## Title

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# The Golgi-associated PDZ Domain Protein PIST/GOPC Stabilizes the β1-Adrenergic Receptor in Intracellular Compartments after Internalization*

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**Background:** PIST/GOPC is a Golgi-associated protein that interacts with several G-protein-coupled receptors via its single PDZ domain.
**Results:** PIST retains β1-adrenergic receptors in intracellular compartments and interferes with receptor degradation after endocytosis.
**Conclusion:** PIST stabilizes the receptor in an intracellular compartment.
**Significance:** PDZ proteins associated with intracellular membranes confer specific features to the subcellular targeting of interacting receptors.

**Many G-protein-coupled receptors carry C-terminal ligand motifs for PSD-95/discs large/ZO-1 (PDZ) domains; via interaction with PDZ domain-containing scaffold proteins, this allows for integration of receptors into signaling complexes. However, the presence of PDZ domain proteins attached to intracellular membranes suggests that PDZ-type interactions may also contribute to subcellular sorting of receptors. The protein interacting specifically with Tc10 (PIST; also known as GOPC) is a trans-Golgi-associated protein that interacts through its single PDZ domain with a variety of cell surface receptors. Here we show that PIST controls trafficking of the interacting β1-adrenergic receptor both in the anterograde, biosynthetic pathway and during postendocytic recycling. Overexpression and knockdown experiments show that PIST leads to retention of the receptor in the trans-Golgi network (TGN), to the effect that overexpressed PIST reduces activation of the MAPK pathway by β1-adrenergic receptor (β1AR) agonists. Receptors can be released from retention in the TGN by coexpression of the plasma membrane-associated scaffold PSD-95, which allows for transport of receptors to the plasma membrane. Stimulation of β1 receptors and activation of the cAMP pathway lead to relocation of PIST from the TGN to an endosome-like compartment. Here PIST colocalizes with SNX1 and the internalized β1AR and protects endocytosed receptors from lysosomal degradation. In agreement, β1AR levels are decreased in hippocampi of PIST-deficient mice. Our data suggest that PIST contributes to the fine-tuning of β1AR sorting both during biosynthetic and postendocytic trafficking.**

Postendocytic sorting of G-protein-coupled receptors (GPCRs)[3] is an important determinant of signal transduction (*e.g.* see Ref. 1). Many receptors are subject to agonist-dependent endocytosis, mostly via a clathrin- dependent pathway (2, 3). After uptake into endosomes, the fate of receptors is determined by factors interacting with the intracellular/cytosolic parts of receptors (4, 5). Whereas several receptors are sorted into a lysosomal, degradative pathway, others may be subject to recycling to the plasma membrane to allow for a new round of receptor activation and signaling. C-terminal PSD-95/discs large/ZO-1 (PDZ) ligand motifs, which enable selected receptors to bind to PDZ domain-containing proteins, have been established as important determinants for recycling (4) or degradation (6). Whereas some PDZ domain proteins serve as scaffolds for GPCR-associated signaling complexes at the plasma membrane (7, 8), it is now becoming clear that receptors are handed over to other PDZ domain proteins during their passage through intracellular compartments (9). Several recent studies have now begun to elucidate PDZ domain-containing proteins that are involved in postendocytic sorting. Thus sorting nexin 27 (SNX27) is a major determinant for recycling of receptors containing a PDZ ligand in general (10) and for GPCRs in particular (9, 11–13). SNX27-mediated recycling involves components of the retromer, which was initially described as the machinery for retrograde endosome to Golgi trafficking (12).

Similar to SNX27, PIST (protein interacting specifically with Tc10; also known as GOPC, CAL, or FIG), is a PDZ domain protein involved in intracellular trafficking of receptors. PIST exhibits different effects on its associated transmembrane proteins, which in most cases associate via the PDZ domain. Thus, PIST targets the associated CFTR toward lysosomal degradation (14–16). With respect to GPCRs, we and others have

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[3] The abbreviations used are: GPCR, G-protein-coupled receptors; SNX27, sorting nexin 27; β1AR, β1-adrenergic receptor; RFP, red fluorescent protein; IBMX, isobutylmethylxanthine; ctr, control.

reported that PIST retains associated receptors at the trans-Golgi network during the biosynthetic pathway, leading to a reduced number of cell surface receptors (9, 17, 18). The relevance of PIST during postendocytic sorting of receptors is so far unclear. PIST binds to GPCRs of different classes, including metabotropic glutamate receptors (6, 19), the somatostatin receptor subtype 5 (9, 18), and the β1-adrenergic receptor. As PIST does not bind to the closely related β2 receptor (17), this difference between two otherwise closely related receptors enabled us to assess the specific role of PIST by comparing differences between the two receptors in their biosynthetic pathway and postendocytic sorting mechanisms. We report here that PIST specifically affects β1 receptors in two different ways: 1) PIST retains β1 at the Golgi apparatus during the biosynthetic pathway, and 2) PIST protects internalized β1 receptors from lysosomal degradation by stabilizing it in an intracellular compartment.

## EXPERIMENTAL PROCEDURES

*Antibodies*—Antibodies against the antigens listed were obtained from the following sources: monomeric RFP, GFP, and the GFP trap matrix from Chromotek (Munich, Germany); β-actin from Abcam (Cambridge, UK); pErk1/2 and Erk1/2 from Cell Signaling Technologies (Frankfurt, Germany); GAPDH from Merck; FLAG from Sigma; SNX1 from BD Biosciences; Giantin from ENZO Life Sciences (Lörrach, Germany). A guinea pig antiserum against the C-terminal portion of PIST has been raised in our laboratory (18, 20).

*Expression Constructs*—An expression construct for GFP-tagged PIST has been described before (9). Monomeric RFP-tagged β1 and β2 adrenergic receptors were expressed using constructs coding for a signal peptide (peptide sequence MVLWLQLALLALLLPTSLAQGEVDI) followed by the monomeric RFP cDNA (obtained from Roger Tsien, UCSD San Diego, CA) (21) and the β1 or β2 receptor coding sequence. Constructs coding for a signal peptide (MKTIIALSYIFCLVFA) followed by a FLAG sequence and the β1 or β2 receptor coding sequence have been described (12).

*Tissue Culture*—Human embryonic kidney 293 cells and 293T cells stably expressing the large T-antigen were obtained from the ATCC and cultured in DMEM containing 10% fetal calf serum. Transfection of 293 cells was performed using Lipofectamine 1000 from Invitrogen according to the manufacturer's protocol. Transfection of 293T cells was performed using Turbofect (Fermentas) according to the manufacturer's protocol.

*RNA Interference*—For knockdown of PIST in 293 cells, predesigned siRNAs (FlexiTube; obtained from Qiagen, Hilden, Germany) were tested for silencing efficiency against PIST by transfection of HEK293 cells with 20 nmol of siRNAs using RNAiMax (Invitrogen). Knockdown efficiency was determined by cell lysis and Western blot 72 h after transfection. siRNA #9 for PIST (target sequence CAAGGTGTTGGTCCAATTAGA) was most effective and used for further experiments. A non-targeting siRNA (AllStars Negative Control siRNA, Qiagen) was used as a negative control.

*Cell Surface Biotinylation*—Determination of cell surface receptors was performed according to Turvy and Blum (22). Cells were washed 3 times in ice-cold Hanks' balanced salt solution and then exposed to 0.5 mg/ml Sulfo-NHS-SS-biotin (Pierce) for 30 min at 4 °C. To quench excess Sulfo-NHS-SS-biotin, cells were washed 3 times in Hanks' balanced salt solution with 5 mM Tris (pH 7.4). For the isolation of biotinylated proteins, cells were lysed in radioimmune precipitation assay buffer and centrifuged for 20 min at 20,000 × *g*. Clear supernatants were either used for Biotin-Streptavidin-ELISA according to Turvy and Blum (22) or incubated with EZview Red Streptavidin Affinity Gel (Sigma) for 4 h at 4 °C. Beads were sedimented by centrifugation at 1000 × *g* for 5 min and washed 4 times with radioimmune precipitation assay buffer. Aliquots of input and precipitate samples were analyzed by Western blot using appropriate antibodies.

*Degradation Assay*—293T cells were cotransfected with SP-FLAG-β1AR and PIST-GFP or GFP (ctr). After 48 h translation was blocked by incubation with 10 μg/ml cycloheximide. After 30 min the cells were stimulated with 10 μg/ml isoproterenol. At different times after stimulation, the cells were lysed, and the remaining amount of receptors was determined by Western blot. The values for SP-FLAG-β1AR were normalized to GAPDH, and the time point $t = 0$ was set to 100%.

*Recycling Assay*—Agonist-dependent internalization and recycling of SP-FLAG-β1AR was determined by flow cytometry as described in Hanyaloglu and von Zastrow (23). Internalization was determined as the difference between steady-state cell surface level (not incubated) and the level after 20 min of incubation with isoproterenol normalized to the steady-state level. For recycling, cells were incubated for another 40 min with the antagonist alprenolol after the initial stimulation with isoproterenol.

*Microscopic Analysis*—293T cells expressing SP-FLAG-β1AR were treated with 10 μg/ml Alexa-647-labeled FLAG-antibody. Then, cells were stimulated with 10 μg/ml isoproterenol for 20 min. After washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Again, cells were washed with PBS and then incubated for 3 min with 0.1% Triton X-100 in PBS for permeabilization. After washing, the cells were blocked with 2% horse serum in PBS for 1 h at room temperature. Cells were then incubated with primary antibody diluted in horse serum, PBS for 3 h followed by appropriate fluorescently labeled secondary antibodies. The cells were analyzed using a PerkinElmer Life Sciences Spinning Disc Microscope. Non-transfected 293T cells were incubated in serum-free media for 16 h and then stimulated with 500 μM IBMX and 50 μM forskolin for 20 min. Afterward, cells were fixed and stained as above.

*Mice*—PIST/GOPC-deficient mice have been described (24). However, these mice were not made available to us despite repeated requests to the authors. Therefore, we obtained the mouse ES cell line EPD0822_2_B04, which carries a targeted exon trap insertion in the second intron of the mouse GOPC gene (coding for mouse PIST) from the EUCOMM consortium (Munich, Germany). After expansion and injection of ES cells, several chimeric animals were obtained that were crossed to obtain heterozygous PIST-deficient animals. Mice were maintained in the C57Bl6 background. Genotype was determined by PCR from tail biopsies using primers CTTTGACGT-

GAAGCTGCCCA (Ff), TGTACAGGCATGCACGAAGAA (Wt rev), and CCCGTCCCCCTTCCTATGTA (KO rev). After several generations of breeding, only three homozygous PIST-deficient animals were obtained, which were analyzed here ($n$ = 222 mice from heterozygous crosses, 81 WT, 138 heterozygous, 3 KO).

*Statistics*—Statistical analysis of data was performed using GraphPad Prism software (Version 6; GraphPad; San Diego, CA); statistical tests were selected based on directions provided by the software manual.

## RESULTS

We previously described that overexpression of PIST leads to intracellular retention of the G-protein-coupled somatostatin receptor 5 (SSTR5; Ref. 9). To test whether this is a general effect we coexpressed an RFP-tagged version of the β1-adrenergic receptor (SP-RFP-β1AR) with its interacting PDZ domain proteins PIST and/or PSD-95 in a human cell line. The amount of cell surface receptors was determined by cell surface biotinylation experiments performed in an ELISA format. We determined that the amount of total cellular receptors was not affected by coexpression of PDZ proteins. In agreement with previous work (17), we observed that overexpressed PIST significantly reduces the number of receptors at the plasma membrane, whereas PSD-95 does not (Fig. 1*A*). Upon coexpression of the β1AR with both PIST and PSD-95, PSD-95 was able to antagonize the effect of PIST on receptor transport to the cell surface, thereby reestablishing control levels of surface-localized β1AR. The effect of PIST was specific to the β1AR, as it did not affect surface localization of the closely related β2AR either when expressed alone or in combination with the β2AR-interacting protein NHERF1 (Fig. 1*B*). This is consistent with the observation that the PDZ ligand motif of the β2AR does not bind to the PDZ domain of PIST (17).[4] The cell surface biotinylation data were confirmed by confocal microscopic analysis of cells expressing RFP-tagged β1AR and GFP-tagged PDZ domain proteins. Here GFP-PIST is localized to a perinuclear compartment, which we identify as the Golgi apparatus due to the known localization of PIST. The overexpression of PIST induces a redistribution of the receptor to the effect that both proteins are colocalized at the Golgi. On the other hand PSD-95 colocalizes with the receptor at the plasma membrane (Fig. 1, *C–E*). Finally, knockdown of PIST by transfection of siRNA increased cell surface levels of full length β1AR (Fig. 1*F*).

As PIST reduces the number of β1 receptors at the plasma membrane, we investigated whether this affects receptor-dependent signaling. For this purpose we analyzed stimulation of the MAPK pathway upon treatment with the agonist isoproterenol by measuring phosphorylation of the Erk1/2 MAPK. In cells overexpressing PIST together with the β1-receptor, we observed a marked reduction of agonist-induced phospho-Erk levels compared with cells coexpressing the GFP control protein, suggesting that reduction of cell surface levels of the β1-receptor indeed leads to a reduced efficiency in signaling (Fig. 2, *A* and *B*). On the other hand, knockdown of PIST leads to a significant increase in agonist-induced Erk1/2 activity (Fig. 2, *C* and *D*). Signaling via a mutant β1-receptor lacking the C-terminal PDZ ligand motif (V477A mutant in Fig. 2, *C* and *D*) was not affected by PIST overexpression or knockdown, indicating that the effect of PIST on agonist-induced MAPK activation is due to the interaction of PIST with the β1AR via the PDZ domain.

The effect of PIST on the agonist-dependent internalization of the β1-receptor was investigated by flow cytometry of cells using a fluorescently labeled antibody directed at the N-terminal FLAG epitope. The amount of internalized receptors was determined as the difference of the signal intensity of non-treated cells and cells treated with the agonist isoproterenol for 20 min. Here PIST had a small but non-significant effect, as the amount of internalized receptors was slightly increased by PIST overexpression and decreased by PIST knockdown (Fig. 3*A*). Performing this assay in the presence of leupeptin (which inhibits lysosomal degradation) or monensin (which inhibits a variety of intracellular trafficking steps including recycling (25, 26)) suggested that some recycling of receptors occurs already during the period of agonist treatment, to the effect that more receptors are removed from the cell surface by endocytosis in the presence of monensin (but not leupeptin) in both control and PIST-overexpressing cells. Again we saw little difference in isoproterenol-induced internalization between control and PIST-overexpressed conditions. In contrast, we observed a strong effect of PIST overexpression on recycling of internalized receptors, which was determined as the reappearance of receptors at the plasma membrane after prolonged incubation in the presence of the antagonist alprenolol. The inhibitory effect of PIST on recycling was specifically evident when looking at the relative proportion of internalized receptors that did return to the cell surface during the recycling period. Overexpressed PIST interfered with recycling of the β1 but not the β2 receptor (Fig. 3*B*). On the other hand, knockdown of PIST had little effect on internalization but led to a significant increase in recycling of the β1 receptor (Fig. 3*C*).

PIST is localized at the trans-Golgi network, whereas internalization and recycling of the β1-receptor is likely to occur via early and recycling endosomes. Thus it is unclear where both proteins meet during endocytic trafficking of the β1AR. To clarify this we labeled cell surface-localized β1AR with the fluorescent anti-FLAG antibody and allowed for internalization of the receptor by treatment with isoproterenol. Cells were then fixed and stained for PIST and SNX1, which as a component of the retromer is involved in endosomal to trans-Golgi network transport. Due to this technical approach and in contrast to the pictures shown in Fig. 1, we focus here exclusively on receptors that have been at cell surface at the onset of agonist treatment. Here we observed that PIST was colocalized with SNX1 at the Golgi apparatus, whereas SNX1 was also present in numerous vesicular structures outside the Golgi where it did not colocalize with PIST (Fig. 4, *A* and *B*). This is consistent with the known endosomal localization of SNX1. The signal for the antibody-labeled β1AR was detected at the plasma membrane, as expected from Fig. 1*C* where we observed the receptor at the cell surface in the absence of PIST overexpression. After treatment with the agonist, part of the β1-specific signal was found in intracellular vesicular structures, some of which colocalized

[4] J. Koliwer, data not shown.

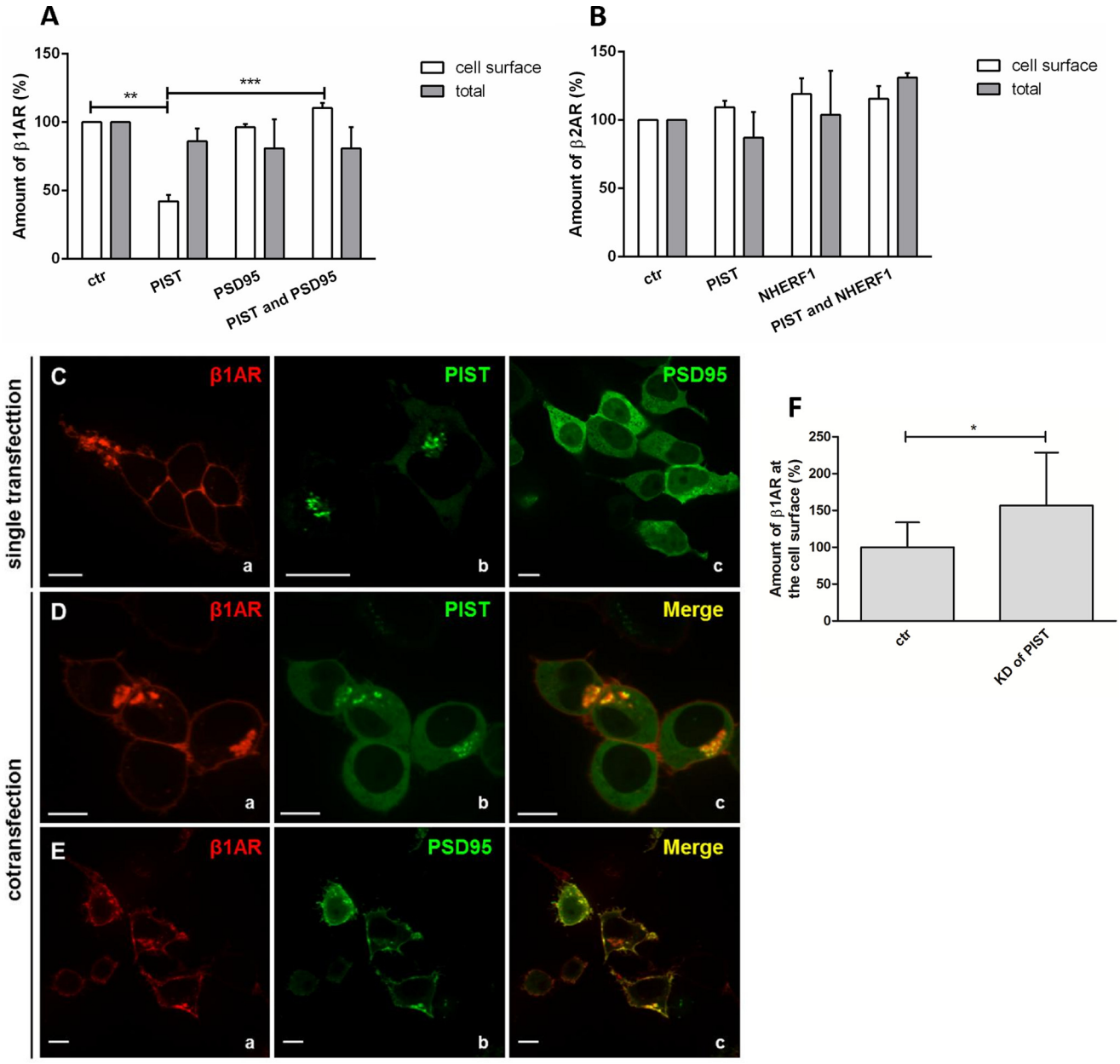



FIGURE 1. **PIST retains β1ARs in an intracellular compartment.** *A*, HEK293T cells expressing SP-RFP-β1AR together with GFP (*ctr*), PIST-GFP, PSD95-GFP, or a combination of PIST-GFP and PSD95-GFP were analyzed by cell surface biotinylation followed by analysis of biotinylated proteins in an ELISA format. The amount of biotinylated β1AR was normalized to biotinylated transferrin receptor. The proportion of cell surface receptors in the control experiment was set to 100%. The number of total receptors was determined by Western blot of cell lysates using anti-monomeric RFP antibody and normalized to the housekeeping protein β-actin. Note that overexpression of PIST does not affect total receptor number but leads to a significant decrease of the amount of β1AR at the cell surface which is rescued by coexpression of PSD95. Mean ± S.E.; unpaired *t* test, two-tailed. $n = 3$. *B*, the same experiment was performed with 293T cells expressing the SP-RFP-β2AR together with GFP (*ctr*), PIST-GFP, NHERF1-GFP, or a combination of PIST-GFP and NHERF1-GFP. No significant difference was observed; $n = 3$. *C*, 293T cells were transfected with plasmids coding for SP-RFP-β1AR (*a*), GFP-PIST (*b*), and GFP-PSD-95 (*c*) and analyzed by fluorescence microscopy. Note that all three proteins exhibit strikingly different subcellular localization. *D*, 293T cells were cotransfected with SP-RFP-β1AR (*a*) and PIST-GFP (*b*) and analyzed in live cell imaging experiments using a Spinning Disc microscope. Colocalization of both proteins in an intracellular compartment was observed (*c*). *E*, 293T cells were cotransfected with SP-RFP-β1AR (*a*) and PSD95-GFP (*b*) and analyzed in live cell imaging experiments using a Spinning Disc microscope. Colocalization of both proteins in small intracellular compartments and at the plasma membrane was observed (*c*). *Scale bar*: 10 μm. *F*, HEK293 cells stably transfected with SP-RFP-β1AR were transfected with PIST siRNA or control siRNA (*ctr*). Cell surface receptors were labeled with biotin and analyzed by biotin-streptavidin precipitation. The amount of biotinylated β1AR was normalized to the input, and control experiments were set to 100%. The knockdown of PIST led to a significant increase of cell surface located β1AR. *, **, ***: significantly different from ctr, $p < 0.05$, 0.01, 0.001, respectively. Mean ± S.D.; unpaired *t* test, two-tailed, $n = 11$.

with SNX1. Interestingly, PIST was distributed much more broadly after stimulation and was also found to be colocalized with SNX1 in endosomal structures. A quantitative analysis of the cellular area covered by PIST-specific immunofluorescence shows that under resting conditions only a small part of the cell shows a PIST signal, in agreement with restriction to the Golgi apparatus. Upon receptor stimulation, this area increases significantly. Thus stimulation of the β1AR appeared to partially release PIST from the Golgi apparatus (Fig. 4, *C* and *D*).

β1 receptors couple to $G_s\alpha$ containing G-proteins leading to an increase in cellular cAMP concentrations. We, therefore,

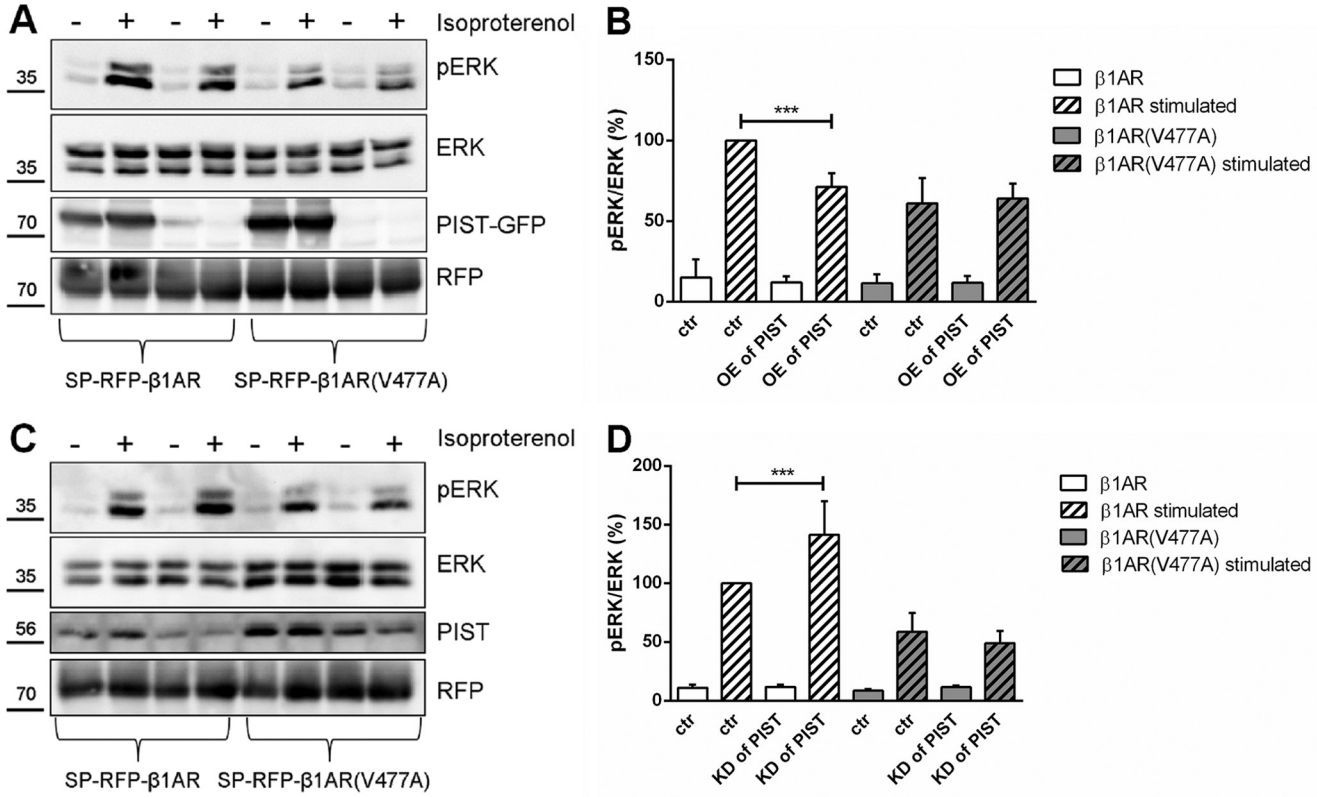



FIGURE 2. **PIST influences the β1AR-dependent MAPK-pathway activity.** HEK293T cells stably transfected with SP-RFP- β1AR or a mutant form lacking the C-terminal PDZ ligand motif (V477A) were cotransfected with PIST-GFP or GFP (*ctr*) (*A* and *B*) or with siRNA leading to KD of PIST or a control siRNA (*C* and *D*). After a 10-h incubation with serum-free medium, the cells were stimulated with 10 μg/ml isoproterenol and analyzed by Western blot. The activity of the MAPK pathway was determined as the ratio of pERK to ERK signals. Control experiments were set to 100%. The overexpression of PIST led to a decrease of the MAPK pathway activity after stimulation of β1AR (*A* and *B*). The knockdown of PIST led to a significant increase in agonist stimulated phosphorylation of Erk1/2 (*C* and *D*). ***, significantly different from ctr, $p < 0.001$. Mean ± S.E.; analysis of variance, followed by Tukey's multiple comparisons, $n = 5$.

tested whether the redistribution of PIST occurs when cAMP levels are increased in a receptor-independent manner by stimulation with a mixture of the adenylate cyclase activator forskolin and the phosphodiesterase inhibitor IBMX. Here we indeed observed a change from an almost exclusive Golgi localization (as exemplified by co-staining with Giantin) to a much broader distribution of PIST, leading to decreased colocalization with Giantin but increased colocalization with SNX1 (Fig. 5). The integrity of the Golgi apparatus itself was not affected by this treatment, as Giantin staining was observed as the familiar cluster of juxtanuclear fluorescence in both treated and non-treated cells.

Interference by PIST with the recycling of the β1AR might favor subsequent targeting of internalized receptors to lysosomes followed by receptor degradation. To investigate this possibility, we studied the stability of both β1 and β2 receptors in cells overexpressing GFP-tagged PIST and compared this to control cells expressing GFP alone (Fig. 6, *A–D*). To avoid synthesis of new receptors during the time period analyzed, cells were treated with the protein synthesis inhibitor cycloheximide. Receptors were quantified by Western blotting of cell lysates, and receptor signals were normalized to GAPDH, which was not noticeably degraded in this time frame, in agreement with published data (27). In these experiments we observed that degradation of both β1 and β2 receptors occurred with a half-life of about 20 h in control cells expressing GFP. This was not altered for the β2 receptor in cells overexpressing PIST, whereas the β1 receptor was significantly stabilized by PIST overexpression as the calculated half-life increased to >60 h. From these data we conclude that PIST stabilizes the β1 receptor against proteolytic degradation and that it does so in an intracellular compartment.

To obtain evidence for *in vivo* relevance of this process, we began to generate and analyze PIST-deficient mice. We obtained a mouse ES cell line harboring an exon trap insertion in the second intron of the gene coding for PIST/GOPC (KO first mode of ES cells distributed by the EUCOMM consortium). Chimeric and eventually heterozygous mice were obtained from these ES cells. However, upon crossing of heterozygous (+/d) animals, only very few knock-out (d/d) mice could be obtained (3 d/d; 138 d/+; 81 +/+), pointing to an essential role of PIST for early mouse development. We analyzed hippocampal lysates of all three KO mice and their WT littermates for the presence of β1 receptors by Western blot. Here we observed that the amount of β1 receptors was indeed reduced in these mice (Fig. 6, *E* and *F*) in support of a role for PIST in stabilizing its interacting GPCRs.

## DISCUSSION

The role of PIST in the intracellular transport of membrane proteins has been enigmatic and, when comparing its effects on different membrane proteins, also controversial. Thus PIST has been shown to promote the transport of the CFTR to lysosomes, thereby contributing to its degradation (14–16). As this reduces the availability of CFTR on the plasma mem-

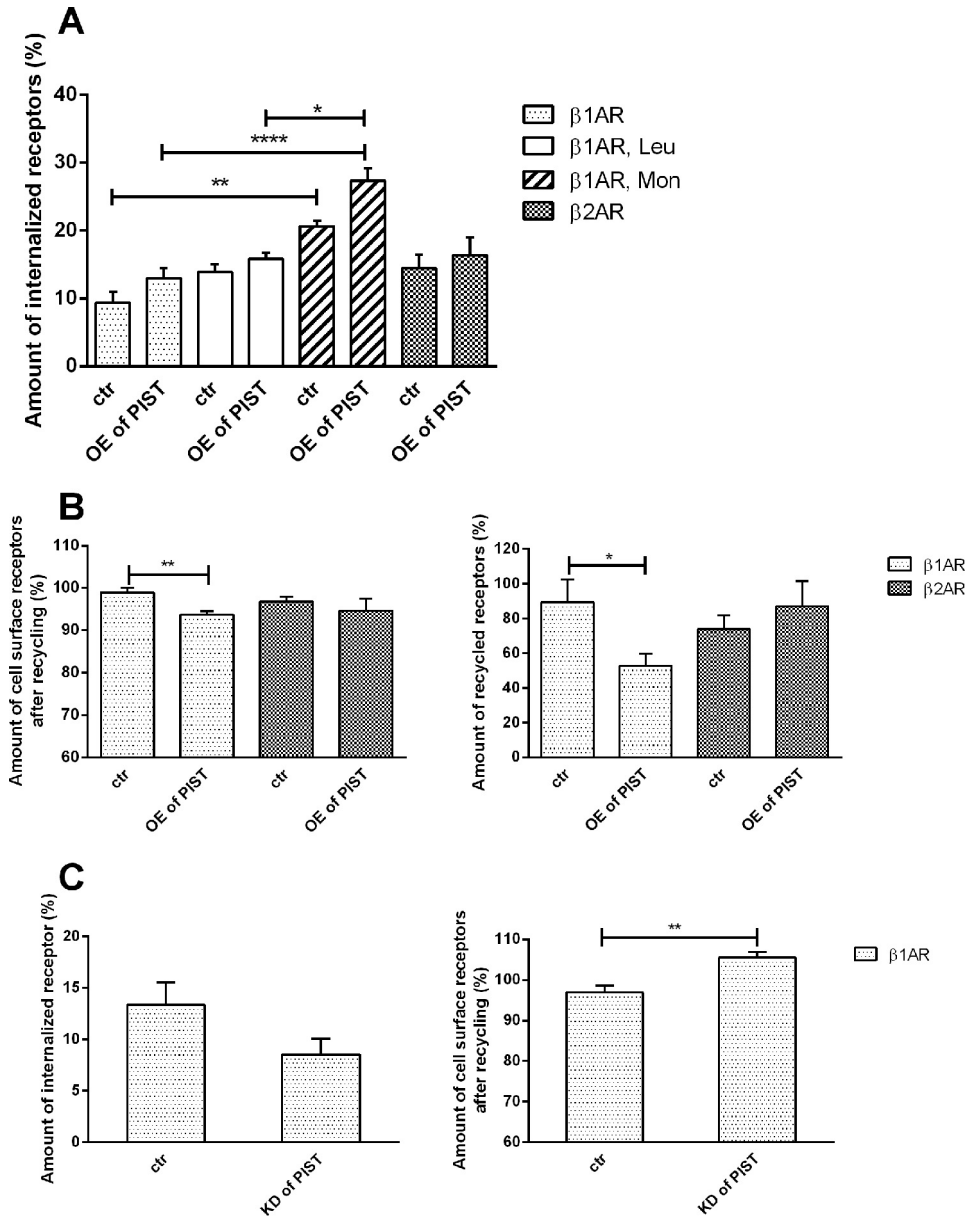


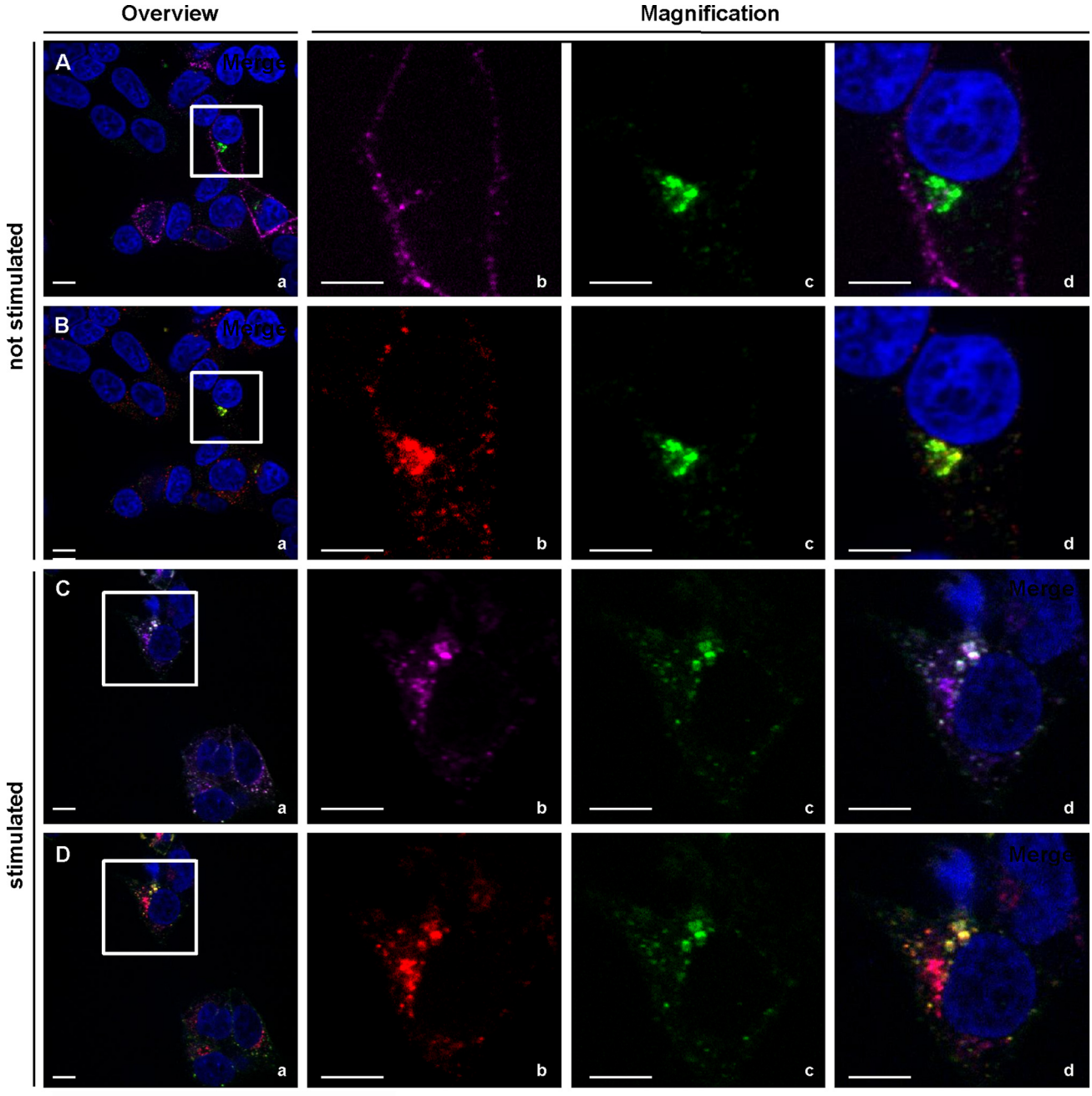

FIGURE 3. **PIST interferes with recycling of the β1-adrenergic receptor.** *A* and *B*, HEK293 cells were cotransfected with either SP-FLAG-β1AR or SP-FLAG-β2AR together with GFP (*ctr*) or PIST-GFP (*OE,* overexpression) and incubated for 20 min with 10 μg/ml isoproterenol (internalization; *A*). Leupeptin (*Leu*, 100 μM; 24 h preincubation) or monensin (*Mon*, 50 μM; 1-h preincubation) were added as indicated. For recycling (*B*) cells were incubated for 20 min with 10 μg/ml isoproterenol and afterward for 40 min with 10 μg/ml alprenolol. Cells were then incubated with a FLAG antibody fused to Alexa-647 for 20 min. The fluorescence of the cells was then analyzed by flow cytometry. The amount of internalized receptors was normalized to the steady-state level (*A*); cell surface receptors after the recycling phase are shown relative to steady-state levels (*B*). In addition, a second panel is shown in *B* where the relative number of recycled receptors is depicted in relation to the amount of internalized receptors. *C*, internalization and recycling of β1 receptors was analyzed under conditions of PIST knockdown. *, **, ***, ****, significantly different from ctr, $p < 0.05$, 0.01, 0.001, 0.0001, respectively. Analysis of variance followed by Tukey's multiple comparisons (*A*; $n = 3$–7); unpaired *t* test (*B* and *C*; $n = 7$).

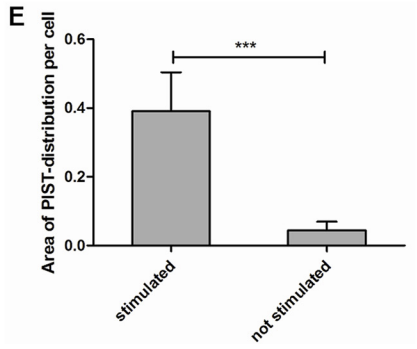
brane, antagonism of PIST has been suggested as a therapeutic principle for cystic fibrosis (28). On the other hand, several membrane receptors are retained by PIST in an intracellular compartment within their biosynthetic pathway (9, 17, 18, 29). The physiological relevance of this phenomenon is unclear.

We show here that PIST affects intracellular trafficking of β1 receptors in two ways; one aspect is the retention of β1 receptors in the Golgi apparatus, in agreement with previous studies on this and other GPCRs (9, 17). The observation that the receptor is released from this retention by other PDZ domain containing proteins such as PSD-95 might provide a clue to the physiological relevance of this phenomenon. As PDZ scaffolds serve to integrate receptors into signaling complexes at the plasma membrane, which are essential for receptor function (5), it would be detrimental if receptors travel to the plasma membrane in the absence of such scaffolding.

The second functional aspect of the PIST/β1-receptor interaction relates to endocytic trafficking. We observed that PIST interferes with recycling of the β1 receptor from an endosomal compartment to the plasma membrane. PIST, which under resting conditions is found almost exclusively at the trans-Golgi network

(20), is redistributed to smaller vesicular structures after receptor stimulation. Here it is colocalized with SNX1. This redistribution of PIST is most likely due to activation of a cAMP-dependent signaling pathway, as it can be initiated also by the combination of forskolin and IBMX, which increases cellular cAMP levels in the absence of β1 receptor activation. Colocalization of PIST and the



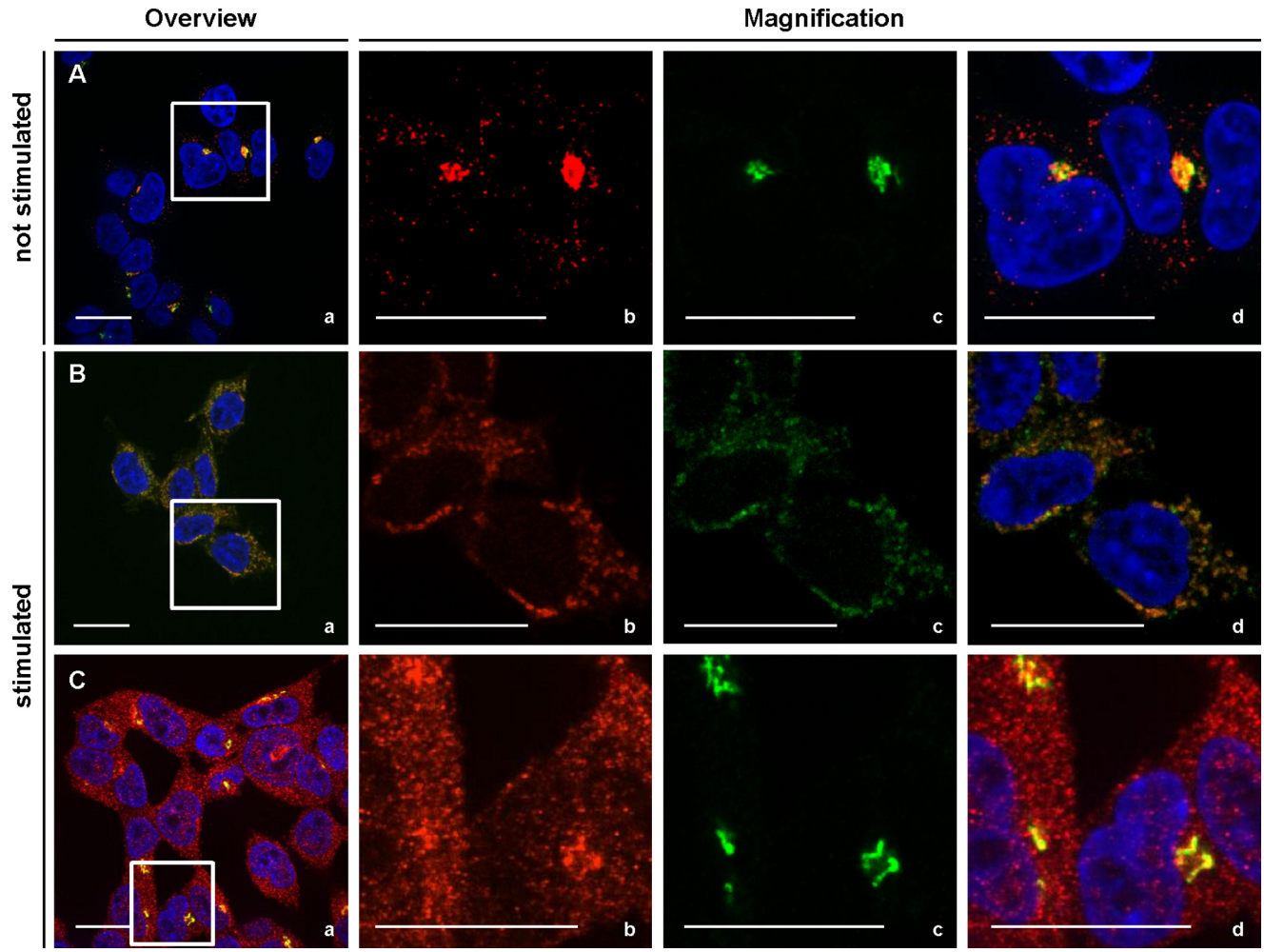



FIGURE 5. **The localization of PIST in the cell changes after stimulation with IBMX and Forskolin.** 293T cells were stimulated with IBMX (500 μM) and forskolin (50 μM) for 20 min (*B* and *C*) or not (*A*). Cells were fixed with 4% paraformaldehyde and stained for PIST and Giantin (*A* and *C*) or SNX1 (*B*). The cells were analyzed using a PerkinElmer Spinning Disc Microscope. Note that in stimulated cells PIST and SNX1 are colocalized and broadly distributed in the cell, whereas PIST is restricted to the Golgi apparatus in non-stimulated cells as identified by colocalization with the Golgi marker Giantin. Giantin itself is not affected by stimulation. *Scale bar*: 10 μm.

endocytosed β1 receptor in this compartment suggests that this is the location where PIST acts on the subcellular trafficking of the receptor. Currently we can only speculate how PIST affects the recycling process of the receptor. As recycling is mediated by SNX27 (12), which binds to the C-terminal PDZ ligand of the β1AR, one possibility would be that PIST competes with SNX27 for binding to the PDZ ligand of the receptor.

Interestingly, interference with the recycling process does not lead to increased lysosomal degradation of the receptor. Previous studies with the CFTR have shown that PIST may target an associated membrane protein for lysosomal degradation (14, 15). However, PIST does not appear to induce degradation of its associated G-protein-coupled receptors (6, 9). In fact we report here that PIST stabilizes the β1 receptor and prevents postendocytic lysosomal degradation. Similarly we have shown before that the somatostatin receptor 5 is not targeted for degradation by its C-terminal PDZ ligand motif, which mediates the interaction with PIST (9). Previous work showed that the β1 receptor is unusually stable toward lysosomal degradation because it is not ubiquitinated in response to agonist treatment and that this resistance is determined by the C-terminal tail of the receptor (30). It appears from our data that the specific interaction with PIST may be partly responsible for this resistance. Thus contrary to its actions on the CFTR, PIST stabilizes G-protein-coupled receptors and protects them from postendocytic degradation.

FIGURE 4. **PIST is colocalized with SNX1 and changes its localization in the cell after stimulation with Isoproterenol.** 293T cells expressing SP-FLAG-β1AR were incubated with Alexa-647-labeled FLAG antibody for 20 min (*magenta*; *A* and *C*). Then the cells were stimulated with 10 μg/ml isoproterenol (*C* and *D*) or not (*A* and *B*). After fixation cells were stained for PIST (*green fluorescence*; *A–D*) and SNX1 (*red*; *B* and *D*) as indicated. Note that the endogenous PIST is colocalized with SNX1 at the Golgi apparatus in non-stimulated cells, whereas there was no colocalization with the β1AR. After stimulation, PIST was distributed more broadly and extensively colocalized with both SNX1 and the β1AR. *Scale bar*: 10 μm. *E*, the *bar graph at the bottom* provides a measure for the cellular area covered by PIST fluorescence signal in stimulated and non-stimulated cells. Therefore, pictures were randomly selected and analyzed for endogenous PIST-distribution by ImageJ. A region of interest corresponding to one cell was selected. The same threshold was chosen for every cell to measure only PIST-positive pixels. The area of the distribution of PIST was calculated as the amount of pixels reaching the threshold relative to the total region of interest. ***, significantly different from ctr, $p < 0.001$. Mean ± S.D.; unpaired *t* test, two-tailed, $n = 10$.

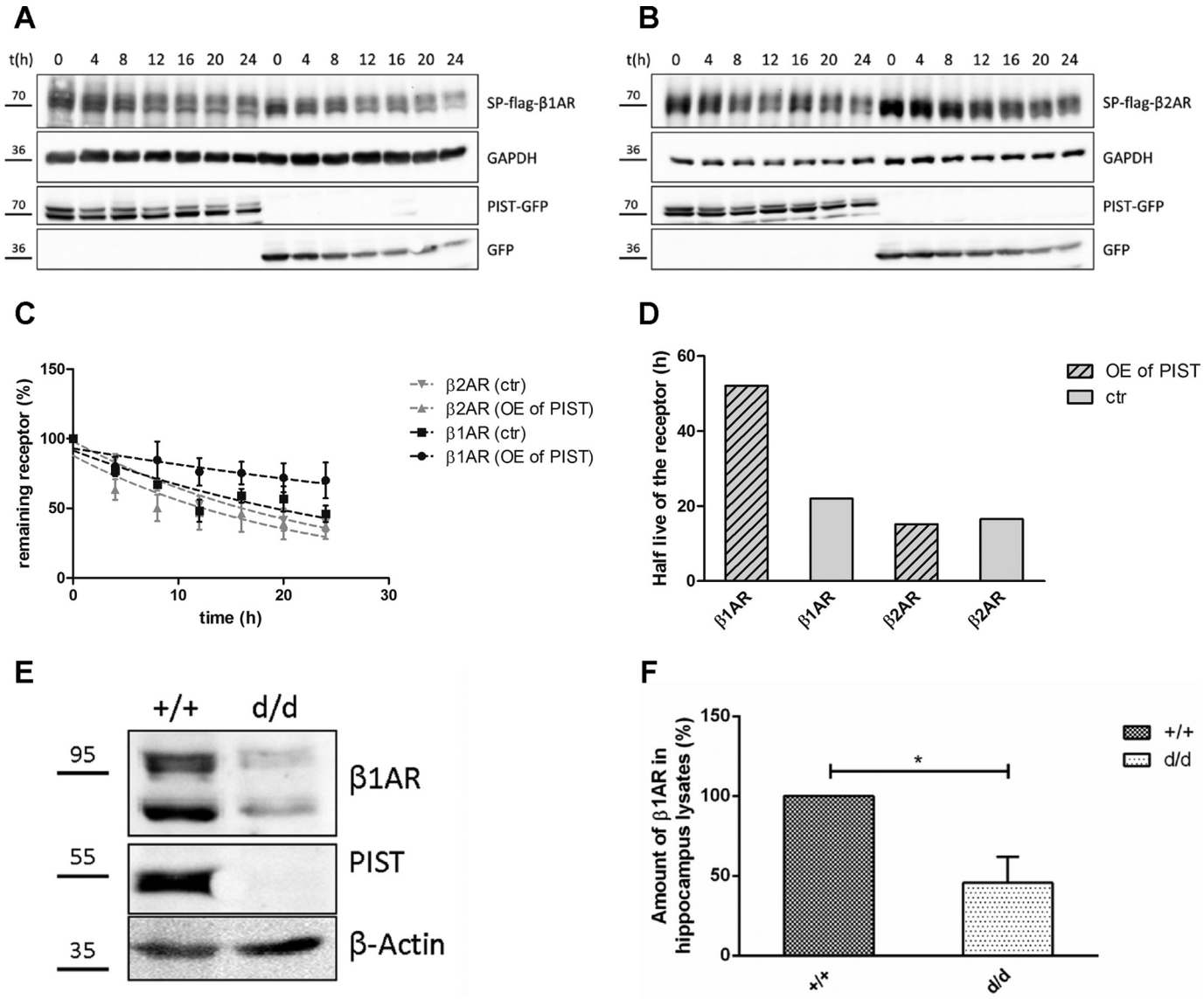



FIGURE 6. **PIST interferes with postendocytic degradation of the β1-adrenergic receptor.** HEK293-T cells were cotransfected with β1AR (*A*) or β2AR (*B*) and PIST-GFP (*OE*, overexpression) or GFP (*ctr*). The cells were preincubated for 30 min with 10 μg/ml cycloheximide and then stimulated with 10 μg/ml isoproterenol in the presence of cycloheximide. After different time periods, the cells were lysed and analyzed by Western blot. *C*, the amount of receptor was normalized to the GAPDH signal, and time point $t = 0$ was set to 100% (mean ± S.E.). *D*, data were fitted to exponential decay curve, and the half-lives of the receptors under different conditions was determined. Whereas the stability of β2AR was not affected by PIST, overexpressed PIST increased the half-life of the β1AR. *E* and *F*, the protein level of β1AR was decreased in the hippocampus of PIST deficient (*d/d*) mice. The hippocampi of PIST d/d mice and WT (+/+) littermates were isolated, and protein lysates were prepared using radioimmune precipitation assay buffer. After removing cell debris by centrifugation, protein lysates were analyzed by Western blot (*E*). The amount of β1AR was determined and normalized to β-actin (*F*). *, $p < 0.05$, paired *t* test, $n = 3$.

This is also supported by initial data on PIST-deficient mice; as PIST is apparently essential for mouse development, only very few mice could be analyzed here. Nevertheless we observed a significant reduction in hippocampal β1 receptor levels in PIST-deficient animals, in agreement with a role of PIST in stabilizing its associated receptors.

Further work will, however, be necessary to determine which of the numerous targets of the PIST PDZ domain is relevant for the early embryonic deficits in PIST-deficient mice. Genetic data from the Decipher database indicate that in human patients copy number variations in the genomic region coding for PIST/GOPC on chromosome 6 are associated with various defects including arrhythmia, autism, and intellectual disability. Some of these defects may be due to dysregulation of β1 receptors caused by a alteration of PIST levels.

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