfb1* was conditionally deleted in bronchial epithelial club cells before intra-nasal
103 administration of either 25μg HDM extract or recombinant murine IL-33 (8). After HDM
104 challenge, lower levels of sADAM33 could be detected in the BALF of *Tgfb1*$^{-/-}$ mice compared
105 to littermate controls (Fig 2A) and it also contained less sADAM33 enzymatic activity (Fig
106 2B). In the same way, when mice were challenged with IL-33, BALF from *Tgfb1*$^{-/-}$ mice had a
107 lower level of sADAM33 immunoreactive protein (Fig 2C) and enzymatic activity (Fig 2D).
108 Of note, exogenous TGF-β alone was ineffective at stimulating shedding of ADAM33 *in vivo*.
109 This might be explained either by the fact that TGF-β could not pass through the intact
110 epithelium of unchallenged mice and/or by the low dose and short half-life of the growth factor
111 which did not allow sufficient time for it to pass through the epithelium to reach the
112 subepithelial mesenchymal cells where ADAM33 is expressed (9).
113
114 Epithelial-derived IL-33 induces rapid release of TGF-β into the airways to enhance migration
115 of ILCs and development of a robust ILC2 response that initiates allergic immunity (9). Since
116 we found that epithelial-derived TGF-β is also required for ectodomain shedding of ADAM33,

it may be speculated that sADAM33 can contribute to the recruitment or activation of ILCs or other innate immune cells, either by affecting matrix turnover or by promoting growth factor or chemokine shedding. Of note, polymorphisms in *TGFB*, *IL33* and *ADAM33* genes have each been associated with asthma susceptibility, yet each has a small overall effect on disease development. The involvement of three susceptibility gene products in epithelial responses to allergens highlights how they may co-operate to amplify the downstream asthmatic responses.

Identification of the involvement of TGF-β in ectodomain shedding of ADAM33 in an *in vivo* model strengthens the case for exploring how human polymorphic variation in the *ADAM33* gene is linked to asthma pathogenesis. Four SNPs (S1, S2, T1, and T2) encode amino acid substitutions in the transmembrane and cytoplasmic domain of ADAM33 and have been associated with asthma (4). Although the intracellular domain of ADAM33 is relatively short, it is very rich in prolines, having a putative SH3 binding site where the T2 SNP is located, a casein kinase I/II phosphorylation site and a MAPK consensus sequence which is likely to be important for regulation of ADAM33 function, especially as we have identified a negative regulatory role for MAPK in our current studies. Further work is required to determine whether this effect is direct and involves ADAM33 phosphorylation or indirect via inhibitors such as TIMP3. Alternatively, one mutation Ala395Val is located within the catalytic domain (4), which may directly affect catalytic activity.

In summary, we have provided direct evidence that epithelial-derived TGF-β is an important regulator of ectodomain shedding of enzymatically active ADAM33 from the mesenchyme. This process appears largely to be autocatalytic and involves SMAD signaling, but is negatively regulated by MAPK signaling. These findings highlight the importance of

epithelial-mesenchymal cross talk in asthma pathogenesis and underscore the potential for co-operation between different asthma susceptibility genes to drive disease pathogenesis.

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Figure Legends

FIGURE 1: Regulation of ADAM33 shedding. Cos-7 cells expressing full length catalytically active murine ADAM33 (A-F) or inactive ADAM33 E347A (A,B) were treated without or with TGF-β (5ng/ml) for 8hr and SMAD (SB431542) (C,D) or MAPK (PD98059) (E,F) inhibitors, as indicated. Soluble ADAM33 immunoreactivity and enzymatic activity in cell-free supernatants were assessed by Western blotting (inset) with densitometry (arbitrary units, AU) (A,C,E) or in a Fluorescence Resonance Energy Transfer (FRET) peptide cleavage assay, respectively; in panel B, enzymatic activity in the supernatants is expressed as a percent of that measured in supernatants from untreated cells expressing full length active ADAM33 while in panels D and F, activity is expressed as a percent of that measured in supernatants from TGF-β-treated cells. Data are expressed as mean + SD (n=4 independent experiments). # $p<0.05$, ## $p<0.01$ *vs* control, *$p<0.05$, **$p<0.01$, ***$p<0.001$

FIGURE 2: Epithelial TGF-β enhances shedding of ADAM33 *in vivo*. Epithelial (Epi)*Tgfb*$^{-/-}$ or littermate *Tgfb*$^{+/+}$ control mice were challenged with intranasal house dust mite (HDM) extract (A,B) or recombinant IL-33 (C,D) and, where indicated, recombinant TGF-β (C,D). Soluble ADAM33 immunoreactivity and enzyme activity in bronchoalveolar lavage fluid (BALF) were assessed by Western blotting (A,C) or FRET peptide cleavage assay, respectively; enzyme activity is expressed as relative fluorescent units per minute (RFU/min) (B,D). Data are mean + SD (n= 4–6 mice per group). Data are representative of 2 independent experiments. ## $p<0.01$, ### $p<0.001$ *vs* control, *$p<0.05$, ***$p<0.001$

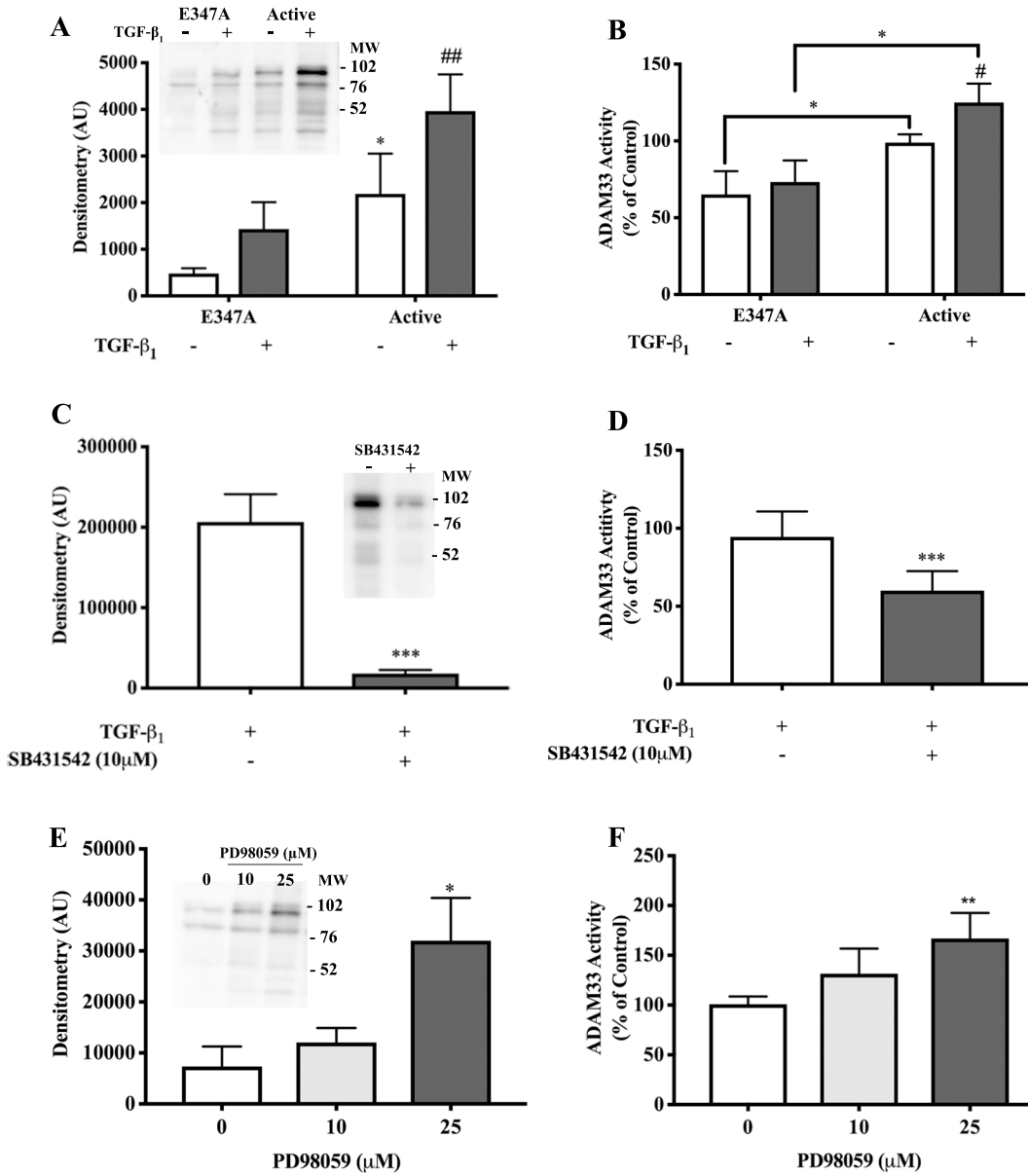
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Figure 1

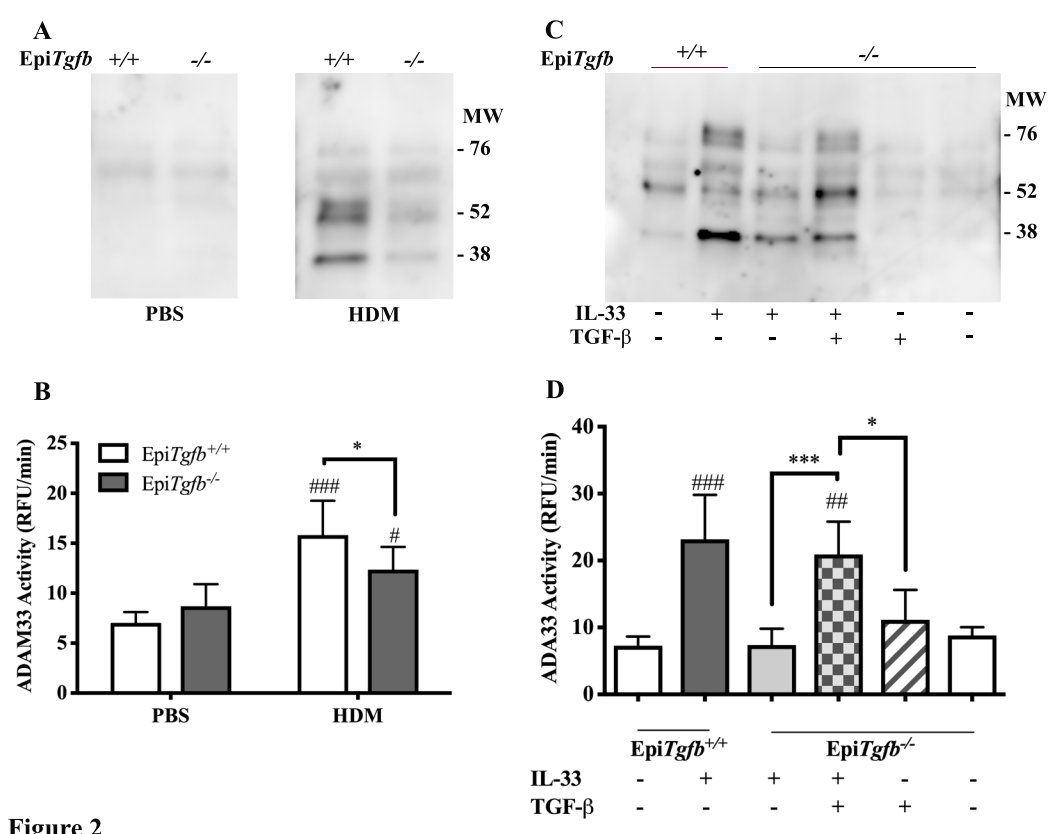
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Figure 2

Online Repository

Methods

Cell culture

The Cos-7 cell line, a fibroblast-like cell line were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, 50μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 1x non-essential amino acids (DMEM/FBS) (all from Life Technologies, Paisley, UK). For transfection, cells were placed in non-supplemented Opti-MEM (Life Technologies).

Transfection of full-length mouse ADAM33

Cos-7 cells were transfected with a plasmid encoding full-length murine *ADAM33* (MR217277 clone, NM_033615; OriGene, MD, USA) or green fluorescence protein (GFP) using X-treme Gene 9 reagent (Roche, Southampton, UK). After 24h, cells were treated with TGF-β_1 (Peprotech, London, UK) for 8h to assess ectodomain shedding of ADAM33. The TGF-β_1 isoform was chosen, as this is the major isoform in adult mice (1). Where indicated, cells were pre-incubated for 1h with the ALK5/SMAD inhibitor SB431542 (10μM; Stratech, Ely, UK), the mitogen-activated protein kinase kinase (MAP2K, MEK) inhibitor PD98059 (10 or 25μM, Sigma, Poole UK), the broad-spectrum metalloprotease inhibitor GM6001 (10μM; Merck, Millipore, Watford, UK) or vehicle control before addition of 5ng/ml TGF-β_1. Cell-free supernatants were harvested and cells were lysed into RIPA buffer.

ADAM33 pull down with concanavalin A beads

Concanavalin A-Sepharose 4B beads (ConA) (Sigma) were used to pull down glycosylated ADAM33 from the supernatants of the transfected Cos-7 cells, as previously described (2). In brief, 1ml supernatant was adjusted to contain 20mM Tris HCl pH 7.4, 0.5M NaCl, and

protease inhibitors (Sigma) before addition of activated ConA beads (20µl beads per ml sample). After binding overnight at 4°C, the beads were washed twice in 20mM Tris HCl pH 7.4 containing 0.5M NaCl and then twice in 20mM Tris HCl pH 7.4 to remove excess salt. The bound protein was then solubilized into SDS sample buffer containing 0.5M methyl α-D-mannopyranoside for SDS-PAGE and Western blotting.

Fluorescence resonance energy transfer (FRET) peptide cleavage assay

An ADAM33 enzymatic assay was performed using a FRET peptide cleavage assay as previously described (3). All assay measurements were made at 37°C in real time using a Bio-Rad CFX96 machine with a 490-nm (6-carboxyfluorescein; FAM) filter, allowing determination of initial rates. Each reaction contained 7µl neat BALF or cell supernatant mixed with 2µl 5x-assay buffer (100mM HEPES pH 7, 2.5M NaCl, 50mM $CaCl_2$ and 1mg/ml bovine serum albumin); the assay was initiated by addition of 44 pmol FRET peptide (DABCYL-YRVAFQKLAE(FAM)K-NH_2) (Severn Biotech, Kidderminster, UK) and 100 pmol $ZnCl_2$ to make a final reaction volume of 10µl. Enzymatic activity was determined by plotting relative fluorescence units (RFU) against time after the background had been subtracted. The rate of the reaction (RFU/min) was determined from the line of best fit in the linear phase of the assay. Control assays utilized human recombinant soluble active ADAM33 (positive control) and mutant ADAM33 (E365A) (negative control) as previously described (2).

SDS-PAGE and western blotting

Solubilized samples from ConA pull down or murine BALF were run on 12.5% acrylamide or 8-20% gradient gels (BioRad, Watford, UK) and transferred onto PVDF membranes. The transferred protein was assessed by Ponceau staining to confirm equivalent protein loading prior to blocking using 2.5% BSA in TBS/Tween and Western blotting. Membranes were

probed with a polyclonal goat antibody against the ectodomain of mouse ADAM33 (AF2434, 1:1000, R&D Systems). Secondary antibody was rabbit anti-goat IgG HRP antibody (Merck; 1:5000). The blots were visualized using enhanced chemiluminescence (Clarity ECL; Bio-Rad) with an Amersham Imager 600 (GE Healthcare, Buckinghamshire, UK); densitometric images were semi-quantified using ImageJ software and data plotted as relative intensity.

The conditional lung epithelial *Tgb1*$^{-/-}$ mouse model

Mice conditionally lacking lung epithelial *Tgfb* expression (*Ccsp*-cre*Tgfb1*$^{-/-}$) were generated as previously described (4). Mice were pre-treated with doxycycline (DOX) (Sigma) (or vehicle as control) to delete *Tgfb1* from the bronchiolar epithelial cells 72h prior to intra-nasal administration of either 25μg HDM extract (1mg/ml protein solution dissolved in PBS) or 25μl of PBS 5 times a week for 3 weeks. Mice were culled 4h after the final challenge. In other experiments, carrier-free recombinant murine IL-33 (1μg/dose in 25μl PBS) (eBioscience, Thermo Fisher Scientific, Hemel Hempstead, UK) was administered 3 times a week for 1 week and mice culled 18h after the final dose. Where required, 50ng recombinant TGF-β_1 in PBS (R&D Systems) was administered intra-nasally without or with IL-33. At the point of killing, BALF was collected by washing the airways three times with 400μl PBS. After centrifugation to remove cells, supernatants were stored at -80°C. The protein content of the BALF was quantified (BCA Assay, Thermofisher) and normalized before use in ADAM33 enzyme assays or solubilization in 2x Laemmli sample buffer (BioRad) for analysis of sADAM33 protein by Western blotting. Mice of both sexes were used between 7 and 12 weeks of age, housed in specific-pathogen-free conditions, and given food and water *ad libitum*. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

Statistics

Normal distribution of the numeric data was evaluated, and appropriate parametric or non-

parametric statistical tests applied. All data are parametric and plotted as mean with one standard deviation (SD). Statistical significance was assessed by using Students t-test (unpaired data) with Welch's correction if SDs were not equal for comparisons between 2 groups. For comparison of 3 or more groups a one-way ANOVA with Dunn's multiple comparison test was used. For comparison of 2 or more groups with 2 independent variables, a two-way ANOVA with Tukey's multiple comparison test was used, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

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Figure Legends

FIGURE E1: TGF-β enhances shedding of murine ADAM33. Cos-7 cells transiently transfected with full length murine ADAM33 or green fluorescent protein (GFP) as control were treated with TGF-β as indicated. Soluble ADAM33 immunoreactivity and enzyme activity in cell-free supernatants were assessed by Western blotting (A) with densitometry (arbitrary units, AU) (B) or FRET peptide cleavage assay, respectively; enzyme activity is expressed as percent of activity found in supernatants from control untreated cells (C). Data are mean + SD and are representative of 4 independent experiments. **p<0.01 ***p<0.001

FIGURE E2: The broad spectrum MMP inhibitor GM6001 does not inhibit ADAM33 shedding. Cos-7 cells transiently transfected with full length murine ADAM33 were pre-incubated with GM6001 (0-10μM) for 1h before addition of 5ng/ml TGF-β for 8h. Cell-free supernatants were assessed for soluble ADAM33 by Western blotting (A) with densitometry (arbitrary units, AU) (B) and by FRET peptide cleavage assay (C). Purified recombinant ADAM33 protein was incubated with GM6001 and tested for activity in a FRET peptide cleavage assay (D). Activity is presented as a percent of that measured in the absence of GM6001. Data are expressed as mean + SD and are representative of 4 independent experiments.

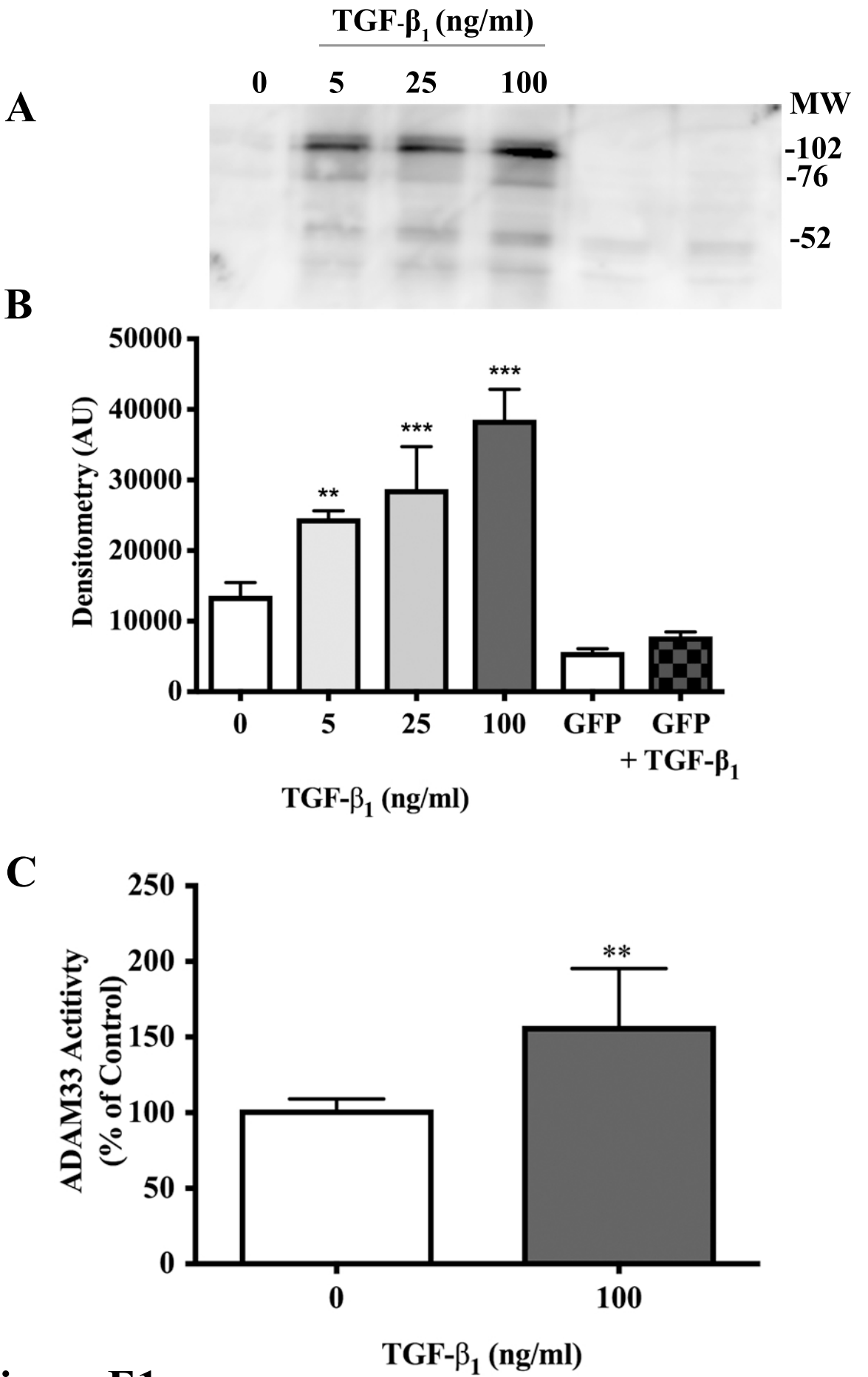
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Figure E1

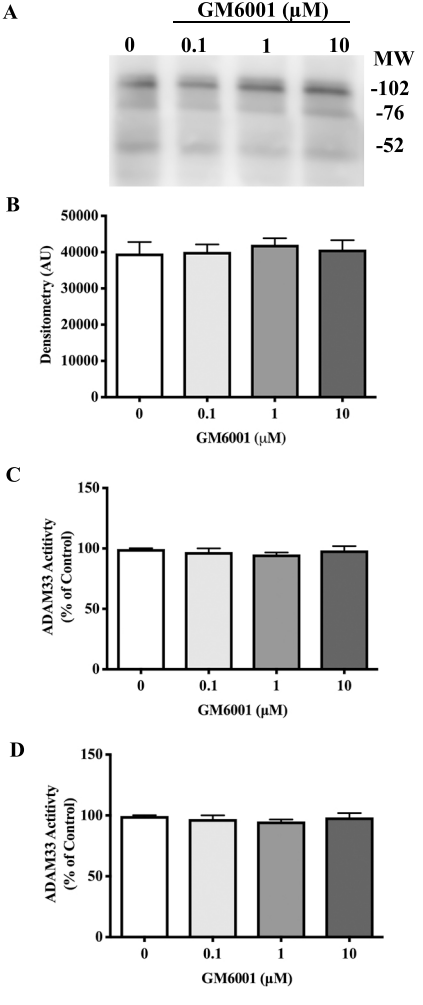
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Figure E2