


RESEARCH ARTICLE

TMEFF2 shedding is regulated by oxidative stress and mediated by ADAMs and transmembrane serine proteases implicated in prostate cancer

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Abstract

TMEFF2 is a type I transmembrane protein with two follistatin (FS) and one EGF-like domain over-expressed in prostate cancer; however its biological role in prostate cancer development and progression remains unclear, which may, at least in part, be explained by its proteolytic processing. The extracellular part of TMEFF2 (TMEFF2-ECD) is cleaved by ADAM17 and the membrane-retained fragment is further processed by the gamma-secretase complex. TMEFF2 shedding is increased with cell crowding, a condition associated with the tumour microenvironment, which was mediated by oxidative stress signalling, requiring jun-kinase (JNK) activation. Moreover, we have identified that TMEFF2 is also a novel substrate for other proteases implicated in prostate cancer, including two ADAMs (ADAM9 and ADAM12) and the type II transmembrane serine proteinases (TTSPs) matriptase-1 and hepsin. Whereas cleavage by ADAM9 and ADAM12 generates previously identified TMEFF2-ECD, proteolytic processing by matriptase-1 and hepsin produced TMEFF2 fragments, composed of TMEFF2-ECD or FS and/or EGF-like domains as well as novel membrane retained fragments. Differential TMEFF2 processing from a single transmembrane protein may be a general mechanism to modulate transmembrane protein levels and domains, dependent on the repertoire of ADAMs or TTSPs expressed by the target cell.

Keywords: ADAM; hepsin; matriptase-1; oxidative stress; TMEFF2

Introduction

TMEFF2, a type I transmembrane protein with two follistatin and epidermal growth factor (EGF) domains, is expressed selectively in the adult brain and prostate (Horie et al., 2000; Liang et al., 2000), with elevated TMEFF2 expression in prostate cancer (PCa) cell lines and clinical samples (Glynne-Jones et al., 2001; Gery et al., 2002; Afar et al., 2004). However, the role of TMEFF2 in PCa development and progression remains unclear, and TMEFF2 activity may depend on disease stage and/or post-transcriptional regulation. The ectodomain of TMEFF2 (TMEFF2-ECD), comprised of the two follistatin

and EGF domains, is cleaved from the cell surface by ADAM17 and the membrane-retained fragment undergoes further processing by the gamma-secretase complex (Ali and Knäuper, 2007). Shedding of TMEFF2-ECD is induced by pro-inflammatory cytokines TNF α and IL-1 β (Lin et al., 2003) or phorbol esters (Ali and Knäuper, 2007), which are known to upregulate ADAM-mediated protein shedding (Brose and Rosenmund, 2002).

Ectodomain shedding may, at least partially, be responsible for pro- or anti-proliferative TMEFF2 functions in PCa. Overexpression of full length, transmembrane TMEFF2 in PCa cells impairs proliferation due to an interaction between

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Abbreviations: ADAM, a disintegrin and metalloproteinase; TMEFF2, transmembrane protein with EGF-like and two follistatin-like domains 2; TTSP, type II transmembrane serine protease

the cytoplasmic domain of TMEFF2 and sarcosine dehydrogenase (SARDH). This interaction results in decreased levels of sarcosine (Chen *et al.*, 2011a; Green *et al.*, 2013), an amino acid associated with PCa progression (Sreekumar *et al.*, 2009). Full length TMEFF2 also attenuates the migratory properties of PCa cells (Chen *et al.*, 2014), indicating a tumour suppressor function. However, the TMEFF2-ECD released, due to shedding, may act as a soluble growth factor. Indeed, treatment of HEK293 cells with purified recombinant TMEFF2-ECD stimulated ERK activation and increased their proliferation rate (Ali and Knäuper, 2007; Chen *et al.*, 2011a). On the other hand, conditioned medium of cells expressing TMEFF2-ECD reduced p-ERK levels in RWPE1 cells in response to PDGF-AA treatment (Chen and Ruiz-Echevarría, 2013) which was also reported to suppress PDGF-AA stimulated growth of NR6 fibroblasts (Lin *et al.*, 2011). While this points to TMEFF2 possessing opposing biological roles the molecular mechanism underlying this dual functionality is unclear. We hypothesise that TMEFF2's biological functions may be regulated by differential proteolysis, which generates not only TMEFF2-ECD but additional protein fragments, which may modulate mitogenic signalling. Indeed, additional soluble TMEFF2 forms can arise from alternative splicing, generating a soluble protein composed of the FS-I module and a truncated FS-II module (Quayle and Sadar, 2006).

To address the hypothesis that TMEFF2 may undergo differential proteolysis we focused our investigation on proteases contributing to the pathogenesis of PCa. These include members of the disintegrin and metalloproteinase family, ADAM9 (Peduto *et al.*, 2005; Fritzsche *et al.*, 2008), ADAM12 (Peduto *et al.*, 2006) and ADAM15 (Lucas and Day, 2009) as well as membrane associated serine proteases implicated in PCa, such as type II transmembrane serine proteases (TTSPs) (Webb *et al.*, 2011) or the GPI-anchored prostatic prostaticin (Chen *et al.*, 2004). Prominent TTSPs involved in PCa progression include matriptase-1, matriptase-2 and hepsin. Matriptase-1 overexpression correlates with Gleason score (Riddick *et al.*, 2005) promoting cell invasion, metastasis and prostate tumour growth (Sanders *et al.*, 2006; Ko *et al.*, 2015) by regulating MET signalling in PCa. Interestingly, matriptase-1 interacts with a close relative of TMEFF2, TMEFF1, where the EGF-like domain of TMEFF1 binds to the matriptase-1 CUB domain (Ge *et al.*, 2006). Matriptase-2 also contains CUB domains and is implicated in PCa cell behaviour (Sanders *et al.*, 2010). Significant overexpression of hepsin is common in 90% of PCa tumours, correlating with Gleason score, serum PSA levels as well as early relapse following radical prostatectomy (Dhanasekaran *et al.*, 2001; Goel *et al.*, 2011). In contrast, prostaticin levels are high in normal prostatic epithelial cells and decrease in PCa (Takahashi *et al.*, 2003). We therefore tested the hypothesis that TMEFF2 is cleaved

at different sites by ADAMs and TTSPs and we provide evidence of complex TMEFF2 proteolysis by these proteases that may impact the biological function of TMEFF2 reported in the literature.

Materials and methods

Reagents

PMA and the NADPH oxidase inhibitor apocynin (APOC) were from Sigma-Aldrich. N-acetylcysteine (NAC), p38 inhibitor (SB203580) and JNK inhibitor (SP600125) and the broad spectrum metalloproteinase inhibitor GM6001 were from Calbiochem. ADAM10 and ADAM17 inhibitors GI254023X and GW280264X were a gift from Dr. Augustin Amour and GlaxoSmithKline. DMEM and Ham's F12 cell media were from Lonza, FBS and hygromycin B from Invitrogen. FuGENE 6 Transfection Reagent was from Roche.

Expression constructs, cell culture, transient transfection and Western blotting

ADAM9 and ADAM12 expression constructs were a kind gift from Dr. Carl Blobel. The cloning of ADAM15 A, B, C isoforms and ADAM15 B E/A inactive mutant into pcDNA4-V5/His vector was described previously (Zhong *et al.*, 2008; Maretzky *et al.*, 2009). The corresponding coding sequences were sub-cloned into pcDNA5/FRT/Flag-His plasmid using *HindIII* and *XhoI*. Matriptase-2 expression plasmid was described in Folgueras *et al.* (2008). Matriptase-1, hepsin and prostaticin plasmids were described in Gray *et al.* (2014) and used to generate constructs expressing inactive S-A mutants by QuikChange mutagenesis (Agilent). Generation of HEK293 cells expressing AP/V5 TMEFF2 and AP/V5 $\Delta_{303-320}$ TMEFF2 was described previously (Ali and Knäuper, 2007) and were maintained in DMEM with 10% FBS and 100 μ g/mL hygromycin B at 37°C in a humidified incubator with 5% CO₂. For shedding experiments 1×10^5 HEK293 cells, expressing alkaline phosphatase tagged wild type or mutant TMEFF2, were plated per well into a 24 well plate and grown overnight in antibiotic free medium. 0.5 μ g of expression plasmid encoding the active and inactive proteases in question were mixed with 1.5 μ L of FuGENE 6 Transfection Reagent added to each well and grown for 2 days prior to shedding experiments in the presence or absence of ADAM 10, ADAM17 or general metalloproteinase inhibitor GM6001 described previously (Ali and Knäuper, 2007). Additional experiments were performed using DU145 cells transiently transfected with AP-TMEFF2. Here, cell lysates were analysed for total AP-activity to normalise the release of soluble AP-TMEFF2 ectodomain into the medium following cell crowding experiments. The data are displayed as percentage of shed TMEFF2.

Cell lysates were harvested at the end of shedding experiments and analysed using 10% SDS-PAGE followed by Western blotting using PVDF membranes.

Statistical analysis

Mean values \pm SD from three independent experiments with four internal replicates were analysed using GraphPad Prism 5.0 and one-way ANOVA with Tukey's test (** $P < 0.01$; * $P < 0.05$). P -values below 0.05 were considered significant.

Results and discussion

Our first aim was to identify whether TMEFF2 could be targeted by other proteases accounting for soluble TMEFF2 (Quayle and Sadar, 2006). We assessed a panel of ADAMs with ADAM9 and ADAM12 overexpression leading to increased shedding of TMEFF2, while the expression of ADAM15 isoforms did not increase AP-activity in media when compared to coexpression of an inactive ADAM15 EA mutant used as a transfection control (Figure 1A). C-terminal TMEFF2 fragments were indistinguishable from previously described ADAM17 fragments (Ali and Knäuper, 2007) (not shown).

We then hypothesised that TTSPs could cleave TMEFF2, as matriptase-1 is known to interact with TMEFF1 (Ge *et al.*, 2006). Overexpression of matriptase-1 or hepsin increased AP-TMEFF2 fragment release into medium by 2–3.5-fold, compared to inactive matriptase-1/hepsin S-A mutant coexpression. Matriptase-2 overexpression was less efficient and increased AP-activity in media 1.5-fold, while prostasin had no effect (Figure 1B). Western blot analysis for remaining membrane-associated fragments following TTSP cleavage showed novel, C-terminal TMEFF2 fragments in matriptase-1 (~24 and ~28 kDa) and hepsin (~20 kDa) expressing cells (Figure 1C). These fragments were absent upon expression of their inactive S-A mutant counterparts, in addition to the ~17 kDa fragment which is due to background ADAM activity previously described (Ali and Knäuper, 2007). Therefore, matriptase-1 and hepsin cleave TMEFF2 in different positions than ADAMs, generating novel transmembrane-retained fragments. No additional C-terminal fragments were detected in cells expressing matriptase-2 (Figure 1C), despite increased AP-activity levels in medium (Figure 1B). Thus, matriptase-2 either cleaves TMEFF2 close to the ADAM cleavage site or alternatively activates ADAMs to induce proteolysis. To address this question, shedding experiments were performed in the presence of selective ADAM10 and ADAM17 inhibitors, GW280264X and GI254023X or the broad spectrum metalloproteinase inhibitor GM6001. The data in Figure 1D showed that matriptase-2 dependent release of AP-TMEFF2-ECD required ADAM activity, whereas

matriptase-1-dependent release did not. Additional experiments were performed to exclude contribution from ADAM9 and other metalloproteinases such as MMPs using the broad spectrum metalloproteinase inhibitor GM6001 (Maretzky *et al.*, 2017) and cells overexpressing matriptase-1 or hepsin, as well as their inactive counterparts. This analysis indicated that matriptase-1 and hepsin were genuine TMEFF2 sheddases as inhibitor treatment was ineffective (Figure 1E).

To corroborate these findings, ADAM-cleavage-resistant AP- $\Delta_{303-320}$ TMEFF2 (Ali and Knäuper, 2007) was used to confirm that matriptase-1 and hepsin cleaved outside and matriptase-2 within the stalk section containing the ADAM cleavage site. Both matriptase-1 and hepsin cleaved AP- $\Delta_{303-320}$ TMEFF2 to a similar extent to wt TMEFF2 (Figure 2A), while matriptase-2 was unable to directly cleave AP- $\Delta_{303-320}$ TMEFF2 lacking the ADAM-cleavage site. TMEFF2 C-terminal fragment analysis in Figure 2B confirmed that matriptase-1 and hepsin cleaved AP- $\Delta_{303-320}$ TMEFF2 by generating novel fragments, showing distinctly different molecular weights, when compared to cleaved wt AP-TMEFF2. Matriptase-1 produced 18 kDa and 20 kDa C-terminal fragments, and hepsin a 25 kDa C-terminal fragment of AP- $\Delta_{303-320}$ TMEFF2. It has to be noted that the AP- $\Delta_{303-320}$ TMEFF2 mutant also lacks two potential TTSPs cleavage site motifs, KKD and VRF (indicated in Figure 2A), as judged by the preferences of TTSPs for P1 arginine or P1' lysine residues (Barré *et al.*, 2014), although other sites can also be cleaved by TTSPs. Potentially, hepsin cleavage occurs at KKD in wild type TMEFF2 to produce the 20 kDa fragment and disruption of this site to the artificial sequence CEKLI then leads to an additional minor cleavage event, as seen when the AP- $\Delta_{303-320}$ TMEFF2 mutant was cleaved in response to hepsin overexpression, producing a novel 25 kDa fragment. We predict possible cleavage sites for matriptase-1 and hepsin in TMEFF2 (Figure 2C), with hepsin cleaving in the stalk sequence, releasing a soluble TMEFF2 fragment composed of the TMEFF2 ectodomain. Matriptase-1 likely cleaves TMEFF2 in two positions, generating soluble proteins containing FS-I or both FS modules, which in conjunction with ADAM dependent cleavage in the stalk region also liberates the EGF-like domain. Thus, these cleavage events may be at least partially responsible for the generation of soluble TMEFF2 forms previously identified by others (Uchida *et al.*, 1999; Quayle and Sadar, 2006).

Soluble TMEFF2 fragments containing FS-domains generated by proteolysis likely modify PDGF-AA growth factor signalling, where PDGF-AA-TMEFF2 complexes modify signalling through PDGFR α , thus full length TMEFF2 or soluble FS-domain containing TMEFF2 fragments may block PDGF-AA signalling (Lin *et al.*, 2011). The FS domains of TMEFF2 also regulate corticotropine-releasing hormone

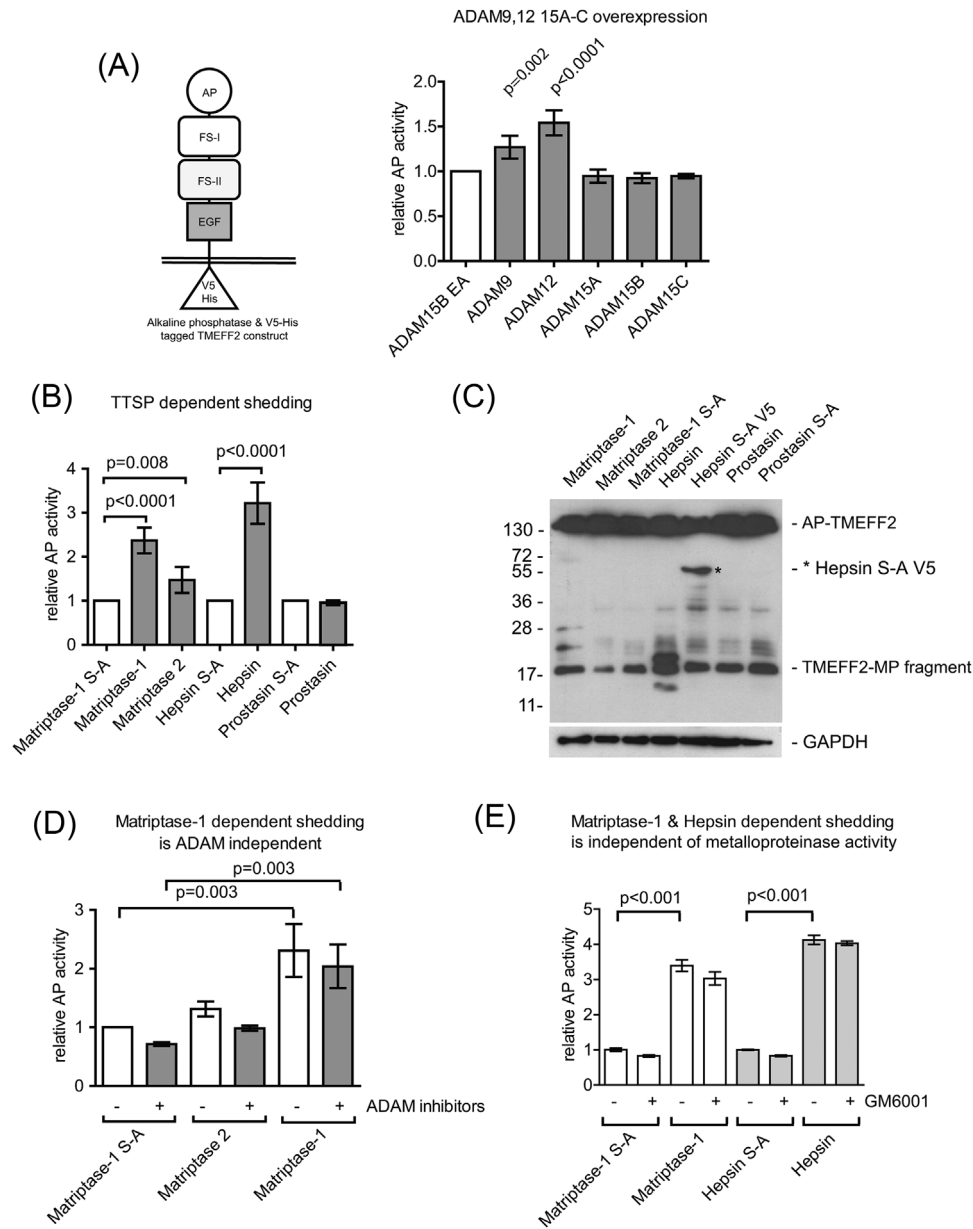


Figure 1 TMEFF2 is a novel substrate for ADAM9, ADAM12, and type II transmembrane serine proteases (TTSPs)—matriptase-1 and hepsin. (A) Schematic representation of AP-tagged TMEFF2 expression construct and ADAM9 and ADAM12-dependent AP-TMEFF2-ECD release into media. (B) Release of AP-TMEFF2-ECD from cells transfected with matriptase-1, matriptase-2, hepsin, prostaticin, or their inactive S-A mutants. (C) WB analysis for the C-terminal V5-epitope of cell lysates demonstrating generation of distinct novel C-terminal TMEFF2 fragments in matriptase-1 (~25 and 28 kDa) and hepsin (~20 kDa) overexpressing cells. (D) The matriptase-dependent release of TMEFF2-ECD is independent of ADAM activity. (E) Matriptase-1 and hepsin-dependent TMEFF2 release is independent of metalloproteinase activity. MP fragment = metalloproteinase fragment.

(CRH) signalling in corticotrope cells, where the production of cAMP, CREB and expression of pro-opiomelanocortin was inhibited, resulting in decreased cell proliferation (Labeur *et al.*, 2015). On the other hand, growth-promoting activity of soluble TMEFF2 has been described (Horie *et al.*, 2000; Ali and Knäuper, 2007; Chen *et al.*, 2011a; Chen and Ruiz-Echevarría, 2013), suggesting that this may be cell type

dependent and potentially regulated by the pattern of ADAM and TTSP expression as well as by the growth factors present in the extracellular environment.

Reactive oxygen species (ROS) influence ADAM expression patterns and activation status (Sung *et al.*, 2006; Willems *et al.*, 2010) and they also regulate TTSP activity, as seen for matriptase-1, which is activated by ROS (Chen *et al.*, 2011b)

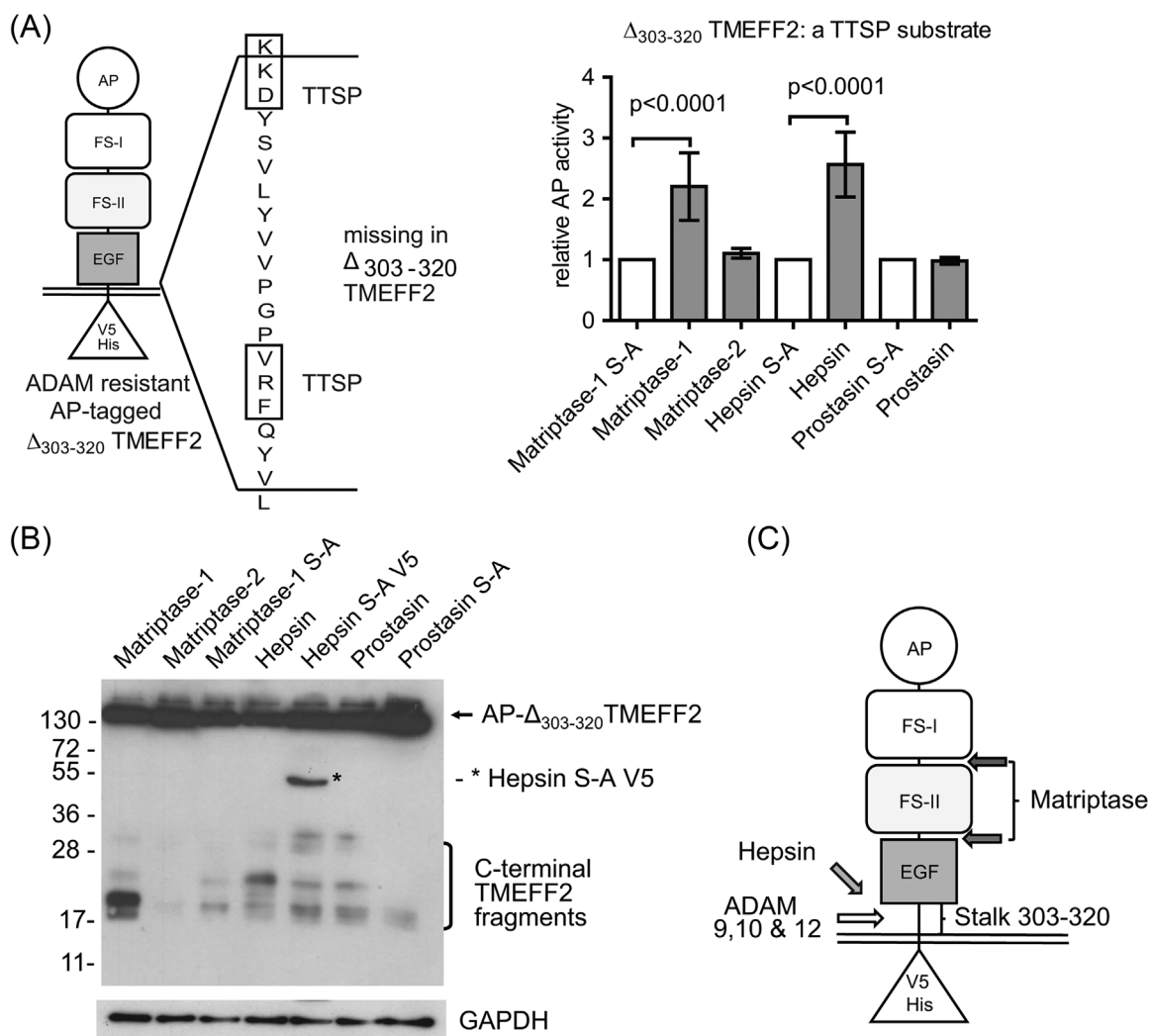


Figure 2 Characterization of TTSPs cleavage sites using the AP- $\Delta_{303-320}$ TMEFF2 mutant lacking the ADAM cleavage site. (A) Schematic representation of AP- $\Delta_{303-320}$ TMEFF2 expression construct and sequence motif deleted in this mutant, showing potential TTSP cleavage sites. Release of AP- $\Delta_{303-320}$ TMEFF2 from cells co-transfected with matriptase-1, matriptase-2, hepsin, prostasin, or their inactive S-A mutants. (B) WB analysis of lysates for AP- $\Delta_{303-320}$ TMEFF2 C-terminal fragments shows distinct cleavage products for matriptase-1 (~17 & 23 kDa) and hepsin (~25 kDa). (C) Model of predicted TMEFF2 cleavage sites for hepsin and matriptase-1.

thus adding additional layers of regulation of shedding events. ROS levels also regulate several signalling pathways in cancer (Hanahan and Weinberg, 2000; Liou and Storz, 2010), including PCa (Khandrika et al., 2009), which led us to hypothesise that ROS levels regulate TMEFF2 shedding. To investigate this hypothesis, we pre-treated AP-TMEFF2 HEK293 cells with the ROS scavenger NAC or the NADPH oxidase inhibitor APOC prior to stimulation with PMA, a known inducer of ROS generation (Datta et al., 2000) and ADAM17 activator (Brill et al., 2009). Both inhibitor treatments resulted in almost complete inhibition of AP-TMEFF2-ECD release, indicating ROS-dependent TMEFF2 processing (Figure 3A). We hypothesised that ROS-induced

TMEFF2 shedding could be mediated by the stress-activated protein kinases JNK or p38 to activate ADAM17. The JNK inhibitor completely blocked AP-TMEFF2 release, whereas the p38 inhibitor reduced TMEFF2 shedding by 50% (Figure 3A) suggesting that TMEFF2 shedding is triggered by oxidative stress signalling. The growth of cells at high cellular density has been reported to be a contributing factor to the increased oxidative stress in cancer (Hanahan and Weinberg, 2000). We investigated whether shedding of TMEFF2 was a pathophysiological response triggered by oxidative stress originating from cells growing in high confluency conditions. Equal number of cells were plated onto 24-well or 6-well plates to obtain 95% and 30%

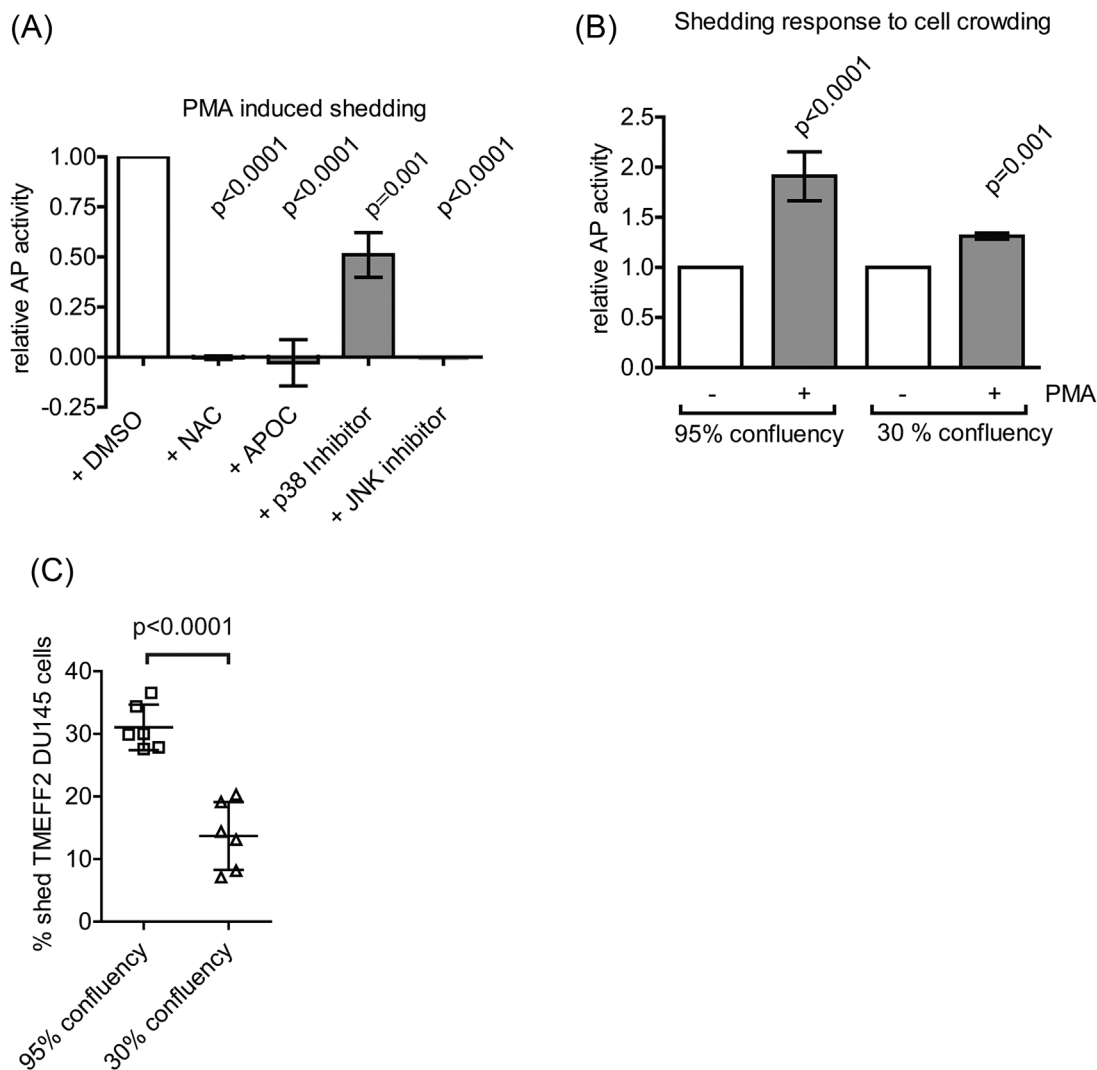


Figure 3 Oxidative stress and ROS-activated ADAMs participate in TMEFF2 shedding. (A) PMA-induced shedding from AP-TMEFF2 HEK293 cells pre-treated for 1 h with NAC, APOC, or p38 and JNK MAPKs inhibitors. (B) PMA-induced shedding of AP-TMEFF2 from 95% and 30% confluent cells. (C) TMEFF2 shedding in DU145 PCa cells is upregulated by cell crowding.

confluency, respectively, and treated with equal volumes of medium containing PMA or control solvent. High cellular density significantly increased the shedding response to PMA activation (Figure 3B). To confirm that TMEFF2 shedding in response to cell crowding was also relevant in PCa cancer cells, we then transiently transfected DU145 cells with AP-TMEFF2 cDNA and analysed shedding responses. AP-activity in cell lysates was determined and AP-activity in medium calculated as % shed TMEFF2 in cells grown at 3% or 95% confluency, respectively (Figure 3C). Data show significant increase in shedding in response to cell crowding, thus confirming the results obtained using HEK293 cells. Collectively, this suggests that cell crowding mimicking conditions frequently found in the tumour microenvironment may trigger

TMEFF2-ECD release, and contribute to the high proliferation rate of cancer cells.

In summary, differential TMEFF2 proteolysis producing various soluble fragments may be a general mechanism of changing its biological activity, dependent on the repertoire of ADAMs or TTSP expressed in the target cell, which in this case is regulated by ROS signalling.

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