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Role of SMURF1 ubiquitin ligase in BMP receptor trafficking and signaling

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ABSTRACT

Heterozygous germline mutations in the bone morphogenetic protein type II receptor gene (*BMPRII*) are associated with hereditary pulmonary arterial hypertension (HPAH). Missense mutations, both in the extracellular ligand-binding and cytoplasmic kinase domains, mostly involve substitution of conserved Cys residues. Singular substitution at any of those Cys residues causes cytoplasmic, perinuclear localization of BMPR with reduced cell surface expression and BMP signaling. The present study examined the effect of Cys residue substitution on BMPR endocytic trafficking and lysosome degradation. We demonstrate that endocytosis/lysosomal degradation of BMPR occurs by two distinct pathways. SMURF1 ubiquitin ligase induces lysosomal degradation of BMPR, while ligase-inactive SMURF1 maintains BMPR protein level and cell surface expression. Substitution of BMPR Cys residues increases lysosomal degradation which is blocked by ligase-inactive SMURF1, elevating protein levels of Cys-substituted BMPRs. Expression of Cys-substituted BMPR suppresses basal BMP signaling activity which is also up-regulated by ligase-inactive SMURF1. Cys-residue substitution thus appears to cause BMPR endocytosis to lysosomes in a SMURF1 ubiquitin ligase-associated pathway. Kinase-activated BMPR undergoes endocytic/lysosomal degradation by a pathway with certain unique properties. Therefore, our results describe a novel mechanism whereby SMURF1 ubiquitin ligase regulates constitutive endocytosis of BMPR which may be mediated by its conserved Cys residues.

1. Introduction

BMP signaling is a critical regulator of vascular development and homeostasis [33]. The function of BMP in angiogenesis is demonstrated in genetic vascular diseases including hereditary pulmonary arterial hypertension (HPAH) in which heterozygous germline mutations are found in the BMP receptor II gene (*BMPRII)* [13,31]. HPAH is characterized by constriction of precapillary pulmonary arteries causing right ventricular hypertrophy and heart failure [19]. Lesions demonstrate medial hypertrophy, plexiform lesions and narrowing of the vascular lumina due to neointima formation from excessive proliferation of endothelial cells (ECs) and smooth muscle cells (SMCs) [29].

PAH is commonly sporadic while HPAH is inherited in the autosomal-dominant mode in 6–10% of patients [12,38]. Both types of PAH present similar phenotypes with reduced BMPR protein expression in pulmonary arteries of patients, thus demonstrating reduced BMP signaling as the primary pathogenesis of PAH [3]. The BMPRII protein is composed of the extracellular ligand-binding, trans-membrane, cytoplasmic kinase, and long tail domains. HPAH-associated mutations are identified in all domains [36]. Missense mutations constitute ~30% of disease mutations, of which all in the ligand-binding domain are substitutions of conserved Cys residues. They all cause perinuclear localization of BMPRII with reduced expression on the plasma membrane and reduced BMP signaling [42,51]. Among missense mutations in the kinase domain, mutations only at Cys residues show cytoplasmic localization, demonstrating that Cys residues, regardless of their location, are important for BMPR trafficking. While BMPRII with Cys substitution is co-localized with endoplasmic reticulum (ER) markers, trafficking to the plasma membrane is elevated by chemical chaperones [53]. Substitutions of conserved Cys residues are thus proposed to retard trafficking to the plasma membrane due to abnormal protein folding [42,51].

BMPRs, members of the TGFβ receptor (TβR) family, are composed of heteromeric BMPRI and II with intrinsic Ser/Thr protein kinase. Upon BMP binding, constitutively active Ser/Thr protein kinase-associated BMPRII recruits and phosphorylates BMPRI, followed by conformational changes and activation of BMPRI Ser/Thr protein kinase for signal transduction [37]. SMURF1 ubiquitin ligase is a negative

Abbreviations: SMURF1, SMAD Ubiquitylation Regulatory Factor 1; ERAD, Endoplasmic Reticulum-Associated Degradation; HPAH, Hereditary Pulmonary Arterial Hypertension; ESCRT, Endosomal Sorting Complex Required for Transport; HRS, Hepatocyte Growth Factor Receptor Substrate; TSG101, Tumor Susceptibility Complex Gene 101; MVB, Multi Vesicular Body

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regulator of BMP signaling and targets BMPR and downstream signaling molecule SMAD1/5 for degradation [18,39,60]. The clathrin endocytic pathway is shown to mediate TGFβ signaling, whereas caveolae-lipid rafts are enriched with SMURF2, a SMURF1 homologue, and SMAD7 which are shown to together facilitate basal receptor turnover [14]. Constitutive recycling and ligand-activated downregulation of TβR are demonstrated to be mediated by distinct endocytic pathways [15,41]. These results demonstrate more than one pathway for endocytosis of the TGFβ receptor family.

HPAH occurs with a low penetrance in which only 10–30% of family members with the disease gene develop overt disease, suggesting genetic/environmental modifiers for PAH clinical manifestation [12,38]. We previously found elevated levels of SMURF1 ubiquitin ligase in pulmonary arteries and tissues of animals with experimental PAH and suggested SMURF1 as a pathogenic mediator of PAH for reducing BMPR and signaling molecules [40]. Elevated levels of SMURF1 were confirmed in human pulmonary vascular cells of patients with primary and secondary PAH [6,50]. SMURF1 knockout was effective in reversing/protecting from PAH in model animals [50].

The extracellular domain of TGFβ family receptors contains eight conserved Cys residues which form disulfide bonds for facilitating the three-finger toxin fold structure [23]. Conserved Cys residues and their disulfide bonds are similarly implicated in protein folding and subunit assembly of membrane proteins, including growth factor receptors, ion channels, and neurotransmitters [1,7,22,55]. However, mutational analyses show that Cys-residue substitutions cause reduced expression on the plasma membrane, intracellular retention, and rapid degradation of acetylcholine receptor, epithelium sodium channel (ENaC), and Na, K-ATPase [7,8,21,55]. Substitution of conserved Cys residues of ENaC and mechanotransducer underlies hereditary diseases, eq. pseudohypoaldosteronism type 1 and non-syndromatic deafness [21,34]. Notch signaling is processed by endocytosis of both Notch receptor and Delta ligand. Substitution of Cys residues in the EGF-like repeat of the Delta extracellular domain is a loss-of-function mutation with defective signaling and endocytosis [44]. Therefore, conserved Cys residues of membrane proteins may have functions in endocytosis and signaling in the intracellular reducing environment.

Trafficking of membrane receptors orchestrates signaling activities and cellular responses [47]. After internalization, membrane receptors are transported to early endosomes/sorting endosomes, from where some are directed to recycling to the plasma membrane and others to late endosomes for lysosome degradation [24]. Receptor recycling maintains cellular responses to ligands, whereas lysosome degradation serves for temporal regulation of signaling activities [32]. Besides bulk membrane protein recycling, some receptors require recycling domains which must be recognized by recycling machinery, although it has not been extensively characterized [32]. Endocytic sorting to lysosomes is suggested to be initiated by receptor ubiquitination upon ligand activation [45,46,49,57]. Ubiquitinated receptors are recognized by the ubiquitin interacting motif (UIM) of the endosomal sorting complex required for transport (ESCRT) components, HRS (hepatocyte growth factor receptor substrate; ESCRT-0 protein) and TSG101 (tumor susceptibility gene 101; ESCRT-1 protein). Through their interaction, receptors are recruited to the ESCRT and transported to multi vesicular bodies (MVBs) for lysosomal degradation [27,30,49]. For example, it has been accepted that EGF receptor-associated tyrosine kinase, activated by ligand binding, phosphorylates the C-terminal domain and recruits Cbl ubiquitin ligase which then ubiquitinates the receptor cytoplasmic domain [32].

We examined endocytosis and degradation of BMPR with Cys residue substitution. Since SMURF1 ubiquitin ligase is mostly responsible for BMPR degradation [18,39,60], we analyzed how SMURF1 and HPAH-associated Cys residues are involved in BMPR trafficking and lysosomal degradation. Our study demonstrates that SMURF1 and substitution of conserved Cys residues both induce BMPR lysosome degradation and that blockage of SMURF1 ubiquitin ligase activity recovers cell surface expression and BMP signaling by Cys-substituted BMPR. These results may implicate both SMURF1 and BMPR Cys residues in constitutive endocytosis/recycling and lysosomal degradation of BMPR.

2. Materials and methods

2.1. Materials

EZ-Link Sulfo-NHS-LC-Biotin (#21335) and EZ-Link Sulfo-NHS-SS-Biotin (#21328) were used as biotin-labeling reagent (Pierce Biotechnology, IL). Streptavidin-agarose (S1638), chloroquine (C6628), poly-L-lysine solution (P4832) and protease inhibitor cocktail (P8340) were obtained from Sigma-Aldrich (St.Louis, MO). Western Lightning Plus-ECL (NEL103001) was obtained from PerkinElmer Inc. (Waltham, MA). MG132 (BPS-27230) was obtained from Biomol Research Lab. (Hamburg, Germany). Antibodies used were; anti-HA tag mAb (#2367), biotinylated anti-phospho-SMAD1/5 Rabbit mAb (Ser463/465) (#9576), HRP-linked anti-mouse IgG Ab (#7076) (Cell Signaling, MA). Anti-Flag rabbit polyclonal (F7425) and anti-actin mAb (A3853) (Sigma-Aldrich, MO). Anti-EEA1 mAb (#610456; BD Biochem Pharmingham (MA). Anti-rabbit IgG-HRP (AP132P; Chemicon International, CA). Anti-human LAMP-1 mAb (H4A3, Developmental Studies Hybridoma Bank, University of Iowa, IA). Anti-SMURF1 mAb (H00057154-M01; Abnova Corporation, Taipei, Taiwan).

2.2. Cell culture

HEK293T and Cos7 cells were cultured in DMEM media (Media Tech Inc., VA) including 10% fetal bovine serum (Sigma-Aldrich, Mo), 100 U/ml penicillin (Media Tech Inc., VA), 100 μg/ml streptomycin (Media Tech Inc., VA), and 2.5 μg/ml amphotericin B (Sigma-Aldrich, Mo).

2.3. Construction of plasmids

The pcDNA3HA-BMPRs and pCMV5B-Flag-SMURF1, respectively, were kindly provided by Kohei Miyazono (Tokyo University, Japan) and Jeffery L Wrana (University of Toronto, Canada). All mutants were generated by PCR-mediated site mutagenesis. Ligase-inactive SMURF1$^{ligase-}$ was similarly constructed by mutagenesis of 726Cys to Ala. EGFP-BMPRI was generated by inserting the *BMPRI* gene into pEGFP-N1. Human cDNA clone for VPS4B (NM_0004869.3; SC324083; Origene, MD) was cloned into pcDNA3HA at *Eco*RI/*Xho*I sites. The ATPase-negative VPS4B was generated by introducing Glu224Gln (224EQ) mutation in the Walker B motif of the AAA$^+$ATPase domain. All DNAs were confirmed by sequencing.

2.4. Transfection and Western blotting

HEK293T cells were transiently transfected with indicated vectors using Superfect (Qiagen, CA) according to the manufacturer's protocol or by the calcium-phosphate precipitation method [52]. For Western blotting, ~1 μg DNA was used in 35 mm plate, and cells were harvested 1 or 2 days after transfection. Cells were lysed with 1% NP40/10 mM Tris-Cl, pH 7.4/0.14 M NaCl or the RIPA buffer containing the protease inhibitor cocktail (Sigma-Aldrich, MO). Following brief centrifugation of lysates, ~30 μg supernatant protein was subjected to 8% SDS-PAGE, Western blotting, and densitometry.

2.5. Fluorescent microscopy

HEK293T cells were transfected by Superfect or calcium-phosphate precipitation method and grown on poly-L-lysine-treated 12 mm cover slips. On next day after transfection, cells were fixed with 4% paraformaldehyde. Cells were blocked by 3% BSA/PBS, made permeable by

0.1% saponin/3% BSA/PBS, and incubated with respective antibody in the presence of 1% BSA and 0.05% saponin in PBS overnight at 4 °C. Primary antibodies were labeled with fluorescent secondary antibodies for 2 h at room temperature. Nuclei were labeled by DAPI. Sub-cellular localization was visualized using 40× objective lens by fluorescent microscopy or 100× objective lens by confocal microscopy.

2.6. Biotin-labeling of plasma membrane proteins

Trafficking of BMPR from the cell surface was followed by labeling cell surface proteins with biotin according to published methods with some modifications [2,9,10]. Cells grown in p35 plates were washed with ice-cold PBS (pH 8) three times and incubated on ice with PBS (pH 8) containing 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin or EZ-Link Sulfo-NHS-SS-Biotin for 30 min. Cells were then suspended in 20 mM NH_4Cl in PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ to quench biotinylation reagents according to the published method. After washing with PBS, cells were incubated in DMEM containing 10% fetal bovine serum at 37 °C, 5% CO_2 for indicated times to chase biotin-labeled, cell surface proteins. Cells were harvested in the RIPA buffer and centrifuged for 5 min at 14,000 rpm. After supernatants were tested for HA-BMPRI expression and biotinylation by Western blotting, duplicates were combined and incubated on rocker with streptavidin-agarose at 4 °C for 2 h in the TBS buffer containing 1% NP40. Beads were collected by centrifugation at 10,000 rpm for 0.5 min and washed with 1.5 M guanidine-HCl three times to remove non-specific binding. After brief wash with TBS containing 1% NP40, beads were suspended in the Laemmli buffer. When cleavable EZ-Link Sulfo-NHS-SS-Biotin was used, cell surface-bound biotin was cleaved by incubation with 100 mM GSH for 1 h at 0 °C, followed by the same procedure as above, except that β-mercaptoethanol was deleted from all reagents.

2.7. Statistical analysis

All numerical data were analyzed by the student *t*-test and ANOVA (GraphPad Prism 7). The significance was evaluated by the *p*-value.

3. Results

3.1. Substitution of conserved Cys residues of BMPRI causes cytoplasmic vesicular localization

HPAH-associated substitutions of Cys residues were identified at the conserved Cys residues both in the extracellular and kinase domains of the *BMPRII* gene [36]. Substitution at any one of these Cys residues demonstrates perinuclear, vesicular localization [42,51]. Since these Cys residues are conserved between BMPRI's and II, substitution was introduced at conserved Cys residues of BMPRI to examine if they have a general role. Among different forms of BMPRI, BMPRIB, which binds specifically to BMP over TGFβ, was used (called BMPRI herein) [37]. Substitution was introduced at Cys346 in the kinase domain (called $BMPRI^{C346A}$). BMPRI Cys346 corresponds to the Cys347 residue mutated in BMPRII of HPAH patients [36]. EGFP-BMPRI and EGFP-$BMPRI^{C346A}$ were constructed and expressed in HEK293T cells. BMPR localization was visualized by analyzing EGFP protein expression by confocal microscopy. As shown for Cys-substitution mutants of BMPRII [42,51], EGFP-$BMPRI^{C346A}$ presented mostly vesicular cytoplasmic localization (Fig.1 Panel A vs. B). On the other hand, EGFP-BMPRI distributed through the cell (Fig.1 Panel A). Substitution of conserved Cys-residues thus causes perinuclear, vesicular localization with both BMPRI and II, indicating that conserved Cys-residues may have a general role in BMPR trafficking.

3.2. Cys substitution causes SMURF1-dependent lysosomal degradation

$BMPRI^{C346A}$ protein expression level was always lower as compared to BMPRI, when expressed using plasmid expression vectors in

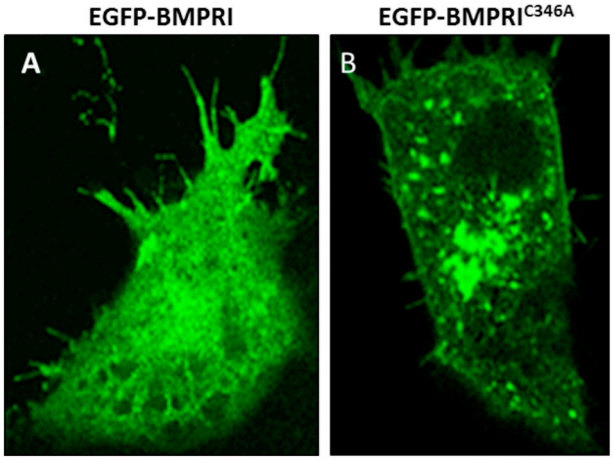

Fig. 1. Effect of Cys substitution on subcellular localization of BMPRI.
EGFP-BMPRI (A) or EGFP-$BMPRI^{C346A}$ (B) was expressed in HEK293T cells and analyzed by confocal microscopy using 100× lense.

HEK293T cells. Since the expression of both BMPR proteins was driven by the same promoter, the reduced protein level most likely resulted from accelerated degradation. SMURF1 ubiquitin ligase, a primary negative regulator of BMP signaling, targets BMPR's for degradation [18,39]. SMURF1 is highly expressed in HEK293 cells (Fig.2). We compared the responsiveness to SMURF1 between wild type BMPRI and $BMPRI^{C346A}$. Steady-state protein levels of HA-BMPRI's were examined by Western blotting after transfection of their respective expression vectors with or without expression of Flag-SMURF1 or Flag-SMURF1$^{ligase-}$. Flag-SMURF1$^{ligase-}$ (Cys752Ala) lacks the active site Cys residue and thus has no ubiquitin ligase activity. When co-expressed together, HA-BMPRI protein was reduced by Flag-SMURF1 and elevated by SMURF1$^{ligase-}$ (Fig.3a Lane 1 vs. 2 & 3). Although Flag-SMURF1 and Flag-SMURF1$^{ligase-}$ were expressed using the same concentration of vectors, the latter was generally expressed higher than Flag-SMURF1, most likely due to auto-degradation of the latter [35]. HA-BMPRI with SMURF1$^{ligase-}$ was higher than HA-BMPRI alone, most likely due to endogenous SMURF1 (a dominant-negative effect). The steady-state protein level of HA-$BMPRI^{C346A}$ was lower than wild type BMPRI (compare Fig.3a Lane 4 vs. 1) and was significantly elevated by Flag-SMURF1$^{ligase-}$ (Fig.3a Lane 1 vs. 3 and 4 vs. 6). The sensitivity to protease inhibitors was compared for HA-$BMPRI^{C346A}$ and SMURF1-induced degradation by HA-BMPRI (Fig.4). SMURF1-induced reduction of HA-BMPRI protein level was suppressed by lysosomal inhibitor

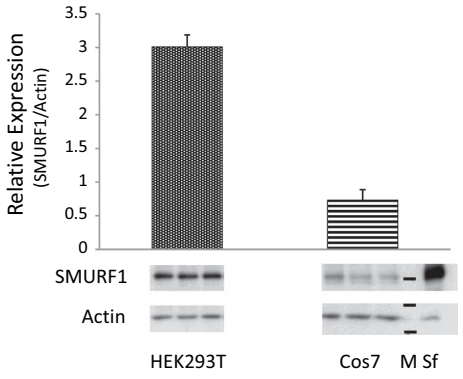

Fig. 2. SMURF1 expression in HEK293T cells.
SMURF1 protein levels were compared between HEK293T and Cos7 cells (means of 3 plates). Densitometric measurements and Western blotting patterns are shown. All are run in a single gel. Sf: FLAG-SMURF1 expressed in Cos7 cells as a standard. M: molecular weight standards for 75, 50 and 35kDa from the top.

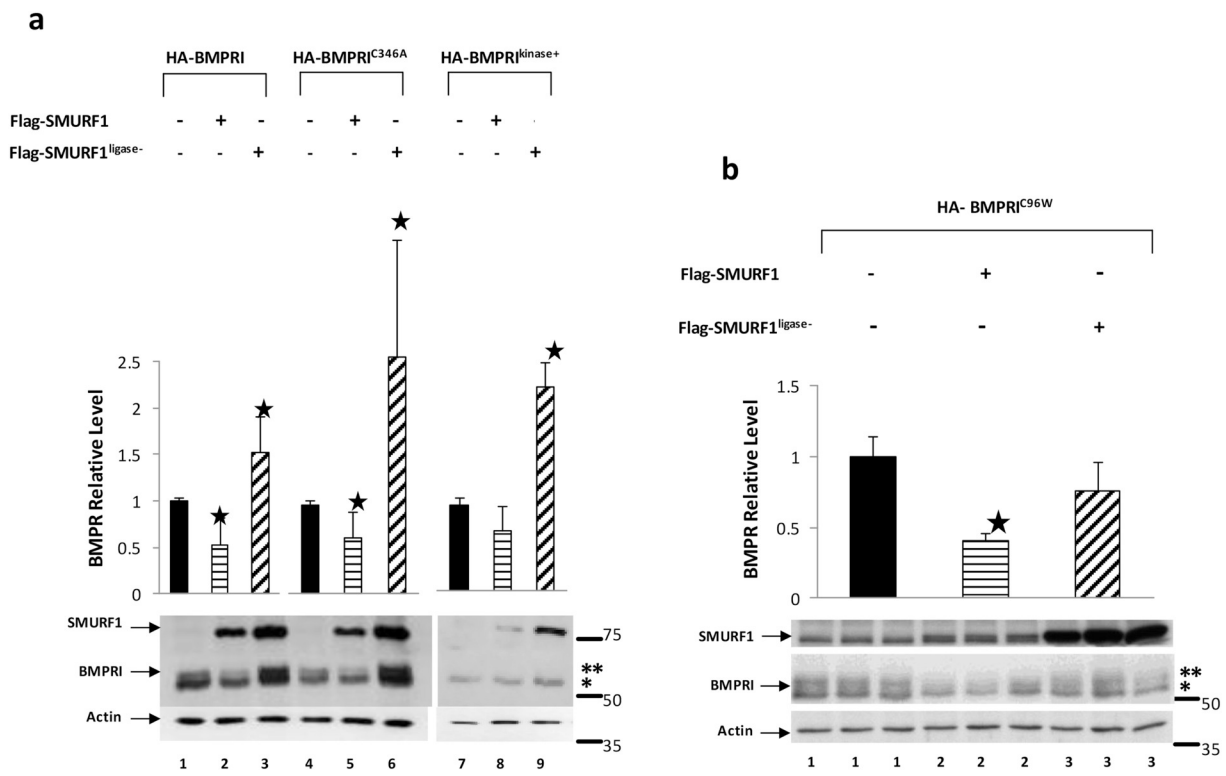

Fig. 3. Steady-state BMPRI protein levels after expression of SMURF1 or SMURF1$^{ligase-}$.
a: HA-BMPRI (Lanes 1–3), HA-BMPRI^{C346A} (Lanes 4–6), and HA-BMPRI$^{kinase+}$ (Lanes 7–9) were expressed with Flag-SMURF1 (Lanes 2, 5, and 8) or Flag-SMURF1$^{ligase-}$ (Lanes 3, 6, and 9) in HEK293 cells. The average densitometric values along with the typical Western blotting patterns for steady-state protein levels are shown from 3 to 5 experiments. Loading control: β-actin. * and **: Fast and slow moving bands of BMPRI. The number 35, 50 and 75 indicate the molecular weight markers in kDa. * indicates the significant difference from the control without SMURF1.
b: HA-BMPRI^{C96W} was expressed without (Lanes 1) or with Flag-SMURF1 (Lanes 2) or Flag-SMURF1$^{ligase-}$ (Lanes 3). The average densitometric values along with the Western blotting patterns for steady-state protein levels in triplicates are shown. Loading control: β-actin. * and **: Fast and slow moving bands of BMPRI. The number 35 and 50 indicate the molecular weight markers in kDa. * indicates the significant difference from the control without SMURF1. The lower band seen under the Flag-SMURF1 is non-specific band seen even in the control without SMURF1 expression.

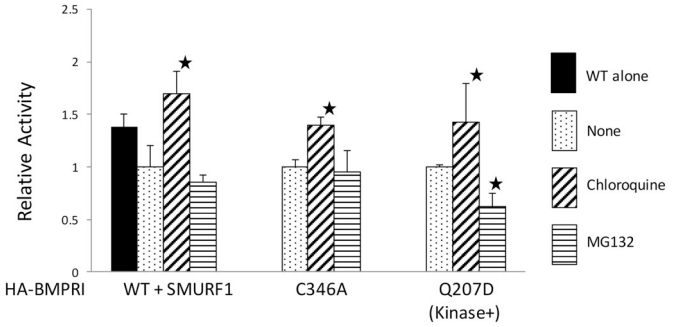

Fig. 4. Effects of protease inhibitors on steady-state protein levels of BMPRIs. HA-BMPRI was expressed without (black bar) or with Flag-SMURF1 (dotted bar as a control for the ones treated with inhibitor). HA-BMPRI^{C346A} (C346A) and HA-BMPRI$^{kinase+}$ (Q207D) were expressed alone. Expressing cells in quadruplicates were treated with 50 μM chloroquine (CHL) or 25 μM MG132 (MG) for 4 h. Average densitometric values of Western blots are shown with standard deviations from three repeated experiments. *: $p < .05$ from the control without protease inhibitor.

(chloroquine), but not by proteasomal inhibitor (MG132). BMPRI^{C346A} protein level was elevated by chloroquine, but not by MG132. These results may support the mechanism that Cys substitution causes lysosomal degradation in a SMURF1-associated pathway, thus causing reduced steady-state protein level (due to endogenous SMURF1) which is elevated by SMURF1$^{ligase-}$. Substitution was also introduced in the ligand binding domain (called BMPRI^{C96W}). BMPRI Cys96 corresponded to the Cys118 residue mutated in BMPRII of HPAH patients [36]. HA-BMPRI^{C96W} with Cys-substitution in the extracellular ligand-binding domain was significantly reduced by SMURF1 (Fig 3b Lanes 2 vs. 1) and normalized to the control level by SMURF1$^{ligase-}$ (Fig.3b Lanes 3 vs. 1). Therefore, substitution of any BMPR conserved Cys residues, regardless of location, induces endocytosis/lysosome degradation in a SMURF1-mediated pathway.

3.3. Ligand-activated Ser/Thr kinase of BMPR induces endocytosis in a distinct pathway

The Ser/Thr-kinase associated with BMPRI is activated by BMPRII-associated kinase upon BMP ligand binding and responsible for downstream signaling. Signaling-competent BMPRI with constitutively-active kinase can be generated by the mutation Gln207Asp (BMPRI$^{kinase+}$) [56,58] and has been commonly used for studies on signaling and endocytic pathways. BMPRI$^{kinase+}$ transduces signals without BMPRII or BMP ligands.

Steady-state protein levels were similarly examined for the kinase-active BMPRI (BMPRI$^{kinase+}$). HA-BMPRI$^{kinase+}$ showed only a fast-moving band on Western blotting (Fig.3a Lanes 7–9 labeled with *). The fast-moving band was previously shown to be generated by deglycosylation of *Golgi*/ER-glycosylated slow-moving band (marked with ** in Fig.3a and b) [42]. Protein expression level was not significantly reduced by expression of Flag-SMURF1 (Fig.3a Lane 7 vs. 8), but significantly elevated by Flag-SMURF1$^{ligase-}$ (Fig.3a Lane 7 vs. 9). The steady-state protein level was elevated by chloroquine, but it was reduced by MG132 (Fig.4). Mechanisms for MG132-activated degradation are not clear, but it might be associated with previous findings that

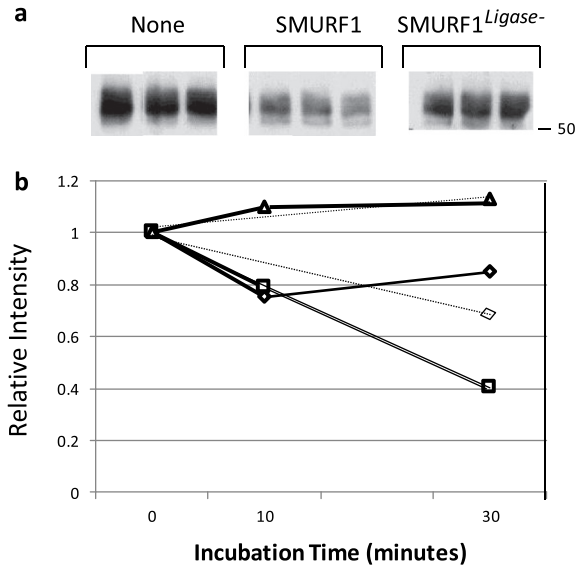

Fig. 5. Effects of SMURF1 and SMURF1$^{ligase-}$ on trafficking of cell surface BMPRI.
HA-BMPRI was expressed without or with Flag-SMURF1 or SMURF1$^{ligase-}$ in HEK293Tcells. Cell surface proteins were labeled with biotin and chased at 37 °C. After isolation of biotin-labeled proteins, HA-BMPRI was identified by Western blotting.
◊ BMPRI, □ : BMPRI + Flag-SMURF1, Δ : BMPRI + Flag-SMURF1$^{ligase-}$. Dotted lines indicate trends. a. Western blots, b. Densitogram.

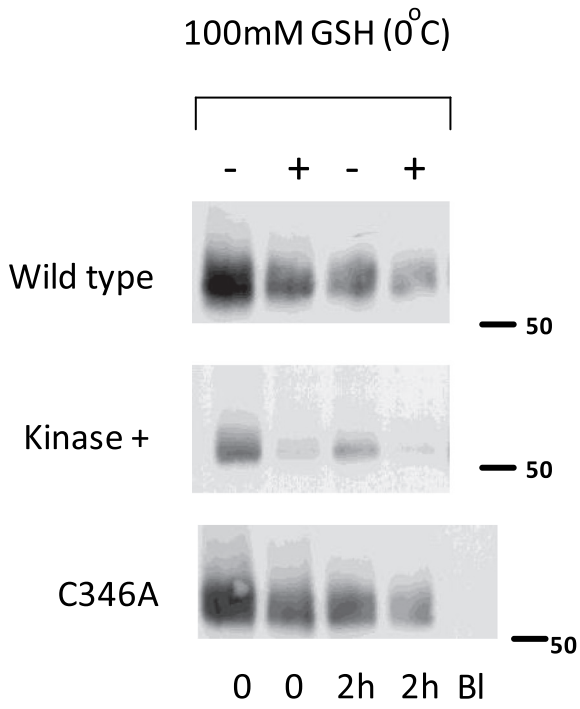

Fig. 6. Intracellular processing of BMPR's from the plasma membrane.
HA-BMPRI, BMPRI$^{kinase+}$ and BMPRI^{C346A} were expressed in HEK293T cells, in which plasma membrane proteins were then labeled with cleavable-biotin and chased. Biotin-labeled, intracellular proteins were then isolated and analyzed. Blank is similarly processed from cells not expressing HA-BMPRI, but labeled with biotin. The number 50 indicates the molecular weight marker in kDa. Each band represents comparative level to the 0-time (−) for each BMPRI type.

proteasome inhibitors influence lysosomal degradation of membrane proteins [11]. Therefore, the ligand-activated Ser/Thr kinase of BMPR may direct it to a distinct endocytic pathway where BMPR may be deglycosylated and show a unique specificity to protease inhibitors. The changes in expression level of BMPRI$^{kinase+}$ by Flag-SMURF1 and Flag-SMURF1$^{ligase-}$ could be due to changes in basal levels caused by SMURF1.

3.4. SMURF1 induces BMPR endocytosis while ligase-inactive SMURF1 elevates surface expression

Since microscopy demonstrates the presence of EGFP-BMPRI in varous cellular compartments, particularly the majority of EGFP-BMPRI^{C346A} in the cytoplasm, trafficking of HA-BMPRI's from the cell surface was examined to evaluate SMURF1 function on endocytosis. Proteins localized on the cell surface were labeled with biotin and chased *in vivo* at 37 °C. Fractions of HA-BMPRI originally present on the cell surface were measured from isolated biotin-labeled proteins. Fig.5a and b demonstrate biotin-labeled HA-BMPRI remaining after incubation at 37 °C. SMURF1 accelerated the decay of biotin-labeled HA-BMPRI from the plasma membrane, whereas SMURF1$^{ligase-}$ stabilized biotin-labeled, surface localized HA-BMPRI. These data confirm that SMURF1 induces BMPR endocytosis, while SMURF1$^{ligase-}$ promotes surface expression.

3.5. Endocytic processing of BMPR

Trafficking from the plasma membrane was examined for three types of BMPR; wild type, Cys-substituted (C346A) and kinase-activated. Cell surface proteins were labeled as for Fig.5, but with cleavable biotin which can be cleaved off when biotin is located on the cell surface, therefore enabling detection of intracellular proteins that originated on the cell surface. HA-BMPRI among biotin-labeled proteins was identified by anti-HA antibody. HA-BMPRI, HA-BMPRI$^{kinase+}$ and HA-BMPRI^{C346A} were expressed in HEK293T cells. Biotin-labeled cells were incubated at 37 °C to follow biotin-labeled proteins. After surface-biotin was stripped off, broad bands of biotin-labeled (intracellular) HA-BMPRI were identified for wild type and Cys mutant before and after the chase period (Fig.6 BMPRI & BMPRI^{C346A}). During biotin-labeling for 30 min on ice, some internalization and endocytosis appeared to occur, thus demonstrating some lower molecular weight forms. With BMPRI$^{kinase+}$, on the other hand, broad bands were present at the 0 time before incubation at 37 °C, whereas only the fast band was identified after a chase period at 37 °C (Fig.6 BMPRI$^{kinase+}$). The fast band was demonstrated to be formed by deglycosylation of the slow-moving band [42]. Thus, the kinase-active form appears to be transported to lysosomes through a pathway where BMPRI$^{kinase+}$ is deglycosylated and which is distinct from that for wild type and Cys mutant BMPR's.

3.6. Dominant-negative effect of BMPR Cys-residue substitution and reversal by ligase-negative SMURF1 on BMP signaling and receptor levels

Expression of BMPRII with Cys-substitution was previously shown to suppress the basal signaling activity by BMP (a dominant-negative effect) [42,51]. We reasoned that the dominant-negative effect was due to Cys-substitution-induced endocytosis of BMPRI and II complexes, thus reducing the cell surface level of endogenous BMPR. If this is the case, the dominant effect on BMP signaling should be reversed by ligase-negative SMURF1. To test this hypothesis, BMPRI and II protein levels were examined with increasing concentrations of BMPRII expressed together with wild type BMPRI. Cys-substitution was introduced at C347 and C118 of HA-BMPRII (HA-BMPRII^{C347A} and BMPRII^{C118W}, respectively), at the kinase- and ligand-binding domains as identified in HPAH patients [36]. HA-BMPRI, at the constant concentration, was expressed with increasing concentrations of HA-BMPRII or HA-BMPRII^{C347A}. Steady-state protein levels of BMPRI and II were analyzed by Western blotting. Protein levels of BMPRI, expressed using the constant vector concentration, decreased when expressed with

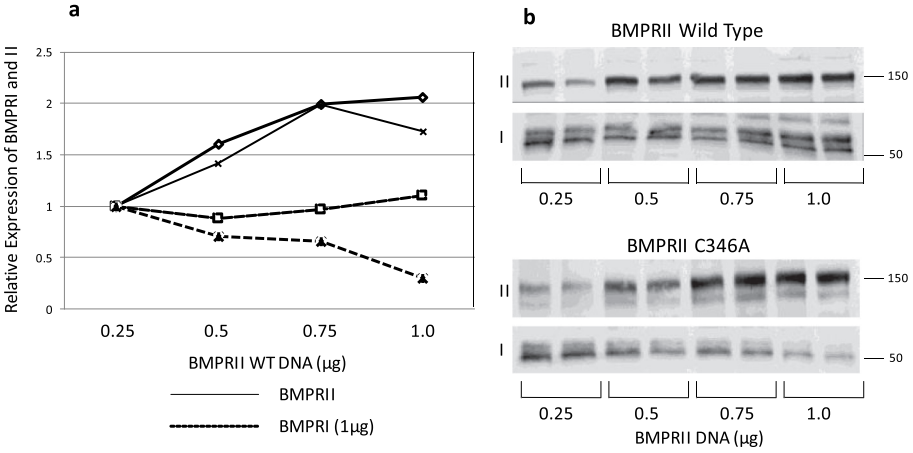

Fig. 7. Dominant-negative effect of Cys-substituted BMPRII on BMPR protein levels.
HA-BMPRI (0.5 μg DNA) was expressed with increasing concentrations of HA-BMPRII or HA-BMPRII-C347A. Steady-state protein levels of BMPRI and II were analyzed. a. Densitometry: ◇—◇ BMPRII, X—X BMPRII^{C347A}, □- -□ BMPRI with BMPRII, ▲- -▲ BMPRI with BMPRII C347A. b. Western blotting pattern in duplicates.

increasing concentrations of HA-BMPRII^{C347A}, while it stayed relatively constant with wild type HA-BMPRII (Fig.7a and b). The expression level of HA-BMPRII^{C347A} protein remained lower than BMPRII wild type as their expression vector concentrations were increased, indicating degradation of the former (Fig.7a and b). Therefore, BMPRI and II appear to be endocytosed in a complex where Cys-residue substitution of BMPRII is dominant in inducing lysosomal degradation of the complex and reducing both BMPRI and II protein levels. These results are consistent with the reported result that wild type BMPRI was found together with Cys-substituted BMPRII in the cytoplasm when expressed together [53].

We demonstrated above that SMURF1$^{ligase-}$ blocked lysosomal degradation and elevated BMPR protein on the plasma membrane (Figs.3a and b, 4 and 5). Then, cell surface expression of Cys-substituted BMPR and BMP signaling activity should be recovered by SMURF1$^{ligase-}$. To test this, wild type and Cys-substituted BMPRII's together with BMPRI were expressed with or without SMURF1$^{ligase-}$. Endogenous BMP signaling activity, as measured from SMAD phosphorylation, was high in the control cells and masked the changes induced by exogenous expression of BMPRIIs. Nonetheless, SMAD phosphorylation was elevated when HA-BMPRII^{C347A} was expressed with SMURF1$^{ligase-}$ (Fig.8a). Furthermore, increasing concentrations of HA-BMPRII^{C118W} or BMPRII^{C347A} with the wild type HA-BMPRI at constant concentration were expressed together with or without Flag-SMURF1$^{ligase-}$ (Fig.8b). SMAD phosphorylation was suppressed in a concentration-dependent manner by expression of HA-BMPRII^{C118W} or BMPRII^{C347A}, demonstrating a dominant-negative effect by Cys-substituted BMPRIIs (Fig.8b). Flag-SMURF1$^{ligase-}$ co-expression elevated protein levels of Cys-substituted HA-BMPRII's and SMAD phosphorylation, more significantly with HA-BMPRII^{C347A} in correlation with expression levels of HA-BMPRII^{C347A} protein (Fig.8b). SMURF1$^{ligase-}$ thus recovers BMP signaling activity by elevating the cell surface expression of Cys-substituted BMPR. Therefore, the dominant-negative effect by Cys-substitution of BMPRII appears to be caused by reduction of the cell surface BMPR complex and is relieved by SMURF1$^{ligase-}$ by raising cell surface expression of BMPR. Furthermore, basal SMAD phosphorylating signaling activity did not change by expression of wild type BMPRII in HEK293T cells, whereas it was suppressed by expression of Cys-substituted BMPRII, demonstrating a dominant negative effect.

3.7. Substitution of conserved Cys residue induces BMPR endocytosis

Since BMPRII with Cys substitution was co-localized with endoplasmic reticulum (ER) markers, substitutions of conserved Cys residues were previously proposed to retard trafficking to the plasma membrane due to abnormal protein folding [42,51]. We examined endocytic/lysosomal trafficking by Cys-substitution of BMPR which might cause reduced expression on the plasma membrane. Subcellular localization of BMPR's was characterized by co-labeling of the early endosome antigen 1 (EEA1) and lysosome antigen membrane protein 1 (LAMP1). HEK293T cells expressing EGFP-BMPRI wild type and C346A were fluorescently co-labeled for EEA1 and LAMP1 (red staining in Fig.9a and b). Co-localization with EEA1, in orange color, was seen significantly with EGFP-BMPRI-C346A (Fig.9a). With LAMP1, orange color localization indicating co-localization was not seen, but EGFP-BMPRI-C346A was seen neighboring LAMP1 in red color (Fig.9b). Thus, the data suggest that substitution of conserved Cys residue induces endocytosis to lysosomes, thus causing co-localization with the endosome marker. When analyzed by confocal microscopy with higher magnification (100× lense), EGFP-BMPRI^{C346A} showed abnormal localization in vesicles resembling aberrant MVBs (Fig.9c Panel D, short arrows). EGFP-BMPRI^{C346A} extensively co-localized with EEA1 (Fig.9c Panel A vs. B).

Vps4 AAA^{+} ATPase is a functional component of the ESCRT pathway. When the ATPase-defective Vps4 mutant is expressed, endocytic membrane receptors accumulate in abnormally enlarged MVBs [4,5]. The ATPase-defective Vps4 (HA-Vps4^{EQ}) was constructed and tested to confirm endocytic trafficking through MVBs to lysosomes. EGFP-BMPRI wild type and C346A as well as EGFP-BMPRI$^{kinase+}$ were expressed with HA-Vps4-EQ in HEK293T cells, and their localizations were examined by fluorescently labeled HA-Vps4^{EQ} (Fig.10). All forms co-localized with HA-Vps4^{EQ} strongly, demonstrating that they are all transported to lysosomes through MVBs.

4. Discussion

The present study demonstrated that substitution of HPAH-associated Cys residues of BMPR induced its endocytosis and lysosomal degradation in a SMURF1-mediated pathway. This conclusion is based on the following observations; 1) Cys residue-substituted BMPR co-localizes with EEA1, 2) Protein level of Cys-substituted BMPR is recovered by lysosomal inhibitor. 3) Protein level of Cys-substituted BMPR is upregulated/normalized to the level of BMPR wild type by ligase-inactive SMURF1. 4) SMURF1 ubiquitin ligase induces lysosomal degradation of BMPR, while ligase-inactive SMURF1 blocks lysosomal degradation and elevates BMPR on the cell surface.

The dominant-negative effect of Cys residue substitution was previously demonstrated on BMP signaling activities [42,51]. Wild type BMPRI was shown to be present in the cytoplasm together with Cys-substituted BMPRII [53]. In the present study, BMPRI protein level was reduced proportionally as the expression of Cys-substituted BMPRII protein was elevated (Fig.7). Therefore, BMPRI appears to be endocytosed in a complex with Cys-substituted BMPRII leading to lysosomal degradation and reduced expression on the plasma membrane. The basal BMP signaling activity, in the absence of exogenous expression of BMPRs, was suppressed by expression of Cys-substituted BMPR

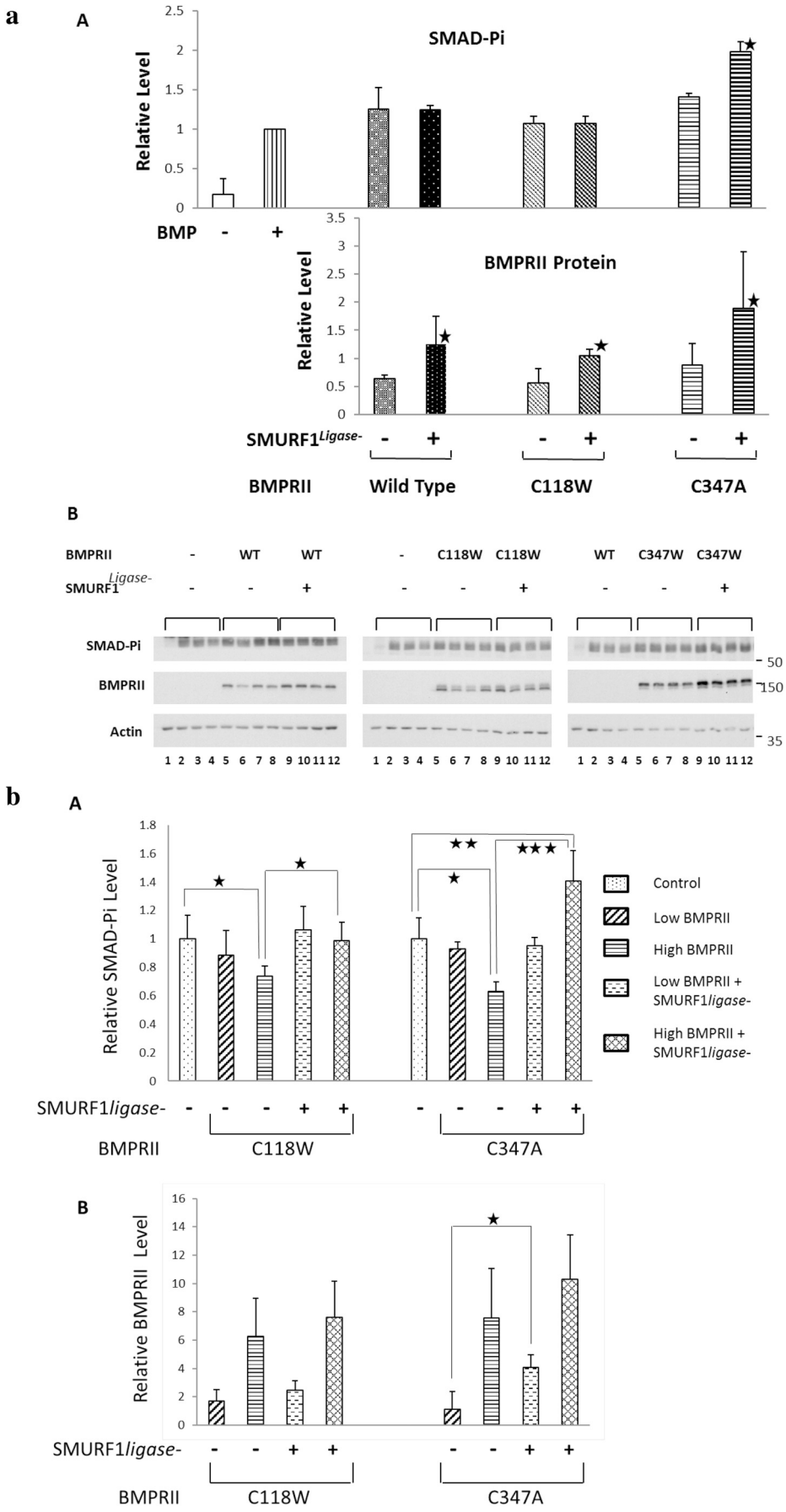

Fig. 8. Elevation of BMP signaling by ligase-inactive SMURF1.

a. HA-BMPRI (0.5 μg DNA) was expressed with HA-BMPRII wild type, C118W or C347A (0.5 μg) with or without Flag-SMURF1$^{ligase-}$ (0.3 μg). Cells were treated with 100 ng/ml BMP2 for 1 h, except the control without BMPR transfection. Phosphorylated SMAD1 was measured as an indicator of BMP signaling activity. Experimental conditions were all performed in quadruplicates. A. Upper panel: Phosphorylated SMAD1, Lower panel: Protein levels of expressed BMPRII's. *: $p < 0.05$. B. Western blot patterns of phosphorylated SMAD1, BMPRII's and β-actin.

b. HA-BMPRI (0.4 μg DNA) was expressed with HA-BMPRII C118W or C347A (0.3 or 0.6 μg) with or without Flag-SMURF1$^{ligase-}$ (0.3 μg DNA). Treatment and analyses were performed as **a**. Upper panel A: Phosphorylated SMAD1, Lower panel B: Protein levels of expressed BMPRII's. *: $p < 0.05$ **:$p < 0.005$ ***:$p < 0.0005$

(Fig.8a and b). On the other hand, BMP signaling activity was activated in parallel to expression levels of Cys-substituted BMPR when upregulated by ligase-inactive SMURF1 (Fig.8a and b). These results were similarly observed with Cys-substitution either at the ligand-binding or kinase domain. Therefore, the dominant-negative effect resulting from Cys-substitution appeared to be caused by downregulation of cell surface BMPR complexes. Blockage of SMURF1 was thus effective for recovering surface expression and signaling activity by Cys-substituted BMPR. It was recently demonstrated that cell surface expression of BMPR was regulated through constitutive endocytosis and lysosomal degradation and can be upregulated by blocking ubiquitin ligases or lysosome inhibitors [17]. The present results are consistent with these

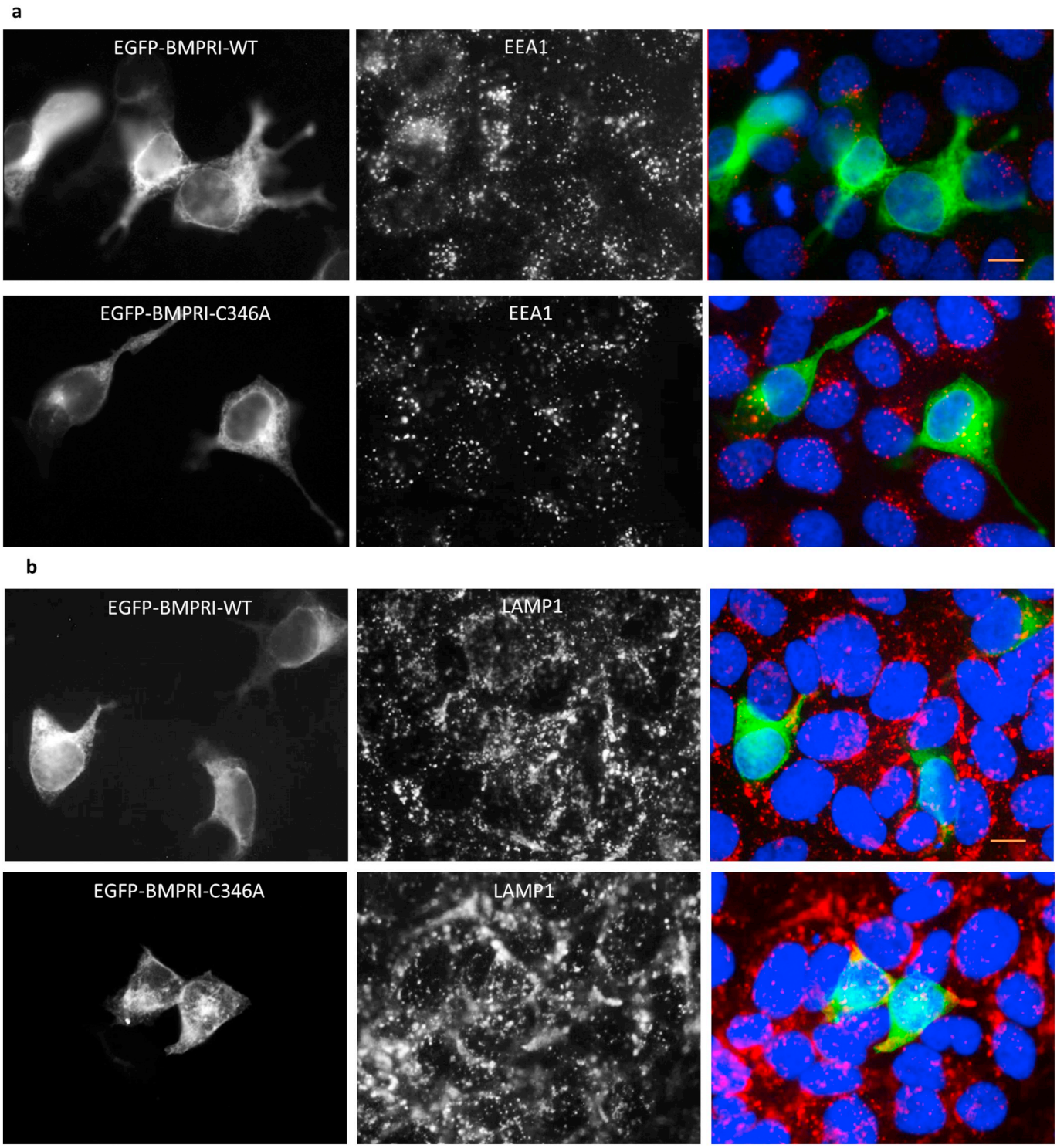

Fig. 9. Co-localization of BMPRI's with EEA1 and LAMP.
EGFP-BMPRI or EGFP-BMPRI^{C346A} was co-labeled for EEA1 and LAMP. They were analyzed by fluorescent microscopy using 40× lens. Green: EGFP. Red: EEA1 **(a)** or LAMP **(b)**. Blue: Nuclei. The scale bar indicates 10 μm. **(c)** Co-labeling of EGFP-BMPRI (A, C) and EGFP-BMPRI^{C346A} (B, D) with EEA1 (A, B) or LAMP1 (C, D) was analyzed by confocal microscopy. EGFP-BMPRIs: green, EEA1 and LAMP1: red. Long arrows: co-localization of EGFP-BMPRI^{C346A} with EEA1. Arrow heads: co-localization of EGFP-BMPRI^{C346A} with LAMP1. Short arrows: abnormal enlarged endosomes.

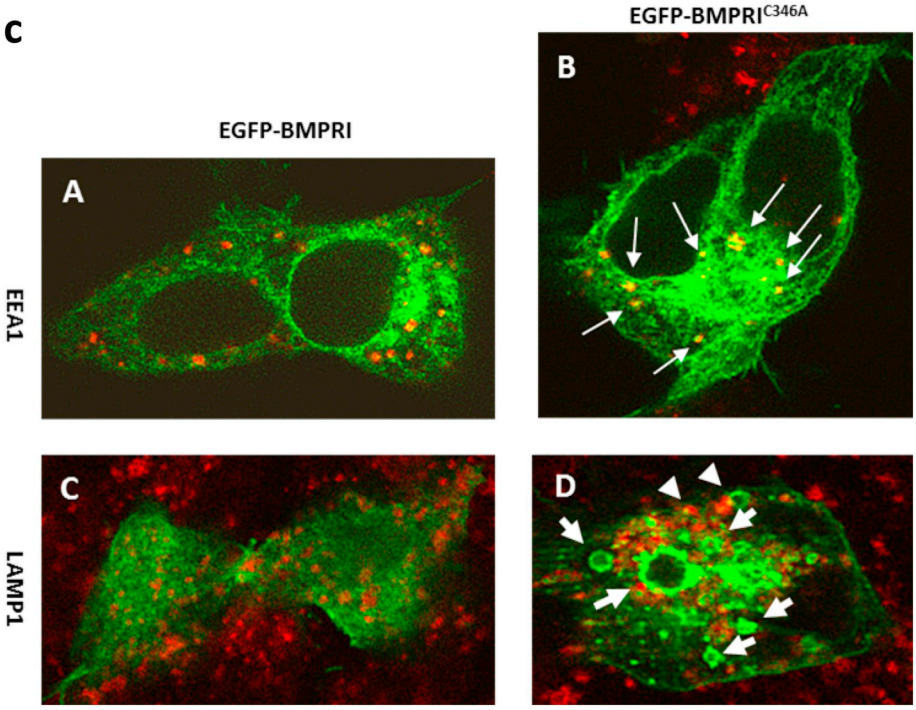

Fig. 9. (*continued*)

findings, indicating that ubiquitin ligases induce constitutive endocytosis of BMPR.

It is believed that membrane receptors, ubiquitinated upon ligand-activation, are recruited to the ESCRT for transport to MVBs and lysosomes [45,46,49,57]. On the other hand, we have demonstrated that ligand-activated Ser/Thr kinase associated with BMPR induces its lysosomal degradation in a pathway which is characterized with de-glycosylation and a unique specificity to protease inhibitors. SMURF1 induces basal endocytosis and lysosomal degradation of BMPR. Ubiquitination of the intracellular Lys-residue adjacent to the transmembrane domain of BMPR, by Kaposi sarcoma-associated herpes viral lytic gene K5 and ITCH ubiquitin ligases, was shown to induce constitutive endocytosis [17]. Pathogenic Cys-residue substitutions have been identified at several different Cys residues over the extracellular ligand-binding and cytoplasmic kinase domains of BMPR in patients with HPAH [36]. Since substitution of any single Cys residue among several conserved Cys-residues induces endocytosis which can be counteracted by ligase-inactive SMURF1, further studies are needed to understand how multiple Cys residues are all involved in SMURF1-dependent trafficking.

Kinase-activated BMPRI appears to be endocytosed and trafficked to lysosomes in a distinct manner. Constitutive endocytosis of BMPR is through the recycling/endocytic membrane pathway which is regulated by SMURF1 ubiquitin ligase. Inactivation of EGFR-associated tyrosine kinase abolishes both EGF-induced signaling and endocytosis/lysosomal degradation while kinase-inactivated EGFR is recycled to the plasma membrane [20,28]. Signaling receptors, such as EGFR and GPCR, are recycled in the absence of endocytosis signals, converting from transient to sustained cell signaling. The Cbl ubiquitin ligase is shown to be recruited for ubiquitination and endocytosis of EGFR whereas, with v-Cbl which is devoid of the ligase domain, the surface expression of EGFR is elevated due to recycling, resulting in constitutive EGF signaling and development of cancer [32]. These results may support a common endocytic mechanism for ligand receptors; constitutive recycling/basal ubiquitin ligase-induced endocytosis and kinase-activated endocytosis to lysosomes.

Cys substituted BMPR colocalized with EEA1 in abnormal cytoplasmic vesicles (Fig.9c). Expression of mutants of ESCRT-0 (HRS) and − 1 (TSG101) as well as VPS4, in addition to interfering with trafficking of membrane receptors to the vacuolar lumen, blocks recycling of membrane receptors to the plasma membrane and causes their accumulation in abnormal endosome structures called the class E compartment [16,25,59]. These similar phenotypes may suggest that conserved Cys residues of BMPR are functionally coupled with constitutive recycling through interaction with ESCRT components. Substitution of Cys residues may thus cause ESCRT-associated dysfunction generating abnormal enlarged endosomes and causing lysosomal degradation. As trafficking pathways going through the endosome compartment, including anterograde trafficking of biosynthetic cargos, appear to be affected in ESCRT mutants [16,43], BMPRII with Cys substitution may co-localize with endoplasmic reticulum (ER) markers, as reported previously [53]. In addition, some overexpressed BMPR Cys mutants could be subjected to ER-associated degradation (ERAD) demonstrating colocalization with ER markers [53].

Endocytosis of signaling receptors have been long regarded to be driven by endocytic machinery where membrane receptors are termed ‘passive cargos’. Recent results however demonstrate cargo-driven control of clathrin dynamics with activated receptors [26,48,54]. The present results have identified conserved Cys residues of BMPR that may facilitate the recycling pathway and the ligand-activated Ser/Thr kinase directing receptors to a unique endocytosis/signaling pathway. Since Cys residues of membrane receptors and transporters have been shown to be associated with abnormal trafficking, signaling, and pathogenesis [7,8,21,34,44], the Cys-residue-driven trafficking function may be a general property of membrane proteins, while Cys residue substitution could have direct impacts on the pathogenesis of genetic diseases.

The present study indicates that SMURF1 ubiquitin ligase controls the steady-state level of BMPR. This is the case in PAH that BMPR protein level and signaling are reduced in pulmonary vascular cells of patients, most significantly HPAH [3], where SMURF1 was shown to be elevated in these cells of PAH patients and model animals [6,40,50]. Blockage of SMURF1 is shown to recover BMP signaling and to suppress phenotypes and development of PAH [6,50]. Therefore, SMURF1 is an excellent therapeutic target against PAH for recovering BMP signaling activity and vascular function.

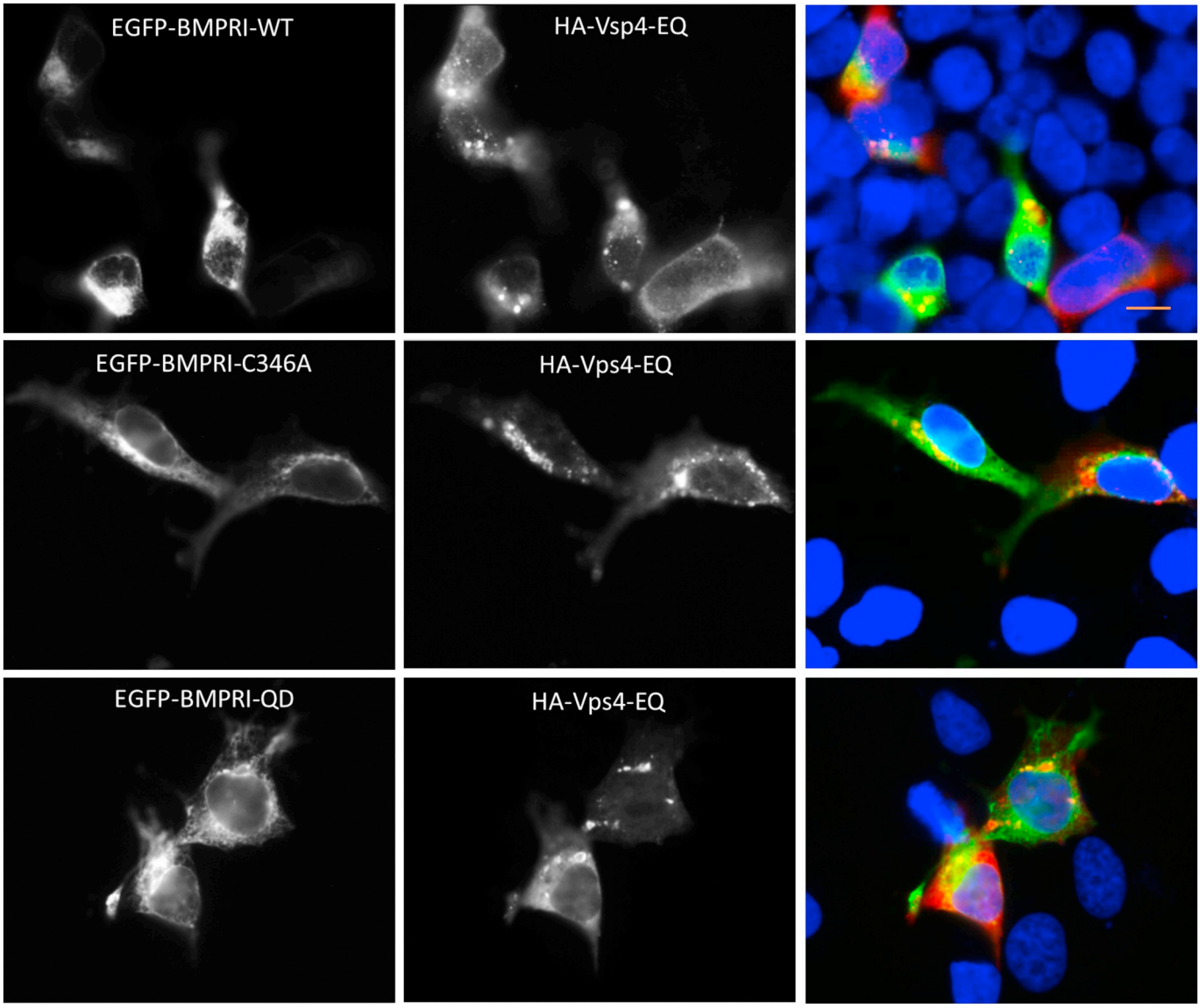

Fig. 10. Effect of dominant-negative VPS4 AAA$^+$ATPase on localization of BMPRI's.
EGFP-BMPRI, BMPRI^{C346A} and BMPRI$^{kinase+}$ were expressed together with the dominant-negative VPS4B (HA-VPS4^{EQ}) in HEK293T cells. Localization of EGFP and HA antigen was visualized by fluorescent microscopy using 40× lense. Nuclei were stained by DAPI. EGFP: green. HA: Red. Nuclei: Blue. The scale bar indicates 10 μm.

5. Conclusions

- Two independent pathways, basal and ligand-activated, are identified for BMPR endocytosis.
- SMURF1 ubiquitin ligase induces basal endocytosis of BMPR.
- Substitution of BMPR conserved cysteine residues induces SMURF1-dependent endocytosis.
- BMPR with cysteine residue substitution demonstrates a dominant-negative effect on BMP signaling which can be recovered by dominant-negative SMURF1.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author's contribution

KM performed all experiments and wrote the manuscript. JDE contributed to the development of this project and writing manuscript.

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