Published in final edited form as:

*Apoptosis*. 2011 February ; 16(2): 162–173. doi:10.1007/s10495-010-0556-y.

# A shear stress responsive gene product PP1201 protects against Fas-mediated apoptosis by reducing Fas expression on the cell surface

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## Abstract

Cells that form vascular system employ different mechanisms to offset deleterious consequences of exposure to cytokines and cells present in blood. Vascular homeostasis is sustained in part by genes, whose expression increases in response to hemodynamic forces in these cells. *PP1201* (also known as *RECS1*) is one such gene whose expression level increases in response to laminar shear stress. Aged mice deficient in *PP1201* are prone to develop cystic medial degeneration (CMD), a form of aortic aneurism manifested with loss of smooth muscle cells and accumulation of basophilic substances. Here we found that higher levels of PP1201 can protect against Fas ligand (FasL)-induced apoptosis. PP1201 interacted with the Fas receptor (CD95/Apo1) and colocalized with it in the Golgi compartment. Unlike its homolog lifeguard (LFG), PP1201 overexpression in several types of cells including primary human aortic smooth muscle cells (AoSMC) decreased the expression of Fas on the plasma membrane without changing the total Fas levels. Only high but not constitutive level of PP1201 controls Fas signaling. Our data suggest that PP1201 functions as an anti-apoptotic protein and its increased expression in vascular cells can contribute to homeostasis by reducing Fas trafficking to the cell membrane.

### Keywords

Apoptosis; Fas; *PP1201*; Golgi; Trafficking; Cystic medial degeneration

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**Conflict of interest** None.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10495-010-0556-y) contains supplementary material, which is available to authorized users.

## Introduction

*PP1201/RECS1* has been identified as a gene responsive to centrifugal force and shear stress (*RECS*) [1]. *PP1201*-deficient mice show no phenotypic abnormalities, but aged knockout mice are prone to cystic medial degeneration (CMD), a pathological condition encountered in aortic aneurysms [2, 3]. These animals display abnormal aortic media with large CMD foci manifested by absence of smooth muscle cells and by the accumulation of basophilic ground substance referred as mucoid degeneration. The aneurysms observed in *PP1201*-deficient mice are noninflammatory as the loss of cells in CMD lesions is not associated with infiltration of T lymphocytes or macrophages [2]. Whereas these studies propose a protective role for PP1201 in vascular homeostasis, they do not address signaling pathways modulated by PP1201 and whether increased levels of PP1201 are obligatory for such a function.

*PP1201* encodes for a protein with seven transmembrane domains that has been reported to localize to endosomal and lysosomal membranes. Detailed phylogenetic analysis indicates that *PP1201* shares lineage with *Lifeguard* (*LFG*) and in vertebrates both these genes are derived from two rounds of duplications of a single ancestor [4]. Expression of LFG is developmentally upregulated in the sciatic nerve [5, 6] and it is known to function as an anti-apoptotic protein by interacting directly with death receptor, Fas and protects neuronal cells against FasL-induced cell death [5–8]. Whereas the association of LFG with Fas is essential for its inhibitory function, this interaction had no effect on Fas trafficking to the plasma membrane [5]. Notwithstanding the similarities between LFG and PP1201, no role for PP1201 in apoptosis has ever been reported. Given that PP1201 levels increase in response to shear stress in vascular cells and aged *PP1201*-deficient aged mice are prone to the development of aortic aneurysms, we hypothesized that upergulation of PP1201 offer resistance against cell death.

Apoptosis is a conserved programmed cell death process triggered in many cell types by exposure to a variety of stimuli including soluble or membrane-bound death ligands such as Fas-L. Apoptosis occurs in all cell types in the vessel wall and is implicated in the normal vasculature and disease conditions including arteriosclerosis, aortic aneurysms, arterial remodeling and medial degeneration [9–11]. Fas expressed on the cell surface predominantly determine the sensitivity of cells to its ligand, and specific stimuli such as p53 activation and interferon-$\gamma$ (IFN-$\gamma$) treatment potentiate apoptosis by increasing cell surface Fas levels [12–14]. Many cell types including vascular cells retain the receptor in the Golgi compartment, thus rendering the cells more resistant to Fas-mediated apoptosis. Very little is known of the mechanisms by which, Fas is retained in the Golgi compartments of vascular cells. In this study we find that high levels of PP1201 can attenuate Fas surface expression and protects cells against Fas-L-induced apoptosis. Our results suggest that increased expression of PP1201 in response to shear stress could help to maintain vascular homeostasis by dampening the effects of Fas signaling.

## Results

### Exogenously expressed PP1201 protects against Fas-mediated apoptosis

PP1201 contains two distinct segments, a 100 amino acid-long cytoplasmic region at the N-terminus and a putative seven-span transmembrane (TM) segment within a 211 amino acid-long C-terminus region (Fig. 1a). NCBI blast search showed a high degree of similarity (53% identity at the amino acid level) between PP1201 and Fas apoptotic inhibitory molecule, LFG (Fig. 1b) mostly in the TM portions suggesting that PP1201 probably share the anti-apoptotic function of LFG. To elucidate the possible mechanisms underlying impaired vascular homeostasis and the loss of smooth muscle cells in aged *PP1201*-deficient mice, we explored the role of PP1201 in cell death signaling pathways. The expression of PP1201 in human primary vascular cells and several tumor cell lines was evaluated by reverse transcriptase (RT)-PCR and Westernblot analyses. Cultured primary human umbilical vascular endothelial cells (HUVEC) and aortic smooth muscle cells (AoSMC) as well as human fibrosarcoma cell line (HT1080), human glioblastoma cell line (U251), human cervical cancer cell line (HeLa) expressed PP1201 at both mRNA and protein levels (Fig. 1c). In contrast, human embryonic kidney cells (293-T) and Jurkat cells had very little mRNA and no protein. We chose Jurkat that expresses little protein and HT1080 cell line and AoSMC primary cells for further experimentation. Jurkat and HT1080 cells, known to be sensitive to Fas-mediated apoptosis, were infected with retroviruses carrying no cDNA (vector control), or cDNAs encoding PP1201. Cells transduced with green fluorescent protein (GFP)-expressing retrovirus in parallel to these transfection assays resulted in nearly 90% GFP-positive cells (data not shown). Pools of cells stably expressing the PP1201-cDNAs were selected and their expression at the protein level in both the cell lines was confirmed by Western blot analysis (Fig. 1d).

As reported in case of LFG, stimulation with different amounts of Fas-L showed increased viability of PP1201 expressing both Jurkat and HT1080 cells compared with vector expressing control cells (Fig. 2a and S1A) [5, 7, 8]. To examine whether the increased survival was the result of reduced cell death upon PP1201 expression, cells were stained with annexin-V and analyzed by flow cytometry. In agreement with the known ability of Fas-L to induce apoptosis in these cell lines, treatment with the ligand had resulted in an increase in the number of annexin-V positive cells in a time-dependent manner (Fig. 2b, c). Annexin V-positive staining was more prominent in vector expressing control cells than PP1201 expressing Jurkat cells (24% vs. 13% in 4 h and 43% vs. 22% in 8 h) (Fig. 2b, c) and HT1080 cells (26% vs. 13% in 6 h) (Fig. S1B and C). In addition, modest reduction in caspase-3 activation in PP1201 expressing cells was found indicating that ecotopic expression of PP1201 protects against FasL-induced caspase activation and apoptosis (Fig. 2d). The resistance conferred by PP1201 against cell-death appears to be selective for Fas signaling as treatment with staurosporine (STS) or another death ligand, TNF-related apoptosis inducing ligand (TRAIL) showed only minimal to moderate effect on the cell viability (Fig. 2e, f). Consistent with this, STS treatment also showed only marginal differences in annexin-V staining between vector and PP1201 expressing cells (54% vs. 43% in 6 h of STS stimulation) (Fig. 2b, c). These results suggest that PP1201 is an anti-apoptotic protein and protects against FasL-induced apoptosis.

### PP1201 interacts with Fas and both molecules localize to the Golgi-compartment

To explore whether PP1201, like LFG can bind directly with Fas, we expressed FLAG-tagged Fas and untagged PP1201 together in 293T cells and evaluated association between them by immunoprecipitation assay. Precipitates of Fas pulled with anti-FLAG antibody contained substantial amounts of PP1201 suggesting that Fas forms a complex with PP1201 (Fig. 3a). Because most of the homology between PP1201 and LFG is confined to their C-terminus TM regions, we tested whether the TM region mediates the interaction of PP1201 with the Fas. As expected, pull-down with anti-FLAG antibody from extracts of the cells expressing FLAG-Fas and V5-tagged PP1201 variant, PP1201 (103–311), precipitated PP1201 fragment without the cytoplasmic domain suggesting that the C-terminus segment containing the TM portion interacts with the Fas (Fig. 3b). Attempts to express PP1201 without the TM (the cytosolic domain alone) detected little protein probably because of the rapid degradation of the expressed peptide (data not shown). In vascular cells, and other normal and cancer cell types the majority of Fas is sequestered in the Golgi apparatus [15]. Given that PP1201 associates with the Fas, we explored the possibility that PP1201 also localizes to the same compartment. It has been reported that PP1201 conjugated with enhanced green fluorescent protein (eGFP) at the C-terminus localizes to endosomal and lysosomal membrane compartments [2].

To determine the intracellular localization of endogenous PP1201, primary HUVEC cells were immunostained with anti-PP1201 antibody. Contrary to earlier reports confocal microscopic analysis revealed localization of PP1201 to mostly perinuclear compartments (Fig. 3c). Immunostaining with an antibody specific for GM130, a protein normally used as a marker to detect the Golgi compartment, showed a substantial colocalization between PP1201 and GM130-positive organelle. Further more, HeLa cells transiently expressing PP1201 conjugated with red fluorescent protein (RFP) at the cytosolic N-terminus, also resulted in the accumulation of PP1201 in the Golgi (Fig. 3d). To explore whether Fas colocalizes with PP1201, we transfected HT1080 cells with Fas conjugated with CFP and RFP-PP1201 and found that both Fas and PP1201 colocalize to perinuclear compartments (Fig. 3e). These results indicate that PP1201 and Fas localizes to the Golgi organelle and bind with each other.

### PP1201 expression reduces Fas surface expression

Given that PP1201 and Fas interact, we explored the possibility that PP1201 regulate the trafficking of Fas from the Golgi compartment by examining Fas surface expression in Jurkat and HT1080 cells that stably express PP1201. The levels of Fas were determined by staining with phycoerythrin-conjugated anti-Fas antibody and subsequent flow cytometry. Correlating with the resistance to FasL-induced death, PP1201 expressing cells showed reduced expression of Fas on the surface (MFI of 96 in Jurkat and 84 in HT1080) compared to vector expressing cells (MFI of 148 in Jurkat and 121 in HT1080) (Fig. 4a, c). In contrast, as was reported earlier [5], LFG expression had very little effect on the Fas surface expression in either Jurkat (MFI 131 vs. 148), or in HT1080 (116 vs. 121) (Fig. 4a, c). Western blot analysis of cellular extracts from both these cell types showed no changes in the total endogenous Fas pool level (Fig. 4b, d) indicating that the reduced surface expression was not the result of altered Fas expression. These results collectively suggest

that unlike LFG, PP1201 exerts its anti-apoptotic function by interfering with Fas protein trafficking that occurs between the Golgi and the plasma membrane.

Because the TM region of PP1201 interacts with Fas, we explored whether this association could interfere with Fas cell surface expression. For this, we cotransfected HT1080 cells with eGFP plasmid along with PP1201 plasmid (either the full-length or the TM) and stained for surface Fas using APC-conjugated anti-Fas antibody. Analysis of cells positive for both eGFP and Fas showed reduced Fas expression in cells transfected with PP1201 full-length (MFI 630) or the TM (MFI 641) compared to vector-transfected cells (MFI 926) (Fig. 4e).

Next we investigated whether PP1201 at constitutive level can regulate Fas trafficking, or the presence of high levels of the protein, which physiologically occurs in response to shear stress is required for its protective function. To address this, we used siRNA strategy to knockdown PP1201 expression in HT1080 that express basal levels of the protein, and in HT1080 cells that stably express PP1201. PP1201-specific siRNA was very effective in substantially reducing the protein in both cases (Fig. 5a, c). Upon transfection with specifc siRNA in HT1080-PP1201 cells at 0.5 nM and 5.0 nM concentrations, the expression of PP1201 was reduced to varying degree (Fig. 5c) and a corresponding increase in surface Fas in these cells was noted (Fig. 5d). In cells transfected with 5.0nM specific siRNA PP1201 protein levels were reduced close to endogenous levels (Fig. 5c) and correspondingly, Fas on the surface of these cells increased and were found to be near to levels constitutively observed in HT1080. In HT1080 cells, loss of the basal PP1201 did not further increase Fas surface expression compared with non-specific control siRNA transfected cells (Fig. 5b, d). In agreement with these results, reduction in PP1201 had no effect on the cell survival upon FasL treatment (data not shown). Finally, to explore whether exogenous expression of PP1201 in primary vascular cells affect the surface Fas levels, AoSMC were transfected with expression plasmids for eGFP along with either PP1201 or with vector as a control, using Amaxa nucleofection procedure and after 48 h of transfection cells were stained for the surface Fas. Although the pool of primary cells expressing eGFP was not large, FACS analysis of these Fas APC and eGFP double-positive cells consistently revealed a decrease in the Fas level on the surface (Fig. 5e). These results collectively suggest that higher levels of PP1201 downregulate the expression of Fas on the surface and protect cells against FasL-induced apoptosis.

## Discussion

Endothelial and smooth muscle cells that constitute the vascular system are constantly exposed to hemodynamic forces and a variety of biological substances including death ligands, cytokines, and cells present in blood. Several mechanisms intrinsic to the vessel wall offset the risk posed by these factors and play pivotal role in the maintenance of vascular homeostasis [16, 17]. These intrinsic mechanisms include the ability of vascular cells in response to shear stress to trigger changes in the activity or the expression of many proteins [18–23]. *PP1201* is one such shear stress responsive gene and mice deficient in *PP1201* are prone to CMD [2]. The molecular mechanisms underlying the loss of SMC and the development of CMD in *PP1201*-null mice are largely unknown. The present study

shows that PP1201 binds with the death receptor Fas, and protects against FasL-mediated death by reducing Fas expression on the plasma membrane. Several types of normal and cancer cells adopt downregulation of surface expression of Fas as a mechanism to attenuate Fas-mediated signaling. It has been reported that dynamin-2 and Fas-associated phosphatase 1 (FAP-1) regulate the export of Fas from the cytoplasm to the cell surface [24, 25]. Interferon $\gamma$ and hyperosmolarity are also reported to sensitize cells to apoptosis by triggering Fas membrane trafficking [26, 27]. Our findings suggest that PP1201-mediated reduction in Fas protein trafficking from the Golgi. Though the details of how PP1201 interfere with Fas surface expression are not clear, high levels of PP1201 could compete with molecules that facilitate Fas trafficking to the plasma membrane.

Fas signaling are implicated in normal vascular development and in a variety of vascular pathologies [9–11]. Complex interactions between varieties of factors largely dictate whether apoptosis follows as a consequence of Fas signaling. For example, a DED-containing intracellular molecule the FLICE-like inhibitory protein (FLIP) associates with pro-apoptotic molecule caspase-8 at the DISC [14, 28, 29]. High levels of FLIP compete out caspase-8 at the activating complex and thus can block Fas-L-induced apoptosis. Other mechanisms that attenuate Fas signaling include expression of receptor molecules without the death domain (decoy receptors) such as DcR3 that compete with Fas for the death signal [30]. Another way cells resist Fas-L effect is by expressing low levels of Fas on the plasma membrane. Cell–cell interactions and mechanical hemodynamic forces in particular also play critical role in vascular cell apoptosis [9, 11]. Fluid shear stress reduces apoptosis, and higher rates of cell death were reported at areas of low shear [31]. Cells in response to fluid force upregulate the expression of many molecules, some of which are believed to be anti-apoptotic in function [1, 16, 17]. PP1201 is one such shear stress responsive gene product and our findings suggest that it can protect cells against Fas-L-induced apoptosis. PP1201 belongs to an evolutionarily conserved family of anti-apoptotic proteins with different tissue expression profiles and sub-cellular localizations. Our data suggest that the protective role of PP1201 is limited to cells with high levels of protein, such as vascular cells in which its expression is upregulated in response to hemodynamic forces.

Contrary to our finding that PP1201 mostly associates with the Golgi, Zhao et al., reported that PP1201 localizes to lysosomal and endosomal vesicles [2]. For localization assays Zhao et al. used a PP1201 construct in which GFP was conjugated to the TM. Because the TM targets the protein to the membrane, to avoid potential interference of GFP with the protein localization we used specific anti-PP1201 antibody to immunostain endogenous PP1201. Consisting with the association of endogenous PP1201 with the Golgi, a PP1201 construct that expresses the TG as free domain with the RFP conjugated at the cytosolic domain also accumulated in the $GM130^{+}$ compartments.

Engagement of Fas with Fas-L also triggers several biological responses such as cell proliferation, chemokine production unrelated to apoptosis [32–35]. Treatment of primary endothelial cells with FasL is known to activate extracellular signal regulated kinase-1 and −2 (Erk1/2), and to induce matrix metalloproteinase (MMP)-9 and probably MMP-2 activity [36, 37]. Both MMP-9 and MMP-2 belong to the gelatinase group of MMPs that play important role in cardio-vascular remodeling [38]. It is possible that suppression of Fas

signaling by PP1201 could also negatively affect MMP production. Interestingly, the reported elevation of MMP-2 and MMP-9 activities in *PP1201*-deficient CMD mice supports such a notion [3]. Thus, enhanced non-apoptotic Fas signaling in *PP1201*-deficient mice might also contribute to the development of aneurysm.

## Materials and methods

### Cell lines and primary cells

Jurkat cells (clone E6–1) were maintained in RPMI media supplemented with 10% FBS (Fetal Bovine Serum). 293 cells and human fibrosarcoma cell line HT1080 were maintained in DMEM media supplemented with 10% FBS. AoSMC (Aortic Smooth Muscle Cells, Catalog No. CC-2571) and HUVEC (Human Umblical Vein Endothelial Cells, Catalog No. CC-2517) were purchased from Lonza and were cultured and maintained in prescribed media SmGM-2 (Lonza, Catalog No. CC-3182) and EGM-2 (Lonza, Catalog No. CC-3162), respectively. All the cells were kept in incubator at 37°C in the presence of 5% $CO_2$.

### RNA extraction and RT-PCR analysis

Actively growing cells were lysed in Trizol reagent from Invitrogen Corporation (Carlsbad, CA) following which; RNA was purified from the aqueous phase, after addition of 0.2 volumes choloform, using RNeasy Mini kit from Qiagen (Valencia, CA). Equal amount of total RNA from each sample was subjected to reverse transcription with oligo dT primers in a 20 μl reaction using the Superscript III cDNA synthesis system, Invitrogen Corporation (Carlsbad, CA). Polymerase Chain Reaction was performed for 22 cycles using equal volume of each first strand reaction mix (3 μl) as template for PCR with PP1201 (F: 5′-CACCGGATC CATGTCCAACCCCAGCGCCCC-3′, R: 5′-ATCGCGCG GCCGCTTAATTGCGATCCCCCATCAG-3′) and GAP-DH (F: 5′-CTGACCTGCCGTCTAGAAA-3′, R: 5′-TTT CTAGACGGCAGGTCAG-3′) primers in a 50ul PCR reaction in (GeneAmp PCR Amplification System 9700, Applied Biosystems) thermo cycler.

### Generation of stable cell lines

Jurkat and HT1080 stable cell lines expressing PP1201 and LFG were generated through retroviral transfection and after selection on puromycin. To produce virus $1.8 \times 10^6$ 293 cells were plated 1 day before of transfection. On next day 3 μg of either pMY-IP (Puromycin marker) or pMY-PP1201 or pMY-LFG were taken along with 3 μg of each pVpack-VSV-G and pVpack-GP (Stratagene) and were tansfected using Lipofectamin 2000 (Invitrogen). Media was substituted with 3 ml fresh media after 6 h. After 48 h media was collected and 1 ml of it was added on 0.1 million of desired cell line. After 24 h 1 ml of fresh media was added in cells and again kept for 24 h. As a control to assess the infection efficiency of virus, GFP containing virus was also used. After 48 h of FACS analysis was performed to check infection efficiency. After that cells were harvested and selected and maintained in the presence of 2 μg/ml of puromycin. Stable expression of PP1201 and LFG in Jurkat cells and in HT1080 cells were detected by immunoblot analysis using anti PP1201 Ab (Catalog No. 200–401-A57, Rockland, PA) and anti LFG Ab (Catalog No. 2285, ProSci Incorporated).

## Cell viability assay

**Cell viability assay was done using cell counting kit 8 (Catalog No. CK04–13, Dojindo)—**According to manufacturer's protocol. Briefly, $1 \times 10^4$ either Jurkat cells or $0.5 \times 10^4$ HT1080 cells (1 day before treatment) were seeded in 96 well plate. Jurkat cells were treated with increasing concentrations of Fas-L to induce apoptosis. Untreated cells were considered as growth control and only media as background. In the case of HT1080 apoptosis was induced by treating cells 20 ng/ml Fas-L along with 0.5 μg/ml cycloheximide to block protein synthesis. Cells treated with cycloheximide only were considered as growth control. Cells were kept in incubator at 37°C for 24 h and on next day 10 μl of Dojindo's cell counting kit reagent was added in each sample and after mixing plate was kept in incubator for 2–4 h or until color develops. Absorbance was measured at 450 nm.

## FACS analysis

**Detection of surface expression of endogenous Fas—**Staining of surface Fas was done by using anti-Fas-PE Ab (BD Pharmingen) according to manufacture's protocol. Briefly 1.0 million cells were taken and washed with 1 ml chilled FACS buffer (1XPBS + 2% FBS) and then finally resuspended into 80 μl FACS buffer. 20 μl of anti-Fas-PE Ab were added to samples. As a control staining was done with 20 μl of non-specific mouse IgG conjugated with PE. Samples were kept on ice for 45 min and then washed twice with FACS buffer and immediately analyzed by flow cytometry (FACS Calibur flow cytometer Becton–Dickinson). In the case of transient transfection with HT1080 and AoSMCs, expression plasmid 5 μg of either pCDNA3 PP1201 or pCDNA3 PP1201-TM (expressing transmembrane region amino acid 103–311) transfected along with 1 μg of peGFP (clonetech). 48 h post transfection cells were harvested and staining for Fas was done using antibody conjugated with APC. Ten thousand cells were acquired and double positive cells were analyzed for the surface expression of Fas.

## Apoptosis assay using Annexin-V and PI

Apoptosis was measured by using Annexin V-FITC Apoptosis Detection Kit-1 (BD Pharmingen) according to manufacturer's protocol. For induction of apoptosis 1 million Jurkat cells were treated with either 20 ng/ml Fas-L (R&D systems, Catalog No. 126-FL) or 0.5 μM staurosporine and kept in incubator at 37°C for different periods and untreated cells were considered as control. In the case of HT1080, 0.5 million cells were seeded 1 day before treatment. On the day of treatment cells were either treated with 1 μg/ml cycloheximide or in combination with 20 ng/ml Fas-L to induce apoptosis and kept in incubator for desired period. Cells treated with only cycloheximide were considered as control. On completion of treatment cells were washed twice with cold PBS then resuspended in 1× Annexin V binding buffer (supplied with kit) at concentration of $1 \times 10^6$ cells/ml. 100 μl of this solution was taken into a 5 ml culture tube and 5 μl of Annexin V-FITC and 5 μl of Propidium Iodide (PI) were added and after mixing sample was kept for 15 min at RT in dark. 400 μl of 1× Annexin V binding buffer was added to this solution and analyzed immediately by flow cytometry.

### Caspase activity

Caspase-3 activity was measured by using EnzChek Caspase-3 Kit (Molecular Probes, Catalog No. MP-13183) according to manufacturer's protocol. Briefly, 2.0 million Jurkat cells were treated with 20 ng/ml Fas-L to induce apoptosis and kept in incubator for desired length of time. Cells were left untreated as a negative control. Upon completion of treatment cells were washed twice with cold PBS and then lysed with the addition of 1× Cell lysis buffer (supplied with kit) and kept on ice for 30 min. After completion of lysis supernatants were taken and apoptosis were assayed as described in detail product literature. As an additional control Ac-DEVD-CHO inhibitor was added to the 6 h Fas induced sample to rule out any artifactual measurement of the enzymatic activity.

### Western blot and coimmunoprecipitation

For coimmunoprecipitation $1.8 \times 10^6$ 293 cells were seeded 1 day before transfection. On the day of transfection 6.0 μg pCDNA3 FLAG-Fas (N terminal FLAG tagged human Fas) expression plasmid and 2.0 μg pCDNA3 PP1201 expression plasmid or pCDNA3 nV5 PP1201-TM (N terminal V5 tagged truncated PP1201 expressing aa 103–311 containing transmembrane region) were transfected alone or in combination using Lipofectamine 2000 (Invitrogen). After transfection cells were kept in incubator for 48 h. After that cells were harvested and washed once with cold PBS then resuspended into 500 μl of 1× cell lysis buffer (Cell Signaling Technology, Catalog No. 9803) and kept on ice for 45 min for lysis to occur. Samples were spun at 13000 RPM for 15 min at 4°C. Small aliquots of these samples were taken and saved for detection of expression levels of transfected expression plasmids. Supernatant was collected and 2 μg non-specific mouse IgG1 (Catalog No. 557273, BD Pharmingen) was added and kept on rotation at 4°C for 30 min. Mean while Protein-G Sepharose beads were equilibrated with PBS followed by 1× cell lysis buffer. 50 μl of equilibrated beads were added to each samples and kept on rotation for another 30 min at 4°C. Samples were spun at 3000 RPM for 1 min and supernatant was collected and 2 μg mouse Anti FLAG M2 (Catalog No. F3165, Sigma) was added to sample and kept on rotation for 4 h at 4°C. After that 50 μl equilibrated beads were added to sample and kept on rotation for another 2 h. Upon completion of rotation samples were spun at 3000 RPM for 1 min and beads were collected and washed thrice with 1× cell lysis buffer. 50 μl of 2× SDS loading dye was added to washed beads and kept at 37°C for 30 min with intermittent vortexing. Supernatant of these samples were loaded on SDS-PAGE and after transfer to PVDF membranes, immunoblotting was done with anti PP1201 Ab or anti V5 Ab. Expression of proteins was also checked in lysates.

### Immunostaining and colocalization

Immunostaining was performed according to protocol described previously (46). For colocalization study HT1080 cells were cotransfected with expression plasmids2.0 μg Cyan Fluorescent Protein (CFP)-Fas and 2.0 μg Red Fluorescent Proten (RFP) PP1201 using Lipofectamin 2000. After 24 h of transfection picture was captured using a Zeiss LSM 510 confocal system using 63X1.4NA Plan-Apochromat oil immersion objective.

### Silencing of PP1201 by siRNA

To silence the expression of PP1201 0.5 million desired cell line or primary cells were seeded 1 day before transfection. On the day of transfection 50 nM ON-Target plus SMART pool (Thermo Scientific Dharmacon RNAi Technologies,Catalog No. L-015095–01) specific to human PP1201 was transfected using Lipofectamine RNAiMAX (Invitrogen) and kept for 24 h, 48 h and 72 h. As controls non-targeting siRNA were also transfected separately along with specific knocking down. Cells were harvested after completion of treatment, cells were lysed in 1× Cell Lysis Buffer (Cell Signaling Technology, Catalog No. 9803) and samples were spun at 13000 RPM for 15 min at 4°C. Supernatants were taken and run on SDS-PAGE then immunoblotted with Rabbit anti PP1201 Ab (Rockland, PA, Catalog No. 200–401-A57) to check expression of PP1201.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by the intramural Research Program of the National Institutes of Health, Center for Cancer research, National Cancer Institute. We thank Richard Siegel for CFP-Fas plasmid, Stanley Lipkowitz for GST-TRAIL and Hiroshi Nojima for human PP1201 cDNA containing plasmids.

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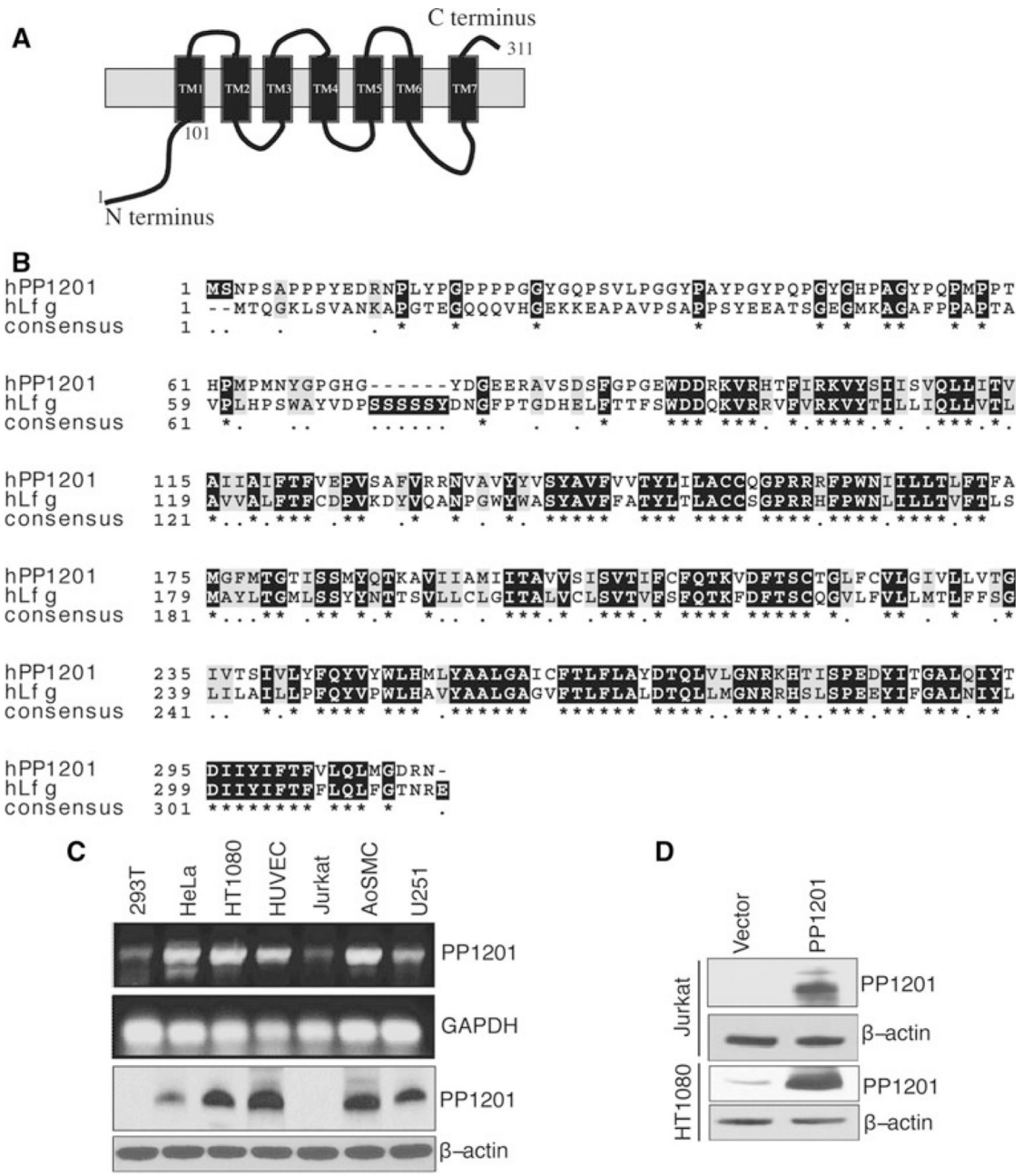


**Fig. 1.**

**a** Schematic diagram shows topology of 311 amino acid long PP1201 protein. PP1201 consists of seven transmembrane segments and an ~100 aa long N terminal hydrophilic region. **b** Comparison and composition of PP1201 with LFG. Protein sequence of human PP1201 (Accession No. # NM_022152) 311 aa and human LFG (Accession No. # NM_012306) 316 aa were aligned using ClustalW software. Black boxes represent the identical and conserved residues within PP1201 and LFG. **c** PP1201 is expressed by various cell lines. Semi-quantitative RT-PCR was performed to quantify the expression level of PP1201 in different cell lines (Upper Panel). Amplifications of glyceraldehyde-3-phosphate dehydrogenase were used as loading controls. Lower panels show endogenous protein levels

in corresponding cell lines and β-Actin was used as loading control for protein. 30 μg of protein was loaded in each well. **d** Generation of stable cell lines. Using retroviral transfection (As described in “Materials and methods”) Jurkat and HT1080 cell lines were used to stably express PP1201. Endogenous expression of PP1201 was present in HT1080 and absent in Jurkat. β-Actin was used as loading control for protein

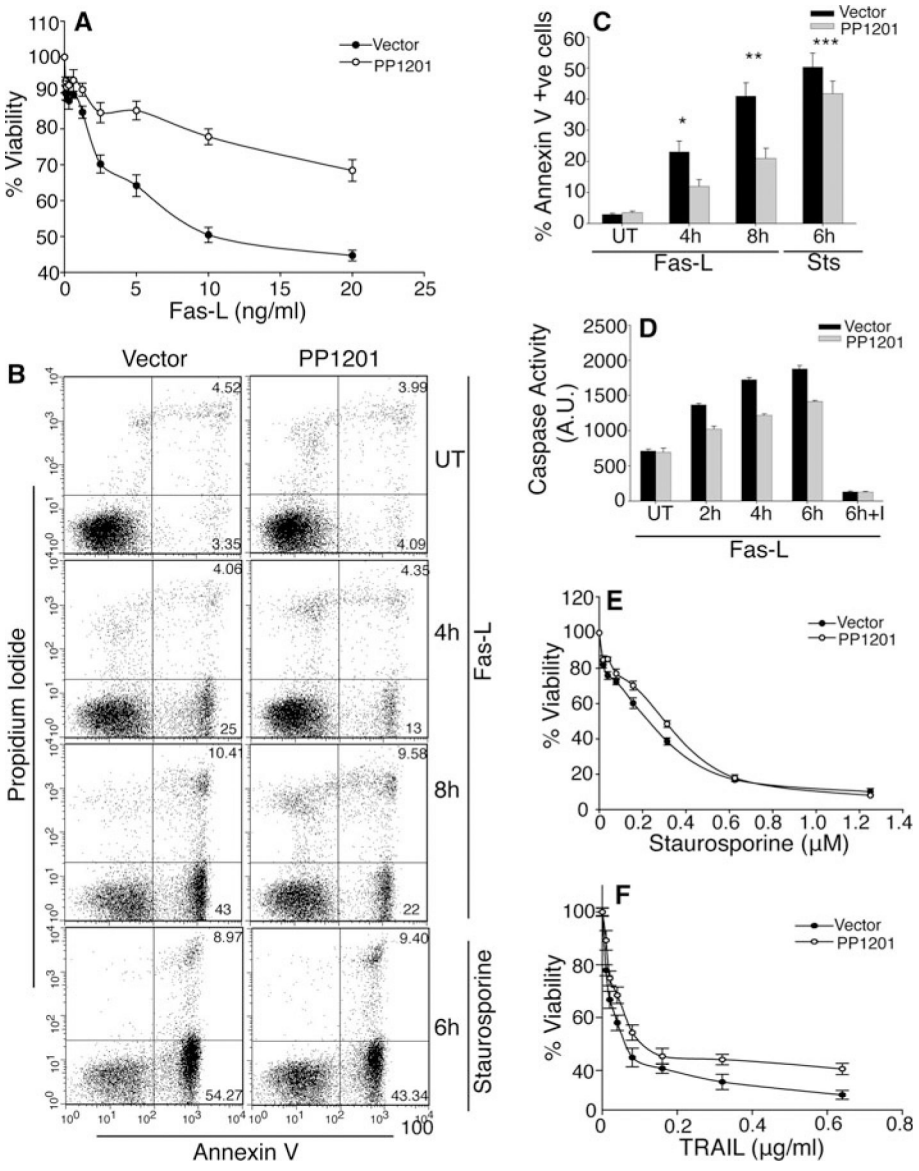


**Fig. 2.**

Overexpression of PP1201 affords protection from Fas mediated Cell Death. **a** Jurkat cell lines stably expressing PP1201 and vector control were treated with Fas-L at indicated doses and cell viability was monitored after 24 h using Dojindos kit (as described in Material and Methods). The results are presented as mean ± SD of three independent experiments. **b** Apoptosis was assessed by flow cytometry using FITC conjugated Annexin-V. In each case about 1 million cells were treated with 20 ng/ml Fas-L for 4 h and 8 h and in the case of Staurosporine (0.5 μM) treatment was done for 6 h. Values mentioned in lower right quadrant represent the percent Annexin-V positive cell population in each case. **c** Comparison of Annexin-V positive cells between Vector control and PP1201 expressing Jurkat cell lines upon treatment with Fas-L and Staurosporine. Bars represent the % Annexin-V positive cells in each case. Values plotted represent average ± SD of three independent experiments. **d** Relative caspase-3 activity in jurkat stable cell lines (vector

control vs. PP1201 expressing cells) upon Fas-L (20 ng/ml) treatment at different time points. Caspase 3 activity was preformed using EnzChek Caspase-3 Assay Kit #1(Molecular probes) according to manufacturar's protocol (details in "Materials and methods"). As a negative control Ac-DEVD-CHO inhibitor for this enzymatic reaction was added in an independent sample after inducing apoptosis by treating cells with Fas-L for 6 h. The values are given as the mean ± SD. **e** and **f** Overexpression of PP1201 very moderately protects Jurkat cells from staurosporine or TRAIL induced apoptosis. Jurkat cells expressing PP1201 and Vector control were challenged with the indicated concentrations of staurosporine for 6 h (**e**) or TRAIL for 18 h (**f**) and then cell viability was monitored as described previously. Values plotted are mean ± SD. *, ** represent $P$ value ≤ 0.0012 for Fas-L treatment and *** represents $P$ value ≤ 0.023 for staurosporine treatment

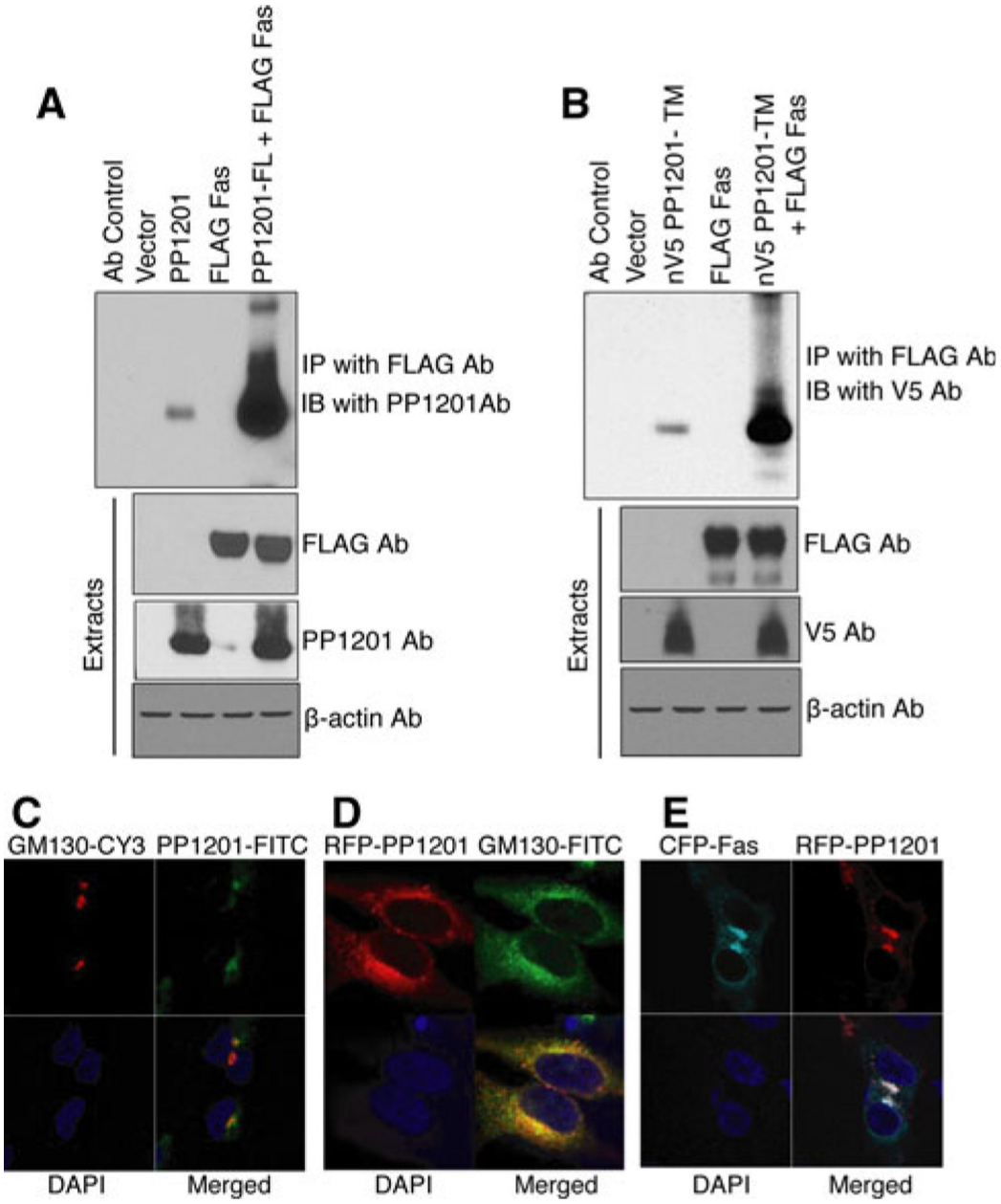


**Fig. 3.**

PP1201 interacts with Fas mainly through transmembrane region and localizes into intracellular compartment. **a** Coimmunoprecipitation of PP1201 with FLAG Fas. Expression plasmids for PP1201 and FLAG Fas were transfected in combination and alone in 293 cells. Cell lysates were immunoprecipitated with mouse anti-FLAG and immunoblotted with rabbit anti-PP1201. Lysates were immunoblotted with anti-FLAG, anti-PP1201 and anti $\beta$-actin. **b** Coimmunoprecipitation of N-terminal tagged V5 truncated PP1201-TM (expressing transmembrane region, 103–311aa) and FLAG Fas. Expression plasmids of these two were transfected in 293-T cell in combination and alone. Cell lysates were immunoprecipitated with mouse anti-FLAG and immunoblotted with mouse anti V5. Lysates were immunoblotted with anti-FLAG, anti-V5 and anti $\beta$-actin. **c** Immunostaining for PP1201 at

endogenous level in HUVEC cells using GM130 as a golgi marker shows that PP1201 partially localized into golgi. **d** Transient transfection of RFP tagged PP1201 in Hela cells and staining with golgi marker GM130 shows PP1201 to be localized into golgi compartment. **e** Transient transfection of CFP (Cyan Fluorescent Protein) tagged Fas along with RFP tagged PP1201 shows colocalization of both mostly into perinuclear spaces like Golgi

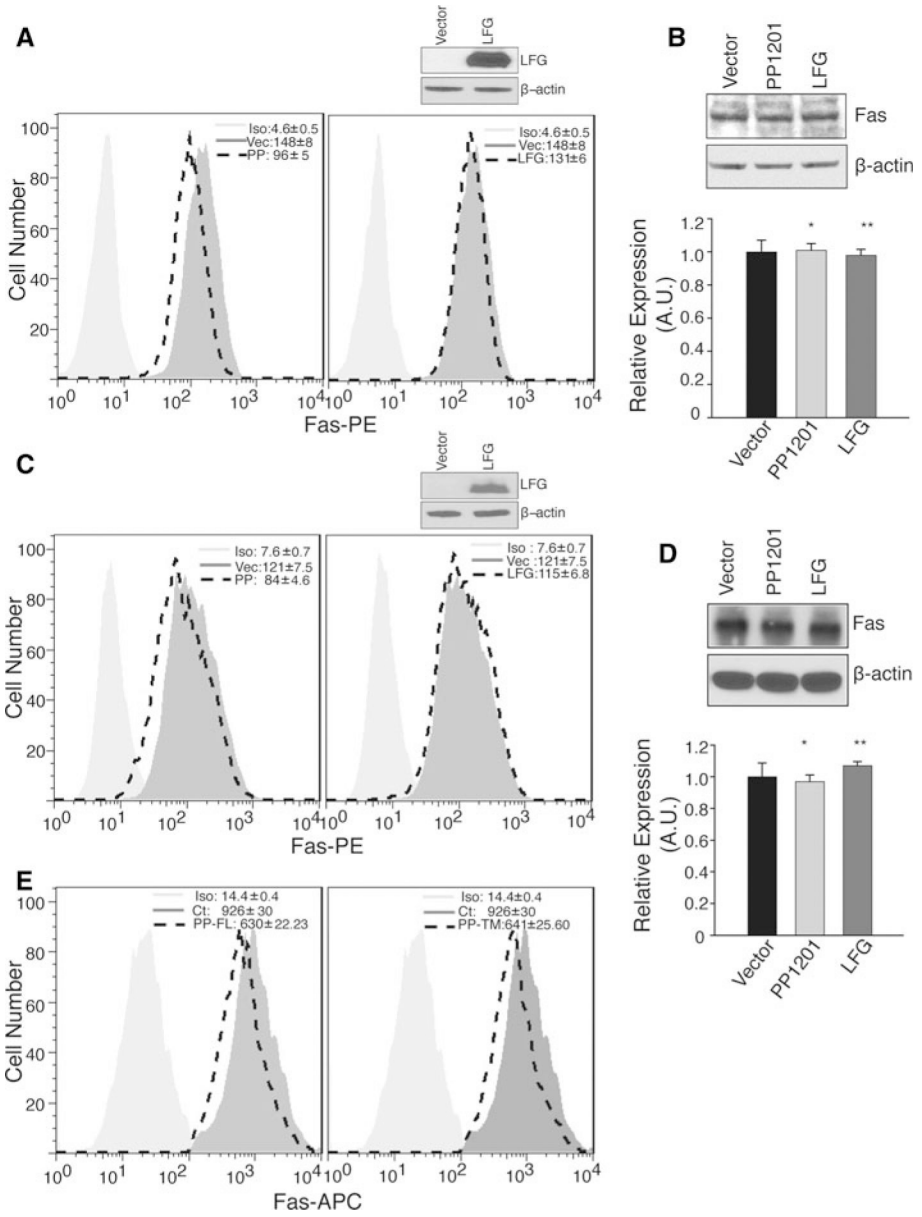


**Fig. 4.**

Surface and total expression level of Fas (CD95) in Jurkat and HT1080 cell lines expressing PP1201 and LFG. **a** Surface Fas levels in Jurkat stable cell lines expressing PP1201, LFG and vector control. Upper panel shows expression levels of LFG in Jurkat stable cell lines. In each case 1 million cells were stained with PE conjugated Fas antibody as described in "Material and methods". Overlay histogram represents one of the triplicates and inset shows average of mean fluorescence intensities (±SD) of triplicate values for Fas staining. **b** and **d** Total endogenous expression of Fas in indicated Jurkat (**b**) and HT1080 (**d**) stable cell lines. Lower panels show relative expression of total expression of Fas by densitometry analysis of 3 different blots (*, ** represent *P* value 0.4275 and 0.3664 (Jurkat) and 0.1516 and 0.3371 (HT1080) by comparing Fas expressions in Vector control versus PP1201 and LFG stable cell lines, respectively. **c** Surface Fas levels in HT1080 stable cell lines expressing PP1201,

LFG and vector control. Upper panel shows expression levels of LFG in Jurkat stable cell lines. In each case 1 million cells were stained with PE conjugated Fas antibody. Overlay histogram represents one of the triplicates and inset shows average of mean fluorescence intensities (±SD) of triplicate values for Fas staining. **d** Total endogenous expression of Fas in indicated HT1080 stable cell lines. E. HT1080 Cells were transfected with eGFP (1 μg) either with PP1201 expression plasmid (5 μg) or with 5 μg truncated PP1201 containing transmembrane region (aa 103–311) expression plasmid. Transfection with 5 μg vector along with 1 μg eGFP was used as a control. 48 h post transfection cells were stained with Fas antibody conjugated with APC and subjected to flow cytometry analysis. Double positive cells were analyzed for Fas staining by overlay histogram plot and by comparing mean fluorescence intensities. Overlay histogram represents one of the triplicates and inset shows average of mean fluorescence intensities (±SD) of triplicate values for Fas staining

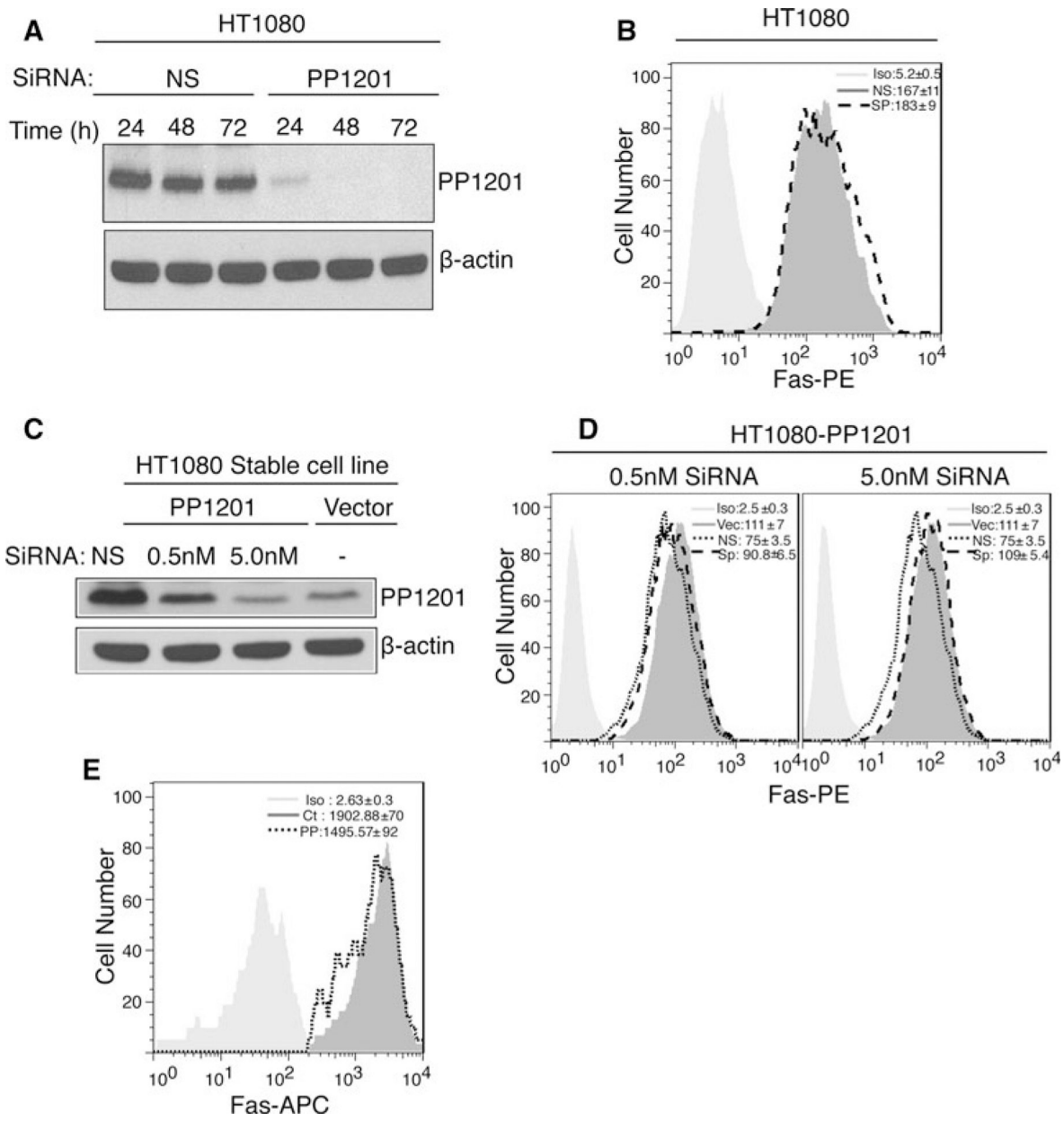


**Fig. 5.**

Silencing of endogenous PP1201 does not influence Cell surface Fas expression but overexpressed PP1201 does. **a** Endogenous expression of PP1201 goes down up to 72 h upon silencing in HT1080 cells. 50 nM of ON-TARGET plus SMART pool (Thermo scientific) specific to human PP1201 was transfected using Lipofectamine RNAiMAX (Invitrogen). As a control 50 nM Non-targeting pool was used. After transfections lysates were prepared of transfected cells for transfection period of 24 h, 48 h and at 72 h and subjected to immunoblot with anti-PP1201 antibody. Blotting with β-actin shows loading control. 30 μg of protein was loaded in each lane. **b** Flow cytometry analysis shows cell surface level of Fas in HT1080 after silencing of endogenous PP1201 at 48 h. Overlay histogram represents one of the triplicate and inset shows average of mean fluorescence

intensities (±SD) of triplicate values for Fas staining. **c** PP1210 expressing HT1080 cells were transfected with the indicated siRNAs and 30 μg of cellular lysates was probed with the indicated proteins. **d** Flow cytometry analysis exhibits surface levels of Fas after silencing to various levels of overexpressed PP1201 in HT1080 stable cell line. Overlay histogram represents one of the triplicates and inset shows average of mean fluorescence intensities (±SD) of triplicate values for Fas staining. **e** Overexpression of PP1201 in AoSMC cells down regulates the cell surface expression of Fas. AoSMC cells were transfected with 1 μg eGFP either with 5 μg vector (control) or with 5 μg PP1201 expression plasmid and after 48 h cells were stained with Fas antibody conjugated with APC and double positive cells were analyzed by FACS. Double positive populations were selected and Fas staining was analyzed by overlay histogram plot and by comparing mean fluorescence intensities. Overlay histogram represents one of the triplicates and inset shows average of mean fluorescence intensities (±SD) of triplicate values for Fas staining