







ARTICLE

Tyrosine phosphorylation of S1PR1 leads to chaperone BiP-mediated import to the endoplasmic reticulum

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Cell surface G protein-coupled receptors (GPCRs), upon agonist binding, undergo serine–threonine phosphorylation, leading to either receptor recycling or degradation. Here, we show a new fate of GPCRs, exemplified by ER retention of sphingosine-1-phosphate receptor 1 (S1PR1). We show that S1P phosphorylates S1PR1 on tyrosine residue Y¹⁴³, which is associated with recruitment of activated BiP from the ER into the cytosol. BiP then interacts with endocytosed Y¹⁴³-S1PR1 and delivers it into the ER. In contrast to WT-S1PR1, which is recycled and stabilizes the endothelial barrier, phosphomimicking S1PR1 (Y¹⁴³D-S1PR1) is retained by BiP in the ER and increases cytosolic Ca²⁺ and disrupts barrier function. Intriguingly, a proinflammatory, but non-GPCR agonist, TNF- α , also triggered barrier-disruptive signaling by promoting S1PR1 phosphorylation on Y¹⁴³ and its import into ER via BiP. BiP depletion restored Y¹⁴³D-S1PR1 expression on the endothelial cell surface and rescued canonical receptor functions. Findings identify Y¹⁴³-phosphorylated S1PR1 as a potential target for prevention of endothelial barrier breakdown under inflammatory conditions.

Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of seven-transmembrane-spanning cell surface receptors that, upon binding to their respective ligands, stimulate heterotrimeric G proteins to rapidly trigger downstream signaling. The critical determinant of GPCR regulation of cellular function is the receptor's cell surface expression (Rosenbaum et al., 2009; Venkatakrishnan et al., 2013). Phosphorylation of GPCRs at C-terminal serine/threonine residues leads to receptor internalization by the canonical dynamin/ β -arrestin-mediated pathway, following which the GPCR either recycles back to the cell surface or undergoes ubiquitin-mediated degradation (Oo et al., 2011). However, several GPCRs in the class A family, such as sphingosine-1-phosphate receptor 1 (S1PR1), contain tyrosine residues within a conserved E/DRY motif located at the boundary between transmembrane domain III and intracellular loop 2 (Cannavo et al., 2013; Rasmussen et al., 2011). Little is known about the molecular determinants of subcellular localization of tyrosine-phosphorylated GPCRs or whether the phosphorylated receptor remains functional after it is internalized.

S1PR1 is widely expressed in several organs, such as lung, brain, heart, and lymphoid glands (Cahalan et al., 2011), but its

expression profile differs among endothelial cells (ECs) from various vascular beds (Cartier and Hla, 2019; Jambusaria et al., 2020). For example, S1P was shown to enhance transendothelial electrical resistance (TEER), a measure of endothelial barrier function, more so in pulmonary microvascular ECs than in pulmonary arterial ECs (Dudek et al., 2004). Single-cell RNA sequencing analysis in a mouse model of atherosclerosis also indicated that S1PR1/ β -arrestin coupling and inflammatory gene expression signature differ between arterial EC subsets at vascular branch points than other EC subtypes at nonbranch points (Engelbrecht et al., 2020).

Upon binding S1P, S1PR1 transmits downstream signaling by coupling with heterotrimeric Gi protein to regulate diverse cellular functions, including maintenance of EC barrier function, as well as an effective immune response by lymphocytes (Rivera et al., 2008). S1PR1 strengthens basal EC barrier function as well as resolves lung injury by mechanisms involving intracellular Ca²⁺ ([Ca²⁺]_i) transient- and Rac1/Cdc42-GTPase-dependent cortical actin assembly, which reanneal adherens junctions (Daneshjou et al., 2015; Mehta et al., 2005; Reinhard et al., 2017). Lymphocyte-expressed S1PR1, on the other hand, regulates

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lymphocyte egress from secondary organs, such as spleen (Matloubian et al., 2004) and lymph nodes (Benechet et al., 2016), based on tissue SIP concentrations. Additionally, cell surface CD69 expression (Cyster and Schwab, 2012), SIPRI-C-terminal phosphorylation, and dynamin (Oo et al., 2011) regulate lymphocyte egress by internalizing SIPRI (Willinger et al., 2015). SIPRI-deficient naive T cells thereby fail to exit the thymus and lymph nodes, leading to impaired immune response after viral infection (Baeyens et al., 2015; Matloubian et al., 2004). Thus, loss of SIPRI function in ECs and lymphocytes is a hallmark of several vascular and chronic inflammatory diseases (Cartier and Hla, 2019; Proia and Hla, 2015).

We showed that in addition to inducing serine phosphorylation at the C-terminus, SIP also mediates SIPRI phosphorylation on tyrosine residue Y¹⁴³ within the ERY motif, following which the receptor is internalized but protected from degradation (Chavez et al., 2015). The Chavez et al. study raised several fundamental questions, namely: (1) How is internalized Y¹⁴³-SIPRI regulated? (2) Is internalized Y¹⁴³-SIPRI functional? And (3) does Y¹⁴³-SIPRI function in a manner like the native receptor in terms of regulating downstream signaling? Here, we express native and phosphospecific SIPRI mutants in ECs to assess the basis of SIPRI phosphorylation at Y¹⁴³ in regulating receptor localization and functions. We demonstrate a unique and functional transformation of SIPRI when phosphorylated on Y¹⁴³. We show that Y¹⁴³ phosphorylation of SIPRI drives the receptor into the ER in a manner dependent on activation of the ER chaperone binding immunoglobulin protein (BiP). While WT-SIPRI recycled back to the EC surface, constitutively phosphomimicking SIPRI (Y¹⁴³D-SIPRI) mutant is retained in the ER by BiP and induces disruption of the endothelial barrier. The present studies have thus identified a previously unknown role of SIPRI in destabilizing endothelial barrier function during vascular injury in a manner dependent on posttranslational modification of the receptor on Y¹⁴³ and ER retention.

Results

Tyrosine-phosphorylated SIPRI localizes at the ER

We cotransfected GFP-tagged WT-SIPRI, Y¹⁴³D-SIPRI (phosphomimicking), or Y¹⁴³F-SIPRI (phosphodeficient) SIPRI mutants along with stargazin, which fluorescently labels the plasma membrane (Inamura et al., 2006) in human umbilical vein ECs (HUVECs) to assess receptor localization. Using total internal reflection fluorescence (TIRF) microscopy, which detects fluorescence within 100 nm from the cell surface and thus detects plasma membrane localization of receptor (Doranzo et al., 2019), as well as confocal microscopy, we observed the predicted colocalization of WT-SIPRI and Y¹⁴³F-SIPRI with stargazin (Fig. 1, A and B; and Fig. S1, A and B). However, Y¹⁴³D-SIPRI failed to colocalize with stargazin and, rather, was found localized in an intracellular compartment (Fig. 1, A and B; and Fig. S1, A and B) distinct from the nucleus (Fig. S1, A and B).

The ER regulates trafficking of newly synthesized or post-translationally modified GPCRs (Drake et al., 2006). We therefore overexpressed WT-, Y¹⁴³D-, and Y¹⁴³F-SIPRI mutants with an mCherry-tagged ER cDNA to test the possibility that SIPRI traffics to the ER upon tyrosine phosphorylation. The ER-

mCherry cDNA has a calreticulin signal sequence at the N-terminus that directs insertion into the ER and a KDEL coding sequence at the C-terminus that is responsible for retention in the ER (Dickens et al., 2016). Interestingly, we found that Y¹⁴³D-SIPRI colocalized with ER-mCherry, while WT-SIPRI or Y¹⁴³F-SIPRI did not (Fig. 1, C and D).

We then stimulated ECs transducing WT-SIPRI or Y¹⁴³F-SIPRI with SIP to assess the time course of SIPRI trafficking to the ER. We found that WT-SIPRI localized to the ER within 2.5 min and remained there for up to 5 min, returning to the cell surface up to 20 min later. In contrast, Y¹⁴³F-SIPRI did not internalize at all, even after SIP stimulation (Fig. 2, A and B). SIP had no effect on Y¹⁴³D-SIPRI internalized localization (data not shown).

To address whether ER localization of GFP-SIPRI is not the result of cleavage of GFP from SIPRI or Y¹⁴³D-SIPRI, we tagged WT-SIPRI or Y¹⁴³D-SIPRI with the photoswitchable fluorescent protein Dendra2, which exhibits a shift in emission spectrum from a 488- to 561-nm maximum wavelength after photoconversion (Chudakov et al., 2007). We irradiated Dendra2 and monitored SIPRI dynamics with or without SIP stimulation. We observed in unstimulated ECs that SIPRI-Dendra2 remained at the cell surface even after photoconversion (Fig. 2, C and D). However, SIP addition rapidly increased SIPRI-Dendra2 inside the cell, demonstrating SIPRI internalization (Fig. 2, C-E). In line with this, we also observed that Y¹⁴³D-SIPRI-Dendra2 remained internalized with or without SIP stimulation following photoconversion (Fig. S1, C and D). These findings demonstrate that upon tyrosine phosphorylation at Y¹⁴³, the receptor is trafficked back from the ER to the EC surface. Consistently, the Y¹⁴³D-SIPRI mutant was constitutively internalized and localized in the ER.

GPCRs homo- and hetero-oligomerize (Ramsay et al., 2002). Previously, we showed that Y¹⁴³D-SIPRI mutant expression in naive or SIPRI-depleted ECs disrupts barrier function (Chavez et al., 2015), raising the possibility that the overexpression of mutated SIPRI may affect the localization and function of endogenous SIPRI. Thus, we coexpressed GFP-tagged WT-SIPRI with vector, HA-tagged Y¹⁴³D-SIPRI, or HA-tagged Y¹⁴³F-SIPRI mutants and determined whether SIPRI mutants compromise GFP localization. We found that GFP-tagged SIPRI is internalized in ECs when cotransduced with HA-tagged Y¹⁴³D-SIPRI. However, GFP-tagged SIPRI remained at the EC surface in ECs cotransducing vector and HA-tagged Y¹⁴³F-SIPRI mutant (Fig. 3, A and B). We also measured TEER in ECs transducing WT-SIPRI-GFP along with HA-tagged Y¹⁴³F- or Y¹⁴³D-SIPRI mutants, respectively. Consistent with previous findings, we observed that Y¹⁴³D-SIPRI disrupted barrier function basally, which was not reversed even after SIP addition (Fig. 3, C and D). This response was not seen in ECs cotransfected with WT-SIPRI-GFP and HA-tagged Y¹⁴³F-SIPRI mutant. Altogether, these findings demonstrate that Y¹⁴³D-SIPRI serves as a dominant negative for endogenous SIPRI and thereby disrupts EC barrier function.

Dynamin pinches off the phosphorylated receptor

Dynamin plays a critical role in endocytosis of GPCRs in a GTP-dependent manner (Ferguson and De Camilli, 2012). To assess the possibility that Y¹⁴³-phosphorylated SIPRI was initially expressed on the cell surface and then internalized in a dynamin-dependent

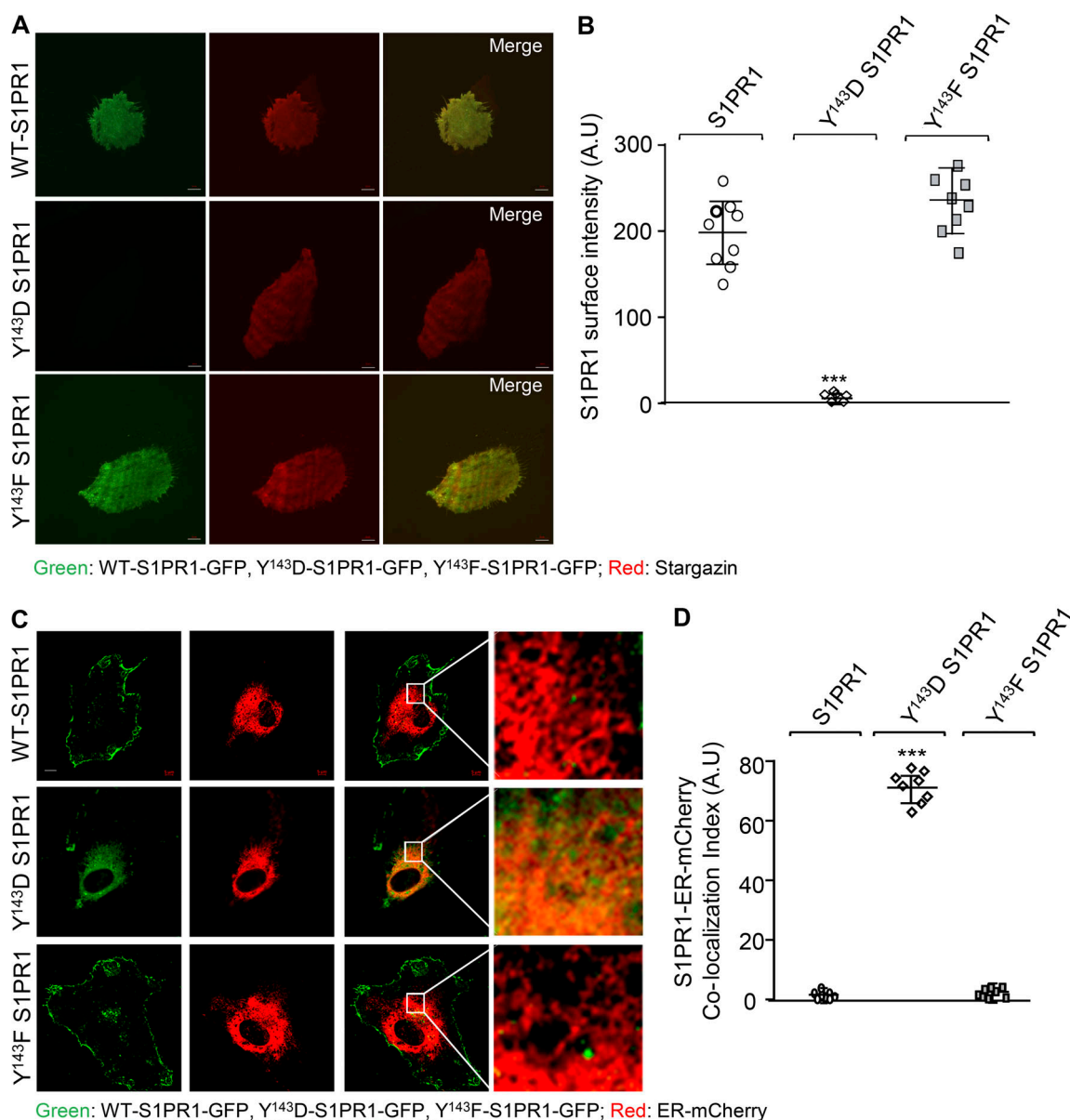


Figure 1. Tyrosine-phosphorylated S1PR1 traffics to the ER. (A) TIRF images of WT-S1PR1, Y¹⁴³F-S1PR1, and Y¹⁴³D-S1PR1 mutants with stargazin taken at 100× magnification. Scale bars, 10 μm. (B) Quantification of the surface expression of S1PR1. *n* = 7–9 cells/group from experiments repeated three times. (C) Confocal images of S1PR1 and ER-mCherry taken at 63× magnification. Inset: 5× magnified image of the area in rectangle. Scale bar, 5 μm and 25 μm in insets. (D) Quantification of the percent colocalization of the S1PR1 with ER-mCherry. *n* = 7–10 cells/group from experiments repeated three times. Data in B and D are shown as mean ± SD. ***, *P* < 0.0001 compared with WT and Y¹⁴³F-S1PR1 by one-way ANOVA with two-tailed paired *t* test. See also Fig. S1, A and B. A.U., arbitrary units.

manner, we treated ECs transducing S1PR1 mutants with dynasore, a specific small-molecule inhibitor of dynamin (Macia et al., 2006), or depleted dynamin using siRNA. Biotinylation assay showed that compared with WT-S1PR1 or Y¹⁴³F-S1PR1, cell surface expression of Y¹⁴³D-S1PR1 was reduced by ~90% (Fig. 4, A and B). Dynasore treatment restored Y¹⁴³D-S1PR1 cell surface expression to the level observed in ECs transducing WT-S1PR1 or Y¹⁴³F-S1PR1 (Fig. 4, A and B). Similar results were obtained in dynamin-depleted ECs (Fig. 4, C and D).

Mutation of Lys 44 to Ala (K44A) in dynamin impairs its GTPase activity and thereby inhibits receptor endocytosis

(Damke et al., 2001). Thus, in other studies, we cotransduced ECs with K44A-mCherry-dynamin along with either GFP-tagged WT-S1PR1, Y¹⁴³D-S1PR1, or Y¹⁴³F-S1PR1 and assessed S1PR1 cell surface expression by confocal microscopy. Overexpression of GTPase-dead dynamin restored Y¹⁴³D-S1PR1 expression on the EC surface (Fig. 4, E and F), demonstrating that dynamin promotes internalization of Y¹⁴³D-S1PR1 in a manner consistent with dynamin-dependent regulation of GPCR trafficking (Willinger et al., 2014).

Rab-GTPases contribute to many steps in vesicular trafficking, including endocytosis and sorting to subcellular compartments

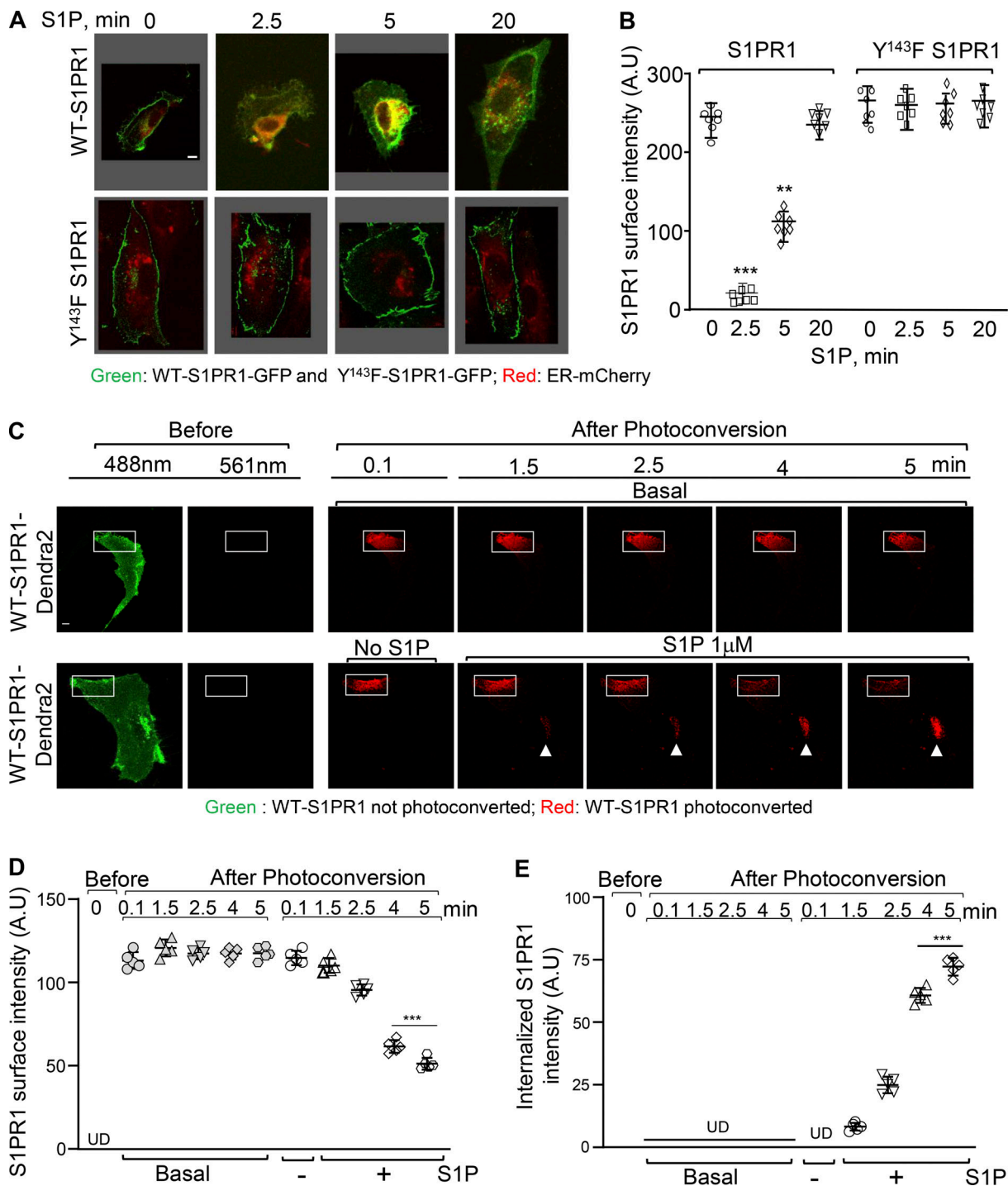


Figure 2. Dynamics of the tyrosine-phosphorylated S1PR1 to the ER. (A) Confocal images of S1PR1 and ER (mCherry) without or with S1P (1 μ M). Gray background indicates the area outside the image. Scale bar, 5 μ m. **(B)** Quantification of the surface expression. $n = 5-7$ cells/group from experiments repeated three times. One-way ANOVA with paired two tailed t test. **, $P < 0.001$; ***, $P < 0.0001$ compared with WT-S1PR1 at 0 and 20 min and Y¹⁴³F-S1PR1 at 0, 2.5, 5, and 20 min. **(C)** Time-lapse images of S1PR1-Dendra2 before and after photoconversion at time 0 (indicated by green, rectangles) and after the addition of S1P (1 μ M; red, rectangles). 488-nm channel (green) = unconverted S1PR1; 561-nm channel (red) = photoconverted S1PR1. Scale bar, 10 μ m. **(D and E)** Quantification using images in C. $n = 5-6$ cells from experiments repeated two times. **(D and E)** One-way ANOVA with paired two tailed t test. ***, $P < 0.0001$ compared with WT-S1PR1-Dendra2 at basal levels or after S1P stimulation at 2.5 min (D) or at 1.5 min (E). Data in B, D, and E are shown as mean \pm SD. See also Fig. S1, C and D. A.U., arbitrary units; UD, undetectable.

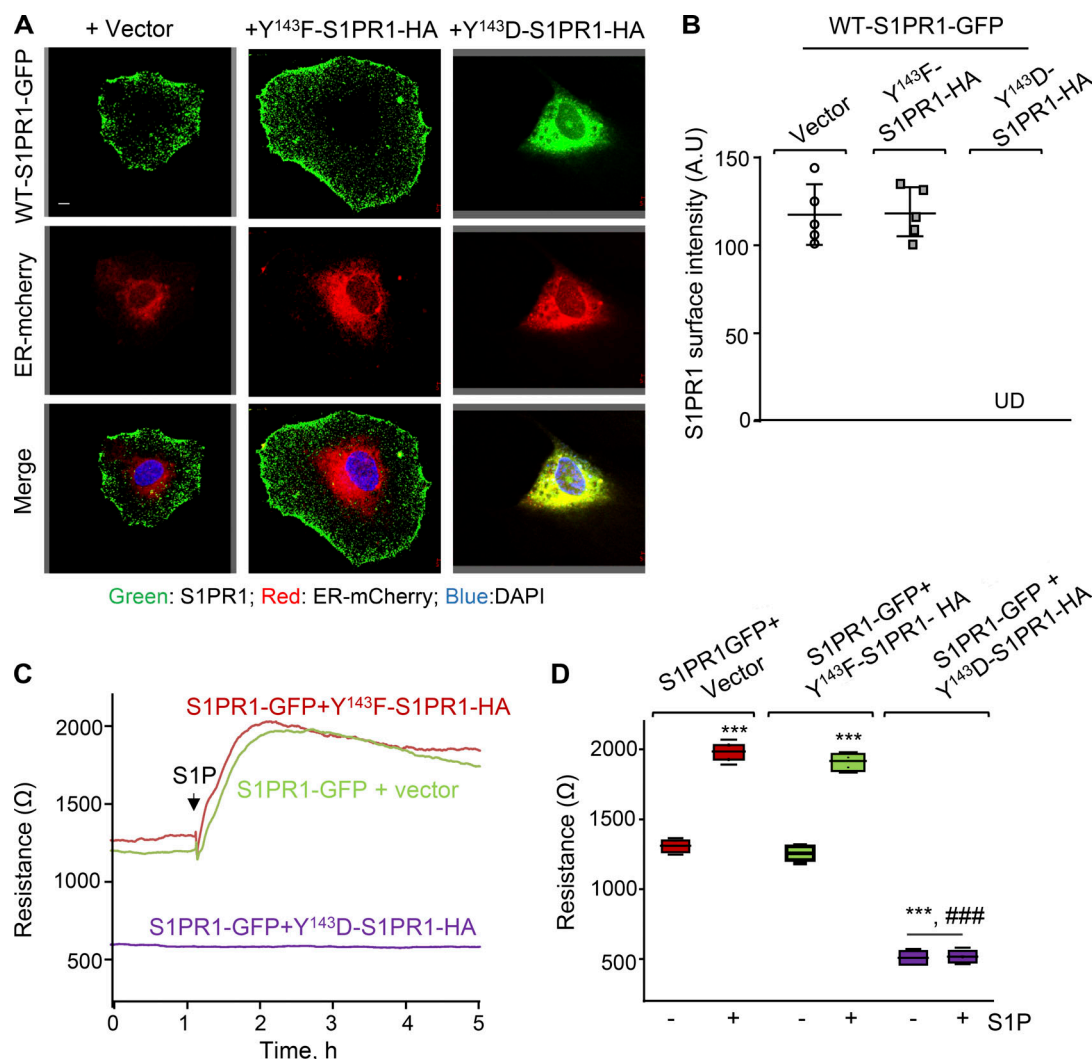


Figure 3. Phosphorylated S1PR1 internalizes endogenous S1PR1. (A) Confocal image at 63× magnification from HUVECs coexpressing GFP-WT-S1PR1 and ER-mCherry along with HA-tagged vector or HA-Y¹⁴³F- or HA-Y¹⁴³D-S1PR1 mutants. Scale bar, 5 μm. (B) Quantification of the S1PR1 surface intensity using images in A. *n* = 5–6 cells/group from experiments repeated two times. (C) TEER trace in ECs coexpressing WT-S1PR1-GFP and HA-tagged vector or S1PR1 mutants. (D) Mean ± SD of TEER using multiple traces in C. Basal TEER (–) = values between 10 and 30 min; S1P-stimulated TEER (+) = values between 2 and 2.5 h. (D) One-way ANOVA with paired two tailed *t* test. ***, *P* < 0.0001 compared with unstimulated cells; ###, *P* < 0.0001 compared with S1P-stimulated WT-S1PR1 + vector or HA-Y¹⁴³F-S1PR1. A.U., arbitrary units; UD, undetectable.

(Hutagalung and Novick, 2011). We therefore assessed whether Rab-GTPases regulate S1PR1 trafficking to the ER. ECs primarily express Rab5, 7, 9, and 11 (Chichger et al., 2016), and previous studies showed that Rab5 and Rab7 are involved in regulating protein sorting and recycling to early endosomes (de Renzis et al., 2002; Gorvel et al., 1991). Thus, we depleted Rab5 and Rab7 and found that their depletion had no effect on S1P-mediated trafficking of WT-S1PR1 to the ER (Fig. S2, A–D). Also, the Y¹⁴³D-S1PR1 mutant remained localized at the ER in control or Rab-depleted ECs (data not shown), ruling out the role of these Rab-GTPases in trafficking S1PR1 to the ER.

Y¹⁴³-phosphorylated S1PR1 interacts with BiP

We previously showed that S1P maximally phosphorylates S1PR1 at Y¹⁴³ within 5 min, leading to receptor internalization followed by receptor dephosphorylation and reappearance on the EC

surface between 15 and 20 min (Chavez et al., 2015). We next harvested lysates from ECs overexpressing WT-S1PR1 at 5 min (maximal phosphorylation) or 15 min (dephosphorylated) after S1P stimulation and performed mass spectrometry of the immunocomplex to identify possible binding partners for tyrosine-phosphorylated S1PR1 (Y¹⁴³-S1PR1). In parallel, we also used lysates from unstimulated Y¹⁴³D-S1PR1- or Y¹⁴³F-S1PR1-expressing ECs. We specifically focused on binding partners that may affect S1PR1 retention in the ER and identified BiP (also known as GPR78) as the key interacting partner for phosphorylated S1PR1 (Fig. S3, A–E). We confirmed this interaction using immunoprecipitation studies in which BiP coprecipitated with WT-S1PR1 at 5 min, whereas the interaction was not detected at 15 min (Fig. 5, A and B). BiP interacted with the Y¹⁴³D-S1PR1 mutant constitutively, and the interaction was not altered after addition of S1P (Fig. 5, A–D). However, BiP failed to interact with

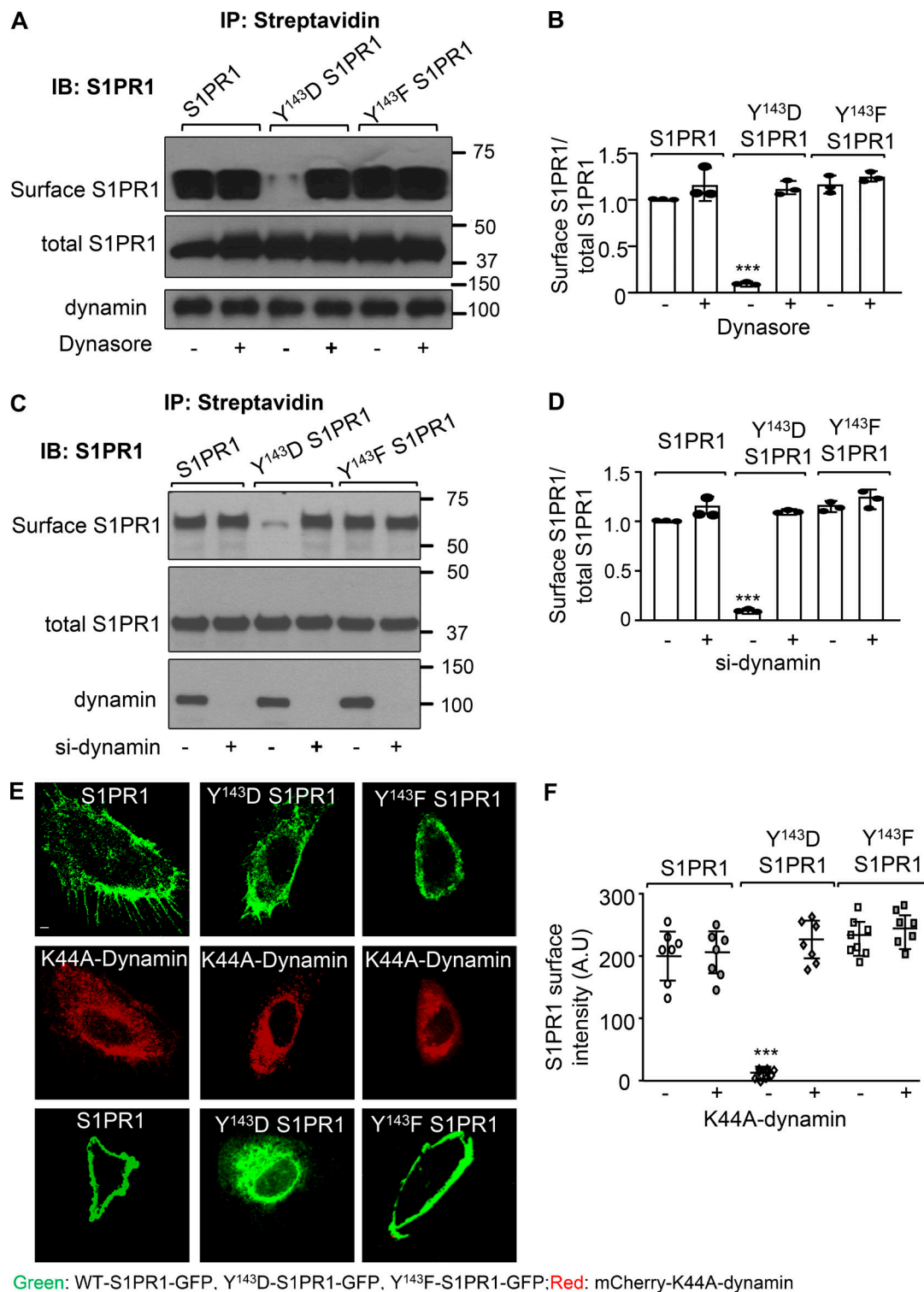


Figure 4. Dynamin internalizes tyrosine-phosphorylated S1PR1. (A) S1PR1 surface expression without (–) or with (+) dynasore (100 μ M) determined using anti-S1PR1 antibody. ECs were first transfected with indicated cDNAs. Dynasore was added within 30 min after transfection, and cells were lysed after 24 h. Total S1PR1 and dynamin expression were quantified using anti-S1PR1 or anti-dynamin antibodies. (B) Densitometry of S1PR1 surface expression over total S1PR1 using blots in A. $n = 3$ independent experiments. (C) S1PR1 surface expression in ECs transfected with control siRNA (–) or dynamin siRNA (+) determined as in A. (D) Densitometry quantified as in B. $n = 3$ independent experiments. (E) Confocal images showing S1PR1 cell surface localization following co-transfection with mCherry-tagged GTPase-defective dynamin (K44A-dynamin). Scale bar, 5 μ m. (F) Quantification of S1PR1 cell surface expression. $n = 5$ –8 cells/group from experiments repeated three times. All data in B, D, and F are shown as mean \pm SD. One-way ANOVA with paired two tailed t test. ***, $P < 0.0001$ compared with WT-S1PR1 or Y¹⁴³F-S1PR1. See also Fig. S2, A–D. A.U., arbitrary units; IB, immunoblot; IP, immunoprecipitation. (A and C) Molecular weight marker in kD.

the Y¹⁴³F-S1PR1 mutant, even after SIP stimulation (Fig. 5, C and D). Imaging studies similarly showed that BiP colocalized with WT-S1PR1 within 5 min of SIP stimulation but not at 15 min. In contrast to the Y¹⁴³F-S1PR1 mutant, the Y¹⁴³D-S1PR1 mutant consistently colocalized with BiP at the ER without or with SIP stimulation (Fig. 5, E and F). These findings suggest that Y¹⁴³ phosphorylation plays a key role in mediating BiP interaction with S1PR1.

ER-localized phosphorylated S1PR1 induces Ca²⁺ signaling in a Gi-dependent manner

SIP increases [Ca²⁺]_i in a Gi-dependent manner (Li et al., 2015; Mehta et al., 2005). Thus, we addressed the possibility that S1PR1 phosphorylation mediates an increase in [Ca²⁺]_i level in response to SIP in ECs transducing WT-S1PR1, Y¹⁴³D-S1PR1, or Y¹⁴³F-S1PR1. ECs were incubated with the Ca²⁺-sensitive dye Fura-2 AM for 15 min, after which the cells were rinsed and [Ca²⁺]_i determined in GFP-expressing ECs (Yazbeck et al., 2017). As expected, SIP increased [Ca²⁺]_i in ECs transducing WT-S1PR1 or the Y¹⁴³D-S1PR1 mutant but only modestly in ECs transducing Y¹⁴³F-S1PR1. However, SIP-induced increase in [Ca²⁺]_i was 3.5-fold higher in ECs transducing the Y¹⁴³D-S1PR1 mutant than ECs expressing WT-S1PR1 or the Y¹⁴³F-S1PR1 mutant (Fig. 6, A and B). These findings indicate that ER-retained Y¹⁴³D-S1PR1 mutant is functional, while the Y¹⁴³F-S1PR1 mutant largely prevents the increase in cytosolic Ca²⁺.

We next inhibited Gi using pertussis toxin (PTX; Gunther et al., 2000) to address its role in regulating the increase in [Ca²⁺]_i. Intriguingly, SIP failed to increase [Ca²⁺]_i in PTX-pretreated ECs transducing WT-, Y¹⁴³D-, or Y¹⁴³F-S1PR1 mutant (Fig. 6, C and D). We also transfected these mutants into human embryonic kidney (HEK) cells as they do not express the S1PR1 receptor (Balaji Ragunathrao et al., 2019) and similarly found that Y¹⁴³D-S1PR1 augmented [Ca²⁺]_i in a Gi-dependent manner (Fig. S4, A and B). In other studies, we performed coimmunoprecipitation experiments using lysates from ECs expressing WT or mutated receptor to address the possibility that Y¹⁴³D-S1PR1 forms a complex with Gi. We found that Y¹⁴³D-S1PR1 pulled down Gi to the same level as S1PR1 or Y¹⁴³F-S1PR1 (Fig. 6, E and F). Thus, these findings indicate that phosphorylated S1PR1 traffics to the ER and induces [Ca²⁺]_i mobilization in a Gi-dependent manner.

SIP may increase the [Ca²⁺]_i concentration by mobilizing the release of Ca²⁺ from ER stores and subsequently activating Ca²⁺ entry through nonselective cation channels (Mehta and Malik, 2006). Thus, we addressed the role of each of these components in enhancing [Ca²⁺]_i in ECs expressing WT or mutated S1PR1. We separated the two phases of the Ca²⁺ transient ER-Ca²⁺ release and store-operated Ca²⁺ entry (SOCE) mediated by plasmalemmal Ca²⁺ channels (Mehta et al., 2005; Yazbeck et al., 2017) by stimulating the cells under Ca²⁺-free bath conditions to deplete the ER-Ca²⁺ store followed by add back of Ca²⁺ to monitor SOCE. ECs transducing the Y¹⁴³D-S1PR1 mutant showed markedly increased SOCE in response to SIP compared with WT-S1PR1-expressing cells (Fig. 6, G and H). ECs expressing Y¹⁴³F-S1PR1 showed negligible SOCE.

We next depleted BiP to assess its causal role in trapping S1PR1 in the ER and inducing SOCE. We found that siRNA

depleted BiP to undetectable levels between 24 and 72 h (Fig. S5, A and B). We then cotransfected ECs with WT- or Y¹⁴³D-S1PR1 along with scrambled (siSC) or BiP siRNA, and after 36 h, S1PR1 cell surface localization was determined using TIRF. Whereas BiP depletion had no effect on WT-S1PR1 cell surface expression, depletion of BiP restored Y¹⁴³D-S1PR1 expression on the EC surface (Fig. 7, A and B). Intriguingly, depletion of BiP prevented SOCE in both WT-S1PR1- and Y¹⁴³D-S1PR1-transducing ECs (Fig. 7, C and D). Thus, BiP was responsible for retention of the Y¹⁴³-phosphorylated receptor in the ER and for augmented Ca²⁺ levels induced by SIP stimulation (Fig. 7, C and D).

To determine whether BiP and Gi were responsible for decreasing endothelial permeability in the Y¹⁴³D-S1PR1 mutant-expressing ECs shown above, we determined TEER in BiP-depleted ECs transducing WT-S1PR1 or Y¹⁴³D-S1PR1. We also inhibited Gi using PTX, as above. Inhibition of Gi failed to alter barrier disruption by the mutant (Fig. S4, C and D). However, depletion of BiP restored basal TEER in ECs transducing the Y¹⁴³D-S1PR1 mutant. Furthermore, addition of SIP enhanced TEER in ECs transducing the Y¹⁴³D-S1PR1 mutant after BiP depletion to a level like EC-expressing WT-S1PR1 (Fig. 7, E and F), thus suggesting that phosphorylated S1PR1, through its interaction with BiP, switches its barrier-enhancing function to a disruptive one.

BiP translocates to cytosol and binds S1PR1 through its ATPase domain

While BiP is known to be an ER-localized chaperone, it is detected in the nucleus, mitochondria, and cytosol (Ni et al., 2011). The BiP ATPase domain (aa 28–405), substrate-binding domain (SBD; aa 422–651), and KDEL motif (aa 651–654) regulate BiP chaperone function (Carrara et al., 2015). To test the possibility that SIP induces BiP recruitment to the cytosol to facilitate BiP interaction with endocytosed S1PR1, we assessed alterations in BiP localization and ATPase activity following SIP stimulation of ECs. We also mapped the domain in BiP that binds phosphorylated S1PR1.

Using cell fractionation and imaging, we assessed alteration in cellular localization of BiP following SIP stimulation. We found that SIP increased BiP translocation to the cytosolic fraction within 2.5–5 min, which was no longer apparent at 15 min (Fig. 8, A and B). Similarly, imaging studies showed that SIP induced recruitment of BiP from the ER to the cytosol within 2.5–5 min (Fig. 8, C and D). Interestingly, SIP also increased BiP-ATPase activity (Fig. 8 E) coinciding with S1PR1 interaction with BiP (Fig. 5, E and F).

To map the domain via which BiP binds S1PR1, we cotransduced HA-tagged vector, full-length BiP, BiP containing only the ATPase domain (BiP-ATPase), and BiP containing the SBD (BiP-SBD) along with the GFP-tagged Y¹⁴³D-S1PR1 mutant in ECs (Fig. 8 F). Coimmunoprecipitation studies showed that phosphorylated S1PR1 interacted with BiP through its ATPase domain (Fig. 8, G and H). In addition, ECs expressing Y¹⁴³D-S1PR1 showed an approximately eightfold increase in BiP-ATPase activity above EC-expressing WT-S1PR1 (Fig. 8 I). These data indicate that SIP initiates two events in parallel: SIP phosphorylates S1PR1 at Y¹⁴³ and promotes the translocation of BiP to the cytosol,

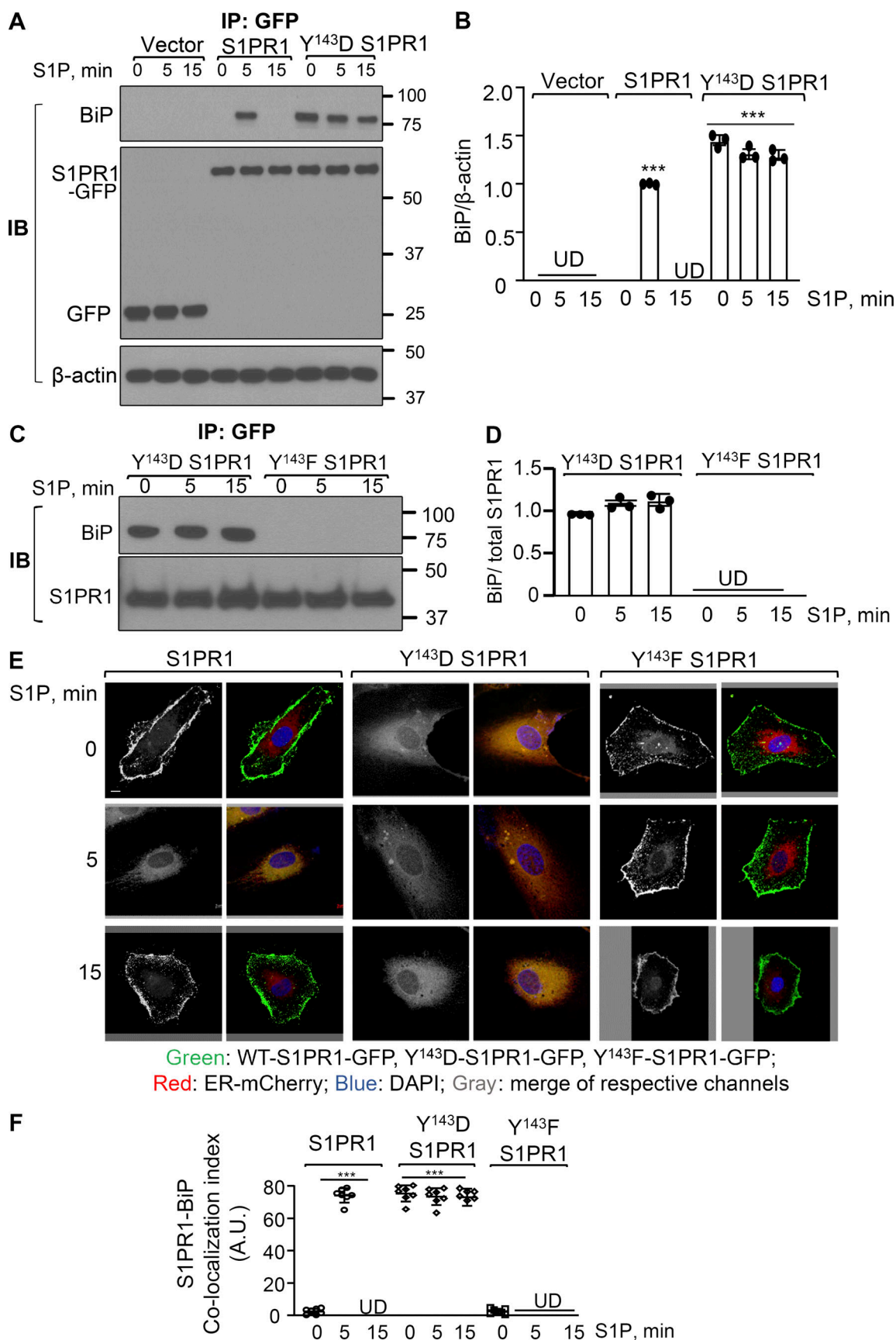


Figure 5. **Phosphorylated S1PR1 interacts with BiP.** (A) Pulldown of BiP from HUVEC transducing vector, GFP-S1PR1, or GFP-Y¹⁴³D-S1PR1 after S1P stimulation at indicated times. Anti-GFP antibody was used to pull down; immunocomplexes were probed with anti-BiP antibody to assess interaction. Anti-GFP

or anti- β -actin antibody was used to confirm total protein. **(B)** Mean \pm SD of BiP interaction with S1PR1 using blots in A and with β -actin as loading control. $n = 3$ independent experiments. ***, $P < 0.0001$ compared with unstimulated WT-S1PR1 by one-way ANOVA with two-tailed paired t test. **(C)** Pulldown of BiP from ECs expressing GFP-Y¹⁴³F- or GFP-Y¹⁴³D-S1PR1 mutants processed as in A. **(D)** Mean \pm SD of the BiP interaction with S1PR1 normalized using total S1PR1. $n = 3$ independent experiments. **(E)** Confocal images illustrating colocalization of S1PR1 with BiP without or with S1P stimulation. Cells were also stained for DAPI to assess nuclei. Gray background indicates the area outside the image. Scale bar, 5 μ m. **(F)** Quantitation of the colocalization index plotted as mean \pm SD. ***, $P < 0.0001$ compared with unstimulated WT-S1PR1 or Y¹⁴³F-S1PR1 by one-way ANOVA with two-tailed paired t test. See also Fig. S3, A–E. A.U., arbitrary units; IB, immunoblot; IP, immunoprecipitation; UD, undetectable. **(A and C)** Molecular weight marker in kD.

leading to increase in BiP-ATPase activity, thereby augmenting its chaperone function and enabling BiP to retain Y¹⁴³D-S1PR1 in the ER.

TNF- α disrupts barrier function by inducing S1PR1 Y¹⁴³ phosphorylation and ER localization

The proinflammatory cytokine TNF- α released during tissue injury disrupts endothelial barrier function by inducing protein phosphorylation and augmenting SOCE (Vandenbroucke et al., 2008). We therefore surmised that increased S1PR1 phosphorylation at Y¹⁴³ may be a common mechanism by which inflammatory mediators, such as TNF- α , disrupt barrier function. Hence, we stimulated ECs with TNF- α for the indicated times and determined S1PR1 phosphorylation on Y¹⁴³. We found that TNF- α induced a fivefold increase in S1PR1 tyrosine phosphorylation within 15 min. Receptor phosphorylation persisted at this level for up to 60 min (Fig. 9, A and B). However, TNF- α did not induce phosphorylation in ECs transducing Y¹⁴³F-S1PR1 (Fig. S5, C and D). In these experiments, we also determined whether the TNF- α -induced increase in S1PR1 phosphorylation enhanced S1PR1's interaction with BiP. We found that TNF- α induced BiP interaction with S1PR1 in a manner dependent on phosphorylation of the Y¹⁴³ residue (Fig. 9, A and B). TNF- α also rapidly induced receptor internalization (Fig. 9, C and D) and disrupted basal barrier function in ECs, which was not rescued by addition of S1P (Fig. 9, E and F). Intriguingly, TNF- α failed to disrupt barrier function in BiP-depleted ECs (Fig. 9, G and H). These results demonstrate that phosphorylation of S1PR1 at Y¹⁴³ and interaction with BiP play a key role in disrupting barrier function under inflammatory conditions.

Discussion

Here, we identify the unique role of S1PR1 Y¹⁴³ phosphorylation in promoting S1PR1 interaction with BiP. BiP in turn translocates Y¹⁴³-S1PR1 to the ER for mediating SOCE and barrier disruption. We show that in response to S1P, the transient nature of S1PR1 phosphorylation at Y¹⁴³ results in rapid internalization of the receptor to ER by BiP followed by receptor reappearance at the cell surface in the next 20 min. However, the non-GPCR agonist TNF- α , by inducing long-lasting phosphorylation of S1PR1 at Y¹⁴³, sustained S1PR1 interaction with BiP at the ER, causing it to disrupt barrier function. We recapitulated these findings using Y¹⁴³D-S1PR1 and Y¹⁴³F-S1PR1 mutants. We show that unlike WT-S1PR1 or Y¹⁴³F-S1PR1, BiP trapped Y¹⁴³D-S1PR1 in the ER, which led to amplified SOCE and barrier disruption.

The large GTPase dynamin plays a critical role in the internalization and trafficking of GPCRs (Ferguson and De Camilli, 2012). Additionally, dynamin GTPase activity is required for

maintaining the activity of internalized S1PR1 required for egress of T lymphocytes from lymphoid and thymus tissues (Benechet et al., 2016; Matloubian et al., 2004). Thus, we focused on dynamin in regulating the fate of phosphoreceptors within ECs. We showed that depletion of dynamin or transduction of GTPase-defective (K44A) dynamin mutant restored Y¹⁴³D-S1PR1 mutant expression on the cell surface, indicating that the Y¹⁴³D-S1PR1 mutant was not degraded but constitutively internalized in a dynamin-dependent manner.

That S1P induces internalization of S1PR1 was known (Anwar and Mehta, 2020). However, the questions of how Y¹⁴³ phosphorylated S1PR1 was shielded from degradation and whether phosphorylation of S1PR1 at Y¹⁴³ was a general mechanism to compromise the EC barrier during inflammation were unknown. Our results defined a novel role of tyrosine phosphorylation of S1PR1 in favoring its localization to a specific organelle in a spatially and temporally defined manner. We showed that WT-S1PR1, upon binding S1P, transiently localized to the ER where it interacted with BiP to induce physiologically relevant Ca²⁺ signaling. The transient nature of this Ca²⁺ signaling might be explained by the activity of PTP1B, a tyrosine phosphatase that resides in the ER (Haj et al., 2002). A plausible scenario consistent with our experimental observations is that PTP1B dephosphorylates Y¹⁴³-phosphorylated S1PR1, thereby disrupting its interaction with BiP, leading to reexpression of WT-S1PR1 on the cell surface. In support of this conclusion, we showed that Y¹⁴³D-S1PR1 mutant remained in the ER and disrupted barrier function while the Y¹⁴³F-S1PR1 mutant remained on the cell surface. We also showed that TNF- α , a potent inflammatory cytokine released during diverse vascular inflammatory conditions and known to induce cSrc activity and inhibit tyrosine phosphatases (DeLalio et al., 2019), promoted the phosphorylation of S1PR1 on Y¹⁴³. Unlike S1P, phosphorylation induced by TNF- α was sustained, leading to retention of the phosphorylated receptor in the ER followed by barrier disruption. Hence, in the case of TNF- α , it is possible that altered phosphatase activity, together with increased cSrc activity, led to long-lasting internalization of the receptor and barrier disruption.

ER store depletion is needed for activation of SOCE, which then induces EC contraction and disrupts EC barrier function by mechanisms involving suppression of Rac1 activity by RhoA (Komarova et al., 2017). Previously, we showed that while Gi inhibited both ER Ca²⁺ release and SOCE following S1P ligation of S1PR1, barrier enhancement by the receptor only required ER Ca²⁺ release (Mehta et al., 2005). Thus, inhibition of Gi is predicted to prevent ER Ca²⁺ release as well as SOCE and endothelial barrier enhancement induced by S1PR1 expressed on the cell surface. In line with this, we show that S1P markedly augmented Ca²⁺ entry (SOCE) in ECs as well as in HEK cells transducing

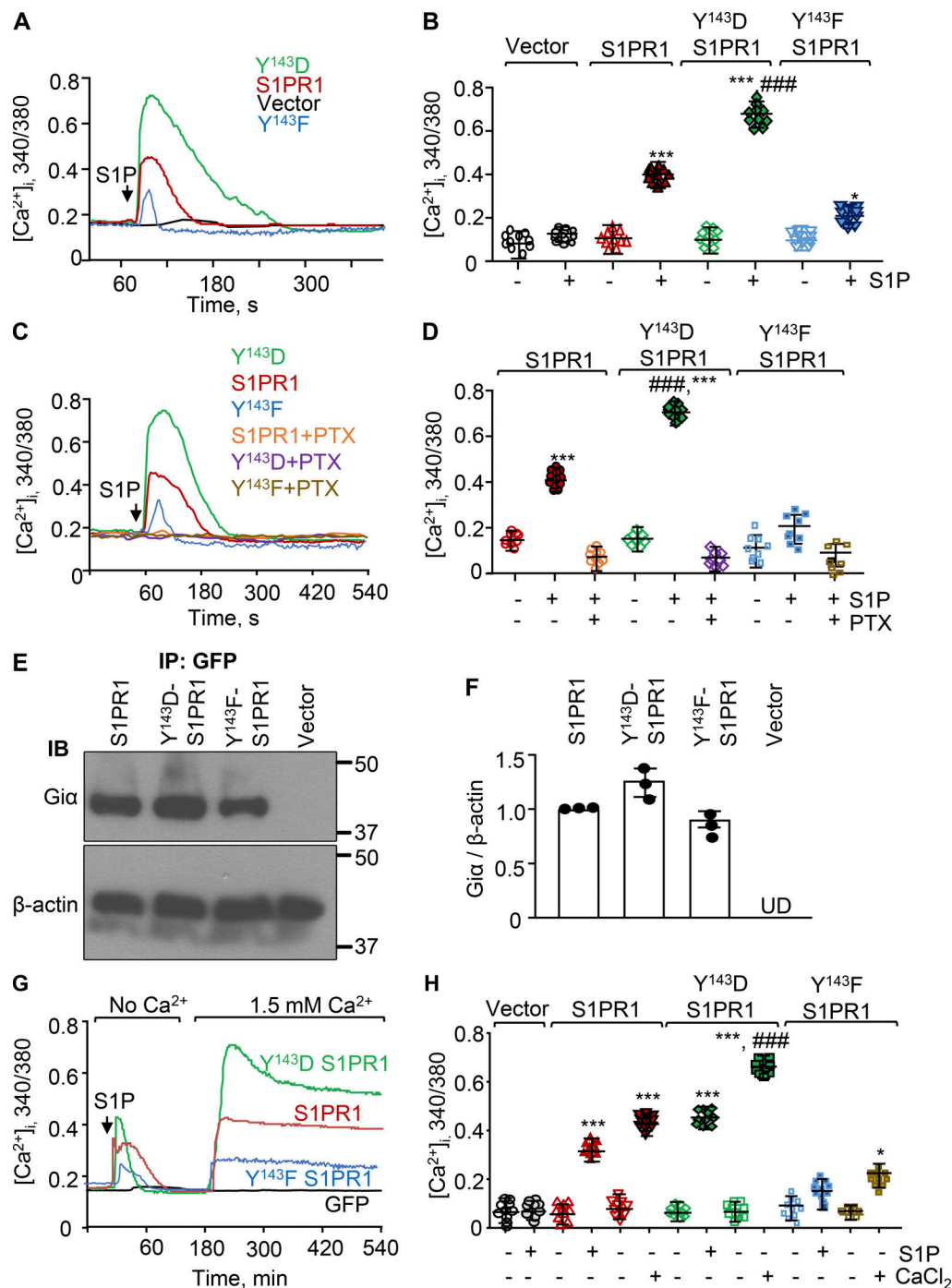


Figure 6. $Y^{143}D$ -S1PR1 augments $[Ca^{2+}]_i$ in a Gi-dependent manner. (A) Traces showing increase in $[Ca^{2+}]_i$ in response to 1 μM S1P from HUVEC transducing vector, GFP-WT-S1PR1, GFP- $Y^{143}F$ -, or GFP- $Y^{143}D$ -S1PR1 mutants. (B) Mean \pm SD of $[Ca^{2+}]_i$ from 6–10 cells in a field from experiments repeated multiple times. Basal Ca^{2+} (–) analyzed at 45 s; peak increase in Ca^{2+} analyzed at 90 s. ***, $P < 0.0001$ compared with unstimulated vector or WT-S1PR1; ###, $P < 0.0001$ compared with S1P-stimulated WT-S1PR1 by one-way ANOVA with two-tailed paired t test. (C) $[Ca^{2+}]_i$ (+) in response to 1 μM S1P from ECs expressing cDNA as in A after pretreatment with PTX (50 μM) for 2 h. (D) Mean \pm SD of $[Ca^{2+}]_i$ from 6–10 cells in a field from experiments repeated multiple times. Basal Ca^{2+} (–) analyzed at 45 s; peak increase in Ca^{2+} analyzed at 90 s. ***, $P < 0.0001$ compared with unstimulated vector or WT-S1PR1; ###, $P < 0.0001$ compared with S1P-stimulated WT-S1PR1 by one-way ANOVA with two-tailed paired t test. (E) Pulldown of Gi with S1PR1 from ECs expressing vector or S1PR1 cDNA as in A. Anti-GFP antibody was used for pulldown. Immunocomplexes were probed using anti-Gi α antibody. β -Actin was used as a loading control. (F) Densitometry of Gi α normalized against β -actin. Experiments were repeated three times independently. (G) ER Ca^{2+} release versus SOCE from ECs transducing cDNAs as in A. ECs bathed in Ca^{2+} -free medium were stimulated with 1 μM S1P to determine Ca^{2+} release (first peak). After Ca^{2+} declined to basal level, 1.5 mM Ca^{2+} was readded to induce SOCE. (H) Mean \pm SD of $[Ca^{2+}]_i$ ($n = 7$ –10 cells/group) from experiments repeated three times. Basal Ca^{2+} (–) was analyzed at 20 s; peak increase in Ca^{2+} from the ER was analyzed at 45 s, while peak of SOCE was analyzed 210 s. *, $P < 0.05$; ***, $P < 0.0001$ compared with unstimulated vector or WT-S1PR1; ###, $P < 0.0001$ compared with WT-S1PR1 after S1P stimulation by one-way ANOVA with two-tailed paired t test. See also Fig. S4, A and B. IB, immunoblot; IP, immunoprecipitation; UD, undetectable. (E) Molecular weight marker in kD.

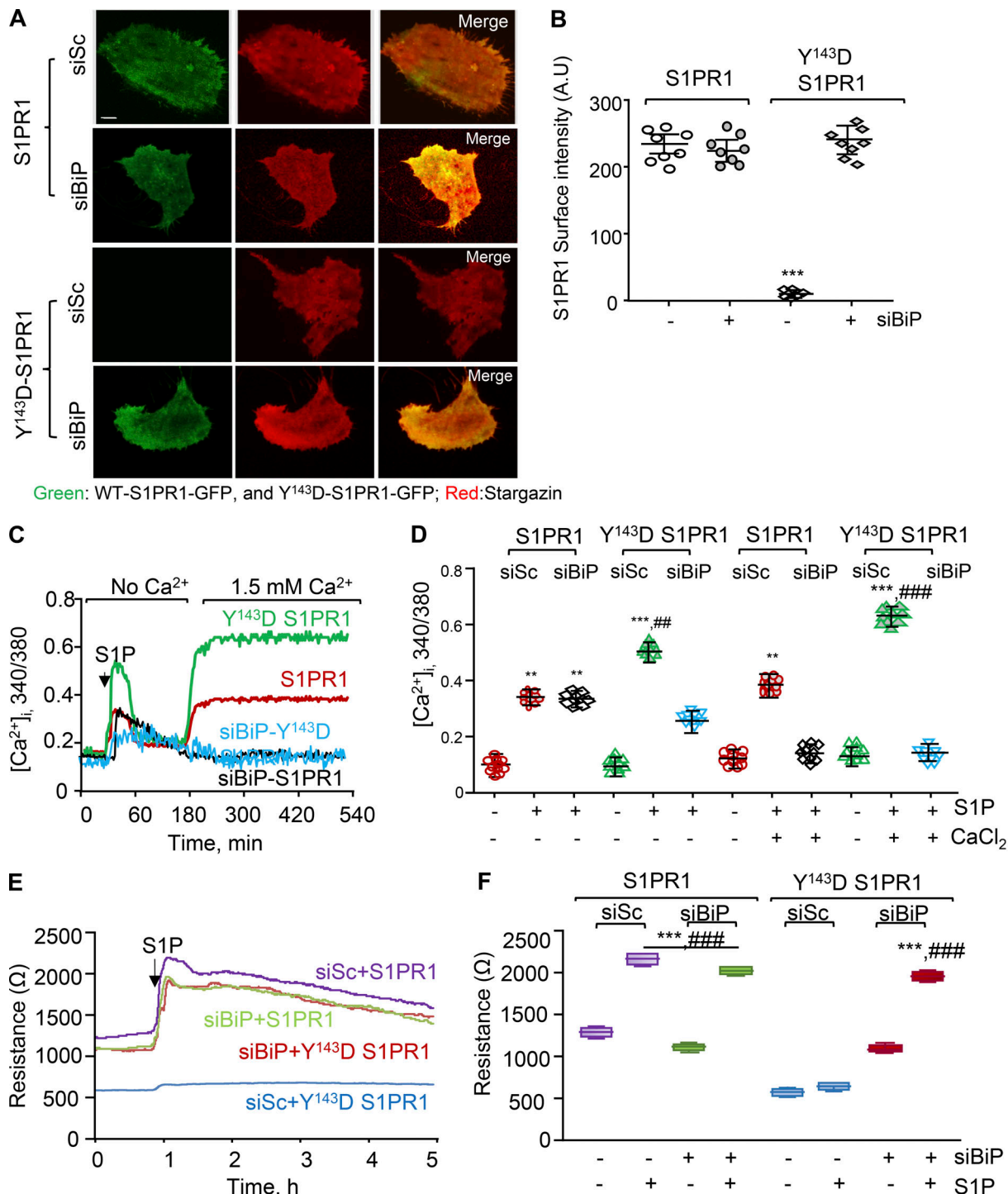


Figure 7. Depletion of BiP restores Y¹⁴³D-S1PR1 cell surface localization and endothelial barrier function. (A) Live cell TIRF images taken at 100× magnification from control siRNA (siSc) or BiP-depleted (siBiP) ECs transducing WT- or Y¹⁴³D-S1PR1. ECs were first transfected with siRNA and at 24 h and retransfected with indicated S1PR1 cDNA, and images were acquired after 48 h. Scale bar, 10 μm. **(B)** Quantification of S1PR1 surface expression ($n = 7$ –10 cells/group) from experiments performed three times independently. ***, $P < 0.0001$ compared with WT-S1PR1 or siBiP plus Y¹⁴³D-S1PR1 by one-way ANOVA with two-tailed paired t test. **(C)** ECs transducing BiP siRNA and S1PR1 mutants as in A were stimulated with 1 μM S1P. Ca²⁺ release (first peak) or SOCE (second peak) was determined as in Fig. 6 G. **(D)** Mean ± SD of increase in Ca²⁺ ($n = 8$ –10 cells/group) from experiments repeated three times. Basal Ca²⁺ (–) analyzed at 20 s; peak increase in Ca²⁺ from ER analyzed at 45 s while peak of SOCE was analyzed at 210 s. **, $P < 0.001$; ***, $P < 0.0001$ compared with unstimulated WT-S1PR1 or Y¹⁴³D-S1PR1; ##, $P < 0.001$; ###, $P < 0.0001$ compared with S1P-stimulated WT-S1PR1 by one-way ANOVA with two-tailed paired t test. **(E)** TEER in response to 1 μM S1P from ECs transducing BiP siRNA and S1PR1 mutants as in A. **(F)** Mean ± SD of TEER from experiments repeated three times. Basal TEER was calculated as mean between 10 and 30 min, while S1P-stimulated TEER was the mean between 1 and 1.5 h. ***, $P < 0.0001$ compared with unstimulated WT-S1PR1 or Y¹⁴³D-S1PR1; ###, $P < 0.0001$ compared with unstimulated siBiP plus WT-S1PR1 by one-way ANOVA with two-tailed paired t test. See also Figs. S4, C and D; and S5, A and B. A.U., arbitrary units.

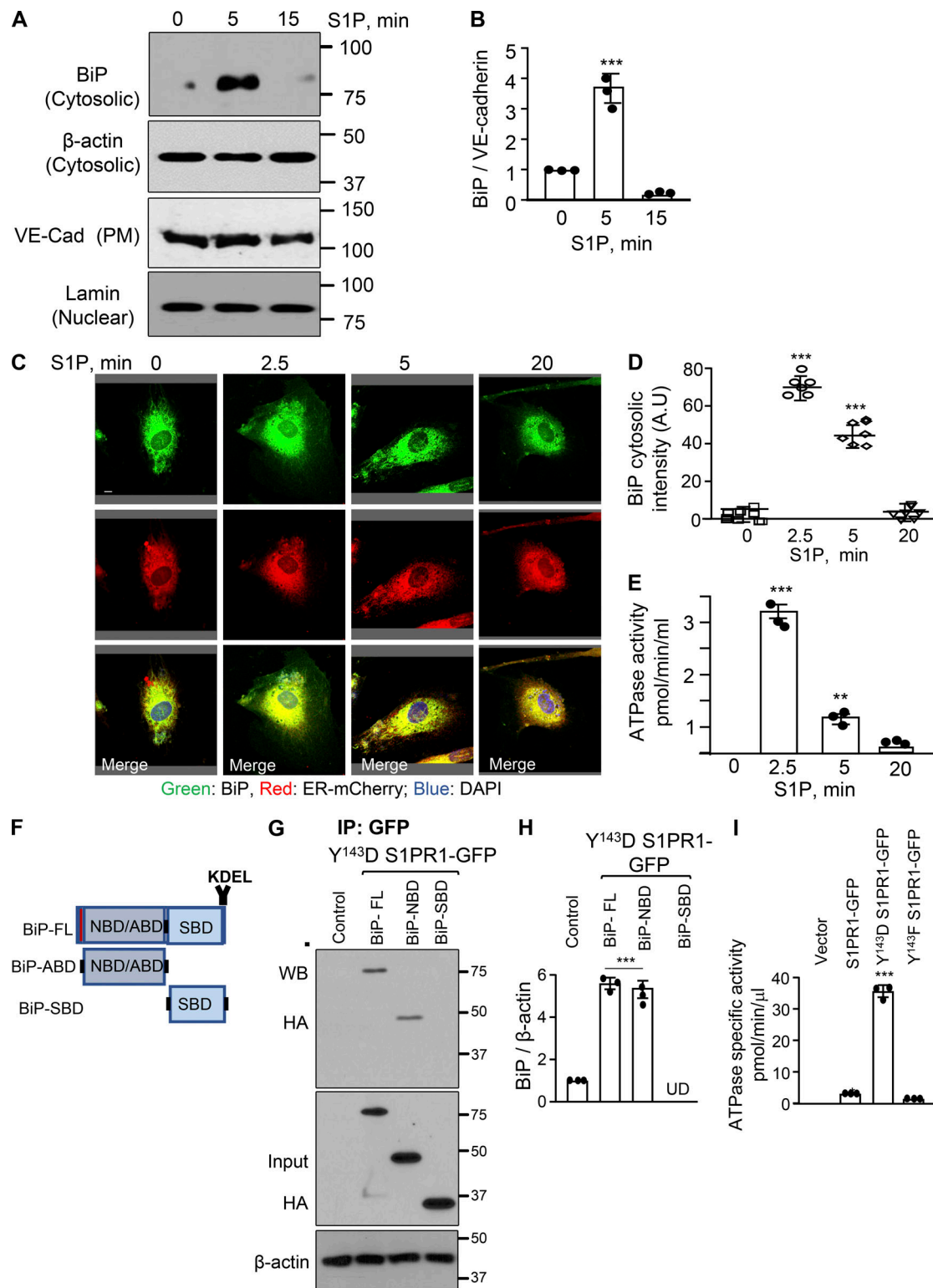


Figure 8. S1P induces BiP recruitment to the cytosol and activates its ATPase activity, which in turn binds tyrosine-phosphorylated receptor. (A) Cell fractionation from unstimulated or S1P (1 μ M)-stimulated HUVECs after indicated times. (B) Mean \pm SD of BiP density in cytosolic fraction, using VE-cadherin as the loading control. Fold increase in BiP/VE-cadherin density was calculated against values at time 0 (no S1P addition). ***, $P < 0.0001$ compared with unstimulated ECs by one-way ANOVA with two-tailed paired t test. (C) Confocal images showing BiP translocation from ECs transducing ER-mCherry without or with 1 μ M S1P stimulation. Cells were stained with anti-BiP antibody and DAPI (to assess nucleus). Images taken at 63 \times magnification. Scale bar, 5 μ m. Gray background indicates the area outside the image. (D) Mean \pm SD of the BiP expression in the cytosol (4–8 cells/group) from experiments repeated multiple times. ***, $P < 0.0001$ compared with unstimulated ECs by one-way ANOVA with two-tailed paired t test. (E) Unstimulated or S1P (1 μ M)-stimulated ECs were immunoprecipitated with anti-BiP antibody, and immunocomplexes were used to measure ATPase activity. **, $P < 0.001$; ***, $P < 0.0001$ compared with unstimulated ECs.

stimulated ECs at 20 min by one-way ANOVA with two-tailed paired *t* test. **(F)** Physical map of full-length BiP and deletion mutants. **(G)** Pulldown of S1PR1 from HUVECs cotransducing either HA-tagged vector (control), HA-full-length BiP, HA-BiP-ATPase domain, or HA-BiP-SBD domain along with Y¹⁴³D-S1PR1-GFP. Anti-GFP was used for pulldown. Immunocomplexes were probed with anti-HA antibody to assess BiP expression. **(H)** Mean \pm SD BiP interaction with S1PR1. Experiments were repeated at least three times independently. ***, *P* < 0.0001 compared with vector (control) by one-way ANOVA with two-tailed paired *t* test. **(I)** Mean \pm SD of BiP ATPase activity in ECs transducing indicated cDNAs was determined as described in E. *, *P* < 0.05; ***, *P* < 0.0001 compared with vector by one-way ANOVA with two-tailed paired *t* test. ABD, ATP-binding domain (ATPase domain); A.U, arbitrary units; Cad, cadherin; FL, full-length; IP, immunoprecipitation; NBD, nucleotide-binding domain; PM, plasma membrane; UD, undetectable; WB, Western blot. **(A and G)** Molecular weight marker in kD.

Y¹⁴³D-S1PR1 compared with WT-S1PR1. Intriguingly, we show that inhibition of Gi also blocked the ER Ca²⁺ release and that SOCE was induced not only by WT-S1PR1 but also by ER-resident Y¹⁴³D-S1PR1, indicating that the mutant signaled in a Gi-dependent manner. Consistent with these functional findings, mutant S1PR1 pulled down Gi as well as the nascent S1PR1. Furthermore, Gi inhibition had no effect on disruption of endothelial barrier function induced by the mutant, indicating the involvement of an intermittent mediator compromising EC barrier function in an SOCE-dependent manner. Previous findings that internalized S1PR1 can function in T cells lends credence to our findings and interpretations (Benechet et al., 2016). However, in T cells, internalized S1PR1 regulated signaling independently of Gi (Baeyens et al., 2015). These findings led us to propose that internalized Y¹⁴³D-S1PR1 mutant interacts with intracellular Gi, consistent with the idea that G proteins localized both at the cell surface and in intracellular compartments can lead to increased Ca²⁺ signaling (Magalhaes et al., 2012).

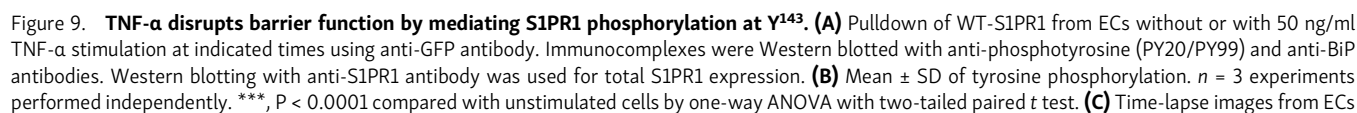
The present work identified the key role of BiP as a mechanism by which Y¹⁴³D-S1PR1 mutant gained access to the ER where it led to augmented SOCE and EC barrier disruption. BiP is known to regulate ER stress, operationally defined as impaired calcium homeostasis (Gardner et al., 2013). A few studies also showed that BiP contributes to the regulation of EC barrier function (Leonard et al., 2019). We show that depletion of Rab-GTPases, which sort endocytosed receptors to subcellular compartments (de Renzis et al., 2002; Naslavsky and Caplan, 2018), had no effect on S1PR1 trafficking to the ER. Interestingly, mass spectrometry and pulldown experiments identified BiP, which primarily resides in the ER due to its KDEL motif (Ni et al., 2011), as a novel partner of phosphorylated S1PR1. We also showed that depletion of BiP reversed SOCE and the barrier disruption caused by the Y¹⁴³D-S1PR1 mutant. Additionally, TNF- α compromised EC barrier function by mediating interaction of Y¹⁴³-S1PR1 to BiP. Thus, knockdown of BiP prevented EC barrier dysfunction by TNF- α . We infer from these findings that long-lasting phosphorylation of the Y¹⁴³ residue and its coupling with BiP at the ER disrupts the barrier by augmenting SOCE. Our findings that ER-resident S1PR1 is functional has precedence. Studies showed that melanocortin 4 receptor, a GPCR expressed in neurons of the hypothalamus central to the control of appetite, localizes to the ER where it avoids desensitization and thereby potently signals in response to α -melanocyte-stimulating hormone stimulation (Granell et al., 2013). Other open questions to be addressed in future studies are the mechanisms by which BiP and ER-resident Y¹⁴³D-S1PR1 induce SOCE to impair barrier function.

BiP can localize to the cytosol as well as to membrane fractions, depending on cellular context (Sun et al., 2006). In

addition to its ER-retaining KDEL motif, BiP contains an N-terminal nucleotide/ATPase domain and SBD (Adams et al., 2019). Studies also show that an increase in BiP ATPase activity is required for its interaction with cellular proteins (Kopp et al., 2019). Findings from the current studies showed that S1P induced BiP translocation to the cytosol. We also showed that S1PR1 interacted with BiP through the ATPase domain in association with an increase in BiP ATPase activity. While the mechanism by which S1P induces BiP translocation to the cytosol and increases its ATPase activity remains unclear, we conclude that BiP binds endocytosed S1PR1 or the Y¹⁴³D-S1PR1 mutant in the cytosol to promote their transport to the ER.

Another unanswered question our study raised is how ER-resident Y¹⁴³D-S1PR1 responds to S1P. While we show that ER-resident S1PR1 responds to S1P in both ECs and HEK cells overexpressing Y¹⁴³D-S1PR1, the topography of ER-localized S1PR1 is unclear. S1P is a polar lipid and cannot easily permeate the inside cell (Hannun and Obeid, 2008; Saba and Hla, 2004). Extracellular S1P is hydrolyzed by lipid phosphate phosphatases to sphingosine, which is taken up by the cell and converted to S1P by SPHK1 or SPHK2. Extracellular S1P also stimulates sphingosine kinase (SPHK) activity, leading to generation of intracellular S1P (Zhao et al., 2007). Both SPHK1 and SPHK2 are shown to be localized in intracellular organelles, including the ER (Maceyka et al., 2012). SPNS2, the lipid transporter, exports SPHK-derived S1P from inside of cells to outside for S1P to ligate S1PR1 or other S1PRs on the outer leaflet of the plasma membrane (Spiegel et al., 2019). Thus, based on the crystal structure of S1PR1, which revealed that S1P binds to its lateral surface (Hanson et al., 2012), we postulate that extracellular S1P or TNF- α stimulates intracellular S1P generation via SPHK1/SPHK2, which can stimulate ER-localized S1PR1, leading to induction of SOCE and barrier dysfunction. This is likely to be the case because depletion of BiP restored the Y¹⁴³D-S1PR1 mutant on the EC surface, inhibited SOCE, and rescued EC barrier function.

The conserved E/DRY motif in GPCRs of the class A family is known to be critically important to the receptor's physiological functions and G protein-binding properties. Mutation of glutamic acid (E) or arginine (R) within this triad induces receptor internalization and dramatically affects its G protein coupling and downstream signaling (Rovati et al., 2007). However, until now, the Y residue within this triad was thought to have a minimal impact on GPCR signaling. Here, we established that phosphorylation of Y¹⁴³ within this triad plays a key role in regulating S1PR1 function. Another unique finding made in this study is that Y¹⁴³D-S1PR1 internalized WT-S1PR1 and disrupted barrier function. Therefore, we conclude from these findings that Y¹⁴³D-S1PR1 functions as a dominant negative subverting



cotransducing indicated cDNAs following stimulation with TNF- α . Image taken at 63 \times magnification. Scale bar, 5 μ m. Gray background indicates the area outside the image. **(D)** Mean \pm SD of the surface expression of S1PR1 (5–7 cells/group) from experiments performed multiple times. ***, $P < 0.0001$ compared with unstimulated cells by one-way ANOVA with two-tailed paired t test. **(E)** TEER was recorded in control ECs after stimulation with vehicle or 50 ng/ml TNF- α . After 2 h, 1 μ M S1P was added to assess the effect of TNF- α -mediated internalization of S1PR1 (observed in C and D) on barrier function. **(F)** Mean \pm SD of the TEER from experiments performed three times. Basal TEER was calculated as mean between 10 and 20 min and between 3 and 4 h after S1P addition. ***, $P < 0.0001$ compared with unstimulated cells by one-way ANOVA with two-tailed paired t test. **(G)** TEER was recorded in BiP-depleted ECs after addition of a single dose of 50 ng/ml TNF- α . **(H)** Mean \pm SD of the TEER from experiments performed three times. Basal TEER (–) in control and BiP-depleted ECs was analyzed as mean between 10 and 20 min. TNF- α (+)-induced loss of EC barrier function in control or BiP-depleted ECs was calculated as mean between 3.5 and 4 h. ***, $P < 0.0001$ compared with unstimulated siSc by one-way ANOVA with two-tailed paired t test. **(I)** Model shows the effect of tyrosine phosphorylation (Y^{143}) of S1PR1 within ERY motif in regulating receptor function. S1P or TNF- α binds S1PR1 (i) and phosphorylates S1PR1 at Y^{143} (ii; represented as red circle). Dynamin pinches off the phosphorylated S1PR1 (iii). S1P also induces BiP ATPase activity and its translocation to cytosol (not shown). Phosphorylated S1PR1 (Y^{143} D-S1PR1) interacts with BiP in the cytosol (iv). BiP imports the receptor to the ER (v). WT-S1PR1 induces Ca^{2+} signaling and returns to the cell surface after dephosphorylation (not shown). BiP traps the Y^{143} D-S1PR1 mutant at the ER where it augments SOCE and induces barrier-disruptive signaling in a Gi-dependent manner. See also Fig. S5, C and D. A.U., arbitrary units; IB, immunoblot; IP, immunoprecipitation; p-Tyr, phosphorylated tyrosine; UD, undetectable. **(A)** Molecular weight marker in kD.

the canonical barrier maintaining function of S1PR1 in ECs. Indeed, findings from the current study show that during inflammation, sustained S1PR1 phosphorylation at Y^{143} through its interaction with BiP induces counterproductive S1PR1 signaling to disrupt barrier function. Whether Y^{143} D mutation in S1PR1 is the cause of vascular leak in susceptible lung injury patients remains to be investigated. We have thus identified phosphorylation of the S1PR1-ERY motif as a new and targetable mechanism of endothelial barrier breakdown common to lung and other organs.

In conclusion, our studies address the fundamental mechanisms regulating the fate of tyrosine phosphorylated S1PR1, as shown in Fig. 9 I, because it is evident that Y^{143} D-S1PR1, in contrast to the native receptor, plays a critical role in triggering barrier-disruptive rather than barrier-enhancing signaling, and that has great potential as a therapeutic target. The widespread expression of BiP, along with the conserved E/DRY motif in class A GPCRs, suggests that the regulatory functions of the Y residue within the triad extend well beyond the physiological effects of S1PR1 exemplified in the current study.

Materials and methods

Materials

S1PR1 antibody was purchased either from Alomone Labs (cat. #ASR-011) or Santa Cruz Biotechnology (sc-48356). Dynamin I/II (cat. #2342), Rab5 (cat. #3547), Rab7 (cat. #9367), Lamin A (cat. #86846), HA-tag (cat. #3724), and BiP[C50B12] (cat. #3177) antibodies were procured from Cell Signaling Technologies. α -1 and VE-cadherin antibodies were purchased from Santa Cruz Biotechnology (sc-13533), while β -actin was from Invitrogen (cat. #MA5-15739). Phosphotyrosine (PY99/PY20, sc-7020 and sc-508) and GFP (sc-9996) antibodies and Protein A/G PLUS-Agarose (sc-2003) were from Santa Cruz Biotechnology. Pierce Streptavidin Agarose beads (cat. #20349), Pierce Biotin (cat. #29129), Prolong Gold Antifade (cat. #P10144), SuperSignal West Pico Chemiluminescent Substrate (cat. #34580), and Halt Protease and Phosphatase Inhibitor Cocktail (100 \times ; cat. #78446) were from Thermo Fisher Scientific. S1P, D-erythro (cat. #BML-SL140-0001) was from Enzo Life Sciences. Dynasore hydrate (cat. #D7693-5MG) and PTX (cat. #P7208) were from MilliporeSigma. VECTASHIELD antifade mounting medium with

DAPI (cat. #H-1200) was from Vector Laboratories. Recombinant human TNF- α (cat. #300-01A) was from PeproTech. Fura-2 AM, a Ca^{2+} -selective fluorescence indicator (ab120873) was from Abcam.

Plasmids

All phosphorylation-specific GFP-tagged S1PR1 constructs were created as previously described (Chavez et al., 2015). ER-mCherry was provided by Y. Komarova. WT-dynamin2-mCherry and dominant-negative (K44A) dynamin-mCherry have been previously published (Shajahan et al., 2004). BiP-mCherry-KDEL (cat. #62233) was from Addgene. S1PR1-Dendra2 construct was generated by a PCR-based strategy and subcloning S1PR1-GFP and Dendra2 (Daneshjou et al., 2015). The primer sequences for subcloning S1PR1-GFP and Dendra2 are as follows: forward primer, 5'-TCGAATGGCAGCAAGGAGGCGGACCGGTCGCCACCATGAACACCCCGGAATTAACCTGA-3', reverse primer, 5'-ATGGCTGATTATGATCTAGAGTCGCGGCGCTTTACCACACCTGGCTGGGCAGGGGGCTG-3'. All cDNA sequences were confirmed against their GenBank accession numbers. These cDNAs were transfected in HUVECs or HEK293 cells using Amaxa Nucleofector (Lonza) or FuGENE HD (Roche).

Cell culture

HUVECs obtained from Lonza (cat. #C2519) were cultured by the same procedure as previously described (Yazbeck et al., 2017). Briefly, cells were plated in a T-75 flask (BD Falcon) coated with 0.1% gelatin and cultured in EBM-2 media (Lonza) supplemented with growth factors and 10% FBS (Thermo Fisher Scientific). Cells were cultured in a 37°C humidified incubator in the presence of 5% CO₂ and 95% O₂ until they formed a monolayer and achieved the desired confluence. HUVECs between passages 5 and 6 were used for these studies. The HEK293 cell line (American Type Culture Collection) was cultured in DMEM (Gibco) supplemented with 10% FBS (Thermo Fisher Scientific) and 5% penicillin/streptomycin (Thermo Fisher Scientific). HEK293 cells were transfected with indicated cDNAs using FuGENE HD (Roche).

Transfections

All siRNA sequences as well as ON-TARGETplus Non-targeting Control Pool (D-001810-10) sequences were purchased from

Dharmacon. Dynamin-2 or BiP (GRP78) or Rab5 and 7 were depleted using the following siRNA sequences: dynamin-2 antisense sequence, 5'-GACAUGAUCCUGCAGUUAU-3'; BiP siRNA, 5'-...CGAGUGACAGCUGAAGACAAGGGUA-3'; Rab5 siRNA, 5'-GGAAGAGGAGUAGACCUUA-3'; and Rab7 siRNA, 5'-GCUGCGUUCUGGUAUUUGA-3'. Cells were transfected with indicated siRNAs using either a Santa Cruz Biotechnology transfection reagent or an Amaxa Nucleofector (Lonza) electroporation system as previously described (Chavez et al., 2015; Yazbeck et al., 2017).

ECs were cotransfected with WT-S1PR1-GFP, Y¹⁴³D-S1PR1-GFP, or Y¹⁴³F-S1PR1-GFP cDNAs along with GTPase-defective dynamin (K44A) or control vector using Amaxa reagent. 24 h after transfection, cells were serum starved and then processed either for imaging or for biochemical analysis (Chavez et al., 2015). The transfection efficiency for GFP was 90%, while efficiency for S1PR1, dynamin, and other cDNAs, which include BiP, ER-mCherry, HA-tagged S1PR1 cDNAs and S1PR1-Dendra2, ranged between 40% and 60%.

Immunoprecipitation and Western blotting

For immunoprecipitation analysis, post-SIP or -TNF- α stimulation serum-starved ECs were stimulated with 1 μ M SIP or 50 ng/ml TNF- α . Cells were washed with ice-cold PBS and immediately lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10% lubrol, 1% NP-40, 25 mM MgCl₂, 1 mM PMSF, 25 mM NaF, 1 mM NaVO₄, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail). Lysates were incubated with anti-GFP (1:200) or anti-S1PR1 (1:100) antibodies overnight followed by the addition of agarose beads to pull down the immunocomplexes (Chavez et al., 2015). Proteins were separated by SDS-PAGE and immunoblotted using indicated antibodies. Dilution for each primary antibody used in the study were as follows: S1PR1 (1:500), dynamin I/II (1:1,000), BiP (1:1,000), VE-cadherin (1:500), Lamin A (1:1,000), Rab5 (1:1,000), Rab7 (1:1,000), HA-tag (1:1,000), GFP (1:500), phosphotyrosine (PY99/PY20, 1:250), α -Glia (1:500), and β -actin (1:1,000). Membranes were then incubated with respective secondary antibodies anti-mouse or anti-rabbit (1:10,000) for 2 h, following which the bands were visualized using imager or autoradiographic films and chemiluminescent Western blotting detection substrate.

Biotinylation assay

HUVECs were washed with ice-cold PBS, after which cells were labeled with 0.5 mg/ml sulfo-NHS-SS Biotin in Ca²⁺/Mg²⁺ containing PBS for 30 min at 4°C. The reaction was then quenched using 100 mM glycine for 20 min, after which cells were harvested in RIPA buffer containing 10% lubrol. Equal amounts of protein were incubated with streptavidin agarose resin beads at 4°C overnight followed by three times centrifugation at 2,400 g and rinsing using RIPA buffer at 4°C. Proteins were then eluted using 4 \times Laemmli buffer and Western blotted as described previously (Chavez et al., 2015).

Confocal and TIRF imaging

HUVECs seeded on 35-mm Nunc glass-bottom dishes (Thermo Fisher Scientific) were transfected with indicated cDNA or

siRNA. 24 or 48 h after transfection, cells were serum starved in basal media supplemented with 0.1% FBS for 1–2 h followed by quick rinse with PBS. Cells were then stimulated with indicated agonists and fixed at indicated times with 2% paraformaldehyde and mounted as described previously (Chavez et al., 2015). In some studies, cells were permeabilized after fixation and immunofluorescently stained with anti-BiP, anti-Rab5, or anti-Rab7 antibodies followed by incubation with appropriate fluorescently conjugated secondary antibodies. Confocal images were acquired using an LSM 880 inverted laser scanning system (Carl Zeiss) equipped with Plan-Apochromat 63 \times /1.4 NA oil immersion objective, argon (λ = 458, 488, 514 nm) and diode-pumped solid-state (λ = 561 nm) lasers, two photomultiplier tubes, and gallium arsenide phosphide detector.

Time-lapse images of S1PR1-Dendra2 were acquired using an LSM 710 Meta inverted laser scanning system (Carl Zeiss) equipped with 63 \times /1.20 NA and 1.46 NA water and oil immersion objectives, diode 405–30 (λ = 405 nm), argon (λ = 458, 488, 514 nm), DPSS 561–10 (λ = 561 nm), and HeNe (λ = 633 nm) lasers. To determine the effect of SIP on the receptor trafficking, Dendra2 was photoconverted with 405-nm laser at 8–12% power for 10 s at a selected region at the plasma membrane, and dual-channel images were simultaneously acquired using a 63 \times /1.20 NA objective every 30 s at λ = 488 nm and λ = 561 nm excitations for green and red states of Dendra2, respectively.

TIRF microscopy was performed using a motorized laser TIRF imaging system (Carl Zeiss) equipped with an ORCA-Flash4.0 V3 Digital CMOS camera (Hamamatsu), and an α Plan-Apochromat 100 \times /1.46 NA objective (Carl Zeiss). For detection of cell surface expression of the receptor, GFP-tagged WT and mutant S1PR1 along with mCherry-tagged stargazin (cell surface marker) were imaged at λ = 488 nm and λ = 561 nm excitation for GFP and mCherry, respectively. Images from green and red channels (excitation 488 nm and 561 nm) were obtained by fast switching the excitation lasers using AxioVision software (Carl Zeiss).

Image processing and analysis

All 16-bit images were analyzed with Fiji image-processing software (ImageJ; National Institutes of Health). For analysis of cell surface expression of S1PR1, multiple regions of interest of the same size were drawn on the plasma membrane, and pixel intensity was calculated. Intensity obtained from multiple regions of interest from the same cell was average (Schindelin et al., 2012).

The surface expression of S1PR1 and its mutants on TIRF images were determined as the average intensity of the plasma membrane after the digital subtraction of the background.

Colocalization between GFP-S1PR1 or its mutants (Y¹⁴³D-S1PR1 and Y¹⁴³F-S1PR1) and mCherry-tagged BiP, ER, or GTPase-defective dynamin (K44A) mutant was quantified using the Colocalization Threshold plugin of ImageJ.

S1PR1 trafficking was assessed by measuring fluorescent intensity of Dendra2 in red state over time after Dendra2 photoconversion at the plasma membrane. Changes in cell surface localization of S1PR1 due to receptor internalization were determined as a loss of red fluorescent signal within the photoconversion region over time after subtraction of the average

background fluorescence values. The levels of internalized S1PR1 were determined as increase of red fluorescent signal over time inside the cell over the cell area after subtracting the background.

Calcium imaging

Increase in $[Ca^{2+}]_i$ was determined using Ca^{2+} -sensitive fluorescent dye Fura-2 AM as described earlier (Sundivakkam et al., 2012; Yazbeck et al., 2017). Briefly, HUVECs or HEK293 cells transfected with indicated cDNAs or siRNAs for 24–48 h were loaded with Fura-2 AM dye for 15–20 min. Cells were rinsed with Ca^{2+} -free HBSS buffer. In this study, only GFP-expressing cells were chosen for Ca^{2+} imaging. Ca^{2+} entry was determined by the readdition of 1.5 mM Ca^{2+} in ECs bathed in Ca^{2+} -free solution and SIP.

Mass spectrometry analysis

HUVECs were transfected with WT-S1PR1, Y¹⁴³D-S1PR1, or Y¹⁴³F-S1PR1 for 24 h. HUVECs expressing WT-S1PR1-GFP were serum starved for 2 h and stimulated with SIP for 0, 5, and 15 min. These time points were chosen based on S1PR1 phosphorylation-dephosphorylation following SIP stimulation (Chavez et al., 2015). ECs expressing Y¹⁴³D-S1PR1 or Y¹⁴³F-S1PR1 for 24 h were also serum starved for 2 h and were left unstimulated. Cells were lysed with 10% lubrol containing RIPA buffer (as above for immunoprecipitation studies), and equal amounts of lysates were immunoprecipitated with anti-GFP antibody overnight at 4°C, after which complexes were pulled down with protein A/G agarose beads. After confirming S1PR1 expression using Western blot, immunocomplexes were separated on 7.5% gel for up to 2 cm, following which the gel was stained using Coomassie brilliant blue and washed using distilled water. Gel lanes were cut and then analyzed at the Harvard mass spectrometry facility. The datasets of spectral counts and intensity scores were calculated and further quantified using Ingenuity Pathway Analysis. Nonunique peptides were excluded from the mass spectrometry data. Only unique candidates in the context of S1PR1 complexes were shown with greater intensities and arbitrarily chosen log₁₀ mean peptide scores. These proteins were evaluated for known interactions with other interaction databases. We focused on BiP (HSPA5) as (1) it is an ER-localized protein and (2) it binds S1PR1 with the highest intensity peptide score among the interactome in the phosphorylated state of S1PR1. Scatter plot of mean intensity versus spectral position was generated using ggplot2. Later, this interaction was further validated using immunoprecipitation studies.

TEER measurements

HUVECs seeded on eight-well gold-plated electrodes (Applied Biosciences) were transfected with either indicated siRNAs (48 h) or cDNAs (24 h). At indicated times after transfection, cells were serum starved for 1–2 h, and basal TEER was recorded. Cells were then stimulated with either 1 μM SIP (Chavez et al., 2015; Tauseef et al., 2008), 50 ng/ml TNF-α, or 50 μM PTX. Note that all the studies were performed in confluent monolayer, which was confirmed

by forming a cell monolayer showing a resistance of ~1,000 Ω.

ATPase activity measurements

ATPase activity was determined using a Malachite Green Phosphate Assay Kit (BioVision) according to the manufacturer's instructions. Reaction mixtures were prepared in triplicate in a final volume of 200 μl, using 10 μg of protein dissolved in 30 mM Hepes-KOH (pH 7.8), 150 mM NaCl, 20 μM ATP, and 2 mM MgCl₂. Samples were incubated for 60 min at 37°C, after which the concentration of phosphate was measured at 620–650 nm using a SpectraMax 340PC Microplate Reader (Bio-Rad). The resulting data were analyzed and kinetic parameters calculated using the Michaelis-Menten equation with GraphPad Prism 5 software (GraphPad Software).

Subcellular localization of BiP

Serum-deprived ECs were stimulated with SIP for indicated times, after which subcellular fractionation was performed using Cell Fractionation Kit (cat. #78840; Thermo Scientific Scientific) and the manufacturer's protocol. Different subcellular protein fractions were run on SDS-PAGE and probed for respective markers.

Quantification and statistics

The statistical analysis was performed using GraphPad Prism 7.0 software. The specific statistical methods used for individual experiments are mentioned in the figure legends with their significance values. Paired *t* tests were performed for experiments containing two groups, while one-way ANOVA was performed in experiments containing multiple groups. Data distribution was assumed to be normal, but this was not formally tested. The following *P* values were used in the study for the significance: *, *P* < 0.05 was considered significant; **, *P* < 0.001 was considered highly significant; and ***, *P* < 0.0001 was considered very highly significant.

Online supplemental material

Fig. S1 shows S1PR1 localization. Fig. S2 shows S1PR1 internalization independent of Rab-GTPases. Fig. S3 shows mass spectrometric analysis of S1PR1 binding partners. Fig. S4 shows the effect of inhibition of Gi on cytosolic calcium and barrier function in response to SIP. Fig. S5 shows that TNF-α fails to phosphorylate Y¹⁴³F-S1PR1.

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performed experiments and analyzed the data. J. Matsche and A. Karginov generated Dendra2-S1PR1 cDNA. R.D. Minshall, G.C.H. Mo, and Y. Komarova helped with imaging and preparation of figures and commented on the manuscript. M. Anwar and D. Mehta interpreted data and wrote the manuscript.

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References

- Adams, B.M., M.E. Oster, and D.N. Hebert. 2019. Protein quality control in the endoplasmic reticulum. *Protein J.* 38:317–329. <https://doi.org/10.1007/s10930-019-09831-w>
- Anwar, M., and D. Mehta. 2020. Post-translational modifications of S1PR1 and endothelial barrier regulation. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids.* 1865:158760. <https://doi.org/10.1016/j.bbalip.2020.158760>
- Baeyens, A., V. Fang, C. Chen, and S.R. Schwab. 2015. Exit strategies: S1P signaling and T cell migration. *Trends Immunol.* 36:778–787. <https://doi.org/10.1016/j.it.2015.10.005>
- Balaji Ragunathrao, V.A., M. Anwar, M.Z. Akhter, A. Chavez, Y. Mao, V. Natarajan, S. Lakshmikantham, M. Chrzanowska-Wodnicka, A.Z. Dudek, L. Claesson-Welsh, et al. 2019. Sphingosine-1-phosphate receptor 1 activity promotes tumor growth by amplifying VEGF-VEGFR2 angiogenic signaling. *Cell Rep.* 29:3472–3487.e4. <https://doi.org/10.1016/j.celrep.2019.11.036>
- Benechet, A.P., M. Menon, D. Xu, T. Samji, L. Maher, T.T. Murooka, T.R. Mempel, B.S. Sheridan, F.M. Lemoine, and K.M. Khanna. 2016. T cell-intrinsic S1PR1 regulates endogenous effector T-cell egress dynamics from lymph nodes during infection. *Proc. Natl. Acad. Sci. USA.* 113:2182–2187. <https://doi.org/10.1073/pnas.1516485113>
- Cahalan, S.M., P.J. Gonzalez-Cabrera, G. Sarkisyan, N. Nguyen, M.T. Schaeffer, L. Huang, A. Yeager, B. Clemons, F. Scott, and H. Rosen. 2011. Actions of a picomolar short-acting S1P₁ agonist in S1P₁-eGFP knock-in mice. *Nat. Chem. Biol.* 7:254–256. <https://doi.org/10.1038/nchembio.547>
- Cannavo, A., G. Rengo, D. Liccardo, G. Pagano, C. Zicarelli, M.C. De Angelis, R. Puglia, E. Di Pietro, J.E. Rabinowitz, M.V. Barone, et al. 2013. β 1-Adrenergic receptor and sphingosine-1-phosphate receptor 1 (S1PR1) reciprocal downregulation influences cardiac hypertrophic response and progression to heart failure: protective role of S1PR1 cardiac gene therapy. *Circulation.* 128:1612–1622. <https://doi.org/10.1161/CIRCULATIONAHA.113.002659>
- Carrara, M., F. Prisch, P.R. Nowak, M.C. Kopp, and M.M. Ali. 2015. Non-canonical binding of BiP ATPase domain to Ire1 and Perk is dissociated by unfolded protein CH1 to initiate ER stress signaling. *eLife.* 4:e03522. <https://doi.org/10.7554/eLife.03522>
- Cartier, A., and T. Hla. 2019. Sphingosine 1-phosphate: Lipid signaling in pathology and therapy. *Science.* 366:eaar5551. <https://doi.org/10.1126/science.aar5551>
- Chavez, A., T.T. Schmidt, P. Yazbeck, C. Rajput, B. Desai, S. Sukriti, K. Giantsos-Adams, N. Knezevic, A.B. Malik, and D. Mehta. 2015. S1PR1 Tyr143 phosphorylation downregulates endothelial cell surface S1PR1 expression and responsiveness. *J. Cell Sci.* 128:878–887. <https://doi.org/10.1242/jcs.154476>
- Chichger, H., J. Braza, H. Duong, G. Boni, and E.O. Harrington. 2016. Select Rab GTPases regulate the pulmonary endothelium via endosomal trafficking of vascular endothelial-cadherin. *Am. J. Respir. Cell Mol. Biol.* 54:769–781. <https://doi.org/10.1165/rcmb.2015-0286OC>
- Chudakov, D.M., S. Lukyanov, and K.A. Lukyanov. 2007. Tracking intracellular protein movements using photoswitchable fluorescent proteins PS-CFP2 and Dendra2. *Nat. Protoc.* 2:2024–2032. <https://doi.org/10.1038/nprot.2007.291>
- Cyster, J.G., and S.R. Schwab. 2012. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu. Rev. Immunol.* 30:69–94. <https://doi.org/10.1146/annurev-immunol-020711-075011>
- Damke, H., D.D. Binns, H. Ueda, S.L. Schmid, and T. Baba. 2001. Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages. *Mol. Biol. Cell.* 12:2578–2589. <https://doi.org/10.1091/mbc.12.9.2578>
- Daneshjoui, N., N. Sieracki, G.P. van Nieuw Amerongen, D.E. Conway, M.A. Schwartz, Y.A. Komarova, and A.B. Malik. 2015. Rac1 functions as a reversible tension modulator to stabilize VE-cadherin trans-interaction. *J. Cell Biol.* 208:23–32. <https://doi.org/10.1083/jcb.201409108>
- de Renzis, S., B. Sönnichsen, and M. Zerial. 2002. Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. *Nat. Cell Biol.* 4:124–133. <https://doi.org/10.1038/ncb744>
- DeLalio, L.J., M. Billaud, C.A. Ruddiman, S.R. Johnstone, J.T. Butcher, A.G. Wolpe, X. Jin, T.C.S. Keller IV, A.S. Keller, T. Rivière, et al. 2019. Constitutive SRC-mediated phosphorylation of pannexin 1 at tyrosine 198 occurs at the plasma membrane. *J. Biol. Chem.* 294:6940–6956. <https://doi.org/10.1074/jbc.RA118.006982>
- Dickens, J.A., A. Ordóñez, J.E. Chambers, A.J. Beckett, V. Patel, E. Malzer, C.S. Dominicus, J. Bradley, A.A. Peden, I.A. Prior, et al. 2016. The endoplasmic reticulum remains functionally connected by vesicular transport after its fragmentation in cells expressing Z- α 1-antitrypsin. *FASEB J.* 30:4083–4097. <https://doi.org/10.1096/fj.201600430R>
- Doronzo, G., E. Astanina, D. Corà, G. Chiabotto, V. Comunanza, A. Noghero, F. Neri, A. Puliafito, L. Primo, C. Spampinato, et al. 2019. TFEB controls vascular development by regulating the proliferation of endothelial cells. *EMBO J.* 38:201798250. <https://doi.org/10.15252/embj.201798250>
- Drake, M.T., S.K. Shenoy, and R.J. Lefkowitz. 2006. Trafficking of G protein-coupled receptors. *Circ. Res.* 99:570–582. <https://doi.org/10.1161/01.RES.0000242563.47507.ce>
- Dudek, S.M., J.R. Jacobson, E.T. Chiang, K.G. Birukov, P. Wang, X. Zhan, and J.G. Garcia. 2004. Pulmonary endothelial cell barrier enhancement by sphingosine-1-phosphate: Roles for cortactin and myosin light chain kinase. *J. Biol. Chem.* 279:24692–24700. <https://doi.org/10.1074/jbc.M313969200>
- Engelbrecht, E., M.V. Levesque, L. He, M. Vanlandewijck, A. Nitzsche, H. Niaz, A. Kuo, S.A. Singh, M. Aikawa, K. Holton, et al. 2020. Sphingosine-1-phosphate-regulated transcriptomes in heterogenous arterial and lymphatic endothelium of the aorta. *eLife.* 9:e52690. <https://doi.org/10.7554/eLife.52690>
- Ferguson, S.M., and P. De Camilli. 2012. Dynamin, a membrane-remodelling GTPase. *Nat. Rev. Mol. Cell Biol.* 13:75–88. <https://doi.org/10.1038/nrm3266>
- Gardner, B.M., D. Pincus, K. Gotthardt, C.M. Gallagher, and P. Walter. 2013. Endoplasmic reticulum stress sensing in the unfolded protein response. *Cold Spring Harb. Perspect. Biol.* 5:a013169. <https://doi.org/10.1101/cshperspect.a013169>
- Gorvel, J.P., P. Chavrier, M. Zerial, and J. Gruenberg. 1991. rab5 controls early endosome fusion in vitro. *Cell.* 64:915–925. [https://doi.org/10.1016/0092-8674\(91\)90316-Q](https://doi.org/10.1016/0092-8674(91)90316-Q)
- Granel, S., B.M. Molden, and G. Baldini. 2013. Exposure of MC4R to agonist in the endoplasmic reticulum stabilizes an active conformation of the receptor that does not desensitize. *Proc. Natl. Acad. Sci. USA.* 110:E4733–E4742. <https://doi.org/10.1073/pnas.1219808110>
- Gunther, E.C., C.S. von Bartheld, L.J. Goodman, J.E. Johnson, and M. Bothwell. 2000. The G-protein inhibitor, pertussis toxin, inhibits the secretion of brain-derived neurotrophic factor. *Neuroscience.* 100:569–579. [https://doi.org/10.1016/S0306-4522\(00\)00309-2](https://doi.org/10.1016/S0306-4522(00)00309-2)
- Haj, F.G., P.J. Verveer, A. Squire, B.G. Neel, and P.I. Bastiaens. 2002. Imaging sites of receptor dephosphorylation by PTP1B on the surface of the endoplasmic reticulum. *Science.* 295:1708–1711. <https://doi.org/10.1126/science.1067566>
- Hannun, Y.A., and L.M. Obeid. 2008. Principles of bioactive lipid signalling: Lessons from sphingolipids. *Nat. Rev. Mol. Cell Biol.* 9:139–150. <https://doi.org/10.1038/nrm2329>
- Hanson, M.A., C.B. Roth, E. Jo, M.T. Griffith, F.L. Scott, G. Reinhart, H. Desale, B. Clemons, S.M. Cahalan, S.C. Schuerer, et al. 2012. Crystal structure of a lipid G protein-coupled receptor. *Science.* 335:851–855. <https://doi.org/10.1126/science.1215904>
- Hutagalung, A.H., and P.J. Novick. 2011. Role of Rab GTPases in membrane traffic and cell physiology. *Physiol. Rev.* 91:119–149. <https://doi.org/10.1152/physrev.00059.2009>
- Inamuro, M., M. Itakura, H. Okamoto, S. Hoka, A. Mizoguchi, Y. Fukazawa, R. Shigemoto, S. Yamamori, and M. Takahashi. 2006. Differential localization and regulation of stargazin-like protein, gamma-8 and stargazin in the plasma membrane of hippocampal and cortical neurons. *Neurosci. Res.* 55:45–53. <https://doi.org/10.1016/j.neures.2006.01.004>
- Jambusaria, A., Z. Hong, L. Zhang, S. Srivastava, A. Jana, P.T. Toth, Y. Dai, A.B. Malik, and J. Rehman. 2020. Endothelial heterogeneity across distinct vascular beds during homeostasis and inflammation. *eLife.* 9:e51413. <https://doi.org/10.7554/eLife.51413>
- Komarova, Y.A., K. Kruse, D. Mehta, and A.B. Malik. 2017. Protein Interactions at endothelial junctions and signaling mechanisms regulating

- endothelial permeability. *Circ. Res.* 120:179–206. <https://doi.org/10.1161/CIRCRESAHA.116.306534>
- Kopp, M.C., N. Larburu, V. Durairaj, C.J. Adams, and M.M.U. Ali. 2019. UPR proteins IRE1 and PERK switch BiP from chaperone to ER stress sensor. *Nat. Struct. Mol. Biol.* 26:1053–1062. <https://doi.org/10.1038/s41594-019-0324-9>
- Leonard, A., V. Grose, A.W. Paton, J.C. Paton, D.I. Yule, A. Rahman, and F. Fazal. 2019. Selective Inactivation of Intracellular BiP/GRP78 attenuates endothelial inflammation and permeability in acute lung injury. *Sci. Rep.* 9:2096. <https://doi.org/10.1038/s41598-018-38312-w>
- Li, Q., B. Chen, C. Zeng, A. Fan, Y. Yuan, X. Guo, X. Huang, and Q. Huang. 2015. Differential activation of receptors and signal pathways upon stimulation by different doses of sphingosine-1-phosphate in endothelial cells. *Exp. Physiol.* 100:95–107. <https://doi.org/10.1113/expphysiol.2014.082149>
- Maceyka, M., K.B. Harikumar, S. Milstien, and S. Spiegel. 2012. Sphingosine-1-phosphate signaling and its role in disease. *Trends Cell Biol.* 22:50–60. <https://doi.org/10.1016/j.tcb.2011.09.003>
- Macia, E., M. Ehrlich, R. Massol, E. Boucrot, C. Brunner, and T. Kirchhausen. 2006. Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell.* 10: 839–850. <https://doi.org/10.1016/j.devcel.2006.04.002>
- Magalhaes, A.C., H. Dunn, and S.S. Ferguson. 2012. Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. *Br. J. Pharmacol.* 165:1717–1736. <https://doi.org/10.1111/j.1476-5381.2011.01552.x>
- Matloubian, M., C.G. Lo, G. Cinamon, M.J. Lesneski, Y. Xu, V. Brinkmann, M.L. Allende, R.L. Proia, and J.G. Cyster. 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature.* 427:355–360. <https://doi.org/10.1038/nature02284>
- Mehta, D., and A.B. Malik. 2006. Signaling mechanisms regulating endothelial permeability. *Physiol. Rev.* 86:279–367. <https://doi.org/10.1152/physrev.00012.2005>
- Mehta, D., M. Konstantoulaki, G.U. Ahmed, and A.B. Malik. 2005. Sphingosine-1-phosphate-induced mobilization of intracellular Ca²⁺ mediates Rac activation and adherens junction assembly in endothelial cells. *J. Biol. Chem.* 280:17320–17328. <https://doi.org/10.1074/jbc.M411674200>
- Naslavsky, N., and S. Caplan. 2018. The enigmatic endosome - sorting the ins and outs of endocytic trafficking. *J. Cell Sci.* 131:jcs216499. <https://doi.org/10.1242/jcs.216499>
- Ni, M., Y. Zhang, and A.S. Lee. 2011. Beyond the endoplasmic reticulum: Atypical GRP78 in cell viability, signalling and therapeutic targeting. *Biochem. J.* 434:181–188. <https://doi.org/10.1042/BJ20101569>
- Oo, M.L., S.H. Chang, S. Thangada, M.T. Wu, K. Rezaul, V. Blaho, S.I. Hwang, D.K. Han, and T. Hla. 2011. Engagement of S1P₁-degradative mechanisms leads to vascular leak in mice. *J. Clin. Invest.* 121:2290–2300. <https://doi.org/10.1172/JCI45403>
- Proia, R.L., and T. Hla. 2015. Emerging biology of sphingosine-1-phosphate: Its role in pathogenesis and therapy. *J. Clin. Invest.* 125:1379–1387. <https://doi.org/10.1172/JCI76369>
- Ramsay, D., E. Kellett, M. McVey, S. Rees, and G. Milligan. 2002. Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): Hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. *Biochem. J.* 365:429–440. <https://doi.org/10.1042/bj20020251>
- Rasmussen, S.G., B.T. DeVree, Y. Zou, A.C. Kruse, K.Y. Chung, T.S. Kobilka, F.S. Thian, P.S. Chae, E. Pardon, D. Calinski, et al. 2011. Crystal structure of the β 2 adrenergic receptor-Gs protein complex. *Nature.* 477:549–555. <https://doi.org/10.1038/nature10361>
- Reinhard, N.R., M. Mastop, T. Yin, Y. Wu, E.K. Bosma, T.W.J. Gadella Jr., J. Goedhart, and P.L. Hordijk. 2017. The balance between G α _i-Cdc42/Rac and G α _{12/13}-RhoA pathways determines endothelial barrier regulation by sphingosine-1-phosphate. *Mol. Biol. Cell.* 28:3371–3382. <https://doi.org/10.1091/mbc.e17-03-0136>
- Rivera, J., R.L. Proia, and A. Olivera. 2008. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat. Rev. Immunol.* 8:753–763. <https://doi.org/10.1038/nri2400>
- Rosenbaum, D.M., S.G. Rasmussen, and B.K. Kobilka. 2009. The structure and function of G-protein-coupled receptors. *Nature.* 459:356–363. <https://doi.org/10.1038/nature08144>
- Rovati, G.E., V. Capra, and R.R. Neubig. 2007. The highly conserved DRY motif of class A G protein-coupled receptors: Beyond the ground state. *Mol. Pharmacol.* 71:959–964. <https://doi.org/10.1124/mol.106.029470>
- Saba, J.D., and T. Hla. 2004. Point-counterpoint of sphingosine 1-phosphate metabolism. *Circ. Res.* 94:724–734. <https://doi.org/10.1161/01.RES.0000122383.60368.24>
- Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, et al. 2012. Fiji: An open-source platform for biological-image analysis. *Nat. Methods.* 9: 676–682. <https://doi.org/10.1038/nmeth.2019>
- Shajahan, A.N., B.K. Timblin, R. Sandoval, C. Tiruppathi, A.B. Malik, and R.D. Minshall. 2004. Role of Src-induced dynamin-2 phosphorylation in caveolae-mediated endocytosis in endothelial cells. *J. Biol. Chem.* 279: 20392–20400. <https://doi.org/10.1074/jbc.M308710200>
- Spiegel, S., M.A. Maczys, M. Maceyka, and S. Milstien. 2019. New insights into functions of the sphingosine-1-phosphate transporter SPNS2. *J. Lipid Res.* 60:484–489. <https://doi.org/10.1194/jlr.S091959>
- Sun, F.C., S. Wei, C.W. Li, Y.S. Chang, C.C. Chao, and Y.K. Lai. 2006. Localization of GRP78 to mitochondria under the unfolded protein response. *Biochem. J.* 396:31–39. <https://doi.org/10.1042/BJ20051916>
- Sundivakkam, P. C., M. Freichel, V. Singh, J.P. Yuan, S.M. Vogel, V. Flockerzi, A.B. Malik, and C. Tiruppathi. 2012. The Ca²⁺ sensor stromal interaction molecule 1 (STIM1) is necessary and sufficient for the store-operated Ca²⁺ entry function of transient receptor potential canonical (TRPC) 1 and 4 channels in endothelial cells. *Mol Pharmacol.* 81:510–526. <https://doi.org/10.1124/mol.111.074658>
- Tauseef, M., V. Kini, N. Knezevic, M. Brannan, R. Ramchandaran, H. Fyrt, J. Saba, S.M. Vogel, A.B. Malik, and D. Mehta. 2008. Activation of sphingosine kinase-1 reverses the increase in lung vascular permeability through sphingosine-1-phosphate receptor signaling in endothelial cells. *Circ. Res.* 103:1164–1172. <https://doi.org/10.1161/01.RES.0000338501.84810.51>
- Vandenbroucke, E., D. Mehta, R. Minshall, and A.B. Malik. 2008. Regulation of endothelial junctional permeability. *Ann. N. Y. Acad. Sci.* 1123:134–145. <https://doi.org/10.1196/annals.1420.016>
- Venkatakrishnan, A.J., X. Deupi, G. Lebon, C.G. Tate, G.F. Schertler, and M.M. Babu. 2013. Molecular signatures of G-protein-coupled receptors. *Nature.* 494:185–194. <https://doi.org/10.1038/nature11896>
- Willinger, T., S.M. Ferguson, J.P. Pereira, P. De Camilli, and R.A. Flavell. 2014. Dynamin 2-dependent endocytosis is required for sustained S1P₁ signaling. *J. Exp. Med.* 211:685–700. <https://doi.org/10.1084/jem.20131343>
- Willinger, T., M. Staron, S.M. Ferguson, P. De Camilli, and R.A. Flavell. 2015. Dynamin 2-dependent endocytosis sustains T-cell receptor signaling and drives metabolic reprogramming in T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 112:4423–4428. <https://doi.org/10.1073/pnas.1504279112>
- Yazbeck, P., M. Tauseef, K. Kruse, M.R. Amin, R. Sheikh, S. Feske, Y. Komarova, and D. Mehta. 2017. STIM1 Phosphorylation at Y361 recruits Orail to STIM1 puncta and induces Ca²⁺ entry. *Sci. Rep.* 7:42758. <https://doi.org/10.1038/srep42758>
- Zhao, Y., S.K. Kalari, P.V. Usatyuk, I. Gorshkova, D. He, T. Watkins, D.N. Brindley, C. Sun, R. Bittman, J.G. Garcia, et al. 2007. Intracellular generation of sphingosine 1-phosphate in human lung endothelial cells: Role of lipid phosphate phosphatase-1 and sphingosine kinase 1. *J. Biol. Chem.* 282:14165–14177. <https://doi.org/10.1074/jbc.M701279200>

Supplemental material

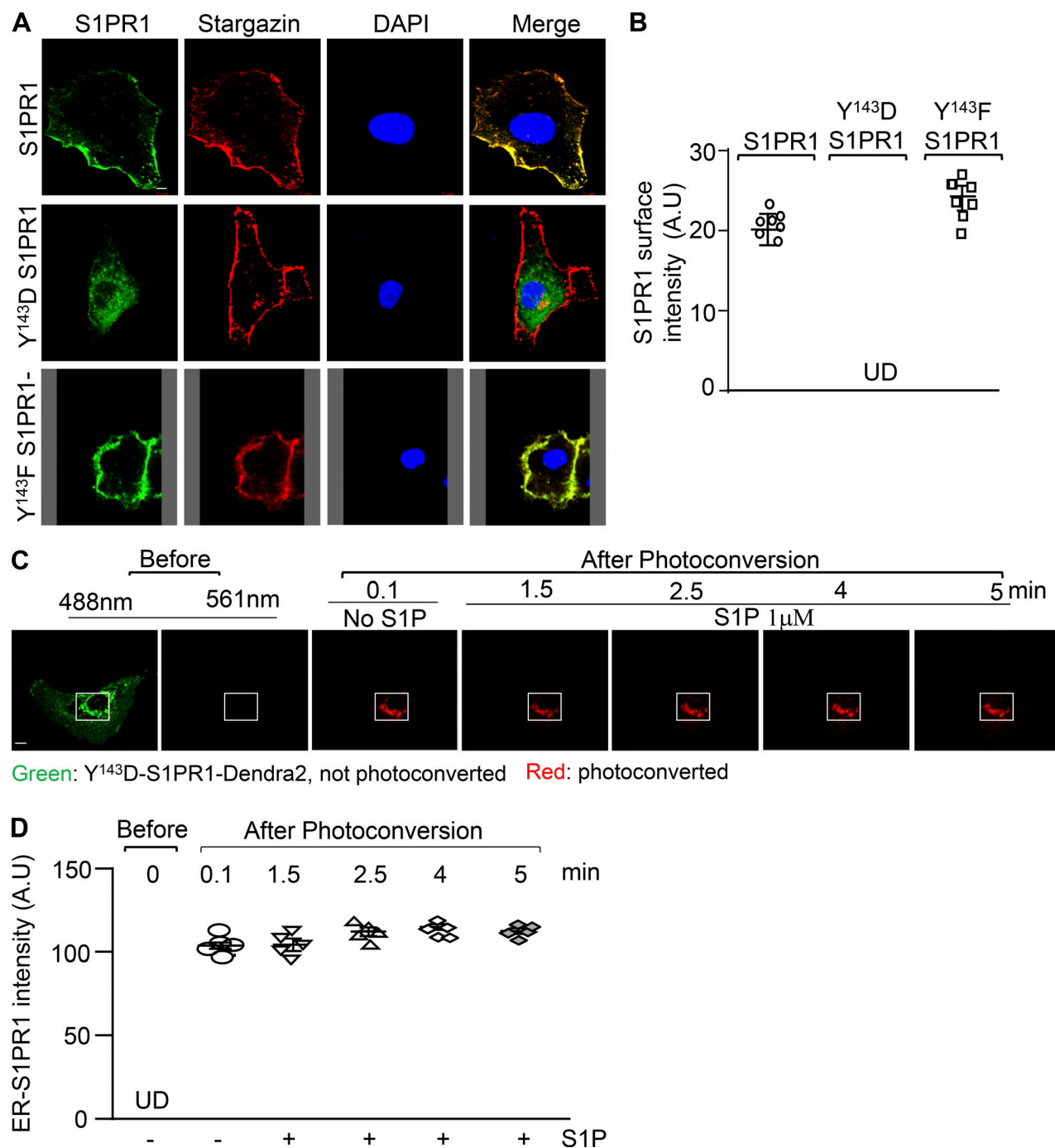


Figure S1. **S1PR1 localization.** (A and B) HUVECs coexpressing WT-S1PR1-GFP and Y¹⁴³D-S1PR1-GFP or Y¹⁴³F-S1PR1-GFP along with stargazin (membrane marker). At 24 h, cells were fixed and stained for DAPI, and images were acquired using a confocal microscope. A representative image of receptor localization in the cell is shown in A, whereas B shows the quantification of the individual data points representing receptor expression along with mean \pm SD (5–8 cells/group). Scale bar, 5 μ m. Gray background indicates the area outside the image. (C and D) Time-lapse images of Y¹⁴³D-S1PR1-Dendra2 before (green, rectangles) and after (red, rectangles) photoconversion at time 0 for basal (C) and after the addition of S1P (1 μ M). Time is given in minutes. A representative image with time lapse of receptor localization in the cell is shown in C, while D shows the quantification of the individual data points representing receptor expression along with mean \pm SD (5–6 cells). Scale bar, 10 μ m. Data are from experiments that were repeated at least two times. Significance determined by one-way ANOVA followed by multiple comparisons between groups. A.U., arbitrary units; UD, undetectable.

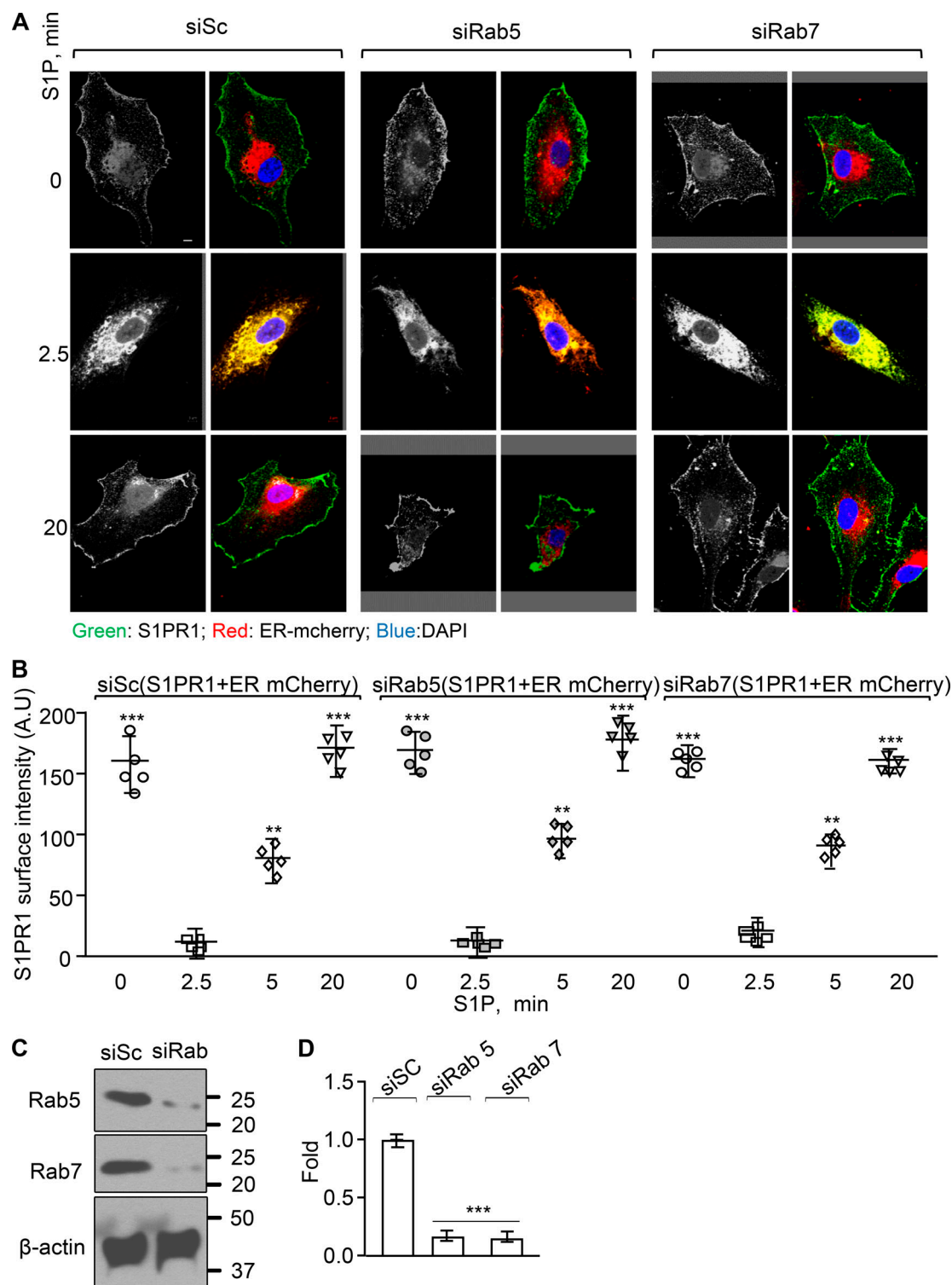


Figure S2. S1PR1 internalizes independent of Rab-GTPases. (A and B) HUVECs were transfected with control siRNA (siSc) or siRNA against Rab5 (siRab5) or Rab7 (siRab7) GTPases. After 24 h, cells were again cotransfected with WT-S1PR1-GFP and ER-mCherry. 48 h after transfection, cells were stimulated with S1P at the indicated time points, fixed, and stained with anti-Rab5 and anti-Rab7 antibodies followed by DAPI (nuclear stain). A shows the representative images (scale bar, 5 μ m), whereas B shows the mean \pm SD quantification of the surface intensity of S1PR1-GFP in HUVECs transfected with siRab5 and siRab7. $n = 4$ –6 cells from experiments that were repeated at least two times. Gray background indicates the area outside the image. **, $P < 0.001$; ***, $P < 0.0001$ compared with S1P-stimulated siSc or siRab5 or siRab7 ECs transfected with S1PR1-GFP at 2.5 min by one-way ANOVA with two-tailed paired t test. **(C and D)** At 48 h after transfection, lysates from control or Rab5/Rab7-depleted ECs were immunoblotted using anti-Rab5 and anti-Rab7 antibodies with β -actin as a loading control. Mean \pm SD Rab5 and Rab7 densities were quantified against β -actin. Blot represents experiments that were repeated multiple times independently. ***, $P < 0.0001$ compared with siSc-transfected ECs by one-way ANOVA with two-tailed paired t test. A.U., arbitrary units. **(C)** Molecular weight marker in kD.

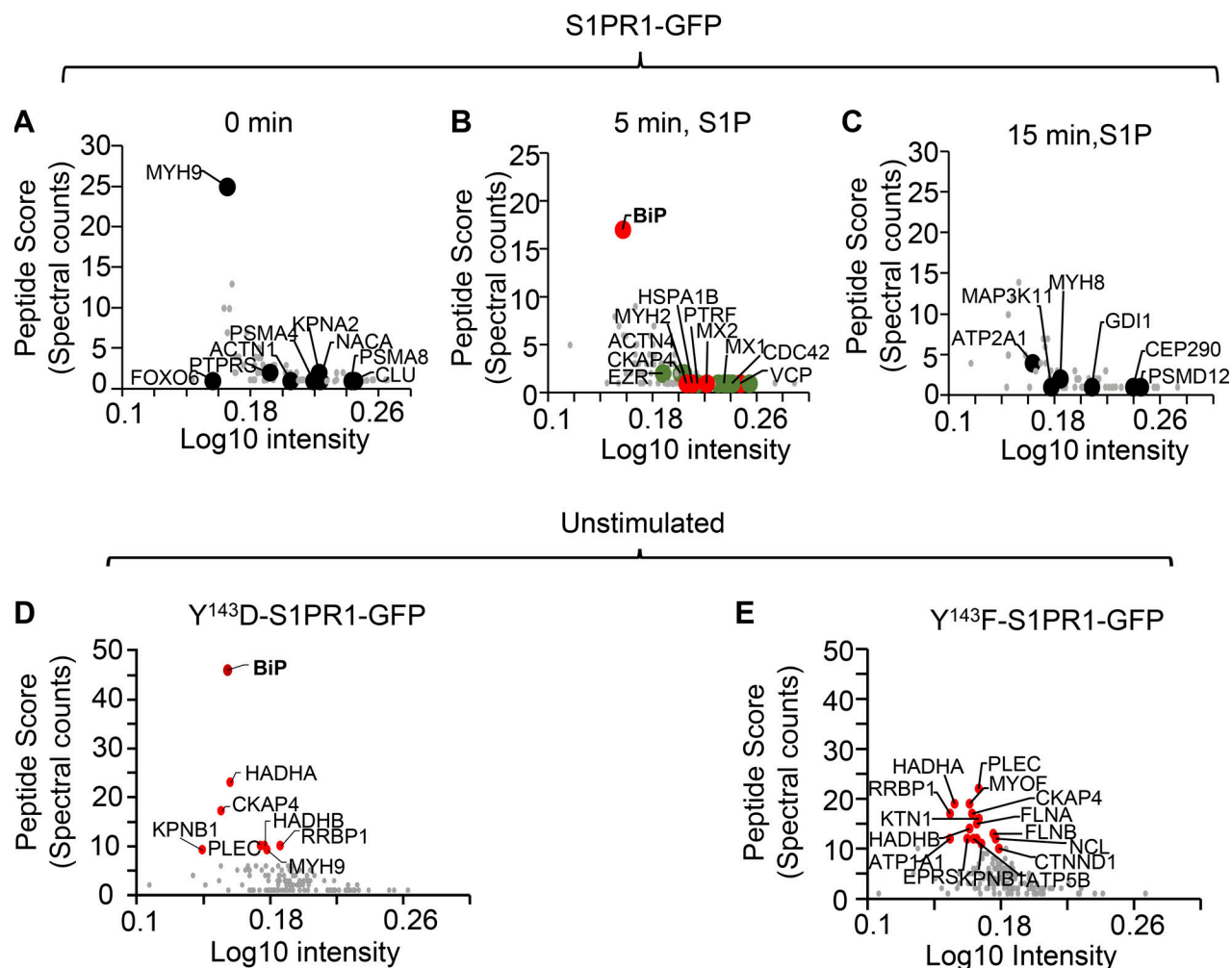


Figure S3. **Mass spectrometric analysis of S1PR1 binding partners.** (A–E) HUVECs expressing vector or GFP-tagged S1PR1, Y¹⁴³D-S1PR1, or Y¹⁴³F-S1PR1 for 24 h were either left unstimulated (A, D, and E) or stimulated with 1 μ M S1P for 5 and 15 min (B and C). Lysates were immunoprecipitated using an anti-GFP antibody. Complexes were separated by SDS-PAGE and analyzed by mass spectroscopy. A scatter plot of mean intensity versus spectral counts is shown. Peptides that were overlapping with GFP were excluded from the mass spectrometry data (see Materials and methods).

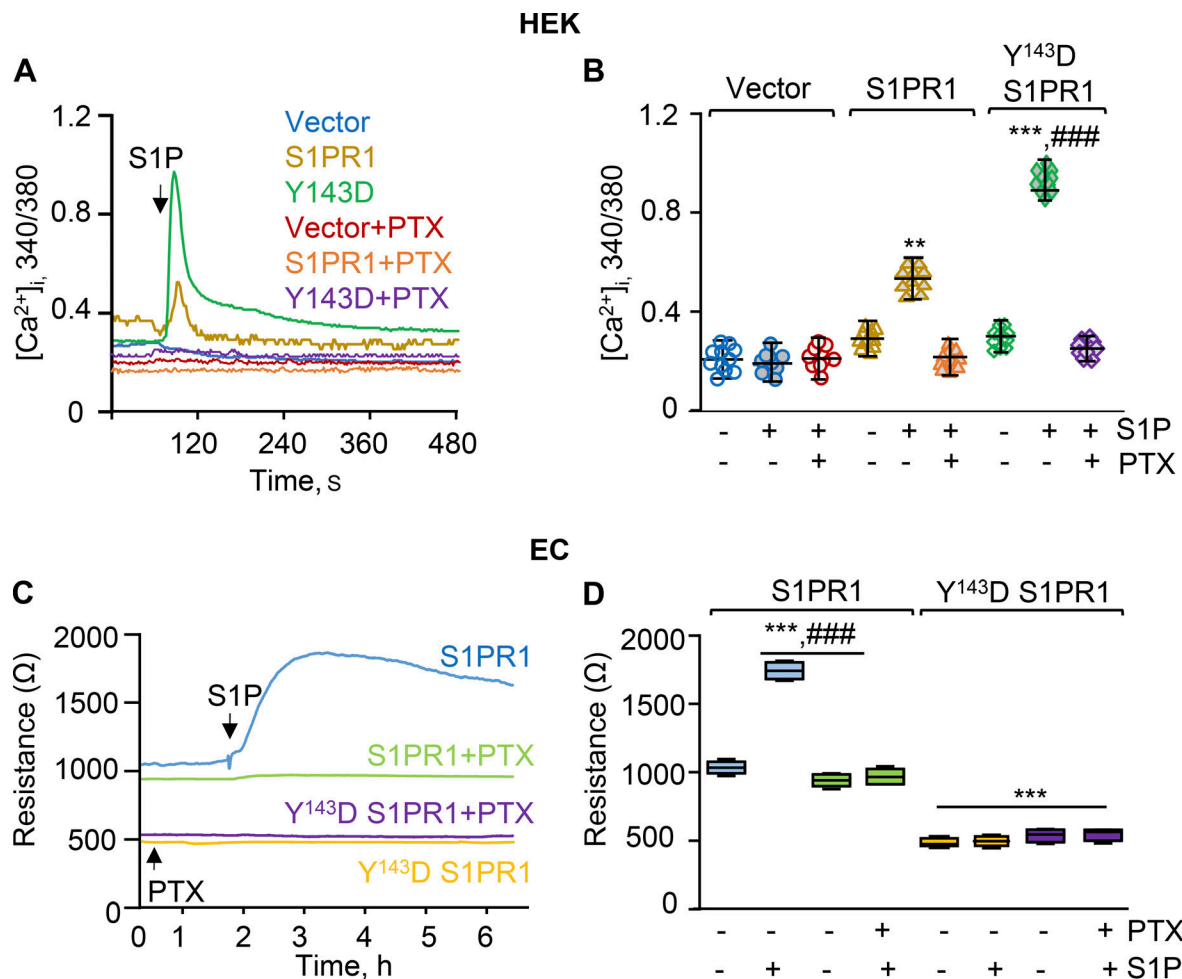


Figure S4. Effect of inhibition of Gi on cytosolic calcium and barrier function in response to S1P. (A and B) After 48 h after transfection, HEK cells transducing vector (GFP), GFP-tagged WT-S1PR1 or Y¹⁴³D S1PR1 cDNAs were left untreated or pretreated with PTX (50 μ M) for 2 h. Cells were then loaded with Fura-2 AM for 15 min. Ratiometric analysis of $[Ca^{2+}]_i$ was then performed after stimulation with 1 μ M S1P. A shows representative traces, whereas B shows individual data points ($n = 8$ –10 cells/group). Basal Ca^{2+} (–) was analyzed at 45 s; peak increase in Ca^{2+} was analyzed at 90 s after S1P stimulation in control or PTX-treated cells. Note that only GFP-expressing cells were chosen to assess $[Ca^{2+}]_i$ release in all experiments. **, $P < 0.001$; ***, $P < 0.0001$ compared with unstimulated cells transfected with vector or S1PR1-GFP; ###, $P < 0.0001$ compared with S1PR1-GFP transfected cells after S1P stimulation by one-way ANOVA with two-tailed paired t test. (C and D) HUVECs seeded on gold-plated electrodes were transfected with S1PR1 or Y¹⁴³D-S1PR1 cDNAs. Cells were pretreated with or without PTX (50 μ M) for 2 h. TEER in real time was determined in response to 1 μ M S1P. An individual TEER trace is shown in C, while D shows the quantification of the TEER from experiments that were repeated at least two times. Basal TEER in control or PTX-treated ECs was quantified as the mean between 10 and 20 min, whereas TEER after S1P addition in these ECs was calculated as the mean between 2 and 3 h. ***, $P < 0.0001$ compared with unstimulated cells transfected with S1PR1-GFP; ###, $P < 0.0001$ compared with S1PR1-GFP-transfected cells treated with PTX and S1P by one-way ANOVA with two-tailed paired t test.

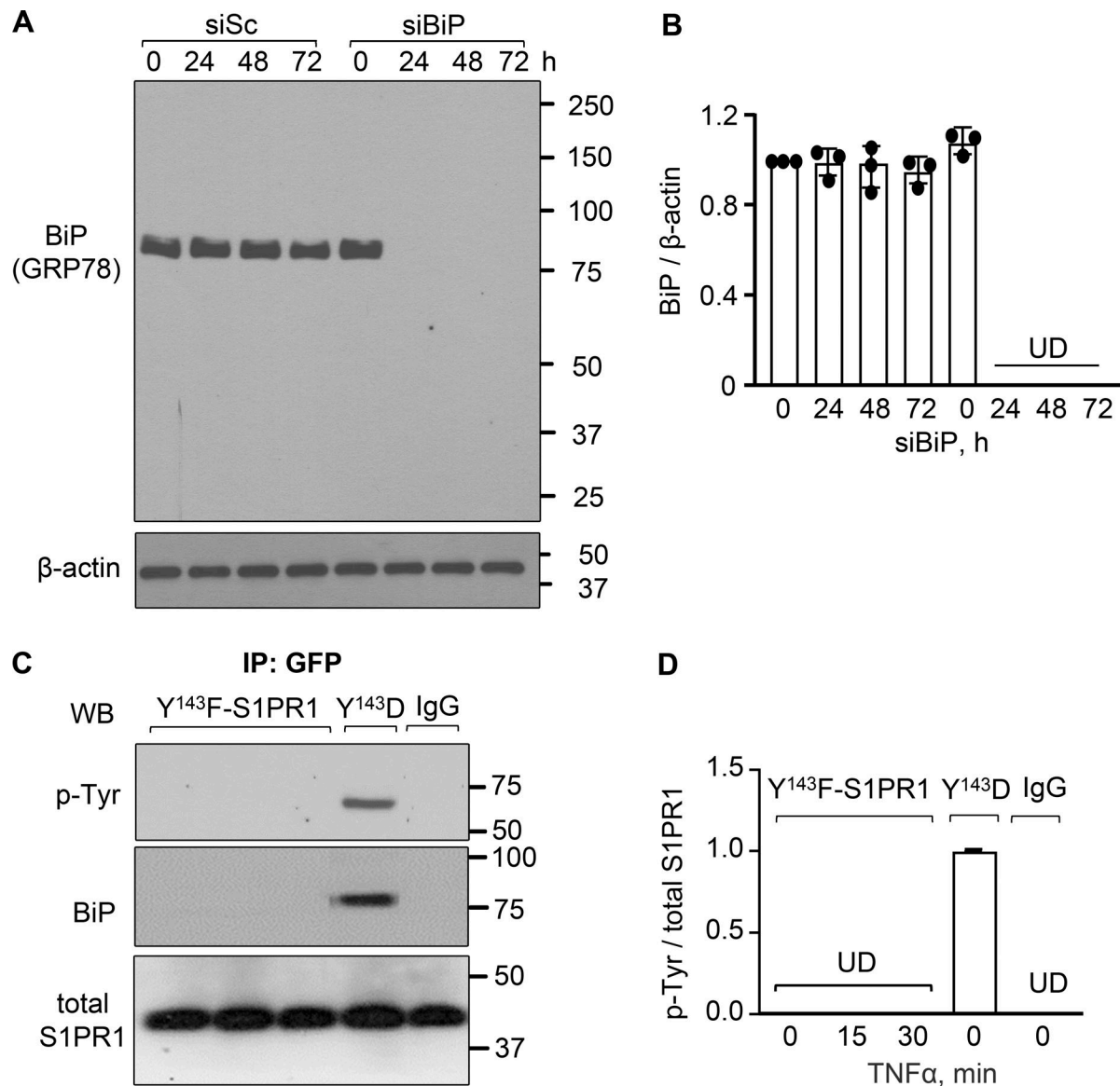


Figure S5. TNF- α fails to phosphorylate Y¹⁴³F-S1PR1. (A and B) HUVECs were transfected with control (siSc) or BiP siRNA (siBiP). Lysates were collected at the indicated times and probed for BiP and β -actin. A shows a representative blot image, whereas B shows the densitometric analysis of BiP depletion with β -actin as control. (C and D) HUVECs were transfected with GFP-tagged Y¹⁴³F-S1PR1 or Y¹⁴³D-S1PR1 cDNAs for 24 h, after which cells were left unstimulated or stimulated with TNF- α for 15 and 30 min in Y¹⁴³F-S1PR1-transducing ECs. Equal amounts of protein lysates were immunoprecipitated with anti-GFP antibody, and S1PR1 phosphorylation on Y¹⁴³ residue and interaction with BiP was determined in immunocomplexes using anti-phosphotyrosine and anti-BiP antibodies. Lysates were probed for total S1PR1 to assess the total protein expression and loading. C shows a representative blot from experiments that were repeated at least three times, while D shows the densitometric analysis of phosphotyrosine with S1PR1 as control. Significance was determined by one-way ANOVA followed by multiple comparisons between groups. (A and C) Molecular weight marker in kD.