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Targeting of FAK Ser910 by Erk5 and PP1 δ in non-stimulated and Phorbol ester-stimulated cells

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Running title: Phosphorylation of FAK by Erk5.

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SYNOPSIS

Ser910 of FAK was phosphorylated in fibroblasts treated with the phorbol ester TPA and dephosphorylated by Phosphatase1 δ , as indicated by shRNA gene silencing. Ser910 was previously reported to be an Erk1/2 target in cells treated with phorbol esters. In contrast, various approaches, including the use of the MEK inhibitors UO126 and CI-1040 to inhibit Erk1/2, pointed to the involvement of Erk5. This hypothesis was confirmed by: 1) shRNA Erk5 gene silencing, which resulted in complete pSer910 loss in non-stimulated and TPA-stimulated cells; 2) Erk5 direct phosphorylation of recombinant FAK; 3) Erk5 activation by TPA. TPA stimulation and Erk5 silencing in MDAMB 231 and 361 breast cancer cells indicated Ser910 targeting by Erk5 also in these cells. Given the proximity of S910 to the FAT regulatory domain of FAK, cell proliferation and morphology were investigated in FAK^{-/-} cells expressing S910A FAK. Cell growth rate decreased and exposure to TPA induced peculiar morphological changes in cells expressing S910A, with respect to wild type FAK, suggesting a role for Ser910 in these processes. This investigation indicated, for the first time, the phosphorylation of Ser910 of FAK by Erk5 and its dephosphorylation by PP1 δ , and suggested a role for Ser910 in the control of cell shape and proliferation.

KEYWORDS: Erk5, Focal adhesion kinase, Protein phosphatase 1, phorbol ester, cell motility, cell survival, protein phosphorylation.

ABBREVIATIONS: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; PDB, phorbol-12,13-dibutyrate; FAK, focal adhesion tyrosine kinase; FRNK, FAK-related nonkinase; PP1, serine/threonine Phosphatase 1; PP2A, serine/threonine Phosphatase 2A; TPCK, L-1-p-tosylamino-2-phenylethyl chloromethyl ketone; GFP, green fluorescent protein.

INTRODUCTION

The focal adhesion kinase FAK is a non-receptor tyrosine kinase that localizes to focal adhesions, the multimolecular structures of contact between cytoskeleton and extracellular matrix. FAK is activated upon cell attachment and spreading and is involved in diverse cellular processes, including cell migration, growth factor signaling and cell survival [1-4]. High FAK levels or deregulation of its signaling may contribute to tumor progression, invasiveness and metastasis [5-7].

FAK is phosphorylated on both tyrosine [1, 2] and serine/threonine [1, 8, 9]. Some serine residues are located in proximity to sites of protein-protein interaction, such as the paxillin-binding site in the FAT domain and the proline-rich domain that binds the SH3-domain of CAS, suggesting their involvement in interactor binding and downstream signaling. Some of the serine residues display consensus sequences for proline-directed kinases and are potentially phosphorylated by members of the MAP kinases or cdks families

[8, 9]. We found that Ser722 is phosphorylated by GSK3 β during cell spreading and migration, and that phosphorylation modulates the FAK kinase activity in a negative way [10]. Ser910 was reported to be phosphorylated by Erk1/2 in cells treated with various agonists, including growth factors and phorbol ester PDB (phorbol-12,13-dibutyrate) [11, 12]. Ser842 and Ser845 of FRNK (the FAK-related non-kinase C-terminal domain of FAK) were found phosphorylated in late mitosis, when FAK undergoes tyrosine-dephosphorylation and inactivation [13]. The proposed role for such phosphorylations is to dissociate FAK from other members of its signaling complex [14], when the focal adhesions are restructured [2] or disassembled in mitosis [15]. Dephosphorylation of these sites is less known. At mitotic exit, FAK serine-dephosphorylation occurs just prior to cells spreading in early G1 and involves the serine/threonine Phosphatase 1 (PP1) [16]. The first indication of a link between FAK and PP1 [17] came from the immunofluorescence detection of PP1 δ in focal adhesions [18, 19]. Subsequently we found that PP1 δ associated with FAK and FRNK in immunoprecipitation and pull-down assays [16]. PP1 is also responsible for Ser722 dephosphorylation in adherent cells [10] and at mitotic exit (unpublished observations).

Erk5, also known as Big MAP kinase, is stimulated by a wide range of mitogens and cellular stresses, and contributes to regulate cell survival and proliferation, and cell differentiation [20-23]. The fact that often Erk5 and Erk1/2 act in synergy and the lack of inhibitors that block Erk5 without affecting Erk1/2 suggest that Erk5 might regulate cellular functions formerly attributed to Erk1/2 [22]. Erk5 is constitutively activated in some tumor cells that overexpress ErbB and contributes to cell proliferation [24]. Erk5 was also reported to be involved, together with Erk1/2, in cytoskeletal disruption, suggesting its potential

relevance to invasion mechanisms [25]. However, the specific Erk5 targets that mediate several of these processes are still unknown.

Due to the proposed relevance of serine phosphorylation to FAK modulation, we treated fibroblasts with the phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate), a PKC activator known to affect both FAK and cytoskeleton, and investigated the changes in the phosphorylation of FAK at Ser910, the kinase and phosphatase involved. While we confirmed the reported increase in pSer910, our investigation brought to discover that Ser910-phosphorylation was due to Erk5 and not to Erk 1/2, under both basal conditions and TPA stimulation. This is the first report of the involvement of Erk5 in the regulation of FAK.

MATERIALS AND METHODS

Materials

The antibodies to detect FAK (C-20), pTyr576/577 of FAK and GFP were from Santa Cruz. The antibodies to detect pSer722, pSer910 and pTyr397 of FAK were from BioSource. The anti-Erk5 antibodies were from Cell Signaling. The anti pErk5 antibodies (anti-pThr218/pTyr220 of Erk5) were from Upstate. The anti Erk1/2, pErk1/2 and α tubulin antibodies were from Sigma Aldrich. The anti-GST antibodies and GH-Sepharose beads were from Amersham Biosciences. The MEK1/2 inhibitor UO126, the p38 inhibitor SB203580, the CDKs inhibitor roscovitine and okadaic acid (K salt) were from Calbiochem. The MEK1/2 inhibitor CI-1040 was a kind gift from Pfizer Inc. Tissue culture and bacteria media and

additives, TPA and protease inhibitors were from Sigma Aldrich. PP1 catalytic subunit was purified from rabbit muscle according to [26]. 1 U of PP1 releases 1 nmol of H_3PO_4 /min.

Cell culture, treatments and lysates

Fisher rat fibroblasts and NIH-3T3 cells were grown in DMEM added with 6% fetal calf serum. MDAMB cells were grown in RPMI added with 10% fetal calf serum [16]. Sub-confluent cells were exposed to TPA or okadaic acid or kinase inhibitors (all diluted in DMSO) or to DMSO alone (control), under the conditions specifically indicated. Following two washes in cold PBS, cells were lysed in 50 mM TRIS-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1 % Triton X-100, 7.5 mM 2-mercaptoethanol (lysis buffer), added with 1 mM orthovanadate (unless when metabolic labeling was performed, Fig. 1A and B), 50 mM NaF or 1 μ M okadaic acid and protease inhibitors (0.02% benzamidine, 0.02 % PMSF, 0.02 % TPCK, 10 μ g/ml soybean trypsin inhibitor and 4 μ g/ml leupeptine).

[³²P]Pi metabolic labeling

The medium was changed into H_3PO_4 -free medium added with 10% H_3PO_4 -free fetal calf serum (previously dialyzed against H_3PO_4 -free medium). After 1 h the medium was replaced with 1 ml of the same medium, added with [³²P] H_3PO_4 (Amersham Biosciences, 1 mCi/150 mm plate) and left for 5 h [16]. 300 nM TPA was added after one h from [³²P] H_3PO_4 addition and left for the remaining 4 h. The extraction buffer contained 1 μ M okadaic acid, to preserve serine and threonine phosphorylations, and no orthovanadate, to favor FAK dephosphorylation on tyrosine (see Fig 1B and [16]).

Gene silencing

For transient PP1 or PP2A gene silencing, rat fibroblasts were transfected with isoform-specific rat siRNA (Rn_Ppp1ca_1HP siRNA, Rn_Ppp1cc_1HP siRNA, Rn_Ppp1cb_3HP

siRNA, Rn_Ppp2ca_1HP siRNA, Rn_Ppp2cb_1HP siRNA from Qiagen, targeting PP1 α , γ 1, δ , or PP2A α or β , respectively) or unrelated control siRNA, using HiPerFect Transfection reagent (Qiagen). The PP2A isoforms were targeted either separately or together (Fig. 2C). Cells were transfected at time 0, day 1 and day 2 (following 1:2 cell splitting). At day 3 cells were treated or not with 300 nM TPA for 1 h and lysed. For stable PP1 δ gene silencing, NIH3T3 cells were transfected with the pLKO.1-puro vector carrying the U6 promoter-mouse PP1 δ (sense-linker-antisense) insert (constructs 97068915TRCN00000618 or 97068917TRCN00000620, Sigma Mission shRNA) or the pLKO.1-puro empty vector, as control, using Lypopfectamine 2000 transfection reagent (Invitrogen). Stable cell lines were established through selection with 5 μ g/ml puromycin, starting from day 1 after transfection [10]. For Erk5 gene silencing, NIH3T3 cells were co-transfected with the Banshee-GFP vector carrying the U6 promoter-mouse Erk5 (sense-linker-antisense) insert [23] or empty vector, (both kindly supplied by Drs. Astar Winoto and Sue J. Sohn, University of California, Berkeley) and the biCS2-puro vector. All the rest was as above. Transfection efficiency was tested by analyzing the expression of the GFP protein. Cells were either lysed at day 3 from transfection or further grown and subcloned by dilution in the presence of puromycin. MDAMB 231 and 361 cells were transfected as above using the pLKO.1-puro vector carrying the U6 promoter-human Erk5 (sense-linker-antisense) insert (constructs TRCN0000010261 or TRCN000001354, Sigma Mission shRNA) or the pLKO.1-puro empty vector, and selected using puromycin, as above. Cells lysates were analyzed by immunoblotting and cells displaying total or sub-total gene silencing were further grown and used in the experiments. α tubulin immunoblot was used to quantify the extracts.

Wild type and S910A FAK stable transfectants

FAK $-/-$ fibroblasts [4] were transfected with the pcDNA/HisMax TOPO vector expressing either wild type or S910A FAK, which contains also the Zeocin-resistance gene (see [10] for construct preparation and FAK $-/-$ cells). Cells were exposed to 100 $\mu\text{g/ml}$ Zeocin starting from day 1 after transfection, grown for 2-3 weeks and subcloned by dilution in the presence of 40 $\mu\text{g/ml}$ Zeocin. Clones expressing equal levels of wild type and S910A FAK were used and two clones for each cell-type were generally used to replicate the experiments.

Cell staining and proliferation assay

Cells were washed in PBS, fixed in 3 % paraformaldehyde, 2 % sucrose in PBS pH 7.5, at room temperature for 10 min, washed in PBS and stained with Giemsa stain. For proliferation assay, cells were fixed as above, stained with Crystal Violet, lysed in 10% (v/v) acetic acid for measurement of optical density at 595 nm.

Bacterial growth, extracts and pull-down assays

Bacteria were grown, induced and extracted as described previously [16]. For pull-down assays, the GST-fusion proteins were bound to 25-50 μl of glutathione-Sepharose beads [16].

Immunoprecipitation and immunoblotting

Anti-FAK or anti-Erk5 antibodies were used for immunoprecipitation with Protein A-Sepharose [16]. Following incubation at 4° C for 90 min with shaking and three washes in cell lysis buffer added with 0.02 % PMSF and 0.02 % benzamidine, the immune complexes were boiled in Laemmli buffer. For PP1 treatment (Fig. 1A) the immune complexes were washed once more with 25 mM imidazole, pH 7.5, resuspended in 25 μl of the same buffer added with 2 mM 2-mercaptoethanol, incubated with 2 units of muscle-purified PP1 catalytic subunit at 30° C for 20 min and boiled in Laemmli buffer [16]. Electrophoresis was on 8.5 %

polyacrylamide-SDS gel and Immobilon-P membranes (Millipore) were used for transblotting. The membranes were probed with the indicated antibodies, followed by protein A-peroxidase or peroxidase-conjugated secondary antibody (Sigma) and the enhanced chemiluminescence ECL system (Amersham Biosciences). For re-probing with another antibody, the membranes were previously incubated in 5 mM phosphate buffer, 2 % SDS and 2 mM 2-mercaptoethanol at 60 ° C for 20 min.

Phosphoaminoacid analysis

[³²P]-labeled phosphoproteins, transferred to Immobilon-P, were hydrolyzed in 6 N HCl at 110 ° C for 1 h and analyzed by two-dimensional high voltage electrophoresis on thin-layer cellulose plates (Merk), followed by autoradiographic detection [27].

Erk5 activity assay

Erk5 was assayed using the Erk5-specific substrate Mef2. BL-21 E. coli were transformed with the plasmid to express GST-Mef2C (kindly supplied by Dr. Eric N. Olson, Southwestern Medical Center, Dallas). The bacteria were cultured, extracted, and Mef2-GST fusion protein was bound to GH-beads as indicated above [16]. Following a final wash in 25 mM imidazole, pH 7.5, the beads were mixed with extract from cells that had been exposed or not to TPA and incubated for 5 min at 30 ° C with shaking, in the presence of 10 mM MgCl₂, 100 μM γ[³²P]ATP (Amersham Biosciences, 2000-2500 cpm/pmol) [10, modified]. The reaction was terminated by adding Laemmli buffer and boiling. The ³²P-labeled Mef2 was subjected to SDS-gel electrophoresis followed by autoradiography. Subsequently, the Mef2 protein bands were excised from the gel and counted in a β-scintillation counter. Control assays, prepared with buffer instead of cell extract, were run in parallel, counted and subtracted from the cpm of the assays.

Phosphorylation of S910 of FRNK

Wild-type or S910A GST-FRNK (GST-fusion protein of the C-terminal of FAK, [10]) was bound to glutathione-Sepharose beads and incubated with immunoprecipitated Erk5 (obtained from fibroblast exposed to 300 nM TPA for 1 h) in the presence of γ [³²P]ATP, 500 μ M ATP and 10 mM MgCl₂. The incubation was carried at 30° C for 30 min with shaking and was terminated by adding electrophoresis sample buffer.

RESULTS

Phosphorylation of FAK at Ser910 in cells treated with TPA

We analyzed the phosphorylation and dephosphorylation of Ser910 (the homologue of chicken Ser911), which was reported to be phosphorylated in Swiss 3T3 cells treated with growth factors or the phorbol ester PDB (phorbol-12,13-dibutyrate; [11, 12]). FAK was immunoprecipitated from rat fibroblasts that had been metabolically-labeled with [³²P]H₃PO₄ prior to and during exposure to the phorbol ester TPA (300 nM); this was followed by immunoblot, autoradiography and phosphoaminoacid analysis of FAK (Fig. 1A and B). The results confirmed that TPA increased FAK-Ser phosphorylation and specifically pSer910 (Fig. 1A and B). pSer910 was further analyzed in lysates of cells exposed to TPA for up to 4 h. The increase in pSer910 reached a maximum within 1 h (Fig. 1C and D), whereas no major changes were detected at the FAK autophosphorylation Tyr397 site, or src Tyr576/577 sites. Additionally, TPA did not affect the phosphorylation of Ser722 (Fig. 1E), a site that we have previously shown to be a GSK3 β -target during adhesion and migration [10].

The phosphatase targeting Ser910

Ser910 phosphorylation was reversed *in vitro* by PP1 (Fig. 1A) and in the cell following TPA removal and further incubation without TPA (Fig. 1E). To investigate which phosphatase targets Ser910 in the cell, we first used the phosphatase inhibitor okadaic acid, which inhibits either PP2A alone or both PP2A and PP1, depending on the concentration [28, 10]. Cells were incubated with or without TPA for 40 min and subsequently with increasing concentrations of okadaic acid for 40 min. In both untreated and TPA-treated cells pSer910 increased only at 0.2 and 0.5 μM okadaic acid (Fig. 2A), suggesting the involvement of PP1 rather than PP2A, generally inhibited at lower concentrations [28]. As a further investigation, cells were incubated with or without 0.4 μM okadaic acid for 20 min, and subsequently with TPA for up to 2 h. The increase in pSer910 in the presence of okadaic acid (Fig. 2B) confirmed that a phosphatase, most likely PP1, was targeting pSer910 throughout the exposure to TPA.

To confirm the involvement of PP1 we silenced PP1 and PP2A in rat fibroblasts by transfecting siRNA targeting either PP1 α , or PP1 γ 1, or PP1 δ or PP2A α and β . The procedure adopted was a modification of the one used by Okada and coll. to silence PP1 [29] and involved three subsequent siRNA transfections, one every 24 h, as further described in the Methods section and Fig. 2 legend. Silencing was achieved in the case of each gene, though often sub-total (see the Discussion section and [29]). One hour before cell extraction, part of the cells were further treated with TPA. As expected, pSer910 levels were very low in untreated cells, and increased following TPA treatment (Fig. 2C). However, silencing brought to a marked increase in pSer910 with respect to TPA treated controls only in the case of PP1 δ

silencing (Fig. 2C). This suggested the involvement of PP1 δ in targeting pSer910, although the additional involvement of other phosphatases cannot be excluded completely (e.g. PP2A, due to its incomplete silencing and a minor increase in pSer910 following TPA treatment).

To confirm the role of PP1 δ , we prepared cells carrying stable gene silencing by transfecting a vector encoding for a PP1 δ -shRNA and further selecting by exposure to puromycin. Due to the lack of a vector to silence the rat gene at the time of the experiments, we used mouse NIH-3T3 cell. Preliminary experiments indicated that TPA dependent phosphorylation of Ser910 occurred also in these cells (not shown). Two constructs, among those tested, targeting different PP1 δ sequences, yielded similar silencing (see Methods section and Fig. 2 legend). Likewise the case of siRNA silencing, exposure to TPA increased pSer910 more in the cells carrying the stable silencing of PP1 δ than in controls (Fig. 2D and E). Altogether, the results indicated the involvement of PP1 δ in targeting pSer910 and excluded the other phosphatases tested.

Erk1/2 is not involved in the TPA-stimulated phosphorylation of Ser910

It has been reported that the activation of protein kinase C by the phorbol ester PDB leads to phosphorylation of FAK at Ser910 by activated Erk1/2 [12]. Also in our system the use of the PKC inhibitors Go6983 and bis-indolylmaleilimide confirmed the involvement of PKC in the TPA-stimulated phosphorylation of FAK at Ser910 (not shown). On the other hand, our investigation led to results that argued against the involvement of Erk1/2, due to the following findings. First, the MEK1/2 inhibitor UO126 [30] indeed decreased pS910, whereas neither the p38 inhibitor SB203580 nor the cdk inhibitor Roscovitin did (Fig. 3A); however, the time-courses of Erk1/2 activation and of Ser910 phosphorylation were quite

different. Erk1/2 activation was maximal at 15 min and subsequently declined (judged by the changes in pErk1/2), whereas FAK phosphorylation at Ser910 peaked at 1 h and was sustained for up to 4 h (Fig. 3B and C). Second, cell-treatment with 1 μ M UO126 (a concentration that inhibited Erk1/2 almost maximally, Fig. 3D) resulted in a pSer910 loss that was much less pronounced than in the case of 10 μ M UO126. Third, incomplete inhibition of Ser910 phosphorylation was obtained also with another MEK1/2 inhibitor, CI-1040, under conditions that inhibited Erk1/2 completely (Fig. 3E). Fourth, the TPA concentrations required to activate Erk1/2 and to phosphorylate Ser910 were quite different. Ser910 was maximally phosphorylated at 300 nM TPA, whereas Erk1/2 was maximally activated at 25 nM TPA, and declined at the higher concentrations (Fig. 3F). In summary, the results suggested the involvement of a kinase different from Erk1/2.

Gene silencing indicates the involvement of Erk5 in Ser910 phosphorylation, in both control and TPA stimulated cells

The MEK inhibitors used to inhibit Erk1/2 are known to inhibit also Erk5, though with higher IC₅₀ [31, 25]. To test the hypothesis of the involvement of Erk5, the gene was silenced and the consequences on pSer910 were investigated. Due to the lack of a vector to silence the rat gene at the time of the experiments, we used mouse NIH-3T3 cell. Cells were transiently transfected with the shRNA-Erk5 construct [23], or with the empty control vector, and the transfection efficiency was tested by analyzing the level of the green fluorescent protein (GFP), encoded by the same vector. To increase the system efficiency, cells were co-transfected with a puromycin-resistance vector and the non-transfected cells (over 80%) were eliminated by exposure to puromycin [10]. Fig. 4A shows the almost complete loss of Erk5

protein within 3 days, with respect to control cells. α tubulin levels confirmed the use of comparable amounts of cell extract. Erk5 silencing was accompanied by loss of pSer910 with respect to control cells (Fig. 4A), thus indicating that Erk5 is the kinase that phosphorylates Ser910, or it is upstream to this site.

To investigate the role of Erk5 in TPA stimulated cells, stable transfectants, carrying either the silenced Erk5 gene or the control vector, were prepared from the puromycin-resistant clones. TPA stimulation did not lead to any change in Ser910 phosphorylation in cells that did not express Erk5, whereas pSer910 increased, as expected, in control cells (Fig. 4B). The results clearly indicated that Erk5 increased pSer910 also following TPA stimulation. Additionally, Erk5 silencing did not affect Erk1/2 levels or the ability of TPA to stimulate Erk1/2 (Fig. 4C), thus confirming that the silencing achieved was specific for Erk5.

Direct phosphorylation of Ser910 by Erk5

To test the ability of Erk5 to phosphorylate Ser910 directly we used GST-FRNK, the GST-fusion protein of the C-terminal domain of FAK. GST-FRNK, either wild type or S910A mutant [10], was bound to GH-Sepharose beads and mixed with Erk5 immunoprecipitated from TPA treated fibroblasts. The reaction was carried-out in the presence of γ [32 P]ATP and the 32 P-labeled GST-FRNK was subjected to SDS-gel electrophoresis, autoradiography and counting in a β -scintillation counter. Wild-type FRNK was phosphorylated to a much higher level than the S910A mutant (Fig. 4D and E), indicating direct phosphorylation of Ser910 by Erk5.

Erk5 undergoes activation in cells stimulated with TPA

The next step was to assess the ability of TPA to activate Erk5. For this purpose rat fibroblasts were exposed to TPA for up to 3 h and the kinase activity of Erk5 was assayed with the specific substrate Mef2, used as GST-Mef2 bound to GH-Sepharose beads. The ^{32}P -labeled Mef2 was subjected to SDS-gel electrophoresis, autoradiography and counting in a β -scintillation counter (Fig. 5A and B). The results indicated that TPA activated Erk5, with a peak at 30 min and 1h. Alternatively, Erk5 was immunoprecipitated from TPA-treated fibroblasts and its phosphorylation at Thr218 and Tyr220, the hallmark of Erk5 activation, was probed (Fig. 5C). Also this approach confirmed maximal Erk5 activation at 30 min and 1h. Altogether the results indicated Erk5 activation by TPA and the time-scale supported its role in the phosphorylation of Ser910. However, Erk5 activation decreased subsequently, whereas pSer910 stayed high for up to 4 h (Fig. 1, 2). This discrepancy might be due to site targeting by a different kinase upon prolonged TPA stimulation or protection from dephosphorylation.

Differential phosphorylation of Ser910 in MDAMB 231 and 361 cells

To investigate the correlation between Erk5 and pSer910 in human cells, we adopted the MDAMB 231 and 361 breast cancer cells. pSer910 was high in non-stimulated 231 cells and little influenced by TPA, whereas in 361 cells pSer910 was very low and increased upon TPA stimulation (Fig. 6A). Likewise the case of fibroblasts, the changes in pSer910 did not parallel Erk1/2 activation (Fig. 6A).

Gene silencing was used to test the involvement of Erk5. Cells were transfected with the shRNA construct targeting human Erk5 or with the empty vector, and stable transfectants were generated following exposure to puromycin. Erk5 silencing by two constructs targeting

different Erk5 sequences (see Methods section) was accompanied by loss of pSer910 in both cell lines (Fig 6B and D), suggesting Erk5 as the kinase that phosphorylates Ser910. As a further test, both stable transfectants and control cells were treated with TPA. Stimulation of Erk5 silenced cells led to little or no change in pSer910, whereas the pSer910 of control cells increased in 361 and to some extent also in 231 cells (Fig. 6C and D). The results further supported the role of Erk5 in the TPA stimulated phosphorylation of Ser910 in both cell lines.

Differential activation of Erk5 in MDAMB 231 and 361 cells

Likewise the case of fibroblasts (Fig. 5), Erk5 activation following TPA treatment was investigated in MDAMB 231 and 361 cells by assaying the Mef2-kinase activity. The TPA induced Erk5 activation and increase in pSer910 were remarkably parallel in each cell line (Fig. 7A and B). In 231 cells both pSer910 and Erk5 activity displayed high basal values and little TPA stimulation (Fig. 6D and 7B), whereas both pSer910 and Erk5 displayed low values and great TPA stimulation in 361 cells. These parallel changes further supported the hypothesis that Erk5 phosphorylates Ser910 also in these cells.

Proliferation and TPA induced morphological changes in FAK $-/-$ cells expressing wild type or S910A FAK.

To investigate the biological functions of Ser910 we adopted FAK $-/-$ fibroblasts in which FAK, either wild type or S910A mutant, had been reintroduced. Stable transfectants were prepared using previously described constructs [10] and the mutation was confirmed by the lack of pSer910 following TPA-stimulation (Fig. 8A). As described previously, the mutation did not affect FAK kinase activity or cell migration in a scratch-wound assay [10],

and pSer910 did not change during cell spreading on fibronectin [10]. All this suggested that the potential role of Ser910 was most likely not related to FAK regulation during the remodeling of focal adhesions that occurs in migration. On the other hand, considering that Erk5 is controlled by proliferation stimuli and that the role of FAK in cell survival involves the FAT domain of FAK, encompassing Ser910, we tested whether the S910A-mutation affected cell proliferation. For this purpose FAK $-/-$ cells or FAK $-/-$ cells expressing wild type or S910A FAK were plated at four different dilutions and tested for cell growth. The summary results (Fig. 8B) indicated that cell growth was delayed in the cells expressing S910A FAK, with respect to cells expressing wild type FAK, suggesting a contribution from Ser910 to the role played by FAK in cell proliferation. Also the mere reintroduction of FAK delayed cell growth (compare FAK $-/-$ cells and cells expressing wild type FAK, Fig. 8B).

TPA induces morphological changes that reflect cytoskeletal rearrangements [32]. In the present case, within 30 min cells expressing wild type FAK looked retracted and cell protrusions appeared, leading to transition from a more polygonal (time 0) to a fibroblast-like shape (Fig. 8C and D). In cells expressing S910A FAK retraction was more pronounced: cell protrusions were longer, thinner and more abundant, leading to a star-like morphology which prevailed at 30 min, was still detected at 1 h and had almost disappeared by 3 h (Fig. 8C and D). In summary, the TPA-induced morphological changes were markedly different whether Ser910 was present or not, suggesting a role for Ser910 in regulating cell shape.

DISCUSSION

The phosphorylation of FAK at multiple tyrosine and serine sites modulates its activity and the association with signaling proteins. However, the kinases and phosphatases that target several of the serine residues are still unknown. This work describes the targeting of Ser910 by Erk5 and PP1 δ , and suggests a role for Ser910 in the regulation of cell proliferation and cell shape.

Erk5 responds to growth factors and stress stimuli, and contributes to differentiation and survival of normal and tumor cells [20-23]. Erk5 has been linked also to cytoskeletal disruption, with potential relevance to invasion mechanisms [25]. However, one problem that has hampered Erk5 studies is the difficulty to differentiate between Erk5 and Erk1/2 functions, since the two enzymes may act in synergy and share substrates, and also the inhibitors used in cellular studies are the same. Moreover, the roles played by Erk5 may vary depending on the cell type and the specific Erk5 targets are mostly unknown. In the present study we found that the phosphorylation of FAK at Ser910, previously attributed to Erk1/2, was due instead to Erk5, at least in the cells examined.

Our experiments brought to exclude the involvement of Erk1/2 in the phosphorylation of Ser910 of FAK, based on: i) the different time-courses of Erk1/2 activation and Ser910 phosphorylation; ii) the different TPA concentrations required to activate Erk1/2 and to phosphorylate Ser910; iii) the incomplete inhibition of Ser910 phosphorylation induced by the MEK inhibitors UO126 and CI-1040, in the presence of total Erk1/2 inhibition. Subsequently, the positive involvement of Erk5 was indicated by: i) Erk5 gene silencing, which resulted in complete loss of Ser 910 phosphorylation in both non-stimulated and TPA-stimulated fibroblasts, and ii) activation of Erk5 in TPA-treated cells, assayed as both Mef2 kinase activity and pSer/pThr-Erk5 phosphorylation. Finally, the direct targeting of Ser910 by

Erk5 was proven by the *in vitro* phosphorylation of Ser910 of FRNK. Our conclusion differs from the one drawn by Hunger-Glaser and coll. [11, 12] indicating Erk1/2 as the kinase that phosphorylates Ser910 in Swiss 3T3 cells stimulated by various agonists, including PDGF, FGF, EGF, bombesin and the phorbol ester PDB. However, they also noticed incomplete inhibition of Ser910 phosphorylation in cells stimulated in the presence of low U0126 concentrations. On the other hand, both Erk1/2 [12] and Erk5 (our data) did phosphorylate Ser910 of FRNK *in vitro*. Altogether, we cannot exclude that, depending on the agonist and cell type, both Erk1/2 and Erk5 may target Ser910.

In MDAMB 231 and 361 breast cancer cells, pSer910 displayed striking differences: it was very high in 231, while almost undetectable in 361 cells. In both cell lines Erk5 activity paralleled Ser910 phosphorylation, suggesting Erk5 involvement, and Erk5 gene silencing induced loss of pSer910, thus confirming Ser910 phosphorylation by Erk5. Considering the role of FAK in the survival of breast cancer cells [6] and the ability of Ser910 to favor cell proliferation (Fig. 8B), Ser910 phosphorylation by Erk5 might contribute to cancer cell proliferation. In some breast cancer cell lines Erk5 was reported to be part of the downstream signaling of ErbB2 [24]. However, in MDAMB 231 cells we detected high basal Erk5 activity, in spite of low ErbB2. The lack of a positive correlation between ErbB2 and Erk5 might indicate that other activated receptors or oncogenes sustain Erk5 activity in these cells.

Several lines of evidence indicated PP1 as a major FAK-serine phosphatase. Initially PP1 δ was detected at focal adhesions and found to associate with FAK [18, 19]. Subsequently it was found that PP1 δ dephosphorylated FAK at mitotic-exit [16] and was specifically targeting Ser722 in adherent cells [10]. Molecular analysis also indicated that the PP1 δ domain interacting with FAK encompassed residues involved in catalysis [33]. The present

data further extended the role of PP1 δ to the dephosphorylation of Ser910. The procedure adopted to silence PP1 and PP2A included repetitive siRNA transfection and was described by Okada and coll. to silence PP1 [29]. In both investigations silencing was not total, a situation that was probably favorable, since it allowed the cells to survive, in spite of interfering with proteins that regulate cell cycle [29]. Altogether, the data suggested a PP1 δ contribution to focal adhesion stability by controlling FAK serine phosphorylation.

The role of FAK serine phosphorylation is still little understood. Previous work from this laboratory described FAK phosphorylation at Ser722 by GSK3 β in cells spreading on fibronectin and during fibroblasts migration [10]. Ser843 was phosphorylated by Ca²⁺/calmodulin-dependent protein kinase following stimulation with G protein-coupled receptor agonists [34]. It has been suggested that serine-phosphorylations contribute to disassemble the FAK signaling complex and prevent the interaction with CAS or paxillin during focal adhesion rearrangements in cell migration [2, 5, 8, 35] and mitosis [15]. Conversely, FAK-serine dephosphorylation was reported to stabilize adhesion in HT-29 colon carcinoma cells [36]. Altogether these observations point to the importance of keeping the phosphorylation of specific serine residues under strict control, in order to preserve the stability of the FAK signaling complex.

In most cells the proliferation response to growth factors depends on cell adhesion to the extracellular matrix. FAK plays a primary role in this process by stimulating various downstream signaling pathways, which depend on both FAK kinase activity and complex formation with adaptor proteins, such as CAS and paxillin. By this means growth factors and integrin signaling cooperate in controlling cell growth and progression through the cell cycle [1, 2, 5, 6]. Several molecular details of this mechanism have still to be unraveled. Our data

suggest a role for Ser910, since its mutation delayed cell proliferation. Given the fact that Erk5 is regulated by growth factors and stress stimuli, and contributes to the cell survival [20-23], it is conceivable that one of the survival pathways involves stimulation of Erk5 and FAK phosphorylation at Ser910.

The morphological changes induced by TPA were more profound and persistent in cells expressing S910A FAK, suggesting a role for Ser910 in the FAK downstream signaling to the cytoskeleton that regulates cell morphology. The involvement of Erk5 strongly supports this view, since also Erk5 has been linked to cytoskeletal disruption, through a mechanism that is not completely clear. The phosphorylation of Ser910 by Erk5 may contribute to fill this gap.

Unraveling the molecular interactions occurring downstream of Ser910 may be more problematic. The location of Ser910 within the FAT domain [1, 2, 5] suggested its involvement in the interaction with paxillin, a scaffold protein linking FAK to the actin cytoskeleton [14, 37]. Additionally, paxillin is phosphorylated in a PKC-dependent way [14] and by Erk1/2 during focal adhesion disassembly [37], suggesting that the paxillin-FAK association might be influenced also by changes at Ser910. The finding that the S910A mutation increased paxillin association seemed to confirm this hypothesis [12]. On the other hand, preliminary data from our laboratory, not included in the present report, did not support this view. We found that the increase in pSer910 induced by TPA was accompanied by increase, rather than decrease, in FAK-paxillin association, whereas loss of Ser910 phosphorylation (due to Erk5 silencing) did not significantly affect the TPA stimulated association. Based on these discrepancies, we suggest that the potential role of Ser910 in the

regulation of the FAK-paxillin association may be more complex and its unraveling requires a deeper investigation.

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FIGURE LEGENDS

Figure 1 - FAK phosphorylation and dephosphorylation in cells treated with TPA.

(A) Metabolic labeling of FAK and phosphorylation at Ser910. FAK was immunoprecipitated from fibroblasts that had been metabolically labeled with [³²P]H₃PO₄ and treated (+TPA) or not with 300 nM TPA for 4 h. The immunocomplexes were prepared in duplicate and one complex from each treatment was further incubated with 2 units of muscle PP1 at 30° C for 20 min (+PP1). Following electrophoresis on SDS-8.5 % polyacrylamide gel and transblotting onto Immobilon-P, the membrane was first exposed to detect the radioactivity (32P-FAK), then probed to detect FAK. In a similar experiment, but without metabolic labeling, FAK was immunoprecipitated, treated with PP1 as above, and used to detect phospho-Ser910 of FAK (pS910) and FAK. **(B) Phosphoaminoacid analysis of labeled FAK.** Two anti-FAK immunoprecipitates were prepared from [³²P]H₃PO₄-labeled cells treated or not with TPA, as in A. The FAK-protein band was cut from the Immobilon-P membrane, hydrolyzed at 110° C for 1 h, analyzed by two-dimensional high voltage electrophoresis and exposed to detect the radioactivity. **(C) FAK phosphorylation at Ser910, Tyr397 and Tyr576/Tyr577.** Cells were treated with 300 nM TPA, collected at the

indicated time points and cell lysates were analyzed by electrophoresis and immunoblot, to detect pS910, FAK phosphorylation at Tyr397 (pY397) and Tyr576/Tyr577 (pY576/7), and FAK. **(D) Quantification of the changes in Ser910 phosphorylation.** The pSer910 bands from cells as in C were quantified by densitometric scanning and the data were normalized by adopting the 1 h value as 100%. Average of 4 independent experiments \pm SEM. **(E) FAK phosphorylation at Ser910 and Ser722 following TPA treatment and subsequent TPA removal.** TPA-treated cells as in A were either collected at 1 and 3 h, or washed twice at 3 h and further incubated for up to 3 h in the absence of TPA. Lysates from cells collected at the indicated time-points were analyzed to detect phospho-Ser 722 of FAK (pS722), pS910 and FAK.

Figure 2 – The phosphatase targeting pSer910 of FAK.

(A) Phosphorylation of Ser910 in cells treated with TPA and further exposed to increasing concentrations of okadaic acid. Cells were incubated with or without 300 nM TPA for 40 min and with the indicated okadaic acid (OA) concentrations for additional 40 min. Cells were collected and lysates analyzed to detect pS910 and FAK. **(B) Phosphorylation of Ser910 in cells treated with okadaic acid and further exposed to TPA.** Cells were incubated with or without 0.4 μ M okadaic acid for 20 min, subsequently with 300 nM TPA for up to 2 h and collected at the indicated time-points. **(C) Effect of transient silencing of PP1 α , γ 1 or δ , or PP2A (α + β) and of TPA on pSer910.** Rat fibroblasts were transfected with siRNA targeting the indicated PP1 or PP2A isoforms (si) or control siRNA (c) at time 0, day 1 and day2, and collected at day 3, as further described in the Methods section. TPA treatment (300 nM) before cell collecting was for 1 h. siRNA targeting

PP2A α and β were co-transfected. Following western blot the membranes were probed to detect the PP1 or PP2A isoforms, α tubulin, pSer910 and FAK. The data are representative of three independent experiments. **(D) Effect of stable silencing of PP1 δ and of TPA on pSer910.** NIH-3T3 fibroblasts were transfected with either hairpin siRNA vector targeting PP1 δ (sh) or control empty vector (O) and stable transfectants were prepared by puromycin selection. Cells exposure to TPA and all the rest was as in C. **(E) Quantification of the effect of TPA on pSer910 in cells carrying stable PP1 δ silencing.** The pSer910 bands from cells as in D were quantified by densitometric scanning and the data obtained were normalized by adopting the control + TPA value as 100%. Average of 4 independent experiments, using two different siRNA constructs, \pm SEM..

Figure 3 - Phosphorylation of FAK Ser910 and Erk1/2 activity in cells treated with TPA and with kinase inhibitors.

(A) Treatment with kinase inhibitors. Cells were treated with 300 nM TPA for 1 h in the presence of the following kinase inhibitors: 10 μ M U0126, to inhibit MEK1/2 (U); 10 μ M SB203580, to inhibit p38 (SB) and 100 μ M Roscovitine, to inhibit cdks (Ro). In addition to pS910 and FAK, the activation level of Erk1/2 (pErk1/2) and Erk 1/2 protein were detected on cell lysates. **(B) Time course of TPA treatment.** Cells were treated with TPA for up to 4 h and analyzes as in A. **(C) Quantification of the time course of TPA treatment.** The pSer910 and pErk 1/2 bands from blots as in B were quantified by densitometric scanning and the data were normalized by adopting the maximal value obtained for each phosphoprotein. as 100%. Average of 4 independent experiments \pm SEM.. **(D) Treatment with U0126.** Cells were treated with 300 nM TPA in the presence or not of 1 or 10 μ M

U0126. **(E) Treatment with CI-1040.** Cells were treated with 300 nM TPA in the presence or not of 1 μ M of the MEK1/2 inhibitor CI-1040. **(F) Treatment with increasing TPA concentrations.** Cells were treated with the indicated TPA concentrations for 1 h.

Figure 4 - Involvement of Erk 5 in the phosphorylation of FAK Ser910.

(A) Transient silencing of Erk5. NIH-3T3 cells were co-transfected with either empty vector (0) or the Erk5 (sh) hairpin siRNA vector together with a vector that expresses the puromycin resistance gene. 24 h after transfection the cells were exposed to 10 μ g/ml puromycin, to kill non-transfected cells, and collected at 72 h. Cell lysates were used to detect Erk5, pS910, FAK, green fluorescent protein (encoded by the transfected vectors, GFP) and α tubulin. **(B-C) TPA treatment of cells carrying stable Erk5 silencing.** Stable transfectants, obtained from cells transfected as in A, were treated with 300 nM TPA for 1 h and analyzed as in A **(B)** or for Erk1/2 activation (pErk1/2) and Erk1/2 protein **(C)**. **(D) Phosphorylation of Ser910 of FRNK by Erk5.** GST-FRNK (GST-fusion protein of the C-terminal of FAK), either wild-type or S910A mutant, was bound to glutathione-Sepharose beads and incubated with immunoprecipitated Erk5 (obtained from fibroblast exposed to 300 nM TPA for 1 h) in the presence of γ [³²P]ATP/MgCl₂. This was followed by electrophoresis, Coomassie blue staining and exposure to detect the radioactivity incorporated into FRNK (32P-FRNK). Wild-type and S910A-FRNK were detected also as GST-fusion proteins in the bacterial lysate used for the pull-down. **(E) Quantification of 32P-FRNK.** FRNK bands were excised from the gel (see D) and counted (cpm/FRNK band). Average of 3 independent experiments \pm SEM.

Figure 5 - Activation of Erk5 in cells treated with TPA.

(A-B) Erk5 activity assayed as Mef2 kinase. (A) GST-Mef2 was bound to glutathione-Sepharose beads and incubated with γ [32 P]ATP/MgCl₂ and cell extract from rat fibroblast exposed to 300 nM TPA for the indicated time. This was followed by electrophoresis, Coomassie blue staining (Mef2) and exposure to detect the radioactivity incorporated into Mef2 (32P-Mef2). **(B) Quantification of 32P-Mef2.** Mef2 bands were excised from the gel (see A) and counted (cpm/Mef2 band)). Average of 3 independent experiments \pm SEM. **(C) Erk5 activity assayed as pErk5.** Erk5 was immunoprecipitated (Erk5 ip), followed by electrophoresis, probing with antibodies recognizing the phosphorylation of Thr218 and Tyr220 of Erk5 (pErk5) and membrane staining with Coomassie blue (Erk5). Control immunoprecipitates (control ip) are also shown.

Figure 6 - Role of TPA and Erk5 in the phosphorylation of FAK Ser910 in MDAMB 231 and 361 cells.

(A) Phosphorylation of Ser910 and Erk1/2 activity in cells treated with TPA. Cells were treated with 300 nM TPA and collected at the indicated time-points. Lysate analysis was as in Fig. 4. **(B) Phosphorylation of Ser910 following Erk5 silencing.** Stable Erk5 silencing was obtained from cells transfected with either the empty vector (0) or the Erk5 (sh) hairpin siRNA vector. **(C) Phosphorylation of Ser910 following TPA treatment of cells carrying silenced Erk5.** Cells were treated with 300 nM TPA for 1 h and analyzed as in B. **(D) Quantification of the effect of TPA on pSer910 in cells carrying Erk5 silencing.** The pSer910 bands from cells as in C were quantified by densitometric scanning and the data obtained were normalized by adopting the control + TPA value as 100%. Average of 4 independent experiments, using two different siRNA constructs, \pm SEM..

Figure 7 - Activation of Erk5 in MDAMB 231 and 361 cells treated with TPA.

(A-B) Erk5 activity assayed as Mef2 kinase. (A) Cells were treated and analyzed as in Fig. 5A. **(B) Quantification of 32P-Mef2.** Mef2 bands were excised from the gel (see A) and counted (cpm/Mef2 band). Average of 3 independent experiments \pm SEM.

Figure 8 - Effects of the expression of S910A FAK and TPA treatment in FAK -/- cells.

(A) Phosphorylation of Ser910 in cells treated with TPA. Cells were treated with 300 nM TPA for 1 h and cell lysates were analyzed for pSer910, FAK and α tubulin. **(B)**

Proliferation of cells expressing wild type or S910A FAK. The indicated amounts of FAK -/- cells expressing wild-type (open circle) or S910A-FAK (filled triangle), or of control FAK -/- cells (filled circle), were seeded in 24-well Costar plates, grown for 48 h, fixed, stained with Crystal Violet and analyzed spectrophotometrically. Average of 5 experiments, using 2 different clones for each cell type (3 experiments in the case of FAK -/- cells) \pm SEM. **(C)**

Morphological changes in cells treated with TPA. Cells treated with TPA as in A were fixed at the indicated time-points and stained with Giemsa stain. The experiment shown is representative of several experiments performed. **(D) Quantification of the morphological changes in cells treated with TPA.** Equivalent fields, displaying cells treated as in C (approximately 20-25 cells/field), were analyzed for the presence of cells with retracted cytoplasm and long, thin extensions (defined as star-like cells, S) and cells with fibroblast shape (defined as fibroblast-like, F). Data are shown as percentage of S and F cells counted in each field. Average of 10 fields from 3 independent experiments \pm SEM.

Figure 1

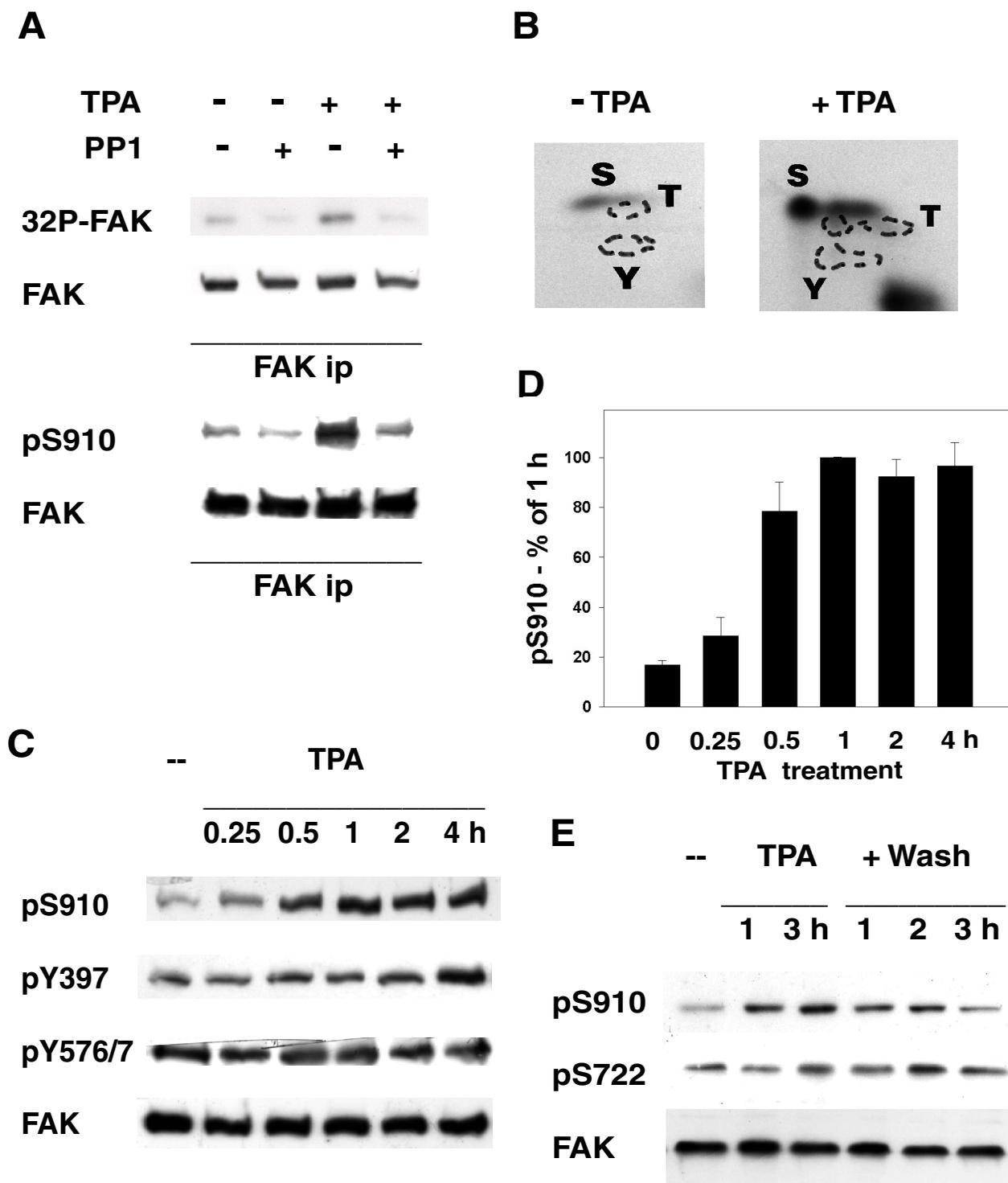


Figure 2

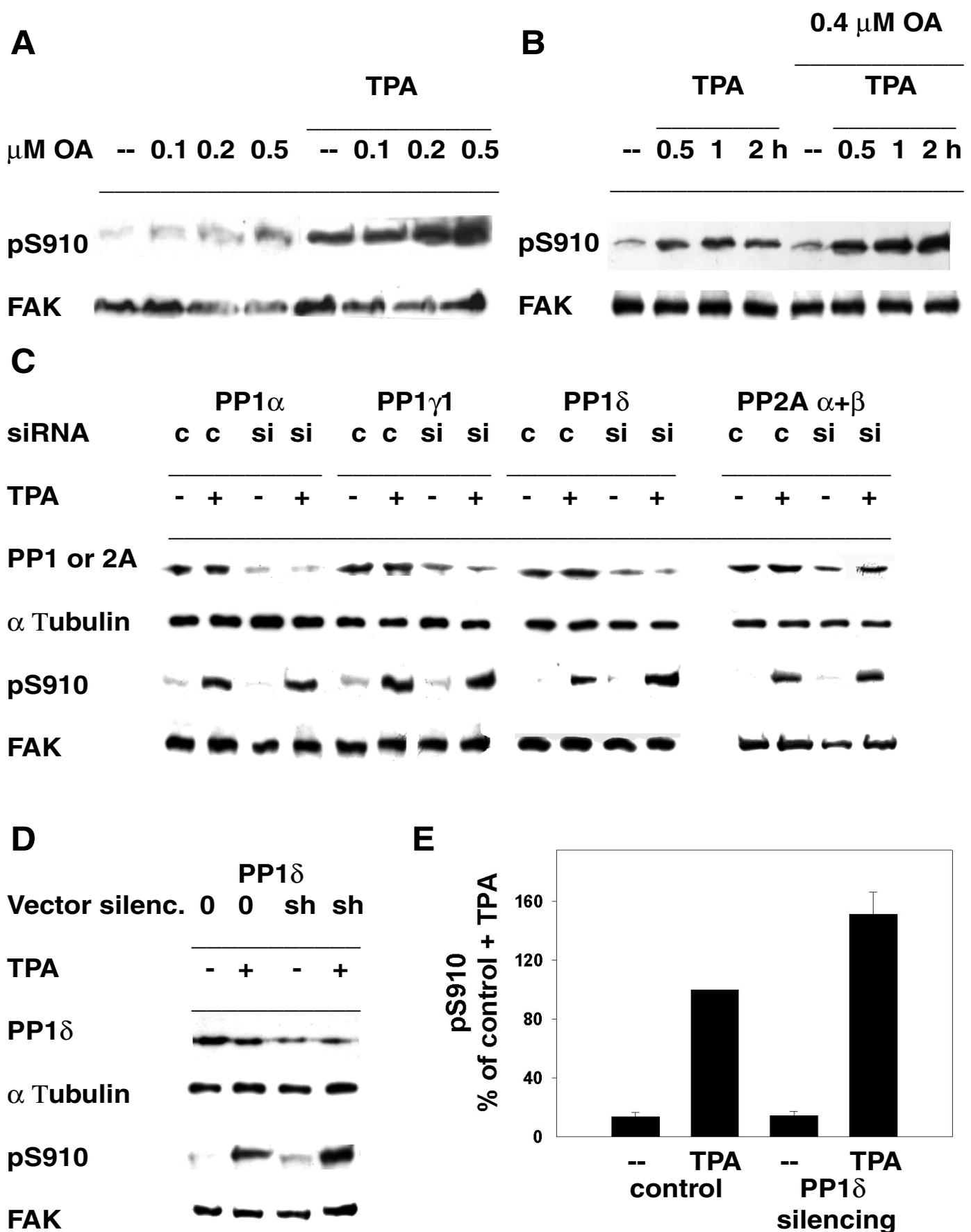


Figure 3

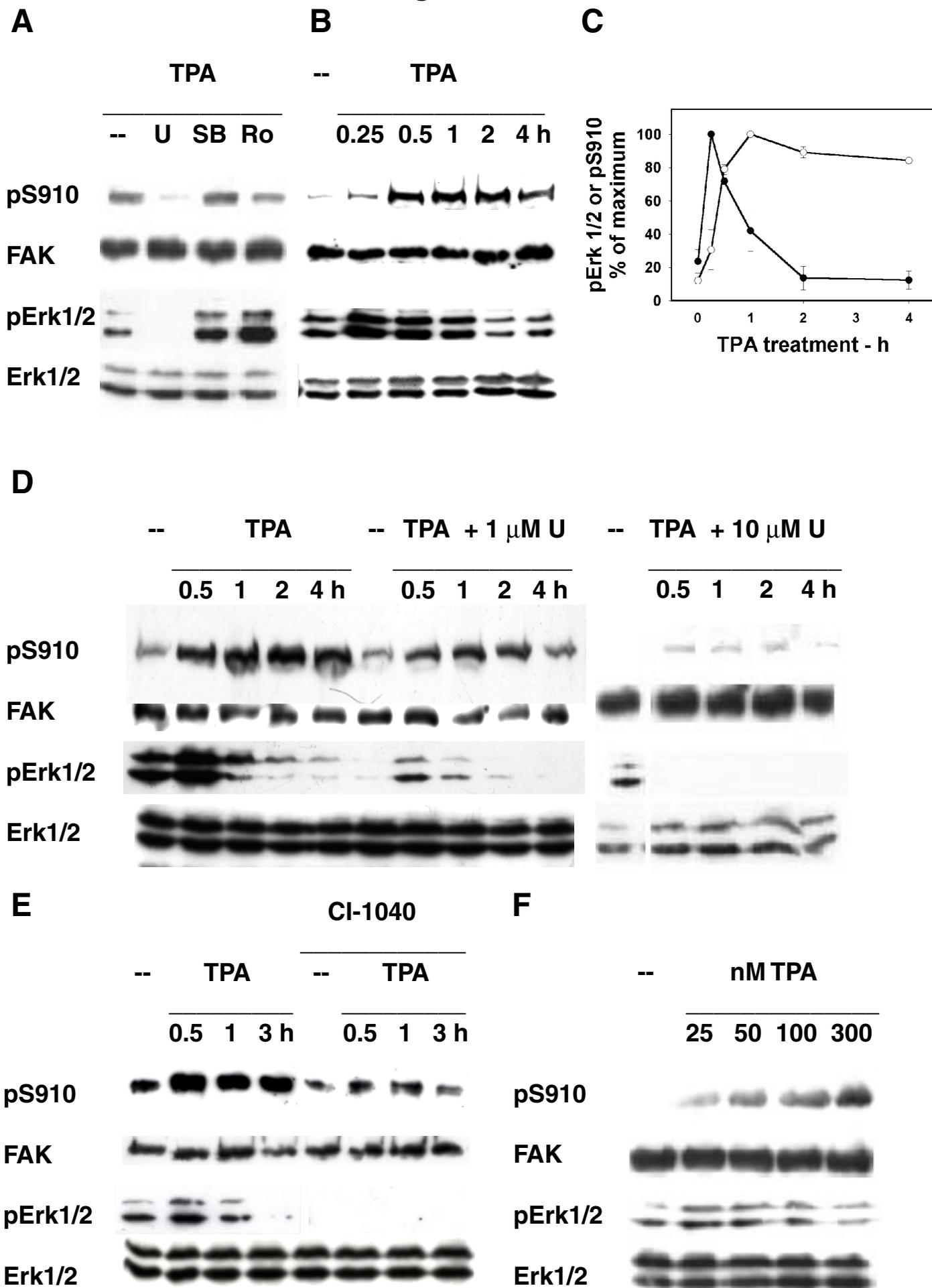


Figure 4

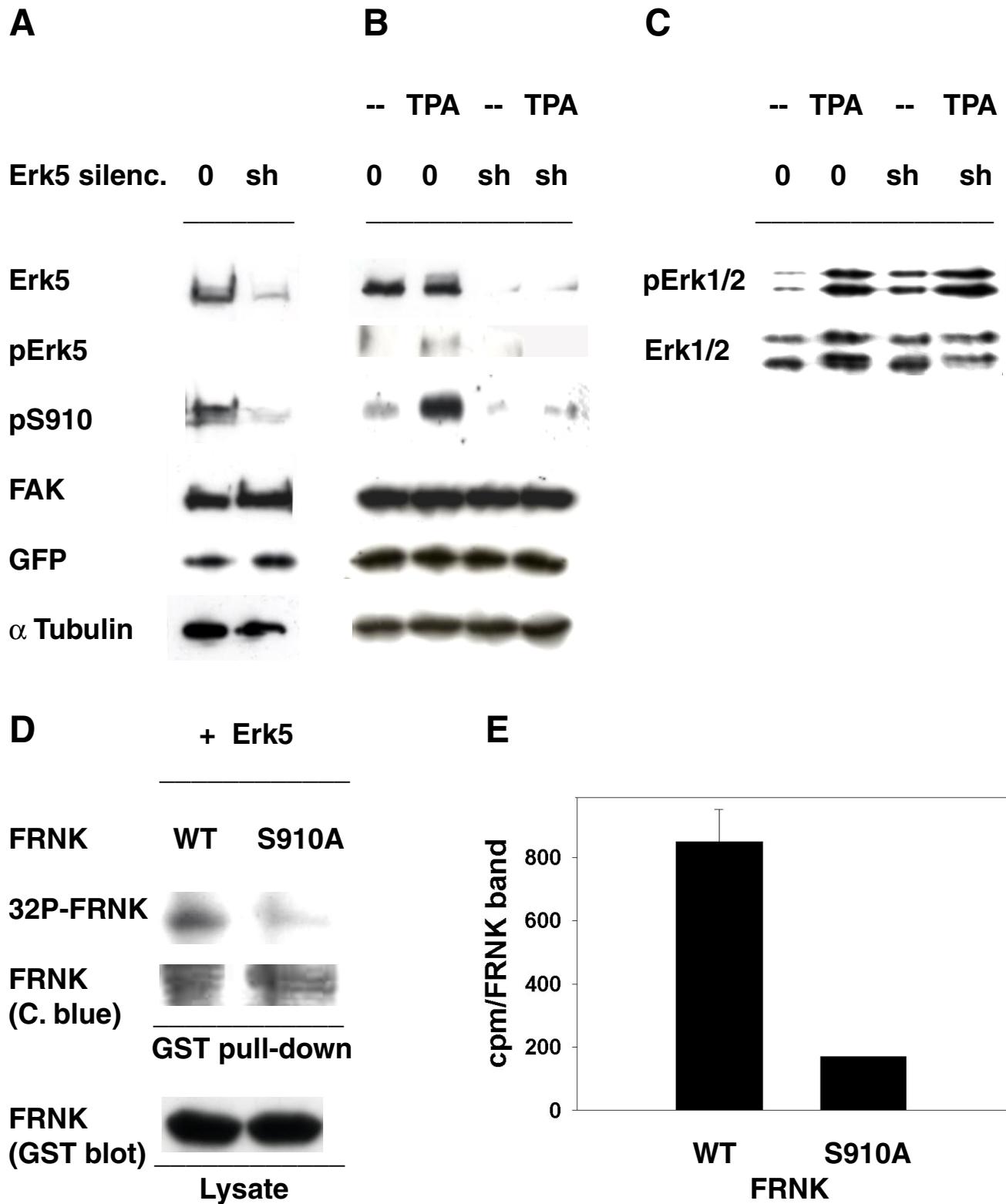


Figure 6

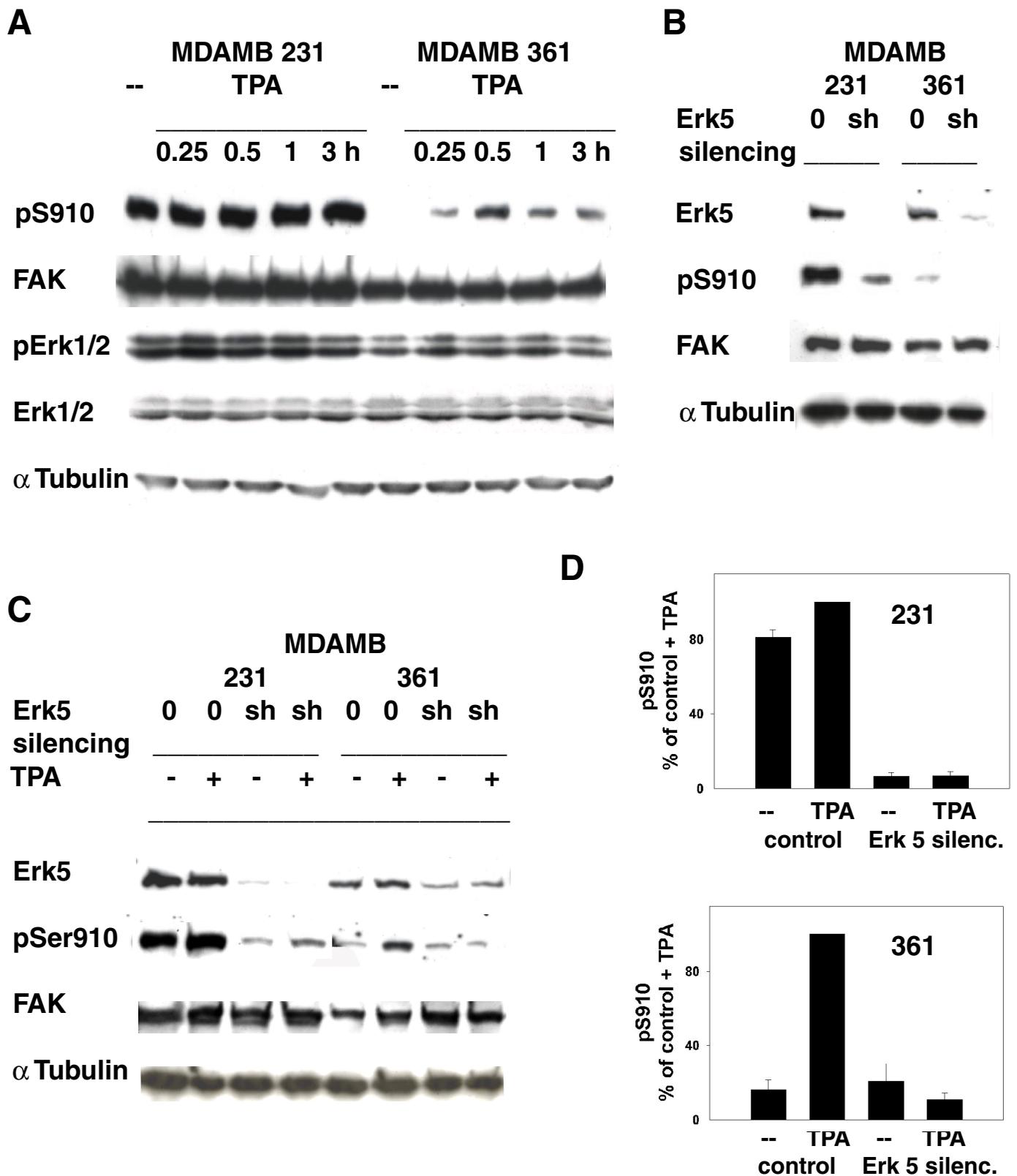
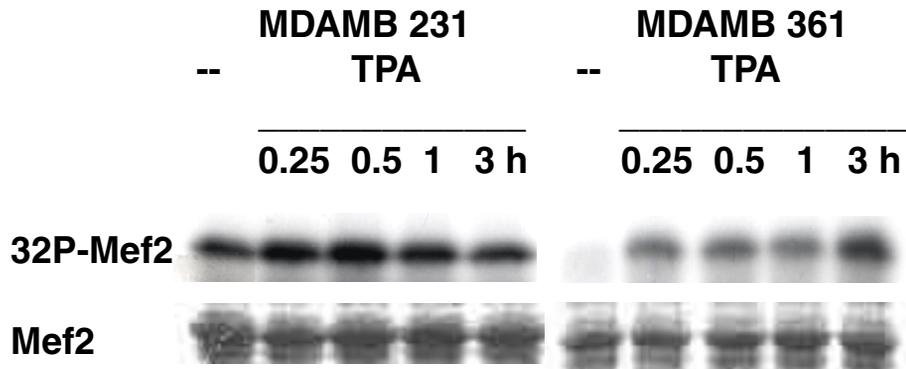


Figure 7

A



B

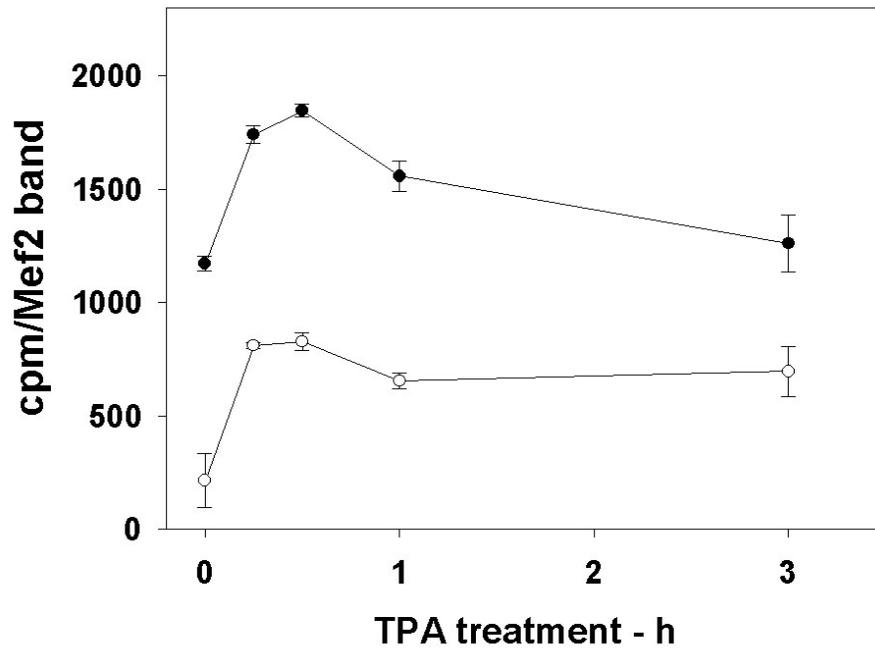


Figure 8

