Peptidyl-prolyl isomerase 1 (Pin1) preserves the phosphorylation state of tissue factor and prolongs its release within microvesicles

Camille Ettelaie[a*], Mary EW Collier[b], Sophie Featherby[a], John Greenman[a] and Anthony Maraveyas[c]

From [a]Biomedical Section, Department of Biological Sciences, University of Hull, Cottingham Road, Hull, HU6 7RX, UK.

[b]Department of Cardiovascular Sciences, University of Leicester, Clinical Sciences Wing, Glenfield General Hospital, Leicester, LE3 9QP, UK.

[c]Division of Cancer-Hull York Medical School University of Hull, Cottingham Road, Hull, HU6 7RX, UK.

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Running title: *Pin1 prolongs TF release in MV*

*Correspondence to Dr 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

The exposure and release of TF is regulated by post-translational modifications of its cytoplasmic domain. Here, the potential of Pin1 to interact with the cytoplasmic domain of TF, and the outcome on TF function was examined. MDA-MB-231 and transfected-primary endothelial cells were incubated with either Pin1 deactivator Juglone, or its control Plumbagin, as well as transfected with Pin1-specific or control siRNA. TF release into microvesicles following activation, and also phosphorylation and ubiquitination states of cellular-TF were then assessed. Furthermore, the ability of Pin1 to bind wild-type and mutant forms of overexpressed TF-tGFP was investigated by co-immunoprecipitation. Additionally, the ability of recombinant or cellular Pin1 to bind to peptides of the C-terminus of TF, synthesised in different phosphorylation states was examined by binding assays and spectroscopically. Finally, the influence of recombinant Pin1 on the ubiquitination and dephosphorylation of the TF-peptides was examined. Pre-incubation of Pin1 with Juglone but not Plumbagin, reduced TF release as microvesicles and was also achievable following transfection with Pin1-siRNA. This was concurrent with early ubiquitination and dephosphorylation of cellular TF at Ser253. Pin1 co-immunoprecipitated with overexpressed wild-type TF-tGFP but not Ser258→Ala or Pro259→Ala substituted mutants. Pin1 did interact with Ser258-phosphorylated and double-phosphorylated TF-peptides, with the former having higher affinity. Finally, recombinant Pin1 was capable of interfering with the ubiquitination and dephosphorylation of TF-derived peptides. In conclusion, Pin1 is a fast-acting enzyme which may be utilised by cells to protect the phosphorylation state of TF in activated cells prolonging TF activity and release, and therefore ensuring adequate haemostasis.

1. Introduction

Precise control of the release of tissue factor (TF) as microvesicle by cells is crucial for adequate coagulation, without the risk of thrombosis. Consequently, the regulation of TF release by cells is subject to a number of mechanisms that act as positive and negative factors. Furthermore, TF appears to have a number of cellular functions, beyond that of initiating the coagulation mechanism [1-3]. Regulation of the incorporation of TF into cell derived microvesicles and also the termination of this process, entails mechanisms that include multiple post-transcriptional modifications of the cytoplasmic domain of TF [3-8]. The phosphorylation of TF by protein kinase c at serine 253 occurs following the de-palmitoylation of the cytoplasmic domain of TF [8] and permits the recruitment of filamin A which is essential for the incorporation of TF into microvesicles [9-11]. In contrast, the delayed phosphorylation of TF at serine 258 by p38 [5] appears to eventually lead to dephosphorylation of serine 253 within TF, by phosphatase PP2A [4]. Therefore an inescapable remaining question concerns the mechanism by which cells gauge the level of TF required/released for appropriate coagulation, and shut down the release process. Interestingly, double-phosphorylation of TF creates a suitable ubiquitination site within the cytoplasmic domain of TF which in turn may promote TF dephosphorylation [6]. Furthermore, the phosphorylation of serine 258 produces a phosphoserine-proline motif (termed an MPM-2 motif) which suggest the involvement of the enzyme peptidy-prolyl trans/cis isomerase 1 (Pin1) [12-14]. This enzyme is known to alter the structure of a number of proteins with a similar motif resulting in wide-ranging outcomes [14-17] including alteration in susceptibility of the target protein to dephosphorylation [16-19], ubiquitination [19,21] as well as the function and stability of the proteins [16,22-24]. In this study we examine the hypothesis that Pin1 is a crucial moderator which acts to regulate the

termination of the release of TF, through a mechanism that is dependent on the level of TF being released and therefore, the level of cellular activation.

2. Materials and Methods

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

MDA-MB-231 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). MDA-MB-231 cells and HCAEC (2×10^5) were transfected with 1 µg of pCMV6-Ac-TF-tGFP plasmid DNA (OriGene, Rockville, USA) or alternatively, mutant forms of the plasmid containing alanine-substitutions at position 258 (Ser→Ala) and position 259 (Pro→Ala) as previously described [4]. Transfection of the cells were carried out using Lipofectin (Invitrogen, Paisley, UK) as previously described in order to express the various types of TF [4]. Cells (2×10^5) were seeded out in 6-well plates and pre-adapted (1 h) to respective serum-free medium prior to experiments [4,9]. To induce microvesicle release, the cells were stimulated with protease activated receptor 2-activating peptide (PAR2-AP); SLIGRL; (20 µM). The released microvesicles were then isolated from conditioned media, resuspended in phosphate-buffered saline (PBS) and assessed according to published procedures [25]. The microvesicles were quantified using the functional assay Zymuphen MP assay kit (Hyphen BioMed/Quadratech Ltd, Epsom, UK) and the microvesicle density determined from the standards provided by the kit. Microvesicle-associated TF antigen and activity were measured using a TF-EIA kit and thrombin-generation assay, respectively [4,26,27]. Cell numbers in the

samples were determined by staining with crystal violet as previously described [28,29]. In some experiments the cells were pre-incubated with Juglone (5-hydroxy-1,4-naphthalenedione) (0-60 mM; Sigma Chemical Co. Ltd, Poole, UK) or Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (0-60 mM; Sigma) prior to activation. Other samples of cells were transfected with a specific set of Silencer®-siRNA (4 nM; Life Technologies, Paisley, UK) to suppress the expression of Pin1, or transfected with a comparable set of control siRNA (4 nM; Life Technologies) prior to activation. The concentration of the Pin1-siRNA was optimised and the downregulation in the expression of Pin1 confirmed prior to the experiments by ELISA and western blot analysis (Supplementary Fig 1). The expression of TF mRNA and protein in the cells as well as cell surface TF were monitored using RT-PCR, TF-EIA and flow cytometry as previously described [9,26].

2.2. Immunoprecipitation of TF protein

TF protein was immunoprecipitated from cell lysates using mouse or rabbit anti-human TF antibodies depending on the target analysis. Therefore, 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 for immunoprecipitation when the western blot analysis involved anti-rabbit antibody. In some experiments using transfected cells, TF-tGFP was immunoprecipitated using an with anti-tGFP-magnetic beads (clone 2H8) (25 µl) as previously outlined [6]. To ensure specificity, appropriate IgG isotypes (Cell Signalling Technology) were also included at identical concentrations alongside, as well as additional controls without any antibody. All samples were incubated at 4°C overnight with gentle shaking. Pureproteome protein A-magnetic beads (10 µl) (Merck-

Millipore) was 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, washed five times with PBST (1 ml) and the samples denatured in SDS-PAGE loading buffer (70 µl) (Sigma).

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 (when IP was carried out using 10H10 antibody) diluted 1:4000 (v/v) in TBST. Alternatively, 10H10 and/or HTF1 (eBioscience/ Thermo Scientific, Warrington, UK) antibodies were used when IP was with FL295 antibody (diluted 1:2000 and 1:4000 (v/v) in TBST respectively). The membranes were then washed with TBST and probed with goat anti-rabbit, or goat anti-mouse alkaline phosphatase-conjugated antibodies (Santa Cruz), diluted 1:4000 (v/v) depending on the source of the primary antibody, and incubated for 90 min. TF bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate (Promega Corp. Southampton, UK) and recorded. No species cross-detection between the primary and the secondary antibodies were observed (Supplementary Fig 2). In addition ubiquitination was detected in samples immunoprecipitated with FL295 antibody, using a mouse anti-human mono/poly-ubiquitin (FK2) antibody (Enzo) diluted 1:4000 (v/v), and developed as above [6]. Phosphorylation of Ser258 was detected in samples immunoprecipitated with 10H10 antibody 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 as previously described [4].

2.4. Pin1 binding assay

Substrate peptides, corresponding to the last 19 amino acids of the cytoplasmic domain of TF were synthesised in biotinylated form (Biomatik, Ontario, Canada). In total 4 peptides were synthesised in non-phosphorylated, single-phosphorylated and double-phosphorylated forms and used as substrates. An additional scrambled peptide (biotin-SWGNVSKLSAPRQGVNKE) was also included alongside. The peptides (5 µM final concentration) 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 to allow binding. The procedure was based on a variation of a previously described binding assay [30]. The wells were washed four times, each time with 300 µl of PBST. HRP-conjugated recombinant Pin1 protein was diluted 1:500 (v/v) in PBST, added to the wells (100 µl) and incubated for 1 h at room temperature. 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 by the addition of 2M sulphuric acid (50 µl) and absorptions measured at 450 nm using a plate reader. The cocnentrations of Pin1 were determined from a standard curve made using HRP-conjugated recombinant Pin1 protein. In addition, the time-course of interaction of Pin1 with the labelled penta-peptides, Succ-Glu-Asn-Ser-Pro-Leu-pNitroanilide and Succ-Glu-Asn-phosphoSer-Pro-Leu-pNitroanilide, was investigated by measuring the alteration in spectroscopic absorption of the solution using a procedure based on that described by Janowski et al [31]. Prior to the experiments the peptides (0.5 µM) were reconsituted in Tris-HCl buffer at a range of pH values ranging between 5.5-9.5. The absorption spectra of the peptide was analysed at 260-360 nm

range and the maximal change in the absorption spectrum was determined to be 315 nm (see results). The absorption spectrum of the recombinant HRP-conjugated Pin1 was also examined which was confimed not to absorb light at 315 nm (not shown). Samples (100 µl) of the peptides were in turn placed in a microcuvette, and recombinant HRP-conjugated Pin1 added (10 nM final concentration). The change in absorption at 315 nm over time was then monitored.

2.5. Determination of cellular Pin1 concentrations by ELISA

The concentrations of Pin1 protein in MDA-MB-231 cells and HCAEC (2×10^5) were determined using a Pin1-ELISA kit (Enzo Life Sciences, Exeter, UK) according to manufacturer's instructions. This procedure was also used to confirm the lack of Pin1 protein after knock out using siRNA as stated above.

2.6. Pin1 binding pull-down assay

A pull-down assay was carried out in a similar procedure to the binding assay with minor alterations. The procedure was carried out using cell lysate (100 µl from 2×10^5 cells) instead of recombinant Pin1-HRP and incubated for 1 h at room temperature. Following binding, the wells were washed with PBST and probed for Pin1 using the HRP-conjugated detection antibody from the Pin1-ELISA kit, as above. The concentrations of Pin1 were determined from a standard curve made using recombinant Pin1 protein from the ELISA kit. In similar experiments, the affinity purified proteins were dissolved in the electrophoresis sample buffer and analysed by western blotting, using a rabbit anti-Pin1 polyclonal antibody (GenTex-Insight Biotechnology Ltd, Wembley, UK) diluted 1:2000 (v/v) in TBST. The membranes were then developed using an alkaline-phosphatase conjugated goat anti-rabbit IgG secondary antibody, and visualised with Western blue substrate as described above.

2.7. Phosphatase assay

The phosphatase assay was carried out as previously described [6], using the biotinylated peptides representing the 19 amino acids of the cytoplasmic domain of TF. The peptides (10 µM) were diluted in phosphate-free Tris-HCl (50 mM) pH 7.5 (100 µl), adsorbed onto the NeutrAvidin 96-well plates and washed fours times with phosphate-free Tris-HCl (300 µl). 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) and $MgCl_2$ (10 mM). A similar set of samples were prepared but devoid of the enzyme. A positive control was prepared as above but containing the peptide KRpTIRR (10 µM) which is recommended as a positive control substrate by the phosphatase PP2A manufacturer. A negative control devoid of any substrate was also included. 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.

2.8. Ubiquitination assay

Ubiquitination reaction was carried out as before [6], using the E2-Screening kit (UBPBio/Caltag Medsystems, Buckingham, UK) and modified as described below to accommodate the various studies. The reactions (25 µl total volume) were set up to contain substrate peptide (5 µM), Ube1 enzyme (0.1 µM), Ube2D enzyme mix (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 recombinant Mdm2 (0.5 µM) (R&D Systems). The reactions were carried out at 37°C for 60 min and 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 60 min 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 (Enzo) diluted 1:500 (v/v) in PBST (100 µl). 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 by the addition of 2M sulphuric acid (50 µl) and absorptions measured at 450 nm using a plate reader.

3. Results

Prior to the study, the suppression of Pin1 using the Silencer® siRNA in MDA-MB-231 cells was optimised (Supplementary Fig 1). Downregulation of Pin1 by siRNA knockdown, or deactivation using Juglone had no influence on TF mRNA or protein levels or cell surface TF exposure compared to control siRNA or cells incubated with Plumbagin (Supplementary Fig 3). Moreover, the rate of cell proliferation was not significantly altered in cells transfected with Pin1 siRNA compared to those transfected with control siRNA (Supplementary Fig 4), although Pin1 is thought to interact with proteins involved in the M-phase of the cell cycle. In addition, the duration of incubation with Juglone/Plumbagin was not long enough to influence cell proliferation and cell numbers were unchanged. Therefore, these data indicate that other cellular alterations arising from Pin1 deactivation or knock-down, were not significant in the present studies.

3.1. Pin1 is required for the prolonged release of TF into microvesicles without affecting microvesicle release

Deactivation of Pin1 in MDA-MB-231 cells by incubation with Juglone, prior to the activation of the cells with PAR2-AP, reduced the release of TF within microvesicles in a Juglone-concentration dependent manner (Fig 1A). A similar pattern was observed in transfected HCAEC expressing TF-tGFP, but with a lower threshold (Fig 1B). In contrast, pre-incubation of the cells with the non-inhibiting analogue Plumbagin, or the vehicle DMSO did not alter the release of TF in either cell type. Importantly, pre-incubation of cells with Juglone did not alter the release of the microvesicles themselves (Fig 1C & 1D). Similarly, suppression of Pin1 expression in MDA-MB-231 cells and HCAEC using specific siRNA significantly reduced the release of TF, while the control siRNA was ineffective (Fig 1E & 1F). Moreover, the suppression of Pin1 using siRNA had no influence on microvesicle release (Fig 1G & 1H).

3.2. Pin1 protects the phosphorylation state of Ser253 in cells and prevents the ubiquitination of TF

The expression of Pin1 was suppressed in MDA-MB-231 cells using siRNA knockdown as above. A set of cells were transfected with control siRNA in parallel. The cells were activated using PAR2-AP and samples were collected at 15, 30 and 45 min. The TF was immunoprecipitated from the cell lysates and examined for TF phosphorylation at Ser253 and Ser258 by western blot analysis with maximal differences observed at 30 min (Fig 2A). It must be noted that due to the procedure used, the immunoprecipitated TF antigen is a purified sample and may not be used to compare the amount of cellular TF in these samples. Also, since no housekeeping control is available, all comparisons were made with respect to the immunoprecipitated TF antigen levels. In the absence of Pin1 the phosphorylation of Ser253 was largely diminished at any of the time points assessed while it was clearly detectable in the control cells at 15 and 30 min (Fig 2B). Interestingly, the phosphorylation state of Ser258 was not

significantly altered following suppression of Pin1 (Fig 2C). Furthermore, treatment of cells with Juglone but not Plumbagin also protected the phosphorylation state of Ser253 without altering the state of Ser258 (Supplementary Fig 5). No TF phosphorylation was detected in any of the samples or control prior to PAR2 activation (not shown). Similar studies using HCAEC produced only small amounts of TF probably due to double transfection procedure (Supplementary Fig 6) and therefore were not pursued further.

3.3. Pin1 preferably interacts with Ser258-phosphorylated TF but also with double-phosphorylated form

MDA-MB-231 cells were transfected to express the wild type-TF-tGFP, or alternatively with alanine-substitution at Ser258 or Pro259. The cell lysates were used to immunoprecipitate the expressed proteins using anti-tGFP-magnetic beads (clone 2H8). An additional sample was immunoprecipitated using anti-TF from the lysate of cells expressing wild type-TF-tGFP, and used for comparison. Examination of the immunoprecipitated products by western blotting, using an anti-Pin1 polyclonal antibody, indicated the co-purification of Pin1 with wild-type TF and the wild-type TF-tGFP (Fig 3A). In contrast, very low levels of Pin1 were detected in the sample containing the TF-tGFP with Pro259→Ala-substitution (<20% of the wild-type TF), and in the sample with TF-tGFP Ser258→Ala-substitution (<10% of the wild-type TF) (Fig 3B). Similar studies using HCAEC produced only small amounts of TF as stated above, and therefore were not pursued further. In order to determine the preferred form of TF as a substrate for Pin1, four peptides corresponding to the 19 amino acids of the C-terminus of TF were synthesised in unphosphorylated, Ser253-phosphoryalted, Ser258-phosphorylated and double-phosphorylated. A scrambled peptide (biotin-SWGNVSKLSAPRQGVNKE) was included. The peptide were prepared with a biotin tag and adsorbed in NeutrAvidin 96-well plates. The plates were then

incubated with a range of concentrations of recombinant HRP-conjugated active Pin1, washed and the interactions detected using TMB substrate. HRP-Pin1 appeared to interact with Ser258-phosphorylated peptide preferentially and to a lesser extent with the double-phosphorylated peptide (Fig 3C). Interestingly, the interaction with Ser253-phosphorylated peptide was the least favourable. An attempt was made to determine the relative dissociation constants (Fig 3D & Table 1). However since Pin1 may interact with peptides differently to the TF molecule, these are relative values only, and are therefore likely to be dissimilar to the real dissociation constants values between Pin1 and TF, or the peptide motifs reported previously [32]. Similarly, incubation of the NeutrAvidin-anchored peptides with cell lysate resulted in the interaction of cellular Pin1 with the peptides (Fig 3E) which also mirrored the pattern observed using the recombinant Pin1. Interestingly, while the values were much lower than those observed with HRP-conjugated recombinant Pin1, the differences between the four peptides were more discernable when examining cellular Pin1 by pull down assay. Analysis of purified proteins by western blot showed the presence of Pin1 as faint bands with a molecular weight of around 18 kDa (Fig 3F) however, the quantity of the purified Pin1 was far too small to permit any meaningful quantification using this technique. In an attempt to determine the speed of the reaction, smaller penta-peptides corresponding to residues 256-260 (encompassing Ser258 but not Ser253) were synthesised with p-nitroanilide on the C-terminus. This procedure permits the measurement of changes in the environment of the nitroanilide by spectroscopic analysis and would indicate any interaction between Pin1 and the peptides [31]. However, the procedure is only relative and indicative, and therefore no attempt was made to derive further kinetic parameters for the interactions. The peptides were synthesised in non-phosphorylated and Ser258-phosphorylated format, and the spectra analysed at various pH values. The wavelength of

maximal change was determined to be 315 nm for both peptides (Fig 3G). Incubation of the Ser258-phosphorylated peptide with Pin1 resulted in rapid change in absorption at 315 nm and was slower in the unphosphorylated peptide (Fig 3H).

3.4. Pin1 can interfere with ubiquitination of TF by ubiquitin ligase complex

Suppression of Pin1 in MDA-MB-231 cells resulted in the early ubiquitination of TF (Fig 4A) which was clearly visible at 15 min in the cells devoid of Pin1 but only became significant at 30 min in the control cells (Fig 4B). This ubiquitination was associated with the activation of the cells and was absent in prior to PAR2 activation, in cells devoid of Pin1 (supplementary Fig 7). Moreover, the examination of the gel did not show multiple banding or smearing associated with poly-ubiquitination (supplementary Fig 8) and is in agreement with our previous study [6]. However, the multiple banding pattern of the oligoubiquitinated TF protein was less visible than previously reported by us. This was deemed to be as a consequence of the lower number of cells used in the experiments, as well as the transient nature of the TF oligoubiquitin chain which was short lived in MDA-MB-231 cells [6]. Additionally, due to immunoprecipitation procedure, the protein content analysed was a fraction of the cellular TF and not representative of the total cellular TF. Again, the amounts of recovered TF from double-transfected HCAEC were too low to determine the values reliably and are not presented. Double-phosphorylated TF peptide was ubiquitinated by the ubiquitin ligase complex containing Ube2D family of E2 ligases and Mdm2 as the E3 ligase [6] in the presence and absence of recombinant Pin1. Addition of recombinant Pin1 (1-10 nM) to the ubiquitination reaction suppressed the ubiquitination of the TF-peptide in a concentration-dependent manner (Fig 4C). However, since the experiment was carried out under static conditions i.e. the product is not removed from the reaction, higher concentrations of Pin1 would result in the increased isomerisation in both directions and were not deemed reliable or

examined further. Analysis of the collected microvesicles did not show any ubiquitination of TF (not shown).

3.5. Pin1 can interfere with TF dephosphorylation

The inclusion of Pin1 into the reaction reduced the ability of recombinant phosphatase PP2Aα to release inorganic phosphate from the double-phosphorylated TF peptide (Fig 5A) but not the Ser253-phosphorylated peptide (Fig 5B). Replacement of the double-phosphorylated TF peptide with a ubiquitinated form liberated a significantly higher amount of inorganic phosphate by PP2Aα (Fig 5C) but was reduced in a similar manner to the non-ubiquitinated peptide, in the presence of Pin1. Finally, to determine the potential of Pin1 to interfere with TF processing within cells, the concentration of Pin1 was measured using an ELISA system. The concentration of Pin1 was determined to be 44.8 nM ± 0.5 in MDA-MB-231 cells while the levels present in HCAEC was determined to be measured 19.3 nM ± 1.7.

3.6. Structural analysis of the outcome of Pin1-mediated isomerisation of TF

Analysis of the NMR structure of the cytoplasmic domain of TF (2CEF) [33] obtained from the databank showed that the Lys255 is present at the surface of the protein (Fig 6A) in 9 out of the 10 prevelent structures (not included). In one remaining structure, Lys255 is only partially covered by the side chain of Asn261. However, since the Asn261 appears exceptionally flexible it would not interefer with the head group of Lys255. Importantly, Lys255 appears to form hydrogen bonds with the backbone carboxy groups from Asn257, phosphoSer258 and Pro259 (Fig 6B) and to a lesser extent with the backbone carboxy group of Glu256 (Table 2). These interactions appear to anchor the ε-amino group of Lys255 allowing only limited movement which was found to be 4.02 Å laterally (across the protein surface), and up to 2.44 Å away from the surface of the protein, measured by calculating the greatest relative movemet of the ε-amino

group betwen all 10 structures. In addition, the acyl group of Lys255 appears to be tightly secured through interaction with Trp254 with little flexibility permitted. The formation of the hydrogen bonds suggests that the protons associated with Lys255 headgroup would face away from the protein surface allowing the exposure of the nitrogen atom. Moreover, the tight control of the position of this ε-amino group indicates that it may be a suitable site for enzyme-mediated nucleopohilic interactions such as ubiquitination by ubiquitin ligases. Interestingly, isomerisation of the phosphoSer258-Pro259 peptide bond by Pin1 (Fig 6C) renders the third carboxy group (Pro259) unavailable for hydrogen bond formation with Lys255 (not shown) and therefore is likely to cause the loss of the availability of the nitrogen atom from Lys255 for amide-esterification, and will hamper ubiquitination.

4. Discussion

Cells are known to respond to damage and stress by releasing procoagulant microvesicles which may contain TF [34-37]. The release of these microvesicles from the activated cells complements the mechanisms that ensure the formation of an adequate clot and containment of the injury. However, since excessive levels of coagulation can be detrimental, it is essential that the cells can adjust the magnitude of their response to that of the injury. Consequently, cells need to detect the level of injury and gauge the amount of released TF accordingly. Moreover, once sufficient clot has been generated, the mechanism of TF release will have to be shut down rapidly. The release of TF as cell-derived microvesicles appears to be regulated through an ordered and time-dependent pathway which include post-transcriptional modifications of the cytoplasmic domain of this protein. As a result, each step permits the interaction of TF with components in the next step resulting in the release or endocytosis of TF. Consequently, there

must also be factor(s) which strictly control the incorporation of TF into microvesicles and terminate the process once a "required amount of TF" has been released. One protein known to be a regulator of post-phosphorylation processes is Pin1 [15-24]. Normally peptide bond *trans*↔*cis* isomerisation occurs at a slow rate in nature since both of these forms are stable and the energy required for isomerisation is relatively high. However, the rotation of the peptide bond between the phosphoserine and the proline (Fig 6C) may be facilitated by enzymes including Pin1. Additionally, since the *trans* configuration is the more prevalent of the two isomers, Pin1 preferentially catalyses the formation of the *cis* isomer. The *cis* isomer is often not accessible to enzymes which bring about subsequent modifications including ubiquitination [19,21] and de-phosphorylation [38]. Conversely, proteins may become more accessible to such alternations as a consequence of isomerisation which promotes the subsequent modification. Therefore, Pin1 can act as a means of accelerating or delaying further processing of proteins [38] and has been shown to be a regulator of diverse mechanisms including cell cycle regulation [22,23,40-42], receptor function [42] and protein stability [16,22-24].

In our study, the ability of Pin1 to alter the incorporation of TF into microvesicles was clearly reduced in a concentration-dependent manner, through deactivation of Pin1 with Juglone but not its non-inhibiting analogue, Plumbagin (Fig 1). These two compounds differ by one methyl group and have similar activities in the cell except in the ability to deactivate Pin1 [44-46]. These data were also confirmed by the suppression of Pin1 expression using specific siRNA. Interestingly, deactivation of Pin1, or the suppression of its expression did not alter the release of microvesicles. This is in agreement with our previous studies which suggest that the incorporation of TF into microvesicles, and the process of microvesicle release, are mediated through distinct and separate mechanisms [4]. Although ultimately, the outcome of these two

mechanisms do overlap. Importantly, the ability of Pin1 to protect TF release appears to be through preserving the phosphorylation state of Ser253 within the cytoplasmic domain of TF (Fig 2). The phosphorylation of this amino acid is required for the recruitment of filamin A which is essential for the incorporation of TF into microvesicles [9-11].

Ser258→Ala-substittion of TF diminished the co-purification of Pin1 (<10%; $p < 0.01$) with mutant TF-tGFP, while Pro259→Ala-substittion of TF-tGFP was less effective (<20%; $p < 0.01$) (Fig 3A & 3B). This is in agreement with the structural analyses since the carboxy group of Pro259 would be needed for TF phosphorylation of Ser258 and possibly ubiquitination of Lys255 (see Fig. 6B). Furthermore, Pin1 preferentially interacted with Ser258-phosphorylated TF peptide and to a lesser extend with the double-phosphorylated peptide, but had a much lower affinity for the Ser253-phosphorylated peptide (Fig 3C-F).

In addition, the inclusion of Pin1 into the *in vitro* phosphatase reaction, hindered the de-phosphorylation of the double-phosphorylated TF peptide by PP2Aα, but was not as effective in protecting the Ser253-phosphorylated peptide (Fig 5). Together these findings suggest that following the phosphorylation of Ser258 within the cytoplasmic domain of TF, the formation of phospho-serine-proline epitope permits the interaction of Pin1 with double-phosphorylated TF. Since, the majority of TF is in the *trans* configuration, isomerisation by Pin1 favours the formation of the *cis* isomer. This form of TF is likely to be capable of interacting with filamin A and therefore be released, since Ser253 is located on the adjacent side of the protein [5,33] and is not directly affected. Moreover, the *cis* isomer appears to be less prone to dephosphorylation by PP2Aα. However, neither the *trans* or *cis* isomers of TF appeared to be ideal substrates for PP2Aα (Fig 5). In contrast, the rate of TF de-phosphorylation was greatly enhanced following ubiquitination of TF. Importantly, Pin1 was capable of effectively disrupting the ubiquitination

of TF peptide by the ubiquitin ligase complex (Fig 4C) as well as the dephosphorylation of the ubiquitinated peptide (5C). This was further supported by the suppression of Pin1 expression which resulted in accelerated ubiquitination of TF in cells (Fig 4B). Together, these observations suggest that the main mode of action of Pin1 is through the prevention of ubiquitination of the cytoplasmic domain of TF, which is essential for the rapid de-phosphorylation of Ser253 within TF, by PP2Aα (Fig 7). Consequently, it is possible that the *cis* isomer of TF may be incorporated and released into microvesicles unhindered. This is not in agreement with our previous report which indicated that endothelial-derived microvesicles do not contain double-phosphorylated TF [4]. Nonetheless, the concentration of Pin1 within normal primary cells was determined to be in the range of 19 nM. Therefore, it is likely that depending on the amount of TF being released, arising from the level of injury/trauma, cellular Pin1 may become saturated. As a result, a small amount of TF may become ubiquitinated, de-phosphorylated and possibly endocytosed [6]. Since Ser258-phosphorylated TF appears to be the preferred substrate for Pin1 (Fig 3), the sequestration of Pin1 to Ser258-phosphorylated TF further reduces the amount of available Pin1 (Fig 7). Consequently, the reduction in the rate of isomerisation of double-phosphorylated TF would lead to the progressively accelerated TF ubiquitination and the abrupt termination of TF release. Therefore at least in normal cells, the majority of TF will be released in the Ser253-phposphorylated form and only a minor amount will be released in the double-phosphorylated form. This explains the lack of detectable phosphorylated Ser258 in endothelial cells as reported in our previous study [4]. Furthermore, it is interesting to note that tumour cells such as MDA-MB-231 cells often contain much higher levels of Pin1 (44.8 nM) which may reach concentrations of up to 500 nM in some cancer cells [39,47-49]. This explains how these cells retain their ability to release TF at high rates even in the presence of augmented expression levels

of TF. In addition, ubiquitinated receptors are often endocytosed through ubiquitin-dependent receptors [49-53] which explains how the mechanism of termination is effective in rodents, without the need for dephosphorylation of a serine residue which lacks in murine.

The regulation of incorporation and release of TF within cell-derived microvesicles appears to involve Pin1 as a means by which cells gauge the level of TF required and released for appropriate coagulation. Although there are some difference in the induction of TF activation/release, the general mechanisms of the incorporation into microvesicles appear to have much in common. As examples, ATP [54] and PAR2-dpendent [9] activation both appear to involve the cytoskeletal protein filamin A as an essential component if TF incorporation into the microvesicles. In fact, we recently have shown that the interaction of filamin A is particularly dependent on the phosphorylation of Ser253 within TF alone and this common mechanism occurs following the activation of cells with either PAR2-agonist or FVIIa [55]. We envisage that our model of PAR2-induced microvesicle release may occur a) by vascular cells in response to local activation of the coagulation mechanisms amplifying the response, and also b) as a consequence of exposure of TF-carrying cancer cells to the bloodstream [56] and may be precursor to tumour-induced thrombosis shown using *in vivo* models [57]. In fact, we have previously shown that TF-containing microvesicles are capable of interacting with extracellular matrices, fibronectin and collagen [28]. Therefore, it is likely that although various types of cells may respond to different agonist, through diverse signalling pathways that converge and culminate in common mechanisms of TF release that play an active role in coagulation/thrombosis. However, these initial signalling pathways may also be the determinants for the incorporation of other receptors, proteins and molecules which instruct the behaviour of the microvesicles and the cells/tissue that they interact with.

In conclusion, Pin1 is a fast acting enzyme capable of interacting with the cytoplasmic domain of TF and protecting this protein from ubiquitination and the subsequent dephosphorylation. Therefore, Pin1 acts as a gauge for the amount of TF being released by the cells and therefore functions to determine the level of injury incurred.

Conflicts of interest

None declared.

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Figure Legends

Table 1. Relative dissociation constants for the interactions of Pin1 with TF-peptide interaction. The substrate peptide corresponding to the last 19 amino acids of the cytoplasmic domain of TF were synthesised in biotinylated form as non-phosphorylated, single-phosphorylated and double-phosphorylated forms. The peptides (5 μM final concentration) were adsorbed onto NeutrAvidin-coated 96-well plate and the wells were washed. The samples were incubated with HRP-conjugated recombinant Pin1 protein diluted in PBST (0-20 nM) and added to wells (100 μl) and incubated for 1 h at room temperature. The wells were then washed a further four times and developed with TMB One Solution (100 μl) and the absorptions measured at 450 nm using a plate reader. The relative dissociation constant values were calculated from the curves and the dissociation constants expressed in terms of Pin1 concentration.

Table 2. Analysis of the interaction of Lys255 with surrounding amino acid groups. The 10 NMR structure of the cytoplasmic domain of TF were obtained from the databank (2CEF) and the distance between the nitrogen of the ε-amino group within Lys255 and the oxygen within the carboxy groups of Glu256, Asn257, PhosphsSer258 and Pro259 were examined. The percentage of incidence, where the two atoms were proximal was determined for each set of amino acid within the 10 structures. In addition, the average distances between the nitrogen of the ε-amino

group within Lys255, and the oxygen within the carboxy groups of respective amino was also calculated.

Fig. 1 The influence of Pin1 on the release of TF within microvesicles. MDA-MB-231 cells and transfected HCAEC expressing TF-tGFP (2×10^5) were pre-adapted to serum-free medium and pre-incubated with Juglone (0-60 nM) or Plumbagin (0-60 nM) for for 60 min. The cells were then activated with PAR2-AP (20 μM) and microvesicles were isolated from the conditioned media of the cells at 30 and 90 min post-activations, respectively. The concentration of TF in A) MDA-MB-231 cells and B) HCAEC was determined using a TF-EIA and differences were assessed by ANOVA (n = 3; * = $p<0.05$ & ** = $p<0.01$ vs the vehicle control sample without any reagent). Microvesicle densities were also determined in C) MDA-MB-231 cells and D) HCAEC using the Zymuphen microparticle assay kit and differences were assessed by ANOVA. (n = 4; ** = $p<0.01$ vs the non-activated sample). MDA-MB-231 cells with a specific siRNA to suppress Pin1 expression or a control siRNA prior and incubated for 48 h. HCAEC (2×10^5) were also co-transfected to express TF-tGFP, together with siRNA to suppress Pin1 expression or a control siRNA, and incubated for 48 h. The cells were then pre-adapted to serum-free medium and activated with PAR2-AP (20 μM). Microvesicles were isolated from the conditioned media of the cells and the concentration of TF in E) MDA-MB-231 cells and F) HCAEC was determined using a TF-EIA and differences were assessed by ANOVA (n = 4; ** = $p<0.01$ vs the non-activated sample # = $p<0.01$ vs the control siRNA sample). Microvesicle densities were also determined in G) MDA-MB-231 cells and H) HCAEC using the Zymuphen microparticle assay kit and differences were assessed by ANOVA (n = 4; ** = $p<0.01$ vs the non-activated sample).

Fig. 2 The influence of Pin1 on TF phosphorylation at Ser253 and Ser258. MDA-MB-231 cells (2×10^5) were transfected with a specific siRNA to suppress Pin1 expression or a confirmed control siRNA prior and incubated for 48 h. The cells were pre-adapted to serum-free medium and then activated with PAR2-AP (20 μM). Cells were lysed in phosphosafe buffer and the TF was immunoprecipitated using a mouse anti-human TF antibody (10H10; 4 μg) and captured using protein A-magnetic beads. The precipitate was washed four times with PBST and briefly boiled in electrophoresis loading buffer and separated by electrophoresis on a denaturing 12% (w/v) polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane and blocked. A) TF phosphorylation at Ser258 was detected using a rabbit anti-phosphoserine-TF antibody 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 diluted 1:2000 (v/v) in TBST buffer. Total TF protein was also detected alongside using a rabbit anti-human TF antibody (FL295) diluted 1:2000 (v/v). After washes with TBST all membranes were developed with goat anti-rabbit alkaline phosphatase-conjugated antibody diluted 1:4000 (v/v) and then visualised using the Western Blue stabilised alkaline phosphatase-substrate and recorded (the micrographs are representative of 3 experiments). B) The band densities of the phosphorylated TF were measured at 15, 30 and 45 min post-activation using the ImageJ programme and calculated as a ratio of total TF protein for B) Ser253-phosphorylated TF and C) Ser258-phosphorylated TF. Differences were assessed by ANOVA ($n = 4$; * = $p<0.05$ vs the respective non-activated sample).

Fig. 3 Analysis of the interaction of recombinant Pin1 with TF-derived peptides. Cells were transfected to overexpress wild-type TF-tGFP or alternatively mutants with substitutions at Ser258 or Pro259. TF-tGFP was immunoprecipitated using anti-tGFP-magnetic beads (clone 2H8) (25 µl). In some samples, TF protein was immunoprecipitated from cell lysates using a rabbit anti-human antibody (FL295) (4 µg per sample) and then purified using Pureproteome protein A-magnetic beads. A) The purified samples were separately probed with a rabbit anti-Pin1 polyclonal antibody, TF (10H10) and tGFP (2H8) by western blot analysis. B) The intensity of the bands were measured and the amount of the co-immunoprecipitated Pin1 was quantified by determining the intensity of Pin1 bands as a ratio the intensity of tGFP. Differences were assessed by ANOVA ($n = 3$; * = $p<0.01$ vs the wild-type TF-tGFP sample/immunoprecipitated with 2H8 antibody). C) The substrate peptide corresponding to the last 19 amino acids of the cytoplasmic domain of TF were synthesised as biotin conjugates, in non-phosphorylated, single-phosphorylated and double-phosphorylated forms. A scrambled peptide was also included. The peptides (5 µM final concentration) were diluted to 100 µl with PBS and distributed (50 µl per well) in a NeutrAvidin-coated 96-well plate and incubated for 2 h at room temperature. The wells were washed four times with PBST (300 µl). HRP-conjugated recombinant Pin1 protein was diluted in PBST (2 nM) and added to wells (100 µl) and incubated for 1 h at room temperature. The wells were then washed a further four times and developed with TMB One Solution (100 µl). The reactions were terminated by the addition of 2M sulphuric acid (50 µl) and absorptions measured at 450 nm using a plate reader. Differences were assessed by ANOVA ($n = 5$; * = $p<0.05$ vs the scrambled peptide). D) The procedure was repeated out in triplicate at a range of Pin1 concentrations (0-20 nM) and the absorptions recorded at each concentration. E) The Pin1-binding experiment was also repeated as above but by subsituting recombinant Pin1

with cell lysate. The samples were then probed with and HRP-conjugated anti-Pin1 antibody, washed and developed with TMB One Solution (100 µl). The recordings were obtained as above and differences were assessed by ANOVA (n = 3; * = $p<0.05$ vs the scrambled peptide). F) Samples of the affinity-purified Pin1 from C were dissolved in the electrophoresis loading buffer and analysed by western blot. The samples were probed using a rabbit anti-human Pin1 polyclonal antibody diluted 1:2000 (v/v) in TBST. The membranes were then washed with TBST and probed with goat anti-rabbit alkaline phosphatase-conjugated antibody diluted 1:4000 (v/v) and incubated for 90 min. Pin1 bands were then visualised using the Western Blue stabilised alkaline phosphatase-substrate and recorded (the micrographs are representative of 3 experiments). G) Labeled penta-peptide Succ-Glu-Asn-Ser-Pro-Leu-pNitroanilide and Succ-Glu-Asn-phosphoSer-Pro-Leu-pNitroanilide were synthesisd and reconstituted (0.5 µM) in Tris-HCl buffer at a range of pH values (5.5-9.5). The absorption spectra of the peptide was analysed at 260-360 nm range and the maximal change in the absorption spectrum was determined. The wavelength with the maximal absorption change was determined to be 315 nm. H) Samples (100 µl) of the peptides were in turn placed in a microcuvette, and recombinant HRP-conjugated Pin1 added (10 nM final concentration). The change in absorption at 315 nm over time was then monitored. (n = 5).

Fig. 4 The influence of Pin1 on TF ubiquitination. MDA-MB-231 cells (2×10^5) were transfected with a specific siRNA to suppress Pin1 expression or a confirmed control siRNA prior and incubated for 48 h. The cells were then pre-adapted to serum-free medium and activated with PAR2-AP (20 µM). Cells were harvested at 15 and 30 min post-activation and lysed in phosphosafe buffer and the TF was immunoprecipitated using a rabbit anti-human TF antibody

(FL295; 4 µg) and captured using protein A-magnetic beads. The precipitate was washed four times with PBST and briefly boiled in electrophoresis loading buffer and separated by electrophoresis on a denaturing 12% (w/v) polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane and blocked. A) TF ubiquitination was detected using a mouse anti-human mono/poly-ubiquitin (FK2) antibody diluted 1:4000 (v/v). Total TF protein was detected using a mouse anti-human TF antibody (10H10) diluted 1:2000 (v/v). The membranes were then washed with TBST and probed with goat anti-mouse alkaline phosphatase-conjugated antibodies diluted 1:4000 (v/v) and TF bands were visualised using the Western Blue stabilised alkaline phosphatase-substrate and recorded (the micrographs are representative of 3 experiments). B) The ubiquitinated TF band densities were measured at 15, 30 and 45 min post-activation, using the ImageJ programme and determined as a ratio of the total TF protein bands. Differences were assessed by ANOVA ($n = 5$; * = $p<0.05$ vs the non-activated sample). C) Ubiquitination reaction was carried out in the presence of a range of concentrations of Pin1 (0-10 nM). The reaction was prepared to contain the double-phosphorylated TF peptide as substrate (5 µM), Ube1 enzyme (0.1 µM), an equal mix of Ube2D enzyme mix (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 recombinant Mdm2 (0.5 µM). The reactions were carried out at 37°C for 60 min and then diluted to 100 µl with PBS and distributed (50 µl per well) in a NeutrAvidin-coated 96-well plate and incubated for 1 h at room temperature. 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). 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. Differences were assessed by ANOVA (n = 5; * = $p<0.05$ vs the sample without any Pin1).

Fig. 5 The influence of Pin1 on TF dephosphorylation by PP2A. Three sets of TF peptides A) Ser253-phosphorylated, B) double-phosphorylated and C) ubiquitinated-double-phosphorylated (10 µM) 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). Pin1 (1-10 nM) was added to each set of the samples Each reaction (25 µl) was prepared by reconstituting 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) and $MgCl_2$ (10 mM). A positive control was prepared as above but containing the peptide KRpTIRR (10 µM) as the recommended control substrate by the phosphatase PP2A manufacturer. A negative control devoid of substrate was also included. 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 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. Differences were assessed by ANOVA (n = 5; * = $p<0.05$ vs the sample without any PP2A; # = $p<0.05$ vs the postive control).

Fig. 6 Structural representation of the cytoplasmic domain of TF and the *trans* and *cis* forms of phosphoserine-proline motif. A) Molecular surface model of the cytoplasmic domain of TF (amino acids 247-263) depicting the position of Lys255 (blue) on the surface, Trp254 (brown) and the carboxy groups of Asn257, pSer258 and Pro259 (red) as well as the phospho group of Se258 (yellow + red). B) stick model of the cytoplasmic domain of TF (amino acids 252-259)

showing the interaction of the head group of Lys255 (blue) with the carboxyl groups of Asn257, pSer258 and Pro259 (red). C) The phosphoserine-proline motif exists in two configurations. The energetically unfavourable isomerisation is catalysed by Pin1 through the rotation of peptide bond (round arrow). The isomerisation alters the direction of the peptide (straight arrows) altering the accessible motifs/domains.

Fig. 7 Proposed mechanism of the regulation of TF release by Pin1. The phosphorylation of the cytoplasmic domain of TF permits its interaction with filamin A and release within microvesicles. Protein isomerisation by Pin1 (A), allows further TF release (B) by preventing the ubiquitination of Lys255 (C) and the subsequent dephosphorylation of Ser253 (D). However, the eventual ubiquitination and dephosphorylation of TF at Ser253 sequesters Pin1 away to the more favourable substrate (Ser258-phosphorylated) resulting the progressively accelerated TF ubiquitination and dephosphorylation (C & D) and termination of TF release.

Table 1

Peptide	Relative Kd (nM)
Unphosphorylated TF peptide	3.25
Ser253-phosphorylated peptide	3.62
Ser258-phosphorylated peptide	0.79
Double-phosphorylated peptide	1.53

Table 2

Amino acid	Number of incidence	Average distance (Å)
Glu256	60%	4.20 ± 0.91
Asn257	80%	3.88 ± 0.95
phospho-Ser258	100%	2.90 ± 0.14
Pro259	70%	3.99 ± 0.64

Figure 1 MDA-MB-231 HCAEC

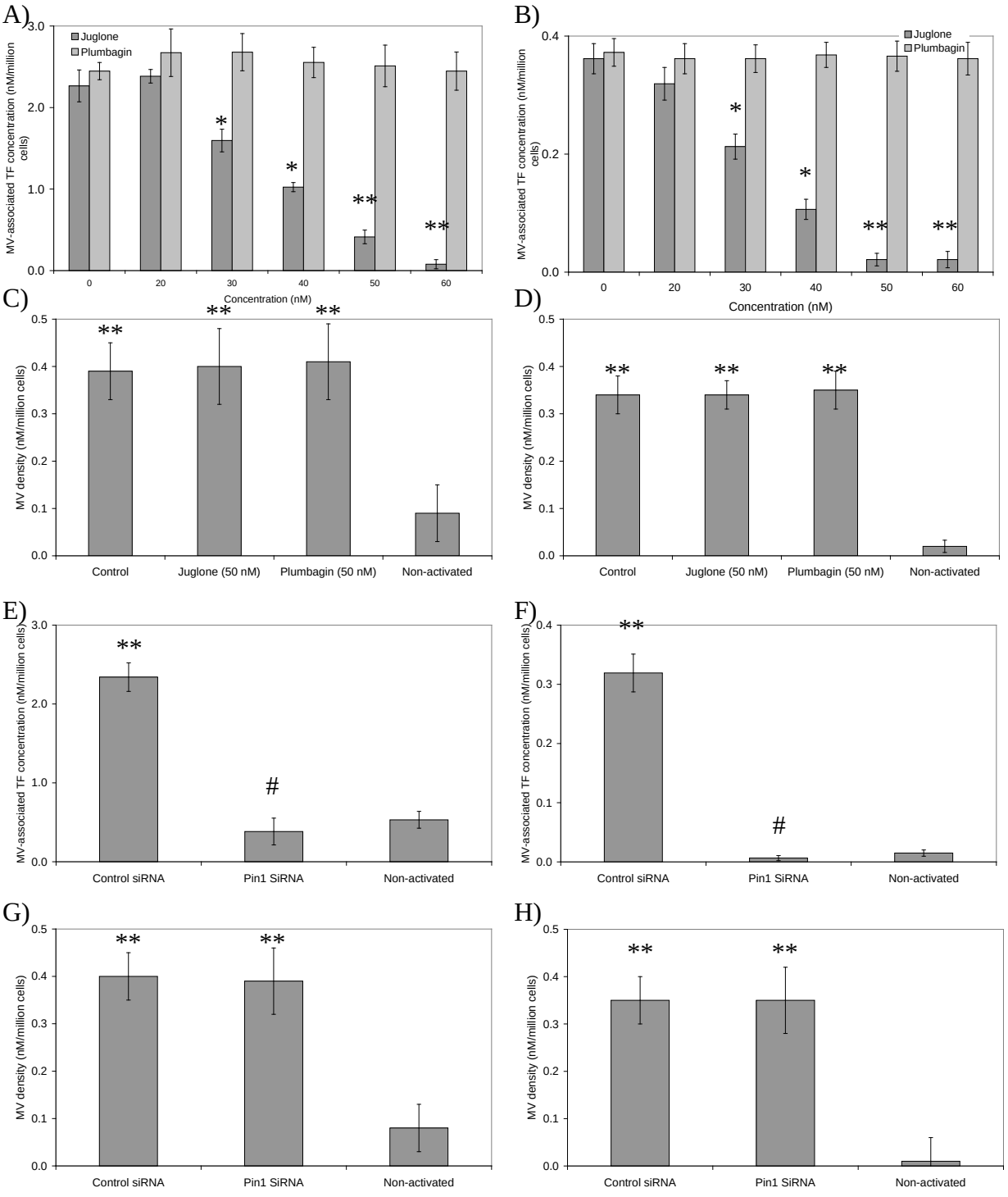
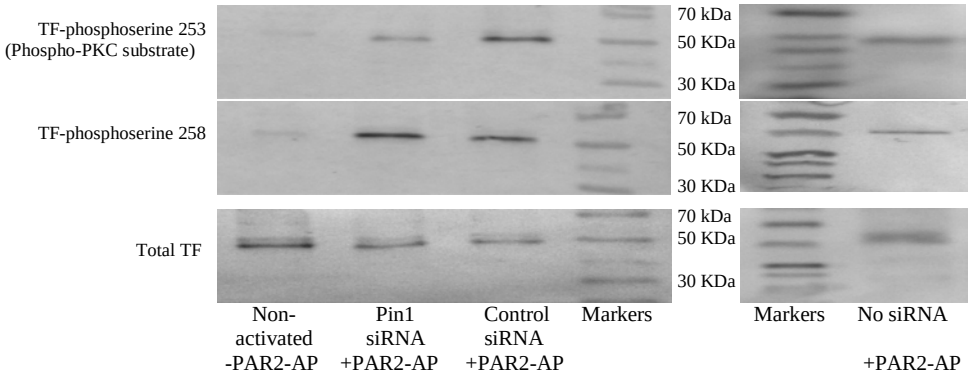
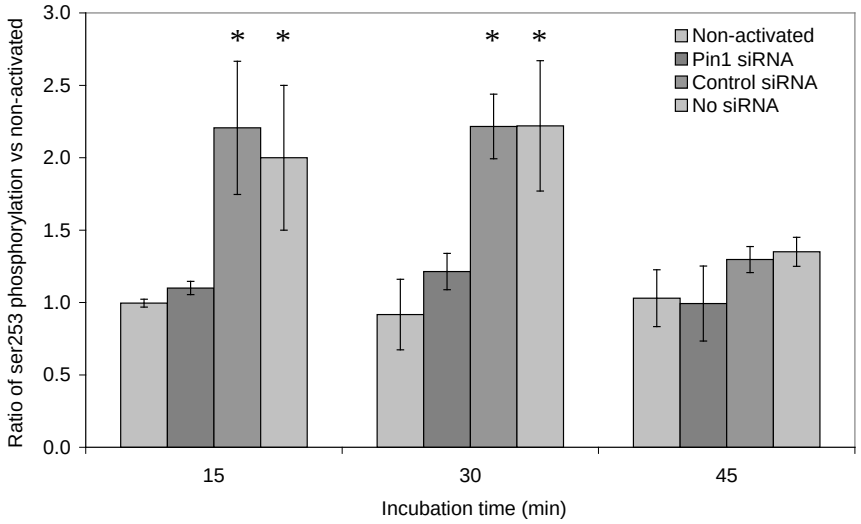


Figure 2

A)


TF-phosphoserine 253
(Phospho-PKC substrate)
TF-phosphoserine 258
Total TF
70 kDa
50 KDa
30 KDa
Non-activated -PAR2-AP
Pin1 siRNA +PAR2-AP
Control siRNA +PAR2-AP
Markers
Markers
No siRNA +PAR2-AP

B)


Ratio of ser253 phosphorylation vs non-activated
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Non-activated
Pin1 siRNA
Control siRNA
No siRNA
15
30
45
Incubation time (min)

C)

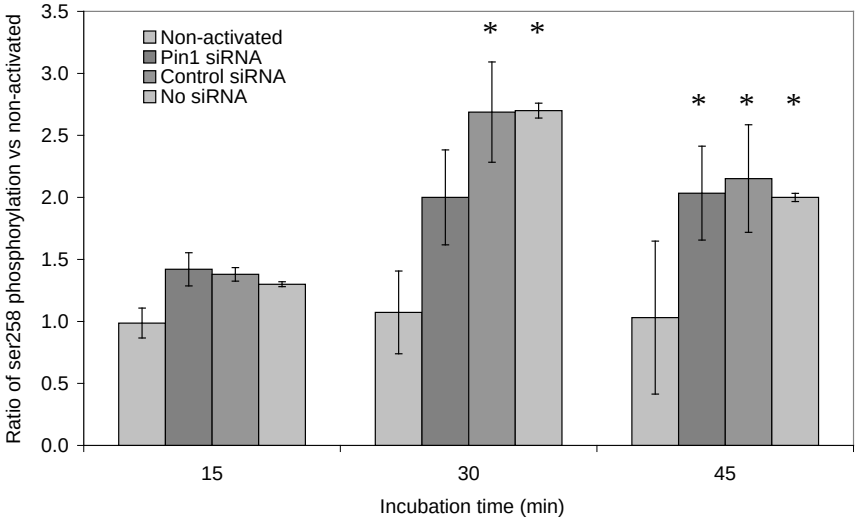

Ratio of ser258 phosphorylation vs non-activated
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Non-activated
Pin1 siRNA
Control siRNA
No siRNA
15
30
45
Incubation time (min)

Figure 3

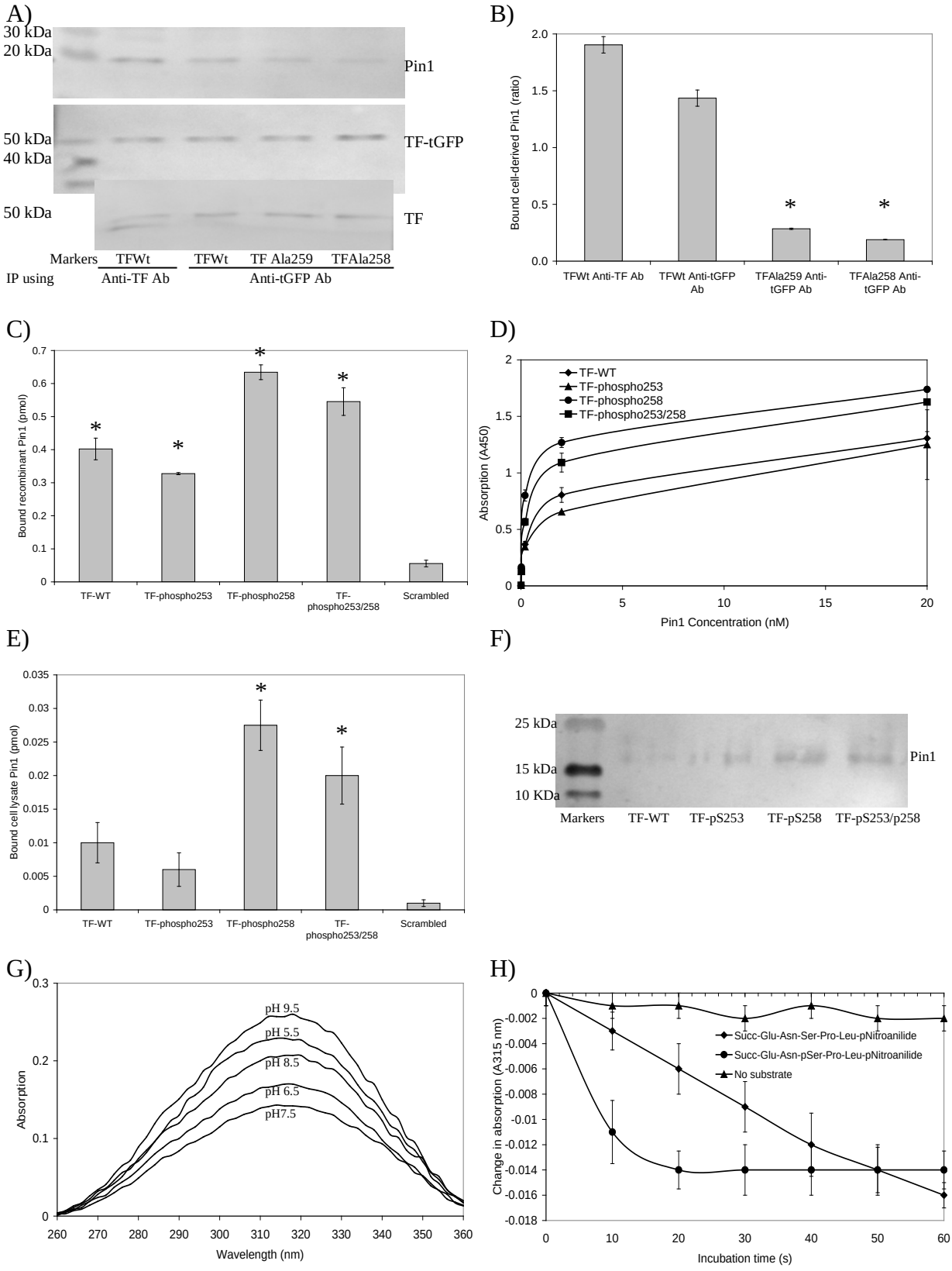
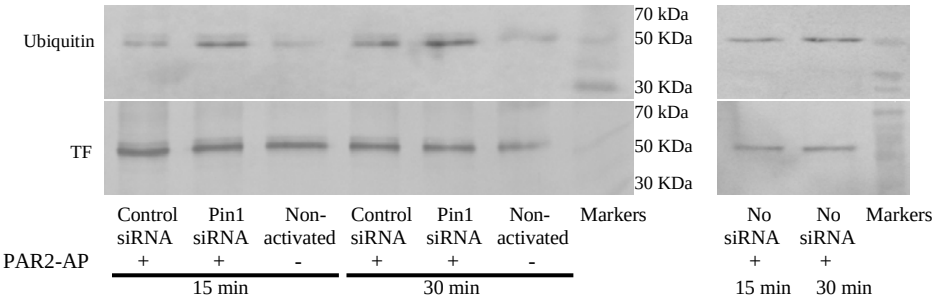
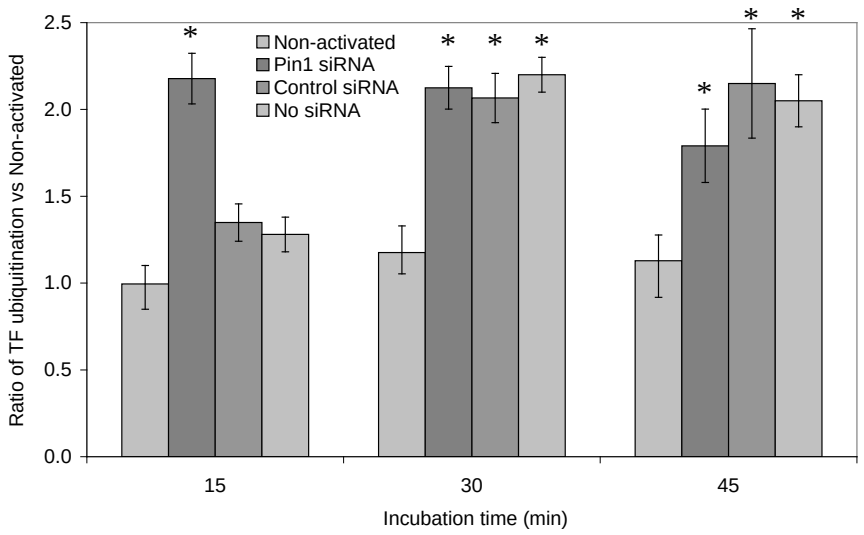

A)
30 kDa
20 kDa
Pin1
50 kDa
40 kDa
TF-tGFP
50 kDa
TF
Markers
TFWt
TFWt
TF Ala259
TFAla258
IP using
Anti-TF Ab
Anti-tGFP Ab
B)
Bound cell-derived Pin1 (ratio)
TFWt Anti-TF Ab
TFWt Anti-tGFP Ab
TFAla259 Anti-tGFP Ab
TFAla258 Anti-tGFP Ab
C)
Bound recombinant Pin1 (pmol)
TF-WT
TF-phospho253
TF-phospho258
TF-phospho253/258
Scrambled
D)
Absorption (A450)
TF-WT
TF-phospho253
TF-phospho258
TF-phospho253/258
Pin1 Concentration (nM)
E)
Bound cell lysate Pin1 (pmol)
TF-WT
TF-phospho253
TF-phospho258
TF-phospho253/258
Scrambled
F)
25 kDa
15 kDa
10 KDa
Pin1
Markers
TF-WT
TF-pS253
TF-pS258
TF-pS253/p258
G)
Absorption
pH 9.5
pH 5.5
pH 8.5
pH 6.5
pH7.5
Wavelength (nm)
H)
Change in absorption (A315 nm)
Succ-Glu-Asn-Ser-Pro-Leu-pNitroanilide
Succ-Glu-Asn-pSer-Pro-Leu-pNitroanilide
No substrate
Incubation time (s)

Figure 4

A)


Ubiquitin
TF
70 kDa
50 KDa
30 KDa
70 kDa
50 KDa
30 KDa
Control siRNA
Pin1 siRNA
Non-activated
Control siRNA
Pin1 siRNA
Non-activated
Markers
No siRNA
No siRNA
Markers
PAR2-AP
+ + - + + -
+ +
15 min
30 min
15 min
30 min

B)


Non-activated
Pin1 siRNA
Control siRNA
No siRNA
Ratio of TF ubiquitination vs Non-activated
2.5
2.0
1.5
1.0
0.5
0.0
15
30
45
Incubation time (min)

C)

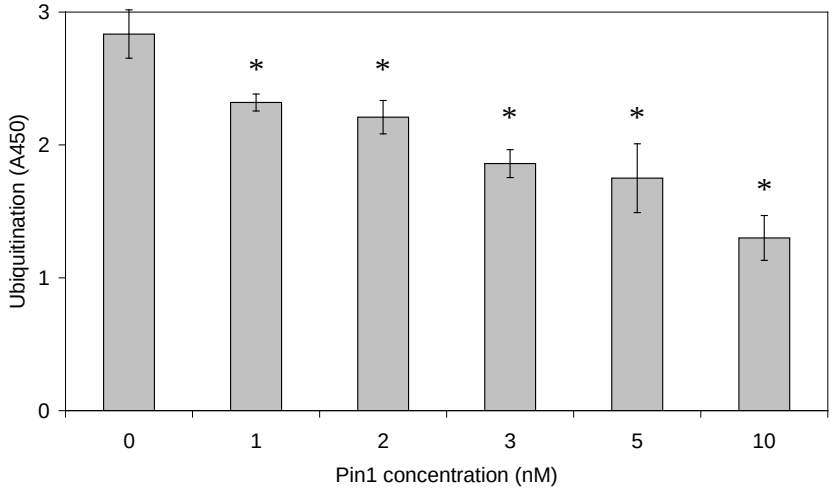
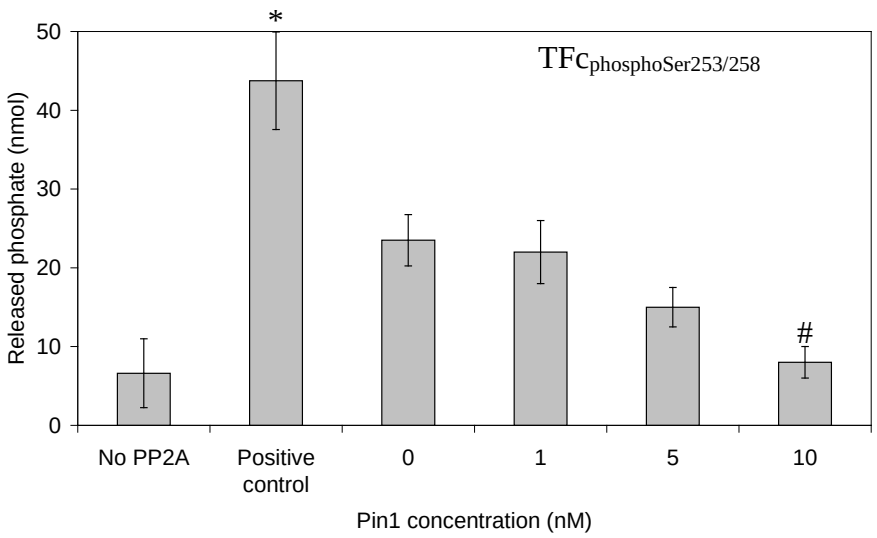
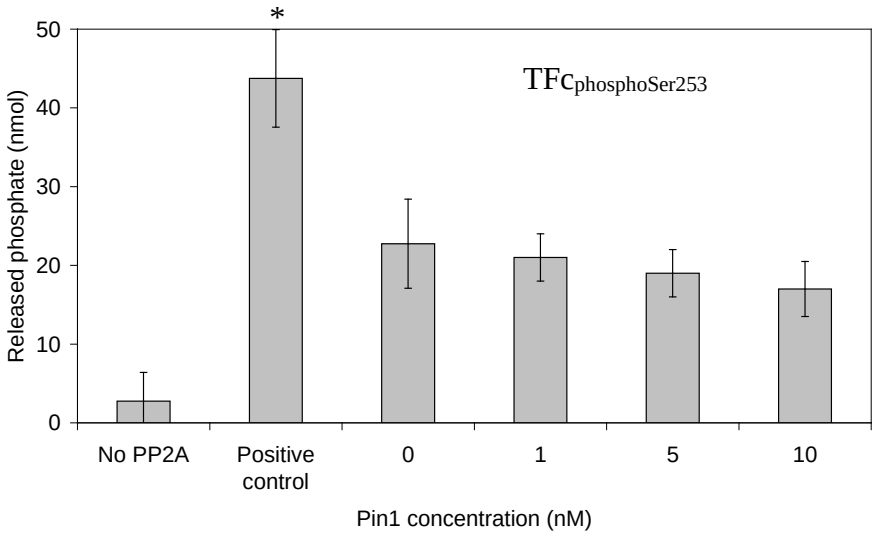

Ubiquitination (A450)
3
2
1
0
0
1
2
3
5
10
Pin1 concentration (nM)

Figure 5

A)


TFc phosphoSer253/258
Released phosphate (nmol)
50
40
30
20
10
0
*
#
No PP2A
Positive control
0
1
5
10
Pin1 concentration (nM)

B)


TFc phosphoSer253
Released phosphate (nmol)
50
40
30
20
10
0
*
No PP2A
Positive control
0
1
5
10
Pin1 concentration (nM)

C)

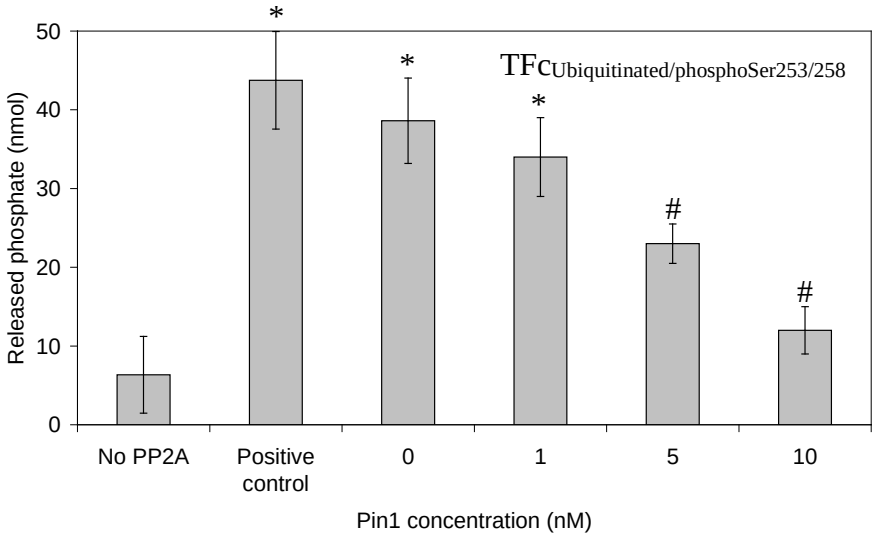

TFc Ubiquitinated/phosphoSer253/258
Released phosphate (nmol)
50
40
30
20
10
0
*
*
*
#
#
No PP2A
Positive control
0
1
5
10
Pin1 concentration (nM)

Figure 6

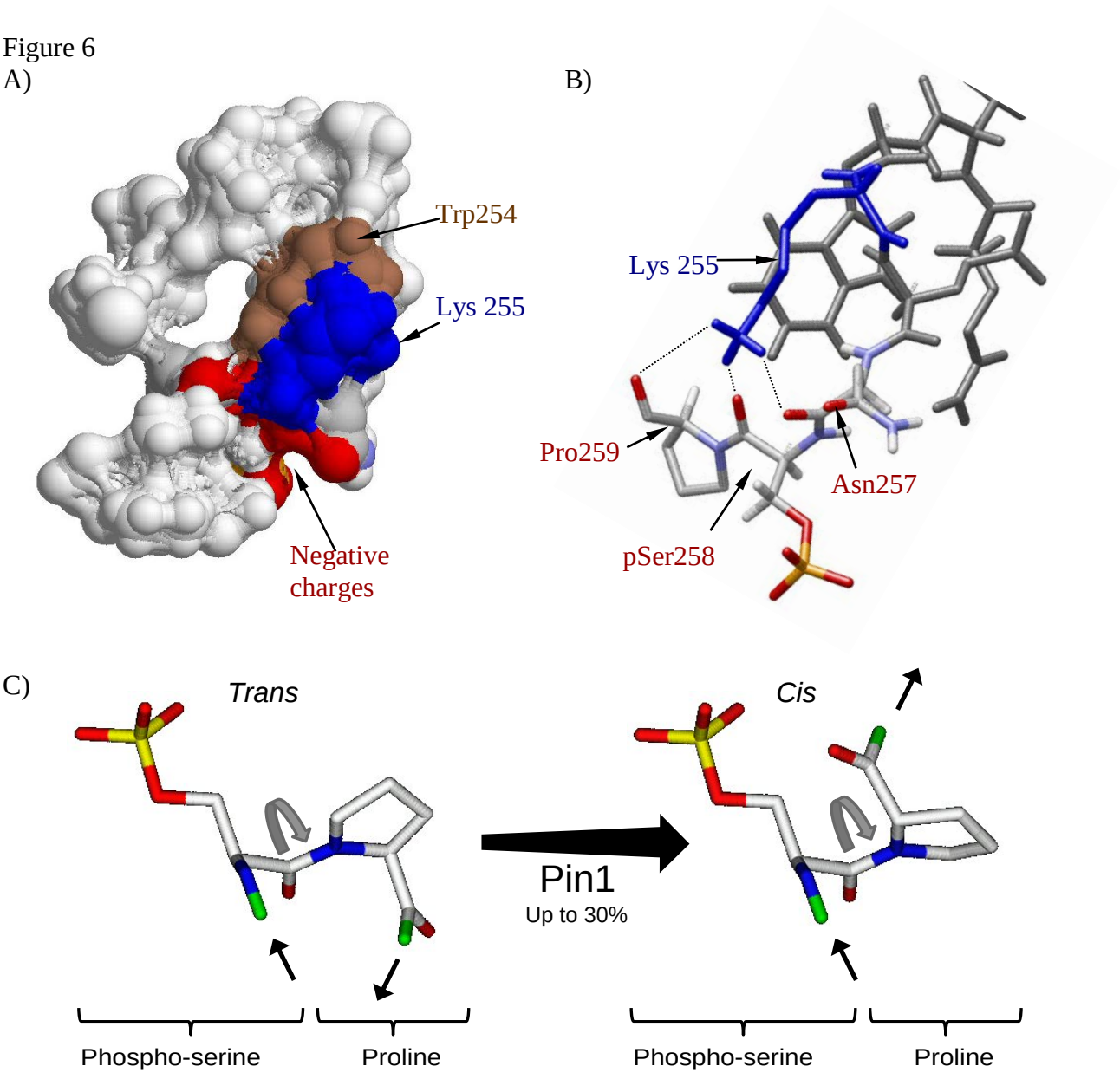
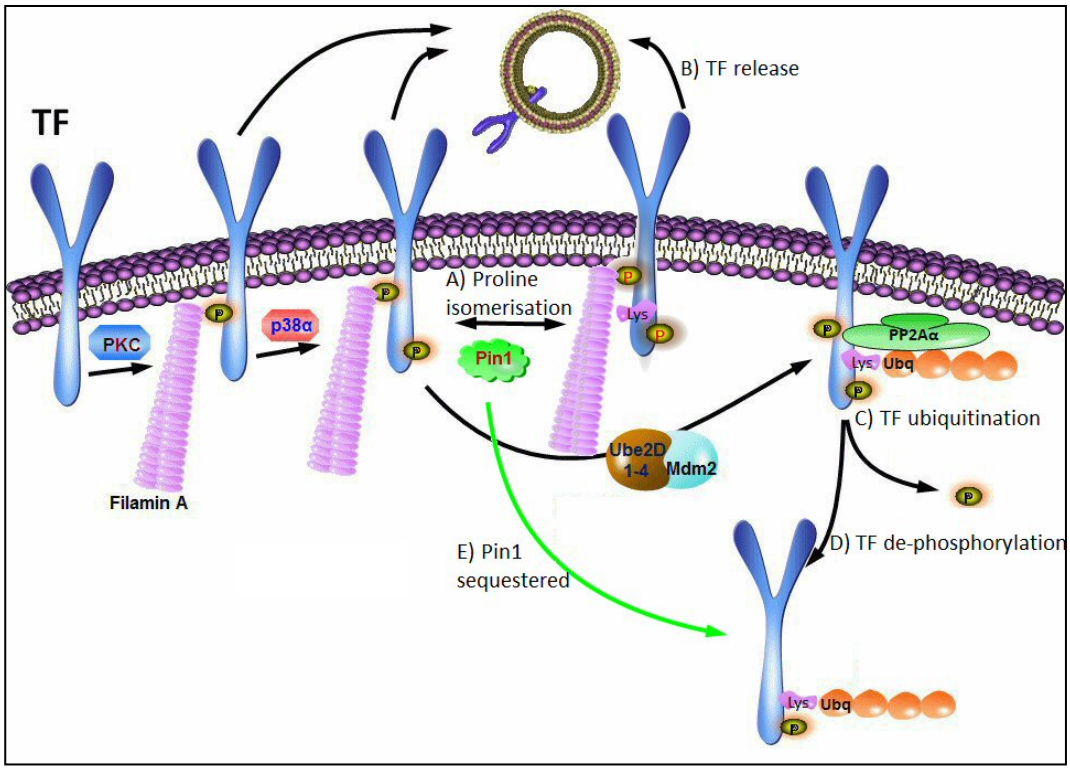

A)
Trp254
Lys 255
Negative charges
B)
Lys 255
Pro259
Asn257
pSer258
C)
Trans
Cis
Pin1
Up to 30%
Phospho-serine
Proline
Phospho-serine
Proline

Figure 7


TF
B) TF release
A) Proline
isomerisation
Pin1
PKC
p38α
Filamin A
Lys
PP2Aα
Lys
Ubq
C) TF ubiquitination
Ube2D
1-4
Mdm2
D) TF de-phosphorylation
E) Pin1
sequestered
Lys
Ubq