Oligoubiquitination of tissue factor on Lys255 promotes Ser253-dephosphorylation, terminates TF release

Ubiquitination of TF terminates TF release

Camille Ettelaie[1], Mary EW Collier[1,2], Sophie Featherby[1], John Greenman[1], Anthony Maraveyas[3]

[1]Biomedical Section, Department of Biological Sciences, University of Hull, Cottingham Road, Hull, HU6 7RX, UK. [2]Department of Cardiovascular Sciences, University of Leicester, Clinical Sciences Wing, Glenfield General Hospital, Leicester, LE3 9QP, UK. [3]Division of Cancer-Hull York Medical School University of Hull, Cottingham Road, Hull, HU6 7RX, UK.

To whom correspondence should be addressed: Camille Ettelaie, Biomedical Section, Department of Biological Sciences, University of Hull, Cottingham Road, Hull, HU6 7RX, UK, Tel: +44(0)1482-465528, Fax: +44(0)1482-465458, Email: C.Ettelaie@hull.ac.uk

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ABSTRACT

Restriction of tissue factor (TF) activity at the cell surface and TF release are critical for prevention of excessive coagulation. This study examined the regulation of TF dephosphorylation and its release through ubiquitination. A plasmid containing the sequence to express the tandem protein TF-tGFP was mutated to include an arginine-substitution at Lys255 within TF. MDA-MB-231 cell line, and HCAEC endothelial cells were transfected and subsequently activated with PAR2-agonist peptide. The wild-type and mutant TF-tGFP were immunoprecipitated from the cell lysates and the ubiquitination and phosphorylation state of TF examined. Analysis of the proteins showed that arginine-substitution of Lys255 within TF prevented its ubiquitination while the wild-type TF-tGFP was oligoubiquitinated. The TF-associated oligoubiquitin chain was estimated to contain up to 4 ubiquitin units, with the linkage formed between Lys63 of one ubiquitin unit, and the C-terminus of the next unit. The Lys255→Arg substitution of TF-tGFP prolonged the phosphorylation of Ser253 within TF, compared to the wild-type TF-tGFP, lengthened the presence of TF-tGFP at the cell surface and extended the duration of TF-tGFP release from cells following PAR2 activation. A biotinylated 19-mer peptide corresponding to the C-terminus of TF (TFc) was used as substrate to show that the ubiquitination of TF was mediated by the Ube2D family of E2-enzymes and involved Mdm2. Moreover, double-phosphorylation of TFc was prerequisite for ubiquitination, with subsequent dephosphorylation of Ser253 by phosphatase PP2A. In conclusion, oligoubiquitination of Lys255 within TF permits PP2A to bind and dephosphorylate Ser253 and occurs to terminate TF release and contain its activity.

1. INTRODUCTION

The ability of cells to release tissue factor (TF) as microvesicles is established as a major participant in haemostasis and its deregulation is thought to be a major factor in the onset of thrombosis [1-5] although there is no definitive association between the concentration of circulating microvesicles and the incidence of thromboembolism [6-8]. Therefore, the precise control of TF activity on the cell surface, and also the termination of release of TF-containing microvesicles is likely to be crucial for preventing thrombosis. The mechanism by which cells regulate the release of TF is dependent on the incorporation of TF into microvesicles, and the release of microvesicles. Previously, we showed that the former process entails the phosphorylation of Ser253 within the cytoplasmic domain of TF [9] which allows the interaction of TF with filamin A and subsequent release of TF [10]. We also reported that the subsequent phosphorylation of Ser258 within this domain results in termination of the incorporation of TF into microvesicles [9,11]. These data also indicated a mechanism that involves the dephosphorylation of Ser253 by phosphatase PP2A. Therefore, there appears to be a sequence of canonical and time-dependent phosphorylation events which first initiate and then terminate the incorporation of TF into microvesicles. The phosphorylation of Ser258 is dependent on, and is preceded by the phosphorylation of Ser253 [9,12]. Moreover, accumulation of TF within the cells, following its activation, appears to further enhance the rate of phosphorylation of TF at Ser258, which in turn leads to the termination of the release [11]. Finally, the phosphorylation of Ser258 appears to participate in the mechanism of dephosphorylation of Ser253 [9]. However, these events do not occur immediately following the phosphorylation of the Ser253, presumably to permit sufficient time for the release of an adequate amount of TF from the cells. The region around Ser258 within the cytoplasmic domain is exceptionally well preserved in a number of

species [9]. As such, the phosphorylation of Ser258 is likely to be via a precisely regulated mechanism, and a critical phase in the regulation of TF exposure on the cell surface and subsequent release. In this study, we have demonstrated an ordered sequence of events by suggesting that the double-phosphorylation of TF results in the ubiquitination and subsequent dephosphorylation of Ser253 by phosphatase PP2A, terminating the release of TF.

2. MATERIAL AND METHODS

2.1 Cell culture, microvesicle preparation and measurement of TF antigen and activity.

MDA-MB-231 breast cancer cell line (ATCC, Teddington, UK) was cultured in DMEM containing 10% (v/v) foetal calf serum (FCS). Human coronary artery endothelial cells (HCAEC) were purchased and cultured in MV media containing 5% (v/v) FCS and growth supplements (PromoCell, Heidelberg, Germany). Cells (2 or 5×10^5) were seeded out in 6-well plates and pre-adapted to respective serum-free medium prior to experiments [9]. To induce microvesicle release the cells were stimulated with protease activated receptor 2-activating peptide (PAR2-AP); SLIGRL; (20 μM) in the presence and absence of recombinant factor VIIa (5 nM; American Diagnostica Inc., Stamford, USA). The released microvesicles were then isolated from conditioned media and resuspended in phosphate-buffered saline (PBS) according to published procedures [13]. The microvesicles were quantified using the functional assay Zymuphen MP assay kit (Hyphen BioMed/Quadratech Ltd, Epsom, UK). Microvesicle-associated TF antigen was measured by adding 1% (w/v) Triton X-100 to the samples and analysis using a TF-EIA kit. TF activity was monitored using a thrombin-generation assay and/or prothrombin time assay using Innovin thromboplastin reagent as standard (approximately 0.55 nM and assumed to contain 1000 U/ml; (Dade Behring, Milton Keynes, UK)) [9,14]. Cell-surface TF antigen levels were determined by a modification of the TF-EIA, as previously

described [15] and cell number in each sample was determined by staining with crystal violet as previously described [16,17].

2.2 Mutagenesis of TF plasmid and immunoprecipitation of TF protein.

The pCMV-AC-TF-tGFP plasmid (OriGene, Rockville, USA) which encodes for turbo-GFP (tGFP) in tandem, on the C-terminal of TF was mutated to substitute arginine instead of Lys255. The mutagenesis was carried out using the Q5-site directed mutagenesis kit (New England Biolabs UK, Hitchin, UK) according to the manufacturer's instructions with forward 5'-CAGAGCTGGAGGGAGAACTCC-3', and reverse 5'-CCCCACTCCTGCCTTTCT-3' primers. Successful mutation was verified by sequencing (MWG Eurofins, Wolverhampton, UK). Depending on the experiment, MDA-MB-231 cells or HCAEC (2×10^5) were transfected with 1 µg of plasmid DNA using Lipofectin (Invitrogen, Paisley, UK) as previously described in order to express wild-type or mutant TF [9,11]. Following transfection, the cells were incubated for 48 h at 37°C to permit the expression of TF. Cells were lysed in 500 µl of Phosphosafe lysis buffer (Merck-Millipore, Nottingham, UK) containing a protease inhibitor cocktail (1% v/v) (Sigma Chemical Company Ltd, Poole, UK) and N-ethylmaleimide (10 mM) (Sigma) to preserve the ubiquitination state of proteins. When using transfected cells, the lysates were immunoprecipitated with anti-tGFP-magnetic beads (clone 2H8) (25 µl) according to the manufacturer's protocol (OriGene) at 4°C overnight with gentle shaking. The tubes were then placed on a magnetic stand, the supernatant removed, and the beads washed five times (1 ml) with PBS-Tween (1% w/v; PBST). The samples were then denatured by boiling in SDS-PAGE loading buffer (70 µl; Sigma). Alternatively, samples were immunoprecipitated from untransfected MDA-MB-231 cell lysates using mouse or rabbit anti-human TF antibodies depending on the target analysis; a rabbit anti-human antibody (FL295; Santa Cruz Technology,

Heidelberg, Germany) (4 µg per sample) was employed when the samples were to be subsequently examined using anti-mouse antibodies by western blot; conversely a mouse anti-TF antibody (10H10; Santa Cruz) (4 µg per sample) was used when the western blot analysis involved anti-rabbit antibodies. Appropriate IgG isotypes (Cell Signalling Technology) were also included at identical concentrations as well as an additional no antibody control. All samples were incubated at 4°C overnight with gentle shaking. Pureproteome protein A-magnetic beads (10 µl; Merck-Millipore) added to all samples and controls and incubated at 4°C for 90 min with shaking. The samples were then placed in a magnetic stand and the supernatant removed. The beads were washed five times (1 ml) with PBST before the addition of and SDS-PAGE loading buffer (70 µl) to denature the samples.

2.3 Western blot analysis.

Electrophoresis was carried out on a 12 % (w/v) denaturing-polyacrylamide gel and the protein bands were transferred onto nitrocellulose membranes and blocked with TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20). Western blot analysis of the samples was carried out to detect TF using FL295 antibody (diluted 1:4000 (v/v) in TBST) or using 10H10 and/or HTF1 antibodies (diluted 1:2000 and 1:4000 (v/v) in TBST respectively), depending on the protocol used in the immunoprecipitation procedure (see above). The membranes were then washed with TBST and probed with goat anti-rabbit, or goat anti-mouse alkaline phosphatase-conjugated antibodies (Santa Cruz) as appropriate (diluted 1:4000 (v/v)) and incubated for 90 min. TF bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate (Promega Corp. Southampton, UK) and recorded. Detection of tGFP bands was carried out using the anti-tGFP antibody (2H8) diluted 1:4000 (v/v) and further developed as above. In addition, ubiquitination was detected using a mouse anti-human mono/poly-ubiquitin (FK2)

antibody (Enzo Life Sciences, Exeter, UK) diluted 1:4000 (v/v), while Lys63-linked ubiquitination was examined using a mouse anti-human Lys63-linked oligoubiquitin antibody (HWA4C4; Enzo) diluted 1:4000 (v/v). Lys48-linked ubiquitination was detected in immunoprecipitated samples using a rabbit anti-human Lys48-linked oligoubiquitin antibody (1001c; Boston Biochem/R&D Systems, Abingdon, UK) diluted 1:4000 (v/v) and developed as above. The specificity of the linkage-specific oligoubiquitin antibodies was also assessed using Lys48- and Lys63-oligoubiquitin multimer proteins (Boston Biochem). Phosphorylation of Ser258 was detected using a rabbit anti-phosphoserine-TF antibody (Abcam, Cambridge, UK) specific for phospho-Ser258, diluted 1:2000 (v/v) in TBST while phosphorylation of Ser253 was detected using a rabbit anti-phospho-PKC-substrate motif antibody (Cell Signalling Technology) diluted 1:2000 (v/v) in TBST buffer according as previously described [9].

2.4 ELISA-based analysis of TF ubiquitination.

The time-course of TF ubiquitination was examined using an ELISA-based procedure which employed the TF capture antibody from the TF EIA kit (Enzyme Research Inc. Swansea, UK). Briefly, the ELISA plates were coated with the sheep anti-human TF capture antibody as previously described [15]. Following blocking and washing the cell lysate samples were loaded into the wells and incubated for 1 h. After further washes with PBST, the wells were probed with an HRP-conjugated mouse anti-human mono/poly-ubiquitin (FK2) antibody (Enzo Life Sciences) diluted 1:500 (v/v) in PBST. The samples were incubated for 1 h, washed with PBST and developed using TMB One Solution (100 µl; Promega Corporation, Southampton, UK). Once the colour was developed the reactions were stopped by the addition of 2M sulphuric acid (50 µl) and absorptions measured at 450 nm using a plate reader.

2.5 De-ubiquitination assay.

De-ubiquitination assay was carried out using the UbiCREST Deubiquitinase Enzyme Kit (Boston Biochem). TF was immunoprecipitated from MDA-MB-231 cell lysate as above but at a larger scale using 20 µg of FL295 antibody and 100 µl of Protein A-magnetic beads and the samples were not eluted. Instead after washes with PBST, the samples were washed once with the "DUB reaction buffer" (1 ml) supplied with the kit and resuspended in the reaction buffer (450 µl). The reactions were prepared on ice, in ten tubes containing the nine DUB enzyme (5 µl) plus one control reaction, and 45 µl of DUB reaction buffer added. The reactions were started by adding 50 µl of magnetic bead slurry, mixed and incubated at 37°C for 30 min. The samples were then placed in the magnetic stand and the supernatant transferred to a fresh tube containing 2 × SDS-PAGE loading buffer (Sigma). The samples were boiled and analysed using western blot by probing with the FK2 anti-ubiquitin antibody.

2.6 Ubiquitination assay.

The substrate peptide corresponding to the last 19 amino acids of the cytoplasmic domain of TF were synthesised in biotinylated form (Biomatik, Ontario, Canada). Four peptides were synthesised in non-phosphorylated, single-phosphorylated and double-phosphorylated forms and used as substrates. An additional double-phosphorylated peptide, but containing arginine at the position occupied by Lys255 was also synthesised. The ubiquitination reaction was carried out using the E2-Screening kit (UBPBio/Caltag Medsystems, Buckingham, UK) and modified as described below to accommodate the various studies. The reactions were set up to contain substrate peptide (5 µM), Ube1 enzyme (0.1 µM), Ube2 enzymes (2 µM), ubiquitin monomer (50 µM), ATP (2 mM), glycerol (5% w/v) and reaction buffer (20 mM Tris, pH 7.6, 50 mM NaCl, 1 mM β-mercapthoethanol and 5 mM $MgCl_2$). In the initial experiments, fraction S100 (2

μM) (Enzo) was added as the Ube3. However, in later experiments this was replaced with recombinant Mdm2 (0.5 μM; R&D Systems) or pVHL (0.5 μM) (Merck-Millipore). Control reactions were also carried out in which one component of the reaction was missing. In total seven Ube2 enzymes were tested according to their relevance to TF-mediated pathways. These enzymes included Ube2D1-4, Ube2E1, Ube2L3 and Ube2N. Once the E2 and E3 enzymes were identified other experiments were carried out where the wild-type ubiquitin was replaced with mutant ubiquitins, with arginine-substitutions at lysine residues 6, 11, 27, 29, 33, 48, 63 or all lysine residues (UBPBio), and a ubiquitin with blocked N-terminus (HA-ubiquitin; Boston Biochem). Finally, studies were carried out with a mutant ubiquitin missing all lysines except Lys63.

All ubiquitination reactions were carried out at 37°C for 60 min and then analysed as follows. The samples were diluted to 100 μl with PBS and distributed (50 μl per well) in a NeutrAvidin-coated 96-well plate (Thermo Scientific, Warrington, UK) and incubated for 2 h at room temperature. In the initial experiments, biotinylated ubiquitin was also included and used as positive control but due to differences in degree of ubiquitination, quantification was not possible. After incubation, the wells were washed four times, each time with 300 μl of PBST and incubated with HRP-conjugated anti-human mono/poly-ubiquitin (FK2) antibody diluted 1:500 (v/v) in PBST. The wells were then washed a further four times and developed with TMB One Solution (100 μl; Promega). Once the colour was developed the reactions were stopped and measured at 450 nm as in section 2.4.

2.7 Phosphatase assay.

The phosphatase assay was carried out using the four peptides representing the 19 amino acids of the cytoplasmic domain of TF, in four different phosphorylation states. Each reaction

(25 µl) was prepared by assembling recombinant phosphatase PP2Aα (0.2 µg; SignalChem/Stratech, Newmarket, UK) in phosphatase reaction buffer (200 mM immidazole pH 7.2, 0.1% (w/v) β-mercapthoethanol and 52 ng/ml bovine serum albumin), $MgCl_2$ (10 mM) and substrate (10 µM). A similar set of samples were prepared but devoid of the enzyme. A positive control was prepared as above but containing the peptide (KRpTIRR) recommended as control substrate by the manufacturer, as well as a negative control devoid of substrate. The samples were incubated at 37°C for 60 min and aliquots of reaction (20 µl) were transferred into a fresh 96-well plate and 150 µl of BioMol (Enzo) added and incubated for 30 min. The absorptions were then measured at 630 nm using a plate reader and concentrations determined from a phosphate-standard curve. In some experiments, the double-phosphorylated TFc peptide was ubiquitinated prior to the phosphatase assay and examined along with the non-ubiquitinated peptide. The peptides were pre-adsorbed onto the NeutrAvidin 96-well plates and washed fours times with phosphate-free Tris-HCl (50 mM) pH 7.5 (300 µl). The phosphatase reaction mixture (25 µl) was then added and incubated for 30 min at 37°C as above. Aliquots (20 µl) were then transferred to an un-coated 96-well plate and assessed with BioMol as above.

2.8 Microscopy

HCAEC (3×10^4) were seeded out into glass base dishes (µ-Dish 35 mm) and transfected with the wild-type and Arg255-substituted pCMV-AC-TF-tGFP plasmids. The cells were incubated for 48 h at 37°C to permit the expression of TF hybrids. The cells were adapted to serum-free medium for 1 h and activated with PAR2-AP or used without activation. After 120 min the cells were washed and fixed. To ensure that the detected TF was associated with cell surface the outer membrane of the activated cells were stained with Alexa Fluor 647-conjugated annexin V (BioLegend/Cambridge Bioscience, Cambridge, UK) as previously described [15].

The cells were analysed for cell-surface TF-tGFP and negatively charged phospholipids, using a Zeiss LSM 710 confocal microscope using a ×63 lens (Carl Zeiss Ltd, Welwyn Garden City, UK) according to procedures detailed previously [15]. Images were acquired using the ZEN software.

3. RESULTS

3.1 Validation of antibodies.

TF antibodies were validated prior to experimentation and no cross-detection of the mouse and rabbit primary antibodies by the secondary antibodies were observed (Supplemental figure 1). Additionally, antibodies against ubiquitin failed to recognise either of the two separate preparations of recombinant human TF used, discounting misrecognition of non-ubiquitinated TF (Supplemental figure 2)

3.2 TF is ubiquitinated following activation of PAR2.

Human coronary artery endothelial cells (HCAEC) were transfected to express TF-tGFP and permitted to express the protein for 48 h. MDA-MB-231 cells and transfected HCAEC were activated with PAR2 agonist peptide. Samples were removed at intervals and TF was immunoprecipitated from the cell lysates. Analysis of ubiquitination of TF by an ELISA-based procedure indicated an increase in ubiquitination of endogenous and overexpressed TF in the cells, upon activation with PAR2-AP. The ubiquitination of TF in MDA-MB-231 cells, measured by ELISA, peaked at 30 min post-activation with a 7-fold increase compared to resting cells (Fig 1A). In contrast, the level of TF ubiquitination in HCAEC rose steadily throughout the duration of assessment also reaching 7-fold increase compared to resting cells but after 120 min (Fig 1B). The duration of assessment for both these cell types were selected to include the

maximal time-point of TF release which were previously determined to be 30 min in MDA-MB-231 cells and 90 min in HCAEC [9,10].

Analysis of immunoprecipitated TF from the cells by western blot were generally in agreement with the time-course data obtained using the ELISA-based study, from the respective cell types. Examination of the blots from MDA-MD-231 cells (5×10^5) showed the presence of a band at around 45-50 kDa and a heavier band at around 70-80 kDa when probed using the anti-ubiquitin antibody (FK2) (Fig 1C). Activation of the MDA-MD-231 cells resulted in the appearance of the heavier band with a peak at 30 min (Fig. 1C). Similar bands were also observed on western blot analysis of HCAEC primary cells (2×10^5) which were transfected to express TF-tGFP (Fig 1D): These bands also had higher molecular weights (75-80 kDa & 100-110 kDa) reflecting the presence of tGFP (Mw = 25 kDa). Moreover, in agreement with the ELISA-based study, the activation of HCAEC resulted in the progressive increase in the amount of ubiquitination of both of these bands although the heavier band was not discernible from the lighter band at later time-points.

Supplementing the medium with factor VIIa intensified the level of ubiquitination as a ratio of the amount of TF in MDA-MB-231 cells at 30 min post-activation with PAR2-AP (Fig. 1E & Fig. 1F).

3.3 TF is ubiquitinated at Lys255.

The cytoplasmic domain of TF contains 3 lysine residues. In order to determine the likely target lysine which may become ubiquitinated, the sequence of TFc was examined using the UBPred [18] analysis tools. Since phosphoserine residues cannot be accommodated by the program, the sequence was analysed with aspartic acid substitutions at positions 253 and 258. Analysis of four sequences indicated that only Lys255 was likely to be a target for ubiquitination

(Table 1). In addition, the probability score was highest if both serine residues were replaced with aspartate. Moreover, since phosphoserine is more negatively charged than aspartate, the likelihood of ubiquitination of Lys255 would be higher. Consequently, a mutant form of TF-tGFP, with Lys255→Arg substitution was expressed in both MDA-MB-231 and HCAEC cells, alongside with cells expressing wild-type TF-tGFP. The cells were activated using PAR2 agonist peptide as above. TF-tGFP was then immunoprecipitated from the lysates using anti-tGFP antibody-conjugated magentic beads and analysed by western blot for TF, tGFP, ubiquitin, phosphoSer253 and phosphoSer258 using different antibodies. Analysis of the cell lysates showed similar levels of tGFP, TF and phosphorylation at Ser258 in MDA-MB-231 cells (Fig. 2) but was less visible in HCAEC due to lower expression of the protein (supplemental figure 3). However, the mutant form of TF lacked any ubiquitination and remained phosphorylated at Ser253 at a time-point when the wild-type TF was de-phosphorylated (100 min for HCAEC; 45 min for MDA-MB-231 cells not shown).

3.4 TF ubiquitination is mediated by the Ube2D family of proteins and Mdm2.

The large size and the hydrophobic nature of TF makes the use of full-length recombinant TF in an *in vitro* ubiquitination assay impossible. In addition, it is not possible to obtain a phosphorylated form of recombinant full-length TF. However, the cytoplasmic domain peptide of TF possesses sufficient structural [19] and functional [11] properties to be used in enzymatic assays. Therefore, a synthetic form of TFc, with phosphorylated groups at residues equivalent to Ser253 and 258 was used in the next studies. The initial analysis of the level of ubiquitination of double-phosphorylated TFc peptide was carried out using the customised E2-screening kit and with nuclear fraction S100 as the source of E3 enzyme according to the instructions of the kit. Positive data were confirmed in later experiments using recombinant E3 enzyme as described

below. Seven E2 enzymes were chosen on the basis of their target substrates, and also cellular mechanisms that were relevant to TF. The analysis of the seven E2 enzymes demonstrated similar and significant levels of ubiquitination of double-phosphorylated TFc peptide by Ube2D1-4 enzymes (Fig. 3A). In contrast, none of the other E2-enzymes showed significant ability to ubiquitinate the same TFc peptide. Additionally, since all the Ube2D enzymes are capable of employing Mdm2 and pVHL as the E3-ligase, the experiments were repeated with recombinant Mdm2 and pVHL instead of fraction S100. Inclusion of Mdm2 in the above reaction resulted in almost three-fold increase in ubiquitination efficiency observed while inclusion of pVHL did not significantly increase the ubiquitination beyond that observed with fraction S100 (Fig. 3B). These observed activities were authenticated by repeating the reaction, but omitting individual components to validate the specificity of the ubiquitination reaction (Fig. 3C).

3.5 Ubiquitination of TF requires the double-phosphorylation of TF.

To determine the preferred form of TFc as substrate for the ubiquitination reaction, separate reactions were carried out containing TFc peptides either in non-phosphorylated, 253-phosphorylated, 258-phosphorylated or double-phosphorylated TFc formats. The reactions were carried out using a mixture of Ube2D enzymes at equal amounts. Analysis of the ubiquitination of the four forms of TFc peptide showed that the double-phosphorylated peptide was the only form that was significantly ubiquitinated (Fig. 3D). Furthermore, no significant ubiquitination was detected with the double-phosphorylated peptide containing an Arg-substitution at the Lys255 position.

3.6 TF oligoubiquitination is mediated through Lys63-linkage.

Examination of immunoprecipitated cellular TF from MDA-MB-231 cells (Fig 4A) or TF-tGFP from HCAEC (not shown) by western blot showed the presence of Lys63-linkage but not Lys48-linkage oligoubiquitination. Additionally, neither of the two non-ubiquitinated recombinant TF samples were recognised by the anti-ubiquitin antibodies (Supplemental figure 2). The specificity of the discriminating antibodies was confirmed using synthetic oligo-Lys48-linked and oligo-Lys63-linked oligoubiquitin (Fig. 4B). In addition, digestion of the immunoprecipitated TF from MDA-MB-231 cells, using de-ubiquitination enzymes AMSH and Trabid, resulted in the release of the greatest amounts of mono-ubiquitin while USP appeared to be less active, releasing ubiquitin multimers as well as monomers (Fig. 4C). AMSH is specific for Lys63-linkage while Trabid is capable of hydrolysing Lys63-, Lys33- and Lys29-linkages between ubiquitin units. Moreover, although USP is capable of hydrolysing all ubiquitin linkages, it was less active in further degrading the oligoubiquitin released from TF, or a synthetic Lys63-linked oligoubiquitin chain (not shown) into monomers. The *in vitro* ubiquitination reaction, using the synthetic double-phosphorylated TFc peptide was also performed as described above but using various mutant forms of ubiquitin, each containing a single arginine-substitution of an individual lysine residues, in turn as well as an N-terminal blocked ubiquitin. Analysis of the incorporation of the mutants forms of the ubiquitins indicated that Lys63 within ubiquitin molecule, was essential for linkage formation during oligomerisation of the ubiquitin chain attached to TF (Fig. 4D). In contrast, substitution of any of the other lysines, or the blockage of the N-terminal methionine using HA-ubiquitin (YPYDVPDYA tag) did not alter the level of ubiquitination discounting the linear linkage. Additionally, replacing the wild-type ubiquitin with a mutant in which only the Lys63 was preserved resulted in E2-

concentration dependent ubiquitination of double-phosphorylated TFc peptide, while inclusion of a ubiquitin mutant in which all of the lysine residues were substituted was ineffective (Fig. 4E).

3.7 Ubiquitination of TF leads to dephosphorylation of Ser253 by phosphatase PP2A.

Previously we showed that incubation of cells with calyculin-A prevented the dephosphorylation of TF and prolonged the incorporation and release of TF within microvesicles [9]. In this study, the ability of phosphatase PP2Aα to dephosphorylate the 19-mer cytoplasmic domain of TF peptide in the four phosphorylation formats (as above) was examined. Incubation of the Ser253-phosphorylated and double-phosphorylated TFc peptides with PP2Aα liberated similar amounts of phosphate (Fig. 5A) but was not comparable to the positive control using the recommended PP2Aα-substrate peptide (KRpTIRR). Interestingly, Ser258-phosphorylated form of TFc peptide was not a suitable substrate for PP2Aα under these conditions, although the possibility that this residue may be dephosphorylated by other enzymes or under different conditions cannot be precluded. Furthermore, ubiquitination of the double-phosphorylated TFc peptide prior to the phosphatase reaction, significantly enhanced the dephosphorylation of this peptide to a level comparable to that of the positive-control peptide (Fig. 5B).

3.8 Ubiquitination of TF regulates the termination of TF release.

In order to determine if the ubiquitination of TF is involved in the termination of TF release, only HCAEC were used in the following studies, since these cells do not express detectable levels of TF under normal conditions [9]. The cells were transfected to express the wild-type and Arg255-substituted TF-tGFP and compared together with untransfected cells. Both sets of transfected HCAEC, together with untransfected cells were activated with PAR2 agonist peptide and the microvesicles collected at 0, 90 and 120 min post-activation. The amounts of TF and the microvesicles determined. It must be emphasised that since both the released TF and

microvesicles are taken up by the cells [15], the measured values represent the resultant net flux in TF/microvesicles into the media [9] at the time point of maximal release. Analysis of microvesicles from the three samples indicated the prolonged release of mutant TF antigen compared to that of the wild-type TF (Fig. 6A). In agreement with the levels of TF antigen, the thrombin generation potential (Fig. 6B) and the procoagulant activity, as measured by the prothrombin time assay (Supplemental figure 4), remained higher in microvesicles isolated from HCAEC expressing the mutant TF, compared to those expressing the wild-type TF. However, these differences were less pronounced since this would also be dependent on the release of phosphatidylserine and possibly, other cell-surface proteins which could affect thrombin generation and the coagulation. Interestingly, the release of microvesicles remained unaltered between the three experiments (Fig. 6C). In agreement with the above, the amount of cell-surface TF indicated the persistence of mutant TF on the HCAEC surface for a longer period than the wild-type TF (Fig. 6D). Moreover, in agreement with the above data, the mutant form of TF persisted at the cell surface depicted by the presence of green TF-tGFP associated with the annexin v-labelled (red) outer cell membrane (Fig 6E), while similar amounts of TF and PS labelling were observed at the time of peak TF release (Supplemental figure 5).

4. DISCUSSION

The regulation of TF activity is dependent on its de-encryption on the cell surface as well as its release as microvesicles. These processes are largely initiated by the activation of cells, through various influencing factors including contact with inflammatory cytokines [20-22], hypoxia [23,24] as well as mediators of coagulation signalling [9-11,24-27]. TF also possesses a number of signalling properties that can influence the cells in which it is expressed, and the cells

that it comes into contact with [28-36]. The variety of the cell surface interactions leading to a multitude of events have been reviewed recently [37]. Among the major functional outcomes examined are migration of smooth muscle cells [38,39], endothelial remodelling [40,38] and apoptosis [37,41,42]. Interestingly, while some of these events also require the activation of PAR2 [40,43], events including cross-talk with IGF-1R and Akt signalling appear to be dependent on TF but preclude PAR2 signalling [39,42,44]. Furthermore, it is known that the release of microvesicles can protect the cells from apoptosis [45,46] but notably, the nature of the microvesicle-cargo is changeable [47]. Similarly, cellular outcomes may also entail alterations to the properties of TF, and therefore may have substantial outcomes on the range and nature of procoagulant activity [48], in determining cancer phenotypes [49] as well as influencing host-defence mechanisms [50].

One crucial step in the regulation of TF is the curtailment of TF activity which is largely carried out by TFPI [51-53]. However, in order to prevent prolongation of the TF exposure, it is also necessary to limit the release of TF within microvesicles. In this study we have demonstrated that the ubiquitination of TF following the activation, acts as a step to terminate the release of TF as microvesicles, and possibly initiates the endocytosis of cell-surface TF (Fig. 7). The ubiquitination of TF occurs only subsequent to phosphorylation of both serine residues, therefore permitting the release of a required amount of TF. The controlling factor for determining the "required amount of TF" is still to be established and is currently under investigation in our laboratory. TF is ubiquitinated on Lys255 and the oligoubiquitin chain appears to contain 3-4 units, although we were not able to discount the presence of longer chains. The initial increase in the molecular weight from around 47 kDa to around 75-80 kDa is in line with the addition of 3-4 ubiquitin units. This may also explain the higher molecular weight bands

that are often detected by a number of commercially used antibodies including 4G4 antibody (Lifespan Bioscience), TF9-10H10, H9, FL294 and 2K1 antibodies (Santa Cruz), 323514 antibody (R&D Systems) and OAAF01006 antibody (Aviva Systems Biology), when probing cell lines such as A-431, WI-38, JEG-3, THP-1 and 293 that are capable of releasing high levels of TF. However, the relatively rapid reduction of the size of the protein in MDA-MB-231 cells but not in HCAEC was unexpected and intriguing. This indicates that the oligoubiquitin chain attached to TF may be partially de-ubiquitinated in cell lines but not primary cells. Therefore, this may be an important mechanism in the prevention of accumulation of excessive amounts of TF, consequently avoiding the associated pro-apoptotic influences [36] in cancer cells. This would also explain the doublet which is sometimes observed with some anti-TF antibodies when analysing cellular TF by western blot (see above examples). The oligoubiquitin linkage is mediated through Lys63 on the ubiquitin units. Lys63-linked ubiquitination is a hallmark of receptors that are destined for endocytosis through recognition by proteins such as Epsin, Eps15 and Rap80-tUIM [54-57]. These proteins recruit oligoubiquitinated receptors via clatherin and caveolin-mediated mechanisms into multivesicular bodies for further cellular sorting [54,55,58]. We previously showed that TF is sorted mainly for storage or recycling, within such bodies [15] but unmodified TF lacks a protein sequence which would permit receptor internalisation. Therefore, Lys63-linked ubiquitination may also act as the required signal promoting TF internalisation. Interestingly, the ubiquitination reaction involved the Ube2D-family of E2-ligases and recruited Mdm2 but not pVHL, as a very efficient E3-enzyme. The significance and implications of these findings remain to be clarified in the future. Finally, this study demonstrated that phosphatase PP2Aα was able to specifically dephosphorylate Ser253 although this reaction was only likely to occur following ubiquitination of TF. Oligoubiquitin chains may

form a platform for the interaction of other proteins. Since there is substantial cross-talk between ubiquitination and phosphorylation events [59] it is possible that the oligoubiquitin may also recruit phosphatases which subsequently dephosphorylate the ubiquitinated protein. These data support our previous study showing that pre-incubation of cells with calyculin-A suppressed the dephosphorylation of TF and prolonged TF release as microvesicles [9].

In conclusion, we have demonstrated a canonical mechanism in which activation of cells results in tandem phosphorylation of the two serine residues within TF and promotes Lys63-oligoubiquitination of TF by Ube2D enzymes and Mdm2 (Fig. 7). This process leads to the recruitment of phosphatase PP2Aα which specifically dephosphorylates Ser253 and prevents the release of TF into microvesicles. Moreover, the ubiquitination of TF also appears to trigger interactions which may lead to the removal of this protein from the cells surface.

5. CONFLICT OF INTEREST

No conflict of interest is declared.

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FIGURE LEGENDS

Table 1. Predicted probability of the ubiquitination of the lysine residues within TFc. The suitability of the three lysine residues within TFc as targets for ubiquitination were predicted using the UBPred program. The sequence of TFc was then modified to contain aspartate instead of Ser253, Ser258 or both in order to mimic phosphoserine by providing negative charges; the probabilities were then re-calculated. (* = $p<0.05$). The lysine residues are underlined and the serine/aspartate residues at positions 253 and 258 are shown in bold.

Figure 1. Time-course of ubiquitination of TF following PAR2-activation of cells. MDA-MB-231 cells (5×10^5) were seeded out in 6-well plates and then pre-adapted to serum-free medium prior to experiments and activated with PAR2-AP (20 µM). In addition, HCAEC (2×10^5) were transfected to express TF-tGFP hybrid protein and treated similarly. MDA-MB-231 cell samples were collected prior to activation, and at 15, 30 and 45 min post-activation. Similarly, HCAEC were collected at 60, 90 and 120 min post-activation. The cells were lysed in 500 µl of Phosphosafe lysis buffer containing a protease inhibitor cocktail (1% v/v) and N-ethylmaleimide (10 mM). The samples were loaded into 96-well plates which were pre-coated with a sheep anti-human TF antibody and blocked with BSA. The samples were washed with PBST and probed with an HRP-conjugated mouse anti-human mono/poly-ubiquitin (FK2) antibody diluted 1:500 (v/v) in PBST. The samples were incubated for 1 h, washed with PBST and developed using TMB One Solution (100 µl). The absorptions for MDA-MB-231 cells (A) and HCAEC (B) were determined at 450 nm on a plate reader (n = 4; * = $p<0.05$ vs the untreated sample).

Immunoprecipitation was carried out from lysates by incubating with rabbit anti-human TF antibodies (FL295; 4 µg per sample) and purified using Pureproteome protein A-magnetic beads (10 µl). The purified samples from MDA-MB-231 cells (C) and HCAEC (D) were analysed by western blot and probed with a mouse anti-human ubiquitin antibody (FK2) or a mouse anti-human TF antibody (10H10) diluted 1:2000 and 1:4000 (v/v) in TBST respectively. The membranes were then washed with TBST and probed with goat anti-mouse alkaline phosphatase-conjugated antibodies, diluted 1:4000 (v/v). The bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate and recorded (the micrographs are representative of 7 experiments). A similar set of experiments were carried out using MDA-MB-231 cells (2×10^5) in the presence and absence of recombinant factor VIIa (5 nM) and the samples were collected at 30 min post-activation, and processed as using the ELISA-based procedure (E) (n = 3; * = $p<0.05$ vs the untreated sample; # = $p<0.05$ vs the sample without FVIIa) and western blotting (F) as above.

Figure 2. Analysis of ubiquitination and phosphorylation in wild-type and Arg255-substittued TF. The pCMV-AC-TF-tGFP was mutated to substitute arginine instead of lysine at position 255. MDA-MB-231 cells (2×10^5) were transfected to express wild-type TF or mutant TF together with tGFP and the cells were incubated for 48 h to permit the expression of TF. Cells were lysed in 500 µl of Phosphosafe lysis buffer containing a protease inhibitor cocktail (1% v/v) and N-ethylmaleimide (10 mM) to preserve the ubiquitination state of proteins. The lysates were immunoprecipitated with anti-tGFP-magnetic beads (clone 2H8; 25 µl). The purified samples were then analysed by western blot and probed using a mouse anti-tGFP antibody (2H8), a mouse anti-human TF antibody (10H10) and a mouse anti-human ubiquitin antibody (FK2) diluted 1:4000, 1:2000 and 1:4000 (v/v) in TBST respectively. The membranes

were then washed with TBST and probed with goat anti-mouse alkaline phosphatase-conjugated antibodies, diluted 1:4000 (v/v). Phosphorylation of serine 258 was detected using a rabbit anti-phosphoserine-TF antibody specific for phospho-Ser258, diluted 1:2000 (v/v) in TBST while phosphorylation of serine 253 was detected using a rabbit anti-phospho-PKC-substrate motif antibody diluted 1:2000 (v/v) in TBST buffer. The membranes were then washed with TBST and probed with goat anti-rabbit alkaline phosphatase-conjugated antibodies, diluted 1:4000 (v/v). All membranes were then visualised using the Western Blue stabilised alkaline phosphatase-substrate and recorded (the micrographs are representative of 4 experiments).

Figure 3. Identification of the ubiquitinase enzymes and the ubiquitinated TFc substrate. The biotinylated substrate peptide corresponding to the last 19 amino acids of the cytoplasmic domain of TF were synthesised in non-phosphorylated, single-phosphorylated and double-phosphorylated forms. A) The reactions were set up to contain substrate peptide (5 µM), Ube1 enzyme (0.1 µM), Ube2 enzymes (2 µM), Ubiquitin monomer (50 µM), ATP (2 mM), glycerol (5% w/v) and reaction buffer (20 mM Tris, pH 7.6, 50 mM NaCl, 1 mM β-mercapthoethanol and 5 mM $MgCl_2$) and using fraction S100 (2 µM) as Ube3. In total seven Ube2 enzymes were tested according to their relevance to TF-mediated pathways. These enzymes included Ube2D1-4, Ube2E1, Ube2L3 and Ube2N. The reactions were carried out at 37°C for 60 min, diluted to 100 µl with PBS and distributed (50 µl per well) in a NeutrAvidin-coated 96-well plate and then incubated for 2 h at room temperature. The wells were washed four times with PBST (300 µl) and incubated with HRP-conjugated anti-human mono/poly-ubiquitin (FK2) antibody. The wells were then washed a further four times with PBST and developed with TMB One Solution (100 µl). Once the colour was developed the reactions were stopped by the addition of 2M sulphuric acid (50 µl) and absorptions measured at 450 nm using a plate reader. (n = 6; * = $p<0.05$ vs the

control sample devoid of Ube2 enzyme). B) The experiments were repeated using a mixture of Ube2D enzymes, but substituting recombinant Mdm2 (0.5 μM) or pVHL (0.5 μM) for fraction S100. (n = 6; * = $p<0.05$ vs the control sample without the Ube3 enzyme). C) To validate the ubiquitination reaction reactions were carried out in which individual components of the reaction were in turn omitted. A mixture Ube2D enzymes and recombinant Mdm2 (0.5 μM) were used. (n = 5; * = $p<0.05$ vs the control sample without the substrate). D) The TFc substrate was identified by repeating the reaction but using the non-phosphorylated, single-phosphorylated and double-phosphorylated forms of TFc peptide. A double-phosphorylated peptide containing Arg at the position occupied by Lys255 was also included (n = 5; * = $p<0.05$ vs the control sample without the substrate).

Figure 4. Identification of the linkage type in oligoubiquitinated TF. TF samples were immunoprecipitated from MDA-MB-231 cell lysates using mouse (10H10) or rabbit (FL295) anti-human TF antibodies depending on the target analysis. A) The samples were examined by western blot using a mouse anti-Lys63-linked oligoubiquitin antibody (HWA4C4) and a rabbit anti-Lys48-linked oligoubiquitin antibody (1001c). B) The specificity of the antibodies was confirmed using synthetic oligo-Lys63-ubiquitin and oligo-Lys48-ubiquitin chains. C) The immunoprecipitated TF was also digested with a set of de-ubiquitination enzymes with different specificity for the site of cleavage (as shown on the figure). The release of mono-ubiquitin was then monitored by western blot analysis (the micrographs are representative of 5 experiments). D) The participation of the lysine residue and the N-terminus methionine within ubiquitin in the incorporation into the oligoubiquitinated TF molecule was assessed using mutant ubiquitins, each missing one of the lysine residues (by arginine substitution). In addition, ubiquitin without any lysine residues (K0) or with blocked N-terminus (HA-Ub) were also included. The

ubiquitination reaction contained the substrate peptide (5 µM), Ube1 enzyme (0.1 µM), Ube2 enzymes (2 µM), Ube3 (Mdm2) (0.5 µM), ubiquitin monomer (50 µM), ATP (2 mM), glycerol (5% w/v) and reaction buffer (20 mM Tris, pH 7.6, 50 mM NaCl, 1 mM β-mercapthoethanol and 5 mM $MgCl_2$). The tubes were incubated at 37°C for 60 min, diluted to 100 µl with PBS and distributed (50 µl per well) in a NeutrAvidin-coated 96-well plate and then incubated for 2 h at room temperature. The wells were washed 4 times with PBST (300 µl) and incubated with HRP-conjugated anti-human mono/poly-ubiquitin (FK2) antibody. The wells were then washed a further 4 times with PBST and developed with TMB One Solution (100 µl). Once the colour was developed the reactions were stopped by the addition of 2M sulphuric acid (50 µl) and absorptions measured at 450 nm using a plate reader. ($n = 6$; * = $p<0.05$ vs the wild-type ubiquitin sample). E) Similar reactions were carried out using a mutant ubiquitin in which only Lys63 was intact (Ub63O), one without any lysine residues (K0) and a wild-type ubiquitin. The reactions were carried out using 0-2 µM of the Ube2 enzyme. ($n = 5$; * = $p<0.05$ vs the respective K0 sample).

Figure 5. Analysis of dephosphorylation of TFc by PP2Aα. A) The biotinylated substrate peptide corresponding to the last 19 amino acids of the cytoplasmic domain of TF were synthesised in non-phosphorylated, single-phosphorylated and double-phosphorylated forms and used as potential substrates. Reactions (25 µl) were prepared by assembling recombinant phosphatase PP2Aα (0.2 µg) in phosphatase reaction buffer (200 mM immidazole pH 7.2, 0.1% (w/v) β-mercapthoethanol and 52 ng/ml bovine serum albumin), $MgCl_2$ (10 mM) and substrate (10 µM). A similar set of samples were prepared but without the enzyme. A positive control was prepared as above but containing the peptide (KRpTIRR) recommended as control substrate by the manufacturer as well as a negative control devoid of substrate. The samples were then

incubated at 37°C for 60 min and aliquots of reaction (20 µl) were transferred into a fresh 96-well plate and 150 µl of BioMol added and incubated for 30 min. The absorptions were then measured at 630 nm using a plate reader and concentrations determined from a phosphate-standard curve. (n = 5; * = $p<0.05$ vs the sample without any substrate). B) The double-phosphorylated TFc peptide was ubiquitinated prior to the phosphatase assay and examined along a non-ubiquitinated peptide. The peptides were pre-adsorbed onto the NeutrAvidin 96-well plates and washed 4 times with phosphate-free Tris-HCl (50 mM) pH 7.5 (300 µl). The phosphatase reaction mixture (25 µl) was then added and incubated for 30 min at 37°C as above. Aliquots (20 µl) were then transferred to a fresh uncoated 96-well plate and assessed as above (n = 5; * = $p<0.05$ vs the sample without any substrate).

Figure 6. Comparison of microvesicle-associated TF antigen and activity, and cell-surface antigen in the wild-type and Arg255-substituted TF. HCAEC (2×10^5) were transfected with the wild-type and Arg255-substituted pCMV-AC-TF-tGFP plasmids using Lipofectin and incubated for 48 h at 37°C to permit the expression of TF. A sample of untransfected cells was included in the experiment and measured alongside. The cells were adapted to serum-free medium for 1 h and activated with PAR2-AP or used without activation. Microvesicles were isolated from the media of the cells by ultracentrifugation and resuspended in PBS. The cells were fixed with glutaraldehyde (3% w/v) and then washed five times with PBS. A) Microvesicles-associated TF antigen was measured using a TF-EIA procedure (n = 3; * = $p<0.05$ vs the respective untransfected sample; # = $p<0.05$ vs the samples expressing wild type TF), and B) microvesicles-associated TF activity was assessed using a thrombin generation assay (n = 3; * = $p<0.05$ vs the respective untransfected sample). C) The functional density of the microvesicles was determined using the Zymuphen MP assay kit (n = 3). D) The amount of TF antigen on the

cell surface was determine by probing with the HRP-conjugated anti-TF antibody from the EIA kit (n = 3; * = $p<0.05$ vs the respective untransfected sample; # = $p<0.05$ vs the samples expressing wild type TF). All values were normalised for the number of cells in each well which was determined by crystal violet staining procedure. E) HCAEC (3×10^4) were seeded out into glass base dishes (µ-Dish 35 mm) and transfected with the wild-type and Arg255-substituted pCMV-AC-TF-tGFP plasmids. The cells were incubated for 48 h at 37°C to permit the expression of TF hybrids. The cells were adapted to serum-free medium for 1 h and activated with PAR2-AP or used without activation. After 120 min, the cells were washed, fixed and stained with Alexa Fluor 647-conjugated annexin V (Red). The cells were analysed for cell-surface TF-tGFP (Green) using a Zeiss LSM 710 confocal microscope using a ×63 water-based lens. Images were acquired using the ZEN software. (The micrographs are representative of 3 experiments).

Figure 7. Schematic representation of the regulation of TF release. The phosphorylation of Ser253 leads to the binding of filamin A and release of TF. The subsequent phosphorylation of Ser258 on TF promotes the Lys63-linked oligoubiquitination of TF at Lys255 which may provide a platform for the binding of protein phosphatase PP2Aα. Dephosphorylation of Ser253 by PP2Aα results in the inability of TF to interact with filamin A and terminates TF release into microvesicles. Additionally, it is possible that the ubiquitination of TF may promote the recognition by ubiquitin-dependent receptors resulting in TF endocytosis.

Table 1

Peptide	Score		
	Lys244	Lys247	Lys255
LAISLHKCRKAGVGQ**S**WKEN**S**PLNVS	0.21	0.30	0.58
LAISLHKCRKAGVGQ**D**WKEN**S**PLNVS	0.21	0.30	0.63*
LAISLHKCRKAGVGQ**S**WKEN**D**PLNVS	0.21	0.30	0.63*
LAISLHKCRKAGVGQ**D**WKEN**D**PLNVS	0.21	0.30	0.67*

Figure 1

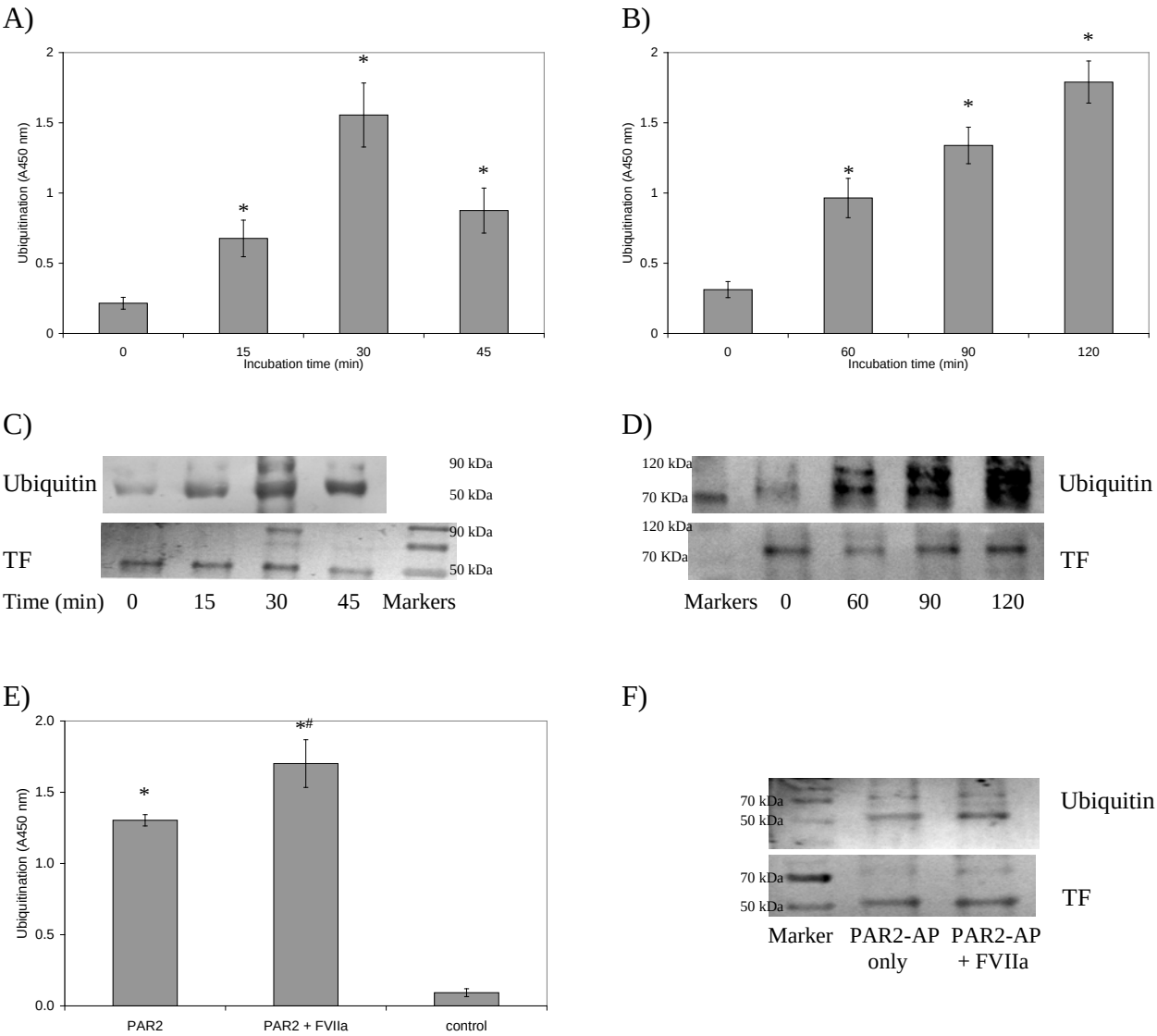


Figure 2

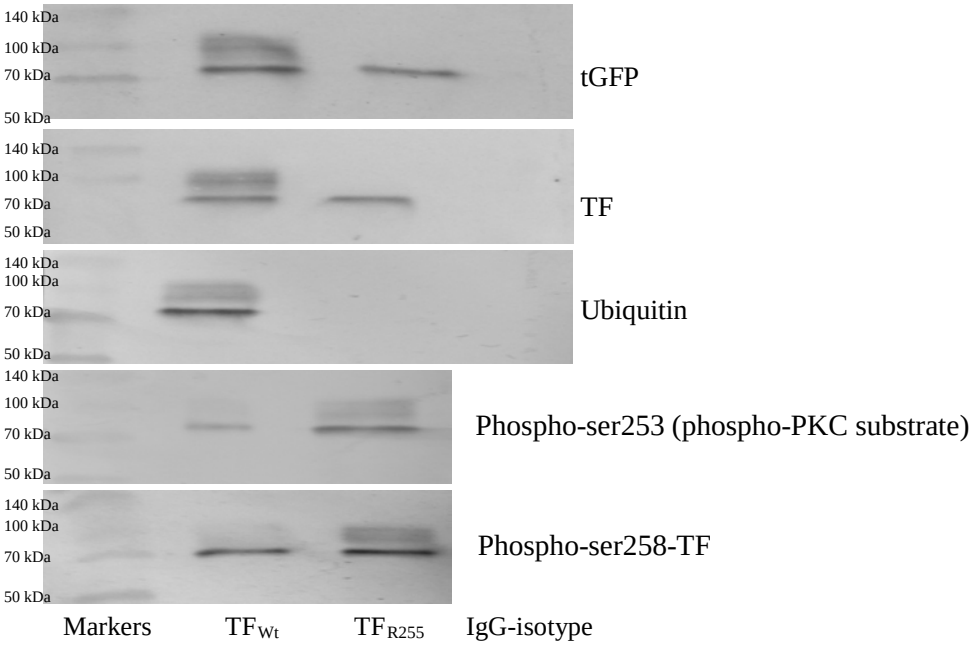


Figure 3

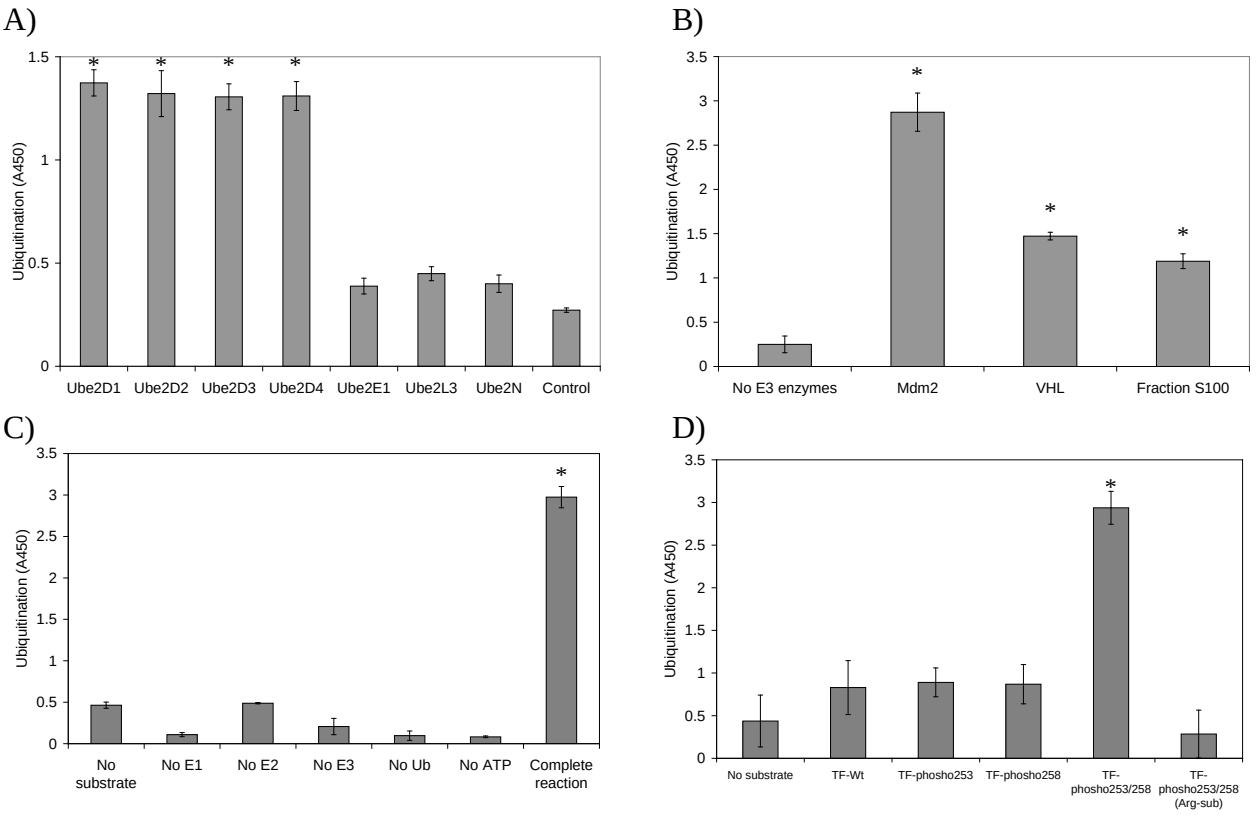
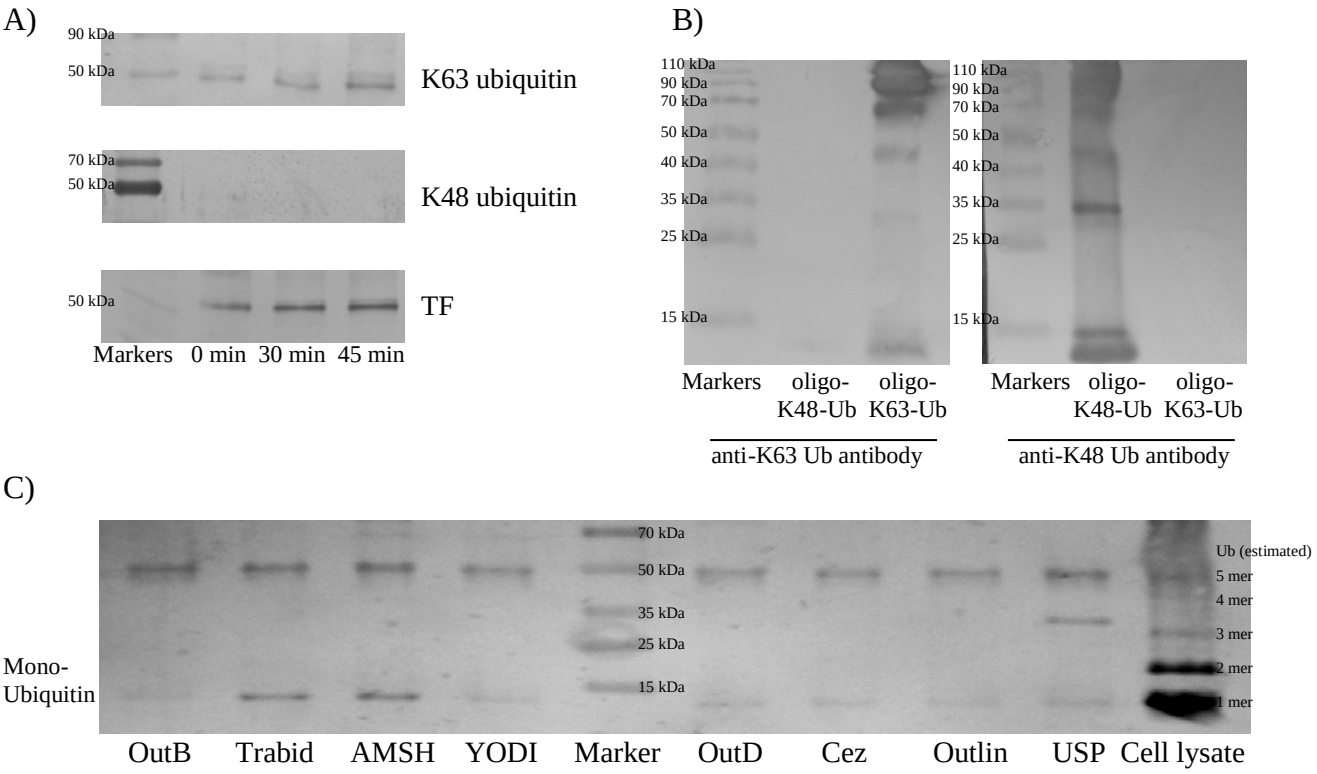


Figure 4



Target	OutB	Trabid	AMSH	YODI	Marker	OutD	Cez	Outlin	USP	Cell lysate
Linear								✓	✓	
K6				✓		✓			✓	
K11				✓		✓	✓		✓	
K27				✓					✓	
K29		✓		✓					✓	
K33		✓		✓					✓	
K48	✓								✓	
K63		✓	✓						✓	

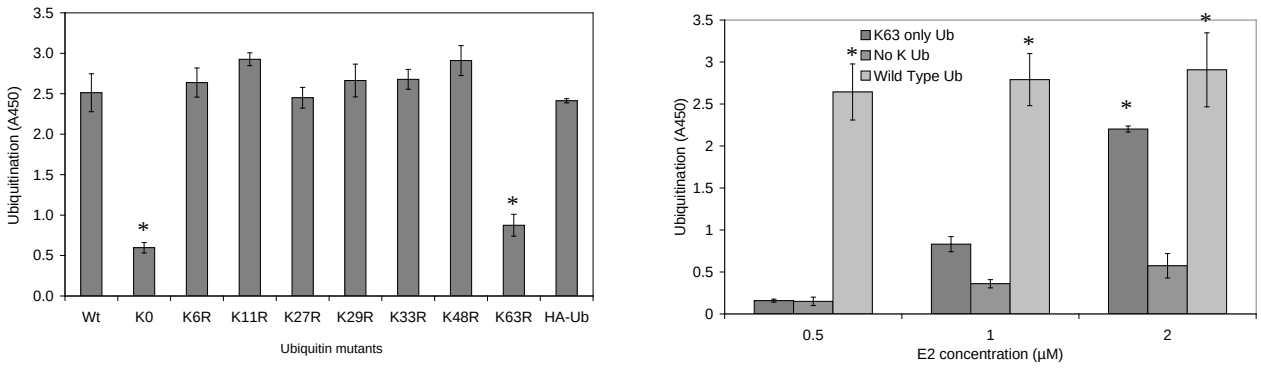
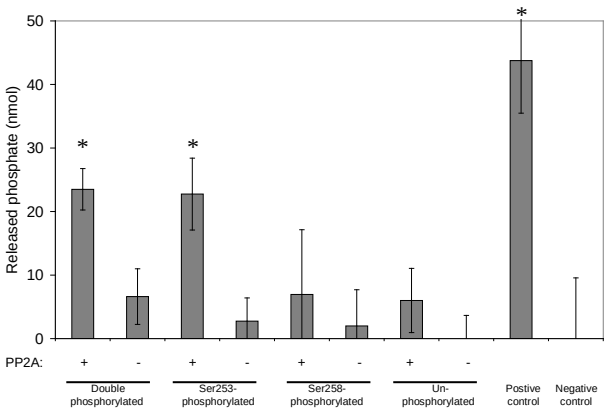


Figure 5

A)



B)

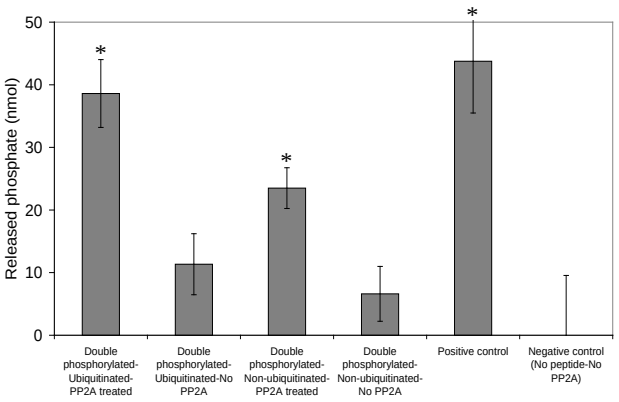
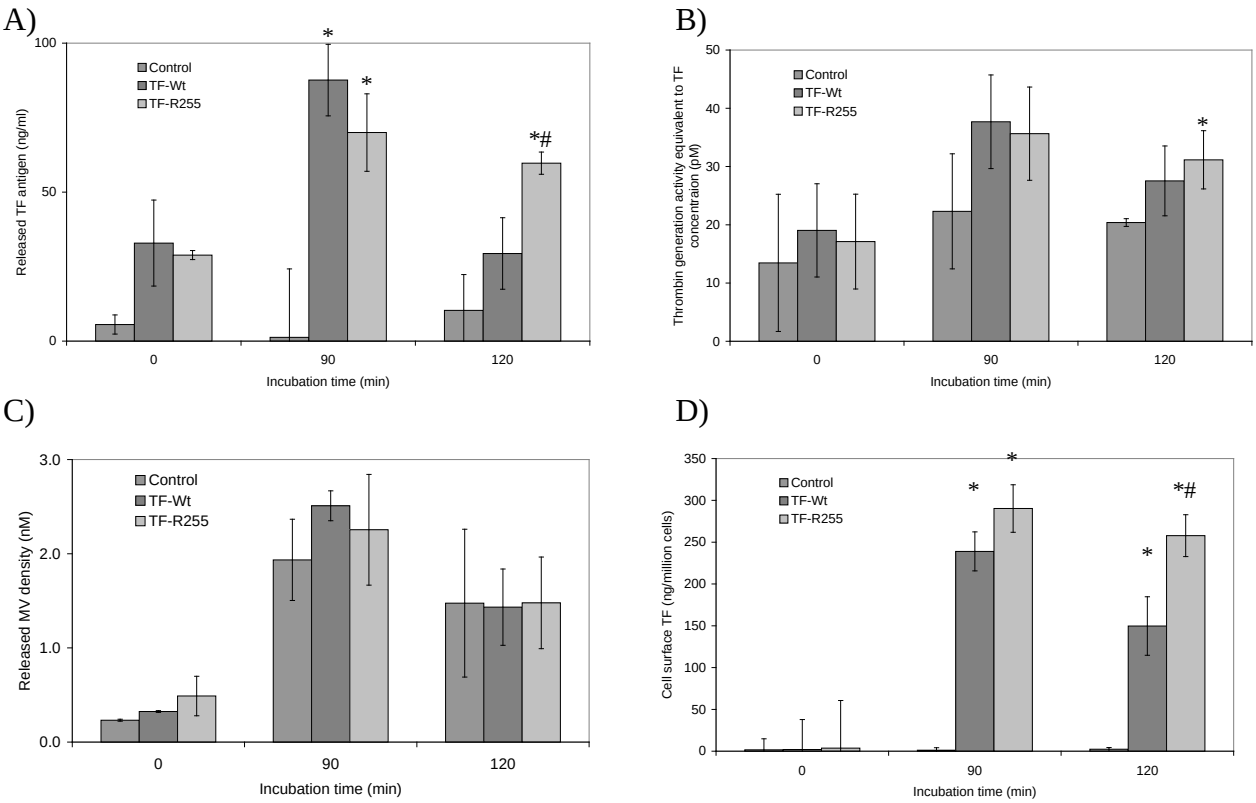
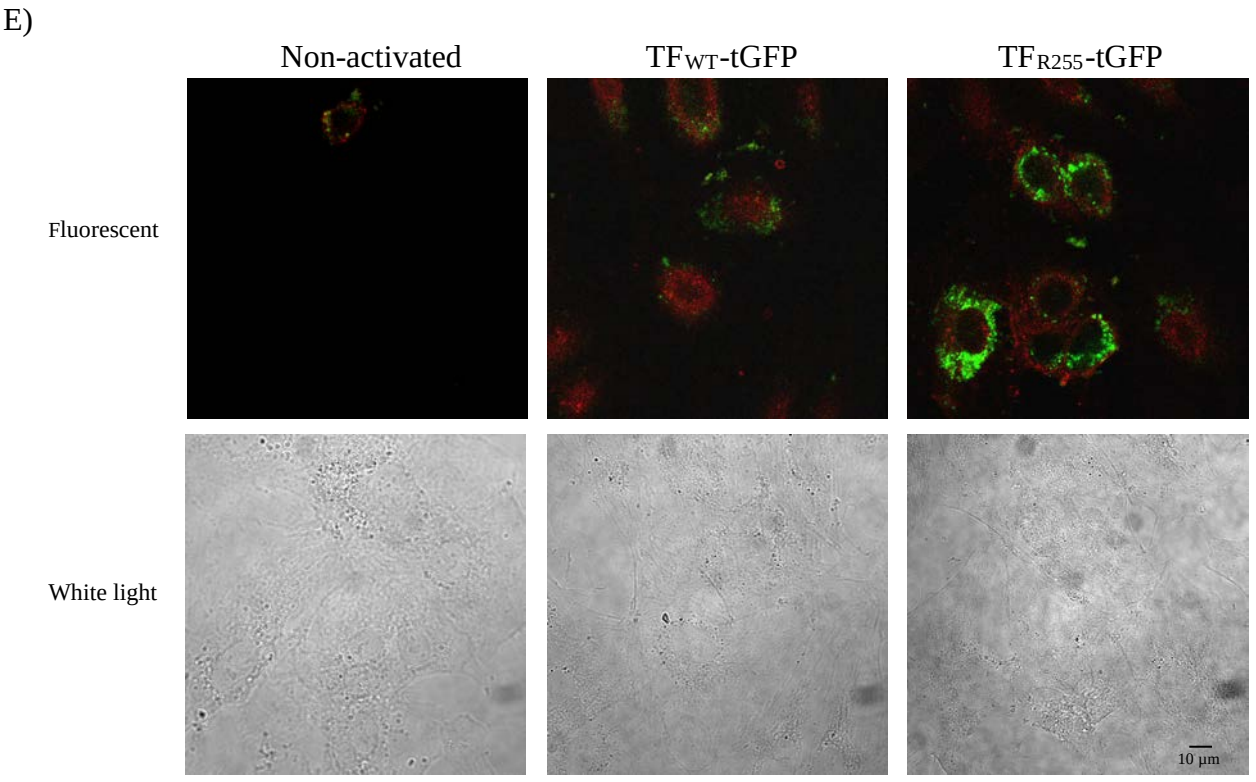


Figure 6





Green = TF-tGFP; Red = Alexa Fluor 647-conjugated annexin V

Figure 7