

PICK1 promotes caveolin-dependent degradation of TGF- β type I receptor

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Protein that interacts with C kinase 1 (PICK1) is a critical mediator of α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor (AMPA) trafficking in neural synapses. However, its ubiquitous expression suggests that it may have other non-neural functions. Here we show that PICK1 antagonizes transforming growth factor beta (TGF- β) signaling by targeting TGF- β type I receptor (T β RI) for degradation. Biochemical analyses reveal that PICK1 directly interacts with the C-terminus of T β RI via its PDZ domain and acts as a scaffold protein to enhance the interaction between T β RI and caveolin-1, leading to enhanced lipid raft/caveolae localization. Therefore, PICK1 increases caveolin-mediated endocytosis, ubiquitination and degradation of T β RI. Moreover, a negative correlation between PICK1 expression and T β RI or phospho-Smad2 levels is observed in human breast tumors, indicating that PICK1 may participate in breast cancer development through inhibition of TGF- β signaling. Our findings reveal a non-neural function of PICK1 as an important negative regulator of TGF- β signaling.

Keywords: PICK1; TGF- β signaling; receptor degradation; caveolin; endocytosis

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Introduction

Transforming growth factor beta (TGF- β) regulates cell proliferation, differentiation, migration and death, and its abnormality has been closely related to pathology, including fibrosis, vascular disorders and cancer development [1-5]. Through a phosphorylation cascade, TGF- β type I receptor (T β RI), which is activated by the type II receptor (T β RII) in the ligand-bound receptor complex, transduces signaling to intracellular molecules, Smad proteins. Then, the activated Smad2/3-Smad4 complex translocates from the cytoplasm into the nucleus, and regulates the expression of target genes [6]. As TGF- β plays vital roles in many physiological processes, its du-

ration and intensity are under tight controls [7].

TGF- β receptors link extracellular stimulation to intracellular responses, so their distributions and stabilities are critical for TGF- β signal transduction [8, 9]. Like many other receptors on cell surface, TGF- β receptors undergo constant internalization, which is mediated by two major endocytic pathways: clathrin-mediated endocytosis and caveolin-mediated endocytosis [10]. The clathrin-mediated endocytosis of TGF- β receptors facilitates phosphorylation of downstream R-Smad and results in enhanced signaling. In contrast, caveolin-mediated endocytosis, which mainly occurs in lipid raft region, inhibits signaling through promoting receptors' ubiquitination followed by degradation [10, 11]. The dynamic balance between these two pathways maintains appropriate response of a cell to TGF- β stimulation.

Protein that interacts with C kinase 1 (PICK1) is a PDZ (PSD-95/Dlg/ZO-1) domain and BAR (Bin/amphiphysin/Rvs) domain containing protein first cloned as a PKC-binding partner through yeast two hybrid system [12]. As a peripheral protein, PICK1 has been reported to interact with a series of membrane proteins including

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α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) [13-16], acid-sensing ion channel (ASICs) [17] and ErbB2/Her-2 [18]. In most of the cases, PICK1 acts as a critical regulator of membrane receptors' subcellular trafficking to modulate neural processes such as learning and memory [19, 20]. PICK1 is widely expressed in brain, testis, heart, lung, liver, kidney and muscle [12]. Its abnormal expression in brain has been linked to schizophrenia and other mental disorders [21-23]. However, the significance of PICK1 in other organs has not been well characterized. Its potential effects on signal transduction and related diseases remain unclear. Recent evidences suggest that PICK1 may affect human cancer development [18, 24-26], in which TGF- β signal-

ing has been reported to play significant roles [5].

A PDZ domain-containing protein GIPC (GAIP-interacting protein, C terminus) has been shown to interact with the TGF- β type III receptor and regulate TGF- β signaling [27], but no PDZ proteins have been reported to interact with T β RI. In this study, we demonstrated that PICK1 antagonized TGF- β signaling and released its inhibition of cell growth. We found that PICK1 bound to T β RI directly and facilitated its internalization through caveolin-mediated endocytic pathway, which led to degradation of T β RI and turnoff of the signal. Moreover, the restriction of PICK1 on TGF- β signaling was observed in human breast cancer, which indicated that PICK1 might affect breast cancer development in a TGF- β -dependent

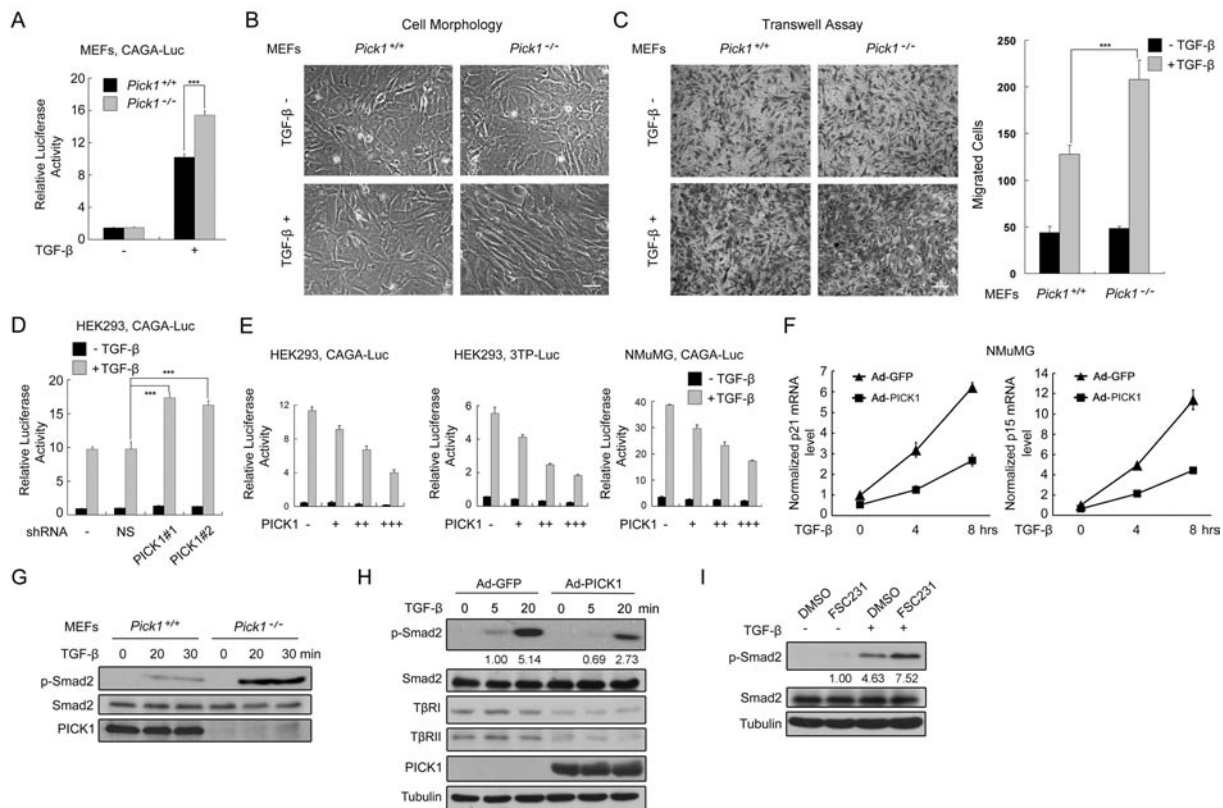


Figure 1 PICK1 attenuates TGF- β signaling. **(A)** MEFs transfected with CAGA-luciferase were treated with 100 pM TGF- β 1 for 16 h and harvested for luciferase measurement. **(B)** MEFs were treated with 200 pM TGF- β 1 for 24 h. Scale bar, 50 μ m. **(C)** MEFs in the transwell were treated with 200 pM TGF- β 1 for 16 h and fixed with methanol. After crystal violet staining, the migrated MEFs were quantitated (right panel). Scale bar, 100 μ m. **(D)** HEK293 cells transfected with CAGA-luciferase and shRNA were treated with TGF- β 1. Nonspecific (NS) shRNA was used as a negative control. **(E)** Cells transfected with different amounts of PICK1 plasmid were treated with TGF- β 1 for 16 h. **(F)** NMuMG cells infected with adenovirus expressing GFP or PICK1 were harvested to examine expression of p21 and p15 using quantitative RT-PCR. **(G)** MEFs were treated with 100 pM TGF- β 1 for various time and harvested for immunoblotting with the indicated antibodies. **(H)** NMuMG cells infected with GFP or PICK1 adenovirus were treated with TGF- β 1 for the indicated time, and harvested for immunoblotting. The band intensity was quantitated with BandScan 5.0. **(I)** NMuMG cells were treated with DMSO or FSC231 for 4 h followed by TGF- β 1 treatment for 30 min. Then, the cells were harvested for immunoblotting. Reporter activity was normalized with co-transfected Renilla and the data represent the mean \pm S.D. ($n = 3$). *** $P < 0.001$.

manner. Not only did this study reveal the relationship between PICK1 and TGF- β signal transduction, but also it provided new insight into physiological and pathological functions of PICK1, especially its effects on tumor development.

Results

PICK1 attenuates TGF- β /Smad signaling

Sequence analysis predicted that the C-terminus of T β RI (QQEGIKM) might function as a potential PICK1 PDZ-binding motif (-X- Φ -X- Φ , where X stands for any residues and Φ stands for hydrophobic residues), thus suggesting that PICK1 may regulate trafficking and function of T β RI. To assess this possibility, we investigated the effect of PICK1 on TGF- β signaling by comparing TGF- β responses between *Pick1*^{+/+} and *Pick1*^{-/-} mouse embryonic fibroblast (MEF) cells. Reporter assay using CAGA-luciferase revealed that TGF- β treatment increased the Smad activation in *Pick1*^{+/+} MEFs, and TGF- β response was markedly enhanced in *Pick1*^{-/-} MEFs (Figure 1A). Consistently, *Pick1*^{-/-} MEFs were more sensitive to TGF- β in the induction of the morphologic change to an elongate shape than *Pick1*^{+/+} MEFs (Figure 1B). Besides, loss of PICK1 promoted the mobility of MEFs upon TGF- β stimulation (Figure 1C). These data indicate that deletion of PICK1 enhances TGF- β response. To verify this, we employed RNA interference to knock down PICK1 expression (Supplementary information, Figure S1) and found that knockdown of PICK1 by shRNAs led to enhanced TGF- β response (Figure 1D). These data further support that disruption of PICK1 expression sensitizes cells to TGF- β responses.

To confirm the negative effect of PICK1 on TGF- β signaling, we overexpressed PICK1 and examined its effect on TGF- β -induced expression of the reporters CAGA-luciferase and 3TP-luciferase. Overexpression of PICK1 inhibited the transcriptional activity of TGF- β in HEK293, NMuMG and HaCaT cells in a dose-dependent manner (Figure 1E and Supplementary information, Figure S2). TGF- β upregulates the expression of p21 and p15 via Smad proteins [28, 29]. The TGF- β -induced expression of p21 and p15 was also attenuated by PICK1 in NMuMG cells, as shown by qRT-PCR (Figure 1F). In agreement with this, the antiproliferative effect of TGF- β was abolished by PICK1 overexpression in NMuMG cells (Supplementary information, Figure S3).

As Smad2/3 proteins are the main mediators of TGF- β signaling and activated by TGF- β receptors via C-terminal serine phosphorylation, we then assessed the effect of PICK1 on Smad phosphorylation. Although the Smad2 level was lower in *Pick1*^{+/+} MEFs, stronger phos-

phorylation was observed in *Pick1*^{-/-} MEFs upon TGF- β treatment (Figure 1G), while overexpression of PICK1 decreased TGF- β -induced Smad2 phosphorylation in NMuMG cells (Figure 1H). FSC231 is a small-molecule inhibitor of PICK1, which abolishes the interaction of PICK1 PDZ domain with other proteins [30]. As shown in Figure 1I, FSC231 enhanced TGF- β -induced Smad2 phosphorylation. Taken together, these data suggest that PICK1 negatively modulates TGF- β /Smad signaling.

PICK1 promotes T β RI degradation

As PICK1 attenuates TGF- β /Smad signaling, we then assessed whether PICK1 regulates TGF- β receptor stability. Immunoblotting analysis revealed that the T β RI protein level was higher in *Pick1*^{-/-} MEFs than in *Pick1*^{+/+} MEFs (Figure 2A). However, the T β RI mRNA level was similar in these cells (data not shown). These data suggest that PICK1 may modulate T β RI stability. To verify this, we followed T β RI turnover after proteins synthesis

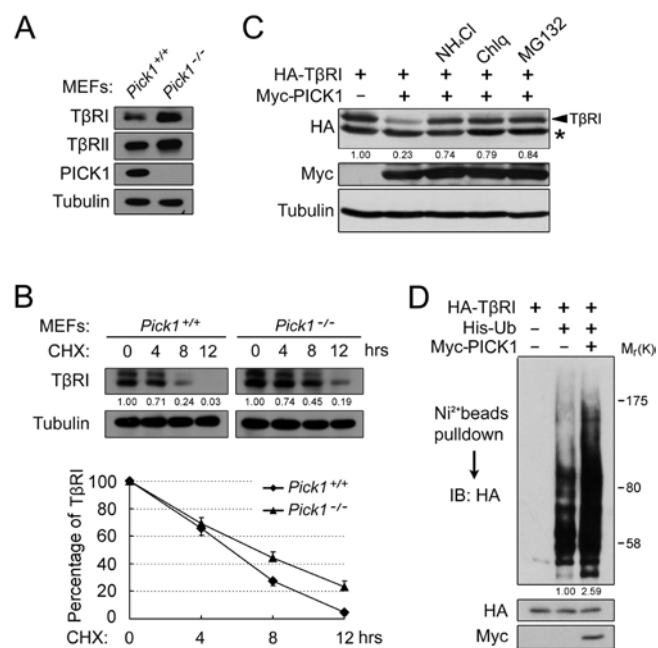


Figure 2 PICK1 enhances T β RI degradation. **(A)** MEFs were harvested for immunoblotting. Tubulin served as a loading control. **(B)** MEFs were treated with cycloheximide (CHX) for the indicated time and harvested for immunoblotting. The band intensity was quantitated and the statistical analysis of 3 independent experiments was provided. **(C)** HEK293T cells transfected with T β RI and PICK1 as indicated were treated with NH₄Cl (25 mM), chloroquine (100 μ M) or MG132 (25 μ M) for 4 h before harvested for immunoblotting. * indicates a nonspecific band. **(D)** HEK293T cells transfected with the indicated constructs were treated with chloroquine and MG132 and harvested for Ni-nitrilotriacetate bead precipitation, followed by immunoblotting.

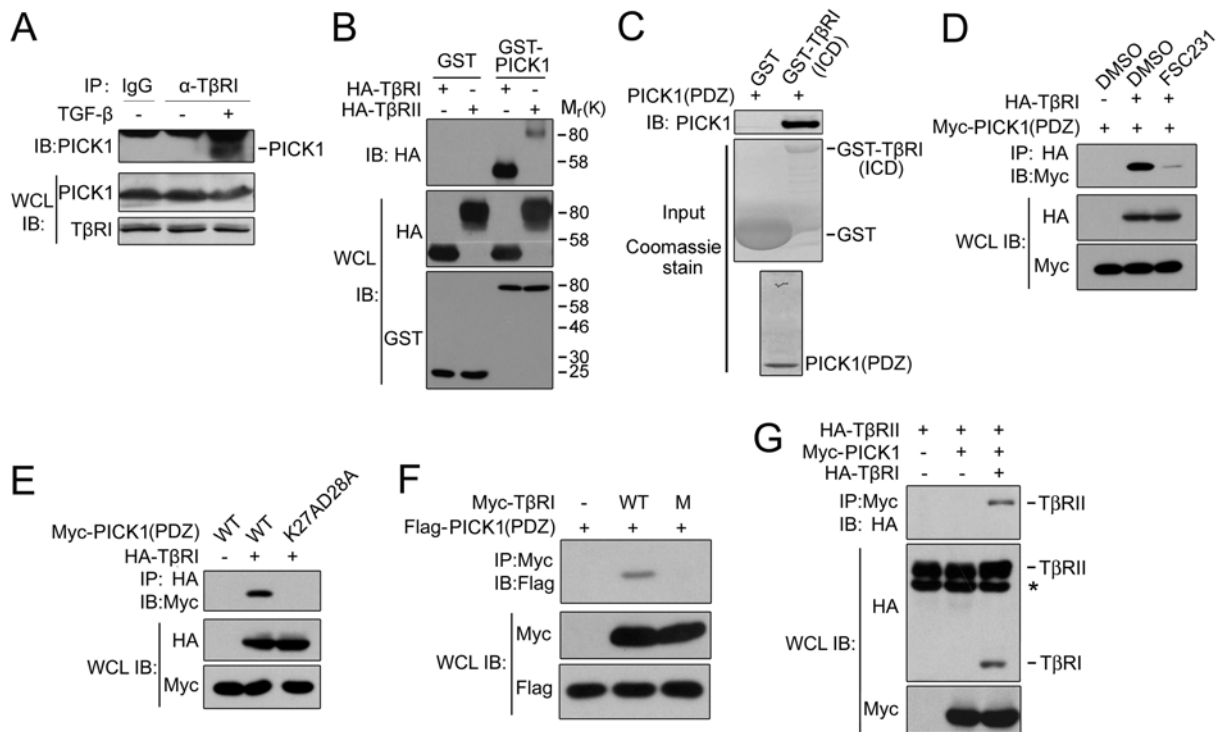


Figure 3 PICK1 directly interacts with T β RI via its PDZ domain. **(A)** HEK293 cells were treated with 100 pM TGF- β 1 for 30 min before harvested for immunoprecipitation (IP) and immunoblotting (IB) analysis. WCL, whole-cell lysate. **(B)** HEK293T cells transfected with GST-PICK1 and HA-tagged receptors were subjected for GST pull-down and immunoblotting. **(C)** Bacterially expressed recombinant GST-T β RI(ICD) and PICK1(PDZ) were subjected for GST pull-down and immunoblotting. GST and GST-T β RI(ICD) inputs were shown by Coomassie staining. **(D)** HEK293T cells transfected with HA-T β RI and Myc-PICK1(PDZ) were treated with FSC231 for 4 h and harvested for anti-HA immunoprecipitation and anti-Myc immunoblotting. **(E-G)** HEK293T cells transfected with the indicated constructs were harvested for immunoprecipitation and immunoblotting. M: mutation of Ile501 and Met503 to Asp at the C-tail of T β RI. * indicates a nonspecific band.

was blocked by cycloheximide (CHX) and found that loss of PICK1 resulted in a prolonged half-life of T β RI in MEFs (Figure 2B).

Consistently, overexpression of PICK1 destabilized T β RI in HEK293T cells, which could be rescued by the lysosome inhibitors NH₄Cl and chloroquine or the proteasome inhibitor MG132 (Figure 2C), indicating that both the lysosome and proteasome degradation pathways are involved in PICK1-mediated degradation of T β RI. As Smurf-mediated ubiquitination of T β RI is required for its degradation [31, 32], we examined whether PICK1 affected T β RI ubiquitination. Indeed, PICK1 enhanced T β RI ubiquitination (Figure 2D).

Protein kinase C is important in regulating cellular functions of PICK1 [33], however, PICK1-mediated degradation of T β RI was not markedly influenced by it (Supplementary information, Figure S4).

PICK1 interacts with T β RI via its PDZ domain

To address how PICK1 controls T β RI turnover, we ex-

amined whether PICK1 interacts with TGF- β receptors. In HEK293 cells, co-immunoprecipitation revealed that T β RI associated with PICK1 at the endogenous protein level upon TGF- β stimulation (Figure 3A). When they were overexpressed, the interaction between PICK1 and T β RI could be detected without TGF- β treatment (Figure 3B).

It has been shown that the PDZ domain of PICK1 mediates its interaction with other proteins [19]. To test whether the PICK1-T β RI interaction was direct, we generated recombinant PICK1 PDZ domain and GST-fused intracellular domain of T β RI(ICD). GST pull-down demonstrated that the PDZ domain could directly bind to T β RI(ICD) (Figure 3C). FSC231 interfered with the interaction between T β RI and PICK1(PDZ) (Figure 3D), further supporting that PICK1 directly interacts with T β RI via its PDZ domain. It has been reported that mutation of Lys27 and Asp28 to Ala can completely disrupt the binding of the PICK1 PDZ domain to other proteins [13, 34]. Consistently, only wild-type PICK1(PDZ), but not the mutant K27A,D28A, was co-immunoprecipitated

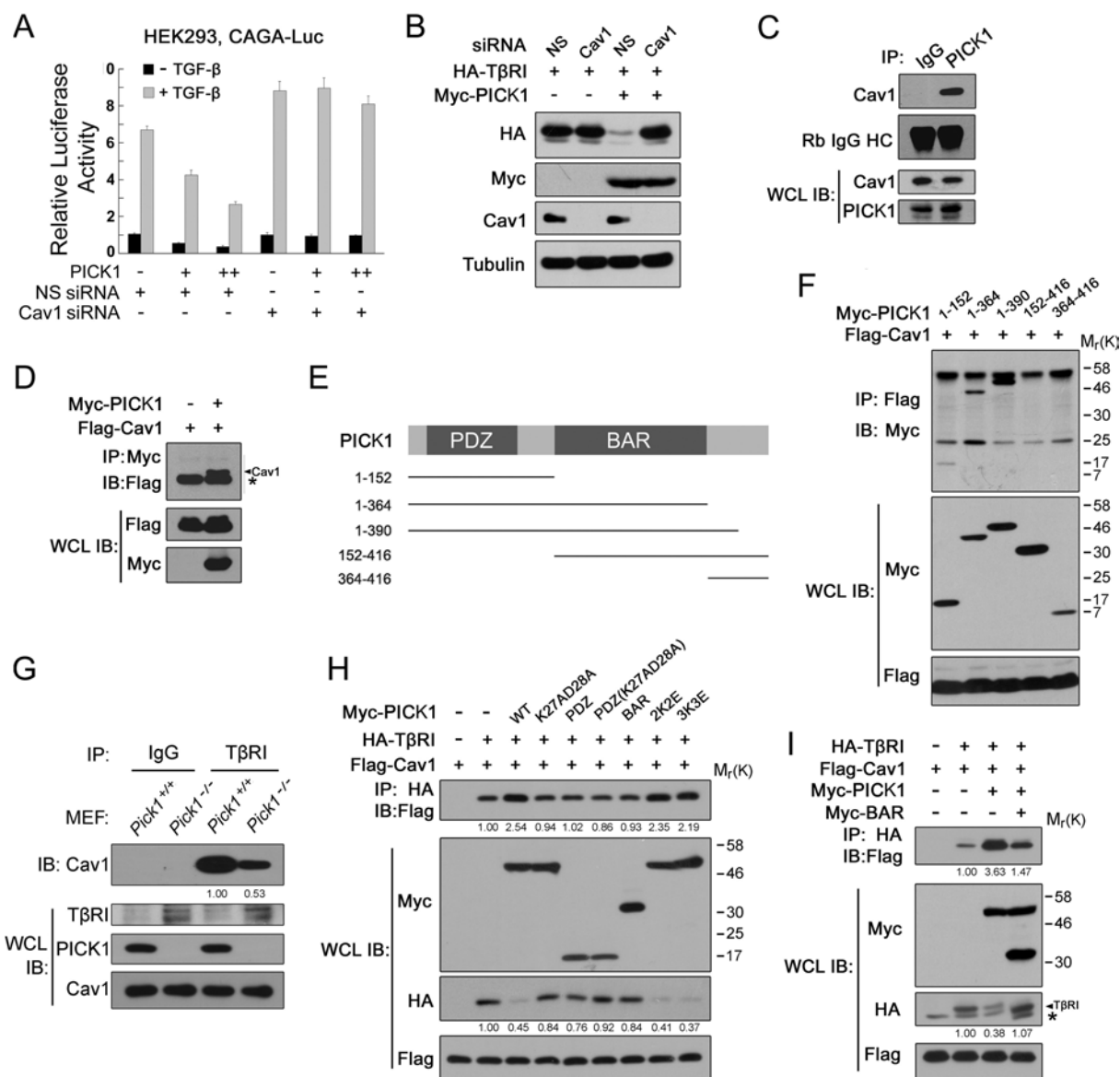


Figure 4 PICK1 promotes the T β RI-caveolin-1 interaction. **(A)** HEK293 cells transfected with CAGA-luciferase and caveolin-1 siRNA were treated with 100 pM TGF- β 1 for 16 h before harvested for luciferase assay. **(B)** HEK293T cells transfected with the indicated constructs or siRNA were harvested for immunoblotting. Tubulin was used as a loading control. **(C, D)** Interaction between PICK1 and caveolin-1. * indicates IgG light chain. **(E)** PICK1 deletion constructs for domain mapping. **(F)** HEK293T cells transfected with the indicated constructs were harvested for immunoprecipitation and immunoblotting. **(G)** The T β RI-caveolin-1 interaction was examined in MEFs with immunoprecipitation and immunoblotting. **(H, I)** HEK293T cells transfected with the indicated constructs were harvested for immunoprecipitation and immunoblotting. * indicates a nonspecific band.

by T β RI (Figure 3E). We further identified the C-terminal tail of T β RI as a PDZ-binding motif as mutation of the residues Ile501 and Met503 to Asp at the T β RI tail abolished its interaction with the PICK1 PDZ domain (Figure 3F).

The data in Figure 3B showed that PICK1 also weakly interacted with T β RII. To explore whether this interac-

tion requires the presence of T β RI, we tested whether PICK1 interacts with T β RII in L17 cells that do not express T β RI [35]. As shown in Figure 3G, PICK1 interacted with T β RII only when T β RI was co-expressed. As overexpressed receptors can form a complex in the absence of TGF- β [36, 37], these results implicate that PICK1 associates with T β RII through T β RI.

Caveolin-1 is essential for PICK1-mediated degradation of T β RI

PICK1 acts as a scaffold protein regulating membrane trafficking of AMPK receptors [19, 20]. It has been suggested that caveolin-1-mediated endocytosis is critical for T β RI degradation [10, 38]. Therefore, we tested

whether caveolin-1 is involved in PICK1-mediated T β RI degradation. First, we examined the effect of caveolin-1 on PICK1 inhibition of TGF- β transcriptional responses. As shown in Figure 4A, caveolin-1 knockdown with siRNA abolished the inhibitory effect of PICK1 on TGF- β -induced expression of CAGA-luciferase. Consistent with

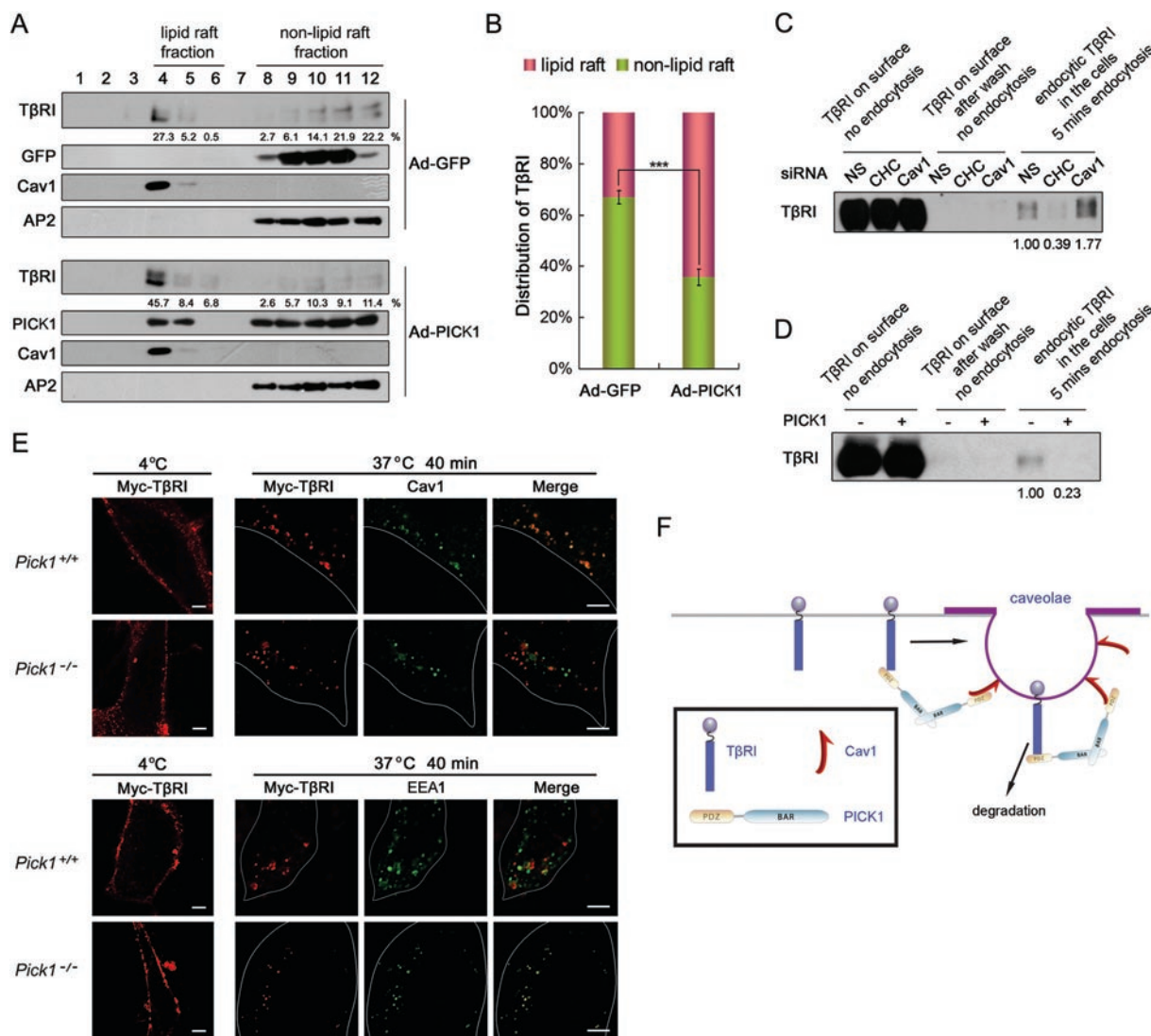


Figure 5 PICK1 facilitates lipid raft localization and caveolin-mediated endocytosis of T β RI. **(A, B)** NMuMG cells infected with GFP or PICK1 adenovirus were harvested for lipid raft purification using sucrose density gradient centrifugation. Fraction aliquots were immunoblotted with the indicated antibodies. Caveolin-1 and AP2 indicate lipid raft/caveolae and non-lipid raft fractions, respectively. Density of T β RI was quantitated and the statistical analysis of 3 independent experiments was shown in **B**. The data represent the mean \pm S.D. ($n = 3$). $***P < 0.001$. **(C, D)** HEK293 cells were transfected with clathrin heavy chain (CHC) or caveolin-1 siRNA **(C)** or PICK1 construct **(D)**, and the cell surface was labeled with biotin. After precipitation with streptavidin beads, T β RI on cell surface and endocytic T β RI were detected with anti-T β RI antibody. **(E)** MEFs transfected with Myc-T β RI were labeled with anti-Myc antibody at 4 °C for 6 h. Then the cells were washed with cold PBS twice, followed by incubation at 37 °C for 40 min. After fixation and permeabilization, the cells were visualized by immunofluorescence with anti-Myc (red) and anti-caveolin-1 (green) or with anti-Myc (red) and anti-EEA1 (green). Scale bar, 5 μ m. **(F)** A working model showing that PICK1 acts as a scaffold protein to enhance the T β RI-caveolin-1 interaction and caveolin-mediated endocytosis and degradation of the receptor.

this, PICK1-mediated degradation of T β RI was prevented when caveolin-1 was depleted (Figure 4B). These data suggest that caveolin-1 is required for PICK1-mediated inhibition of TGF- β signaling and T β RI degradation.

To elucidate the relationship of PICK1 and caveolin-1 in regulation of TGF- β signaling, we investigated whether PICK1 interacts with caveolin-1. Co-immunoprecipitation showed that PICK1 interacted with caveolin-1 at the endogenous protein levels in NMuMG cells (Figure 4C) and at the ectopically expressed protein levels in HEK293T cells (Figure 4D). Domain mapping revealed

that the 1-152 residues of PICK1 containing the PDZ domain was essential for its interaction with caveolin-1 (Figure 4E and 4F). However, the binding ability of the 1-152 fragment to caveolin-1 was weaker than that of full-length PICK1, suggesting that other domains may also contribute to this interaction.

As PICK1 interacts with both T β RI and caveolin-1, it might serve as a scaffold protein promoting the T β RI-caveolin-1 association. Indeed, we observed that the interaction between T β RI and caveolin-1 was greatly attenuated in *Pick1*^{-/-} MEFs (Figure 4G). Consistently,

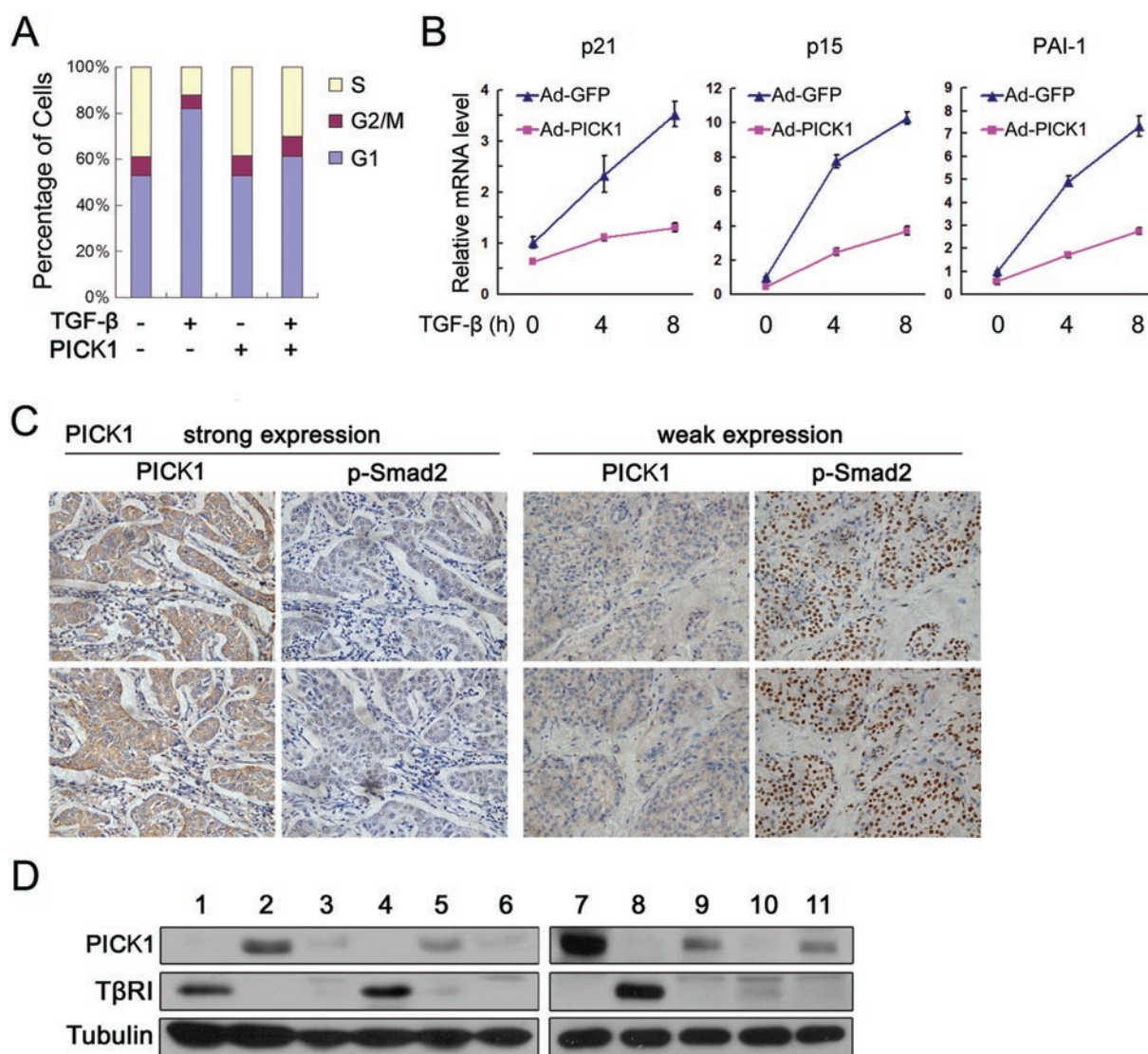


Figure 6 PICK1 protein levels are negatively correlated with TGF- β signaling activities in human breast cancer. **(A)** MCF10A cells were infected with GFP or PICK1 adenovirus and treated with TGF- β 1 for 20 h, followed by FACS analysis. **(B)** Expression analysis of TGF- β target genes by quantitative RT-PCR in MCF10A cells infected with GFP or PICK1 adenovirus. **(C)** Representative images from immunohistochemistry staining of breast cancer tissues with anti-PICK1 and anti-p-Smad2 antibodies in consecutive sections. **(D)** Immunoblotting of 11 fresh human breast cancer samples using the indicated antibodies.

the T β RI-caveolin-1 association was markedly enhanced by wild-type PICK1 although the total T β RI level was lower as the result of PICK1-mediated degradation (Figure 4H). However, none of the PDZ domain, the BAR domain or K27A,D28A mutant had effect on the T β RI-caveolin-1 interaction and T β RI degradation. PICK1 K266E,K268E (2K2E) and K251E,K252E,K257E (3K3E) mutants, which lose the lipid-binding ability [39], could not disrupt the PICK1 activity to interact with T β RI and promote its degradation.

The BAR domain of PICK1 is critical for PICK1 dimerization [40]. Overexpression of the BAR domain could bind to wild-type PICK1 and then disrupt the wild-type PICK1 dimerization efficiently (Supplementary information, Figure S5). To test whether dimerization is important for PICK1 to promote the interaction between T β RI and caveolin-1, we co-expressed the BAR domain and found that it decreased the PICK1-enhanced T β RI-caveolin-1 interaction (Figure 4I), implicating that PICK1 dimerization is critical for its enhancement on the T β RI-caveolin-1 interaction.

PICK1 facilitates lipid raft localization and caveolin-mediated endocytosis of T β RI

As PICK1-enhanced T β RI degradation is dependent on caveolin-1, we assessed whether localization of T β RI in lipid raft is affected by PICK1 in NMuMG cells. In GFP-expressing control cells, T β RI was distributed in both lipid raft (33%) and non-lipid raft fractions, while in the cells expressing PICK1, more T β RI was found in lipid raft fractions (60%) (Figure 5A and 5B).

T β RI can be internalized via both clathrin-mediated and caveolin-mediated endocytosis [10], and caveolin-mediated endocytosis turns off TGF- β signaling by promoting T β RI degradation. These two endocytosis pathways are maintained at a dynamic balance, and blockage of one pathway would lead to amplification of the other [38]. Endocytosis of biotin-labeled T β RI was employed to study the effect of PICK1 on this dynamic balance of the receptor [41]. Clathrin heavy chain siRNA and caveolin-1 siRNA were used to block clathrin-mediated endocytosis and caveolin-mediated endocytosis, respectively (Figure 5C and Supplementary information, Figure S6). We found that most T β RI internalization into the cell within 5 min was mainly through a rapid clathrin-mediated endocytosis (Figure 5C), which was blocked by PICK1 (Figure 5D), suggesting that PICK1 can prevent T β RI from the fast internalization through the clathrin-dependent pathway. In agreement with this, the majority of internalized T β RI was co-stained with the caveolin-1-positive vesicles in *Pick1*^{+/+} MEFs, while the proportion of T β RI-caveolin-1 co-localization was dramatically de-

creased in *Pick1*^{-/-} MEFs (Figure 5E). Consistently, the co-localization between internalized T β RI and EEA1 was increased in *Pick1*^{-/-} cells (Figure 5E). These data suggest that PICK1 can shift the internalization of T β RI from the clathrin-dependent pathway to the caveolin-dependent pathway.

To address whether dimerization is important for PICK1 to mediate caveolin-dependent endocytosis of T β RI, we expressed the BAR domain in HeLa cells and found that disruption of PICK1 dimerization markedly decreased the co-localization between endocytic T β RI and caveolin-1 (Supplementary information, Figure S7).

These results together suggest that PICK1 facilitates lipid raft localization and caveolin-mediated endocytosis of T β RI and thus accelerates T β RI degradation.

PICK1 expression is negatively correlated with TGF- β signaling in human breast cancer

Deregulation of TGF- β signaling contributes to tumorigenesis. It has been reported that PICK1 is highly expressed in breast cancer cells [26]. To investigate whether the negative regulation of PICK1 on TGF- β signaling contributes to breast cancer development, we first examined the effect of PICK1 on the antiproliferative activity of TGF- β in breast epithelial MCF10A cells. As shown in Figure 6A and 6B, overexpression of PICK1 not only attenuated G1 phase arrest induced by TGF- β , but also remarkably decreased mRNA levels of PAI1, p21 and p15, the latter two genes playing pivotal roles in cell proliferation. Immunohistochemistry analysis revealed that PICK1 expression and phosphor-Smad2 level, a TGF- β signaling indicator, showed a significant negative correlation ($P = 4.3e-12$; correlation coefficient = 0.54) in 141 breast cancer samples (Figure 6C). Furthermore, this negative correlation was also observed in 11 fresh breast tumors (Figure 6D). These results suggest that PICK1 may promote tumorigenesis by inhibiting TGF- β signaling.

Discussion

TGF- β signaling is tightly regulated to ensure its appropriate activity in embryo development and tissue homeostasis. PICK1 is a scaffold protein to control trafficking of several membrane receptors, including AMPA receptor and ASIC, and has been implicated to function in nervous system and male fertility [17, 19, 20, 42]. Here, we identify PICK1 as a novel negative regulator of TGF- β signaling by modulating subcellular localization and trafficking of T β RI. By promoting the interaction between T β RI and caveolin-1, PICK1 facilitates caveolin-mediated endocytosis of cell surface T β RI, resulting in T β RI degradation and signaling termination (Figure 5F).

TGF- β receptors are dynamically distributed on the plasma membrane [10]. Interleukin-6 [43], ADAM12 [44] and cholesterol depletion [45] can partition T β RI from the lipid raft microdomains into non-lipid raft regions, while hyaluronan [46] and the polysaccharide heparin sulfate [47] promote lipid raft localization of T β RI. Several lines of evidence showed that caveolin, which is enriched in lipid rafts, is required for T β RI turnover [38, 46, 48, 49]. In the current study, we found that PICK1 could promote caveolae localization of T β RI. This is achieved for PICK1 to function as a scaffold protein to bridge T β RI and caveolin-1. Consistently, less T β RI was found in *Pick1*^{-/-} cells. Interestingly, unlike AMPA receptor, PICK1-mediated regulation of membrane distribution of T β RI is independent of protein kinase C activity. Of note, our previous work demonstrated that lipid raft location of T β RI is also required for TGF- β -induced activation of MAPKs [49]. We observed that loss of PICK1 or knockdown of caveolin-1 led to enhanced phosphorylation level of p38 (data not shown), supporting the notion that T β RI localization in caveolae mainly modulates receptor turnover while its distribution in non-caveolae lipid rafts is important for MAPK activation.

T β RI enters the cell via both clathrin- or lipid raft-mediated endocytosis [10, 50]. These two distinct endocytic pathways predetermine whether T β RI promotes a signaling response or is degraded [9]. Clathrin-mediated endocytosis of T β RI into early endosomes enhances TGF- β signaling while lipid raft/caveolin-mediated endocytosis enhances ubiquitination and degradation of T β RI. Consistent with this, our biotin-labeled T β RI endocytosis assay revealed that rapid internalization of T β RI is mainly through clathrin-mediated endocytosis, indicating that clathrin-mediated endocytosis may be important for immediate cell response to TGF- β stimulation. Furthermore, PICK1 inhibits rapid clathrin-mediated internalization of T β RI, suggesting that PICK1-enhanced caveolae localization of T β RI leads to signal turnoff not only by promoting T β RI degradation, but also by shielding the receptor from entering early endosomes.

PICK1 is ubiquitously expressed in many tissues outside the nervous system [12], however, its non-neural functions remains largely unclear. Increased expression of PICK1 was reported in breast cancer [26]. We observed a negative correlation between PICK1 and p-Smad2 levels in breast cancer samples, indicating that PICK1 may promote breast cancer formation by restricting TGF- β signaling. In agreement with this, our results revealed that overexpression of PICK1 in breast epithelial cells abolished TGF- β -induced growth inhibition.

In summary, this study provides the evidence that PICK1 facilitates TGF- β receptor degradation in a cave-

olin-dependent manner. Our findings extend the understanding of the dynamic regulation of TGF- β receptors and offer new insight into the cellular and pathological functions of PICK1.

Materials and Methods

Plasmids and RNA interference

Human PICK1 was cloned into pCMV-Myc, Flag-pCDNA3.1 and PEBG1 for eukaryotic expression. MBP-PICK1 was generated by subcloning into the *EcoRI* and *SaII* sites of pMal-s (a gift from Jingming Yuan) for prokaryotic expression. pCMV-Myc-PICK1 K27A, D28A, 2K2E and 3K3E were constructed by the mutagenesis of pCMV-Myc-PICK1 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and confirmed by sequencing. For RNA interference, two PICK1 shRNA constructs were generated using pL3.7. The targeted sequences are as follows: 5'-GAAGTACCTGGACGTGAAG-3' and 5'-GAGGAGCTGGAGCGGACCG-3'. siRNAs targeting caveolin-1 and clathrin heavy chain were purchased from GenePharma. The sequences of siRNAs were as follows: negative control, sense 5'-UUCUCCGAACGUGUCACGUTT-3', anti-sense 5'-ACGUGACACGUUCGGA-GAATT-3'; caveolin-1, sense 5'-GGGACAUCUCUACACCGUCC-3', anti-sense 5'-GGAACGGUGUAGAGAUGUCCC-3'; clathrin heavy chain, sense 5'-GUAUCCAAUUCGAA-GACCTT-3', anti-sense 5'-GGUCUUCGAAUUGGAUUAUACTT-3'.

Reagents and antibodies

FSC231 was a generous gift from Dr Ulrik Gether. Propidium iodide, NH₄Cl, chloroquine and cycloheximide (CHX) were obtained from Sigma, MG132 from Calbiochem, TGF- β 1 from R&D Systems, HRV3C from Novagen, Transferrin conjugates (Fluor 488) from Invitrogen. Antibodies were purchased from ABR (α -Adaptin2 and α -PICK1), BD Biosciences (α -caveolin-1), Cell Signaling (α -Myc tag), Millipore (α -Myc tag and α -p-Smad2), Proteintech Group (α -PICK1), Santa Cruz (α -GST tag, α -HA tag, α -PICK1, α -Smad2, α -T β RI, α -T β RII and α -tubulin), Sigma (α -Flag tag M2), eBioscience (Rabbit TrueBlot HRP-conjugated anti-rabbit IgG), GE Healthcare (ECL HRP-linked anti-mouse IgG and ECL HRP-linked anti-rabbit IgG) and Jackson ImmunoResearch (fluorescein isothiocyanate (FITC)-conjugated anti-mouse and tetramethylrhodamine β -isothiocyanate (TRITC)-conjugated anti-rabbit).

Cell lines and transfection

HEK293, HEK293T, HeLa, HaCaT, NMuMG cells and MEFs were maintained in DMEM medium supplemented with 10% FBS (Hyclone) in a 37 °C humidified incubator containing 5% CO₂. L17 cells were maintained in MEM medium with 10% FBS and MCF10A was maintained in MCF10A specific culture medium. Transfection was performed with VigoFect (Vigorous) and Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations.

Pick1^{-/-} mice and MEF isolation

Pick1^{-/-} mice have been reported [42]. MEFs were isolated and cultured as previously described [51].

Adenoviral expression

Ad-EGFP and Ad-PICK1 were generated with AdEasy™ Adenoviral Vector System (Stratagene). PICK1 was cloned into the *XhoI* and *HindIII* sites of pShuttle-CMV, and then transferred to pAdEasy-1 vector using homologous recombination *in vivo* in BJ5183 cells. Linearized pAdEasy-1-PICK1 produced viral stocks after transfection into 293A cells.

Reporter assay, immunoblotting, immunofluorescence, immunoprecipitation and flow cytometry

These assays were performed as previously described [52, 53].

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen) and cDNA was synthesized with Revertra Ace (Toyobo). A Mx3000p Quantitative PCR system (Stratagene) was employed to perform qRT-PCR using EvaGreen dye (Biotium). The primers were: mouse p21 (5'-GTGATTGCGATGCGCTCATG-3' and 5'-TCTCTTGCAGAAGACCAATC-3'), mouse p15 (5'-CCCTGCCACCCTTACCAGA-3' and 5'-CAGATACCTCGCAATGT-CACG-3'), mouse GAPDH (5'-AAGAAGGTGGTGAAGCAG-3' and 5'-TCATACCAGGAAATGAGC-3'), human p21 (5'-TG-GAGACTCTCAGGGTCGAAAA-3' and 5'-GCGTTTGGAGT-GGTAGAAATCTG-3'), human p15 (5'-CACCGTTGGC-CGTAAACTTAAC-3' and 5'-TAATGAAGCTGAGCCAG-GTCT-3'), human PAI1 (5'-GAGACAGGCAGCTCGGATTC-3' and 5'-GGCTCCCAAAGTGCATTAC-3') and human GAPDH (5'-GAAGGTGAAGGTCCGAGATC-3' and 5'-GAAGATGGT-GATGGGATTC-3').

In vitro pull-down assay

MBP-PICK1 and GST-T β RI (intracellular domain, ICD) fusion proteins were expressed in *E. coli* and purified with glutathione-sepharose (Amersham Pharmacia Biotech) or amylose resin (New England Biolabs). PDZ domain of PICK1 was obtained after on-beads-cleavage of MBP-PDZ with HRV3C. The beads binding MBP-PICK1 or GST-T β RI(ICD) were washed extensively with binding buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl) and were incubated with purified T β RI(ICD) or PICK1(PDZ) for 1 h, respectively. Bound proteins were extracted with loading buffer and analyzed by immunoblotting.

Receptor endocytosis assay and quantitative analysis of endogenous T β RI endocytosis by biotinylation

HEK293 cells were placed on ice, washed twice with ice-cold PBS and then labeled with 0.2 mM Sulfo-NHS-SS-biotin (Thermo) in PBS for 30 min. After washing twice with ice-cold PBS and replacing with pre-warmed DMEM, dishes were placed in 37 °C incubator for 5 min, allowing for T β RI endocytosis. All dishes were then returned to ice and washed once with ice-cold PBS to stop membrane trafficking. To strip biotin remaining on cell surface, cells were treated twice with stripping buffer (50 mM cold L-Glutathione reduced, 75 mM NaCl, 75 mM NaOH, and 10% FBS) at 4 °C for 20 min. The cells are lysed in lysis buffer (10 mM EDTA, 1% Triton X-100, 0.1% SDS and protease inhibitors in PBS) and incubated with streptavidin beads (Thermo) for 1 h at 4 °C. Strptavidin-precipitated T β RI protein was detected with immunoblotting. The biotinylated T β RI remaining within the cells should be compared to the total T β RI level on cell surface before

stripping.

Antibody-labeled T β RI endocytosis

Pick1^{+/+} and *Pick1*^{-/-} MEFs were placed on glass coverslips and transfected with T β RI tagged with Myc right after signal peptide. Two days later, cells were washed twice with ice-cold PBS, followed by incubation with α -Myc tag antibody (1:100 in PBS containing 10% FBS) for 6 h at 4 °C. Then cells were washed with cold PBS twice and returned to 37 °C for 40 min, allowing endocytosis to occur. The cells were fixed and prepared for immunofluorescence analysis.

Lipid raft fractionation

Lipid raft fractionation was performed as previously described [49].

Immunohistochemistry and tissue array

Immunohistochemistry was performed with human breast tumor tissue array (grades I-III with normal controls) by Cybrdi Inc (Xi'an, China). A spearman's correlation test was used to analyze the relationship between PICK1 and p-Smad2.

Tumor samples

Fresh breast cancer tumor samples were obtained from the First Affiliated Hospital of Nanchang University by following the Ethics Committee guideline and stored at -80 °C. The frozen tissue was weighed, cut into small pieces and homogenized in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 1% SDS) with Complete Protease Inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche).

Statistic analysis

The band intensity was quantitated with BandScan 5.0. Student's *t*-test was performed in statistic analysis.

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