Ubiquitin ligase SPSB4 diminishes cell repulsive responses mediated by EphB2

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\textbf{ABSTRACT} Eph receptor tyrosine kinases and their ephrin ligands are overexpressed in various human cancers, including colorectal malignancies, suggesting important roles in many aspects of cancer development and progression as well as in cellular repulsive responses. The ectodomain of EphB2 receptor is cleaved by metalloproteinases (MMPs) MMP-2/MMP-9 and released into the extracellular space after stimulation by its ligand. The remaining membrane-associated fragment is further cleaved by the presenilin-dependent γ-secretase and releases an intracellular peptide that has tyrosine kinase activity. Although the cytoplasmic fragment is degraded by the proteasome, the responsible ubiquitin ligase has not been identified. Here, we show that SOCS box-containing protein SPSB4 polyubiquitinates EphB2 cytoplasmic fragment and that SPSB4 knockdown stabilizes the cytoplasmic fragment. Importantly, SPSB4 down-regulation enhances cell repulsive responses mediated by EphB2 stimulation. Altogether, we propose that SPSB4 is a previously unidentified ubiquitin ligase regulating EphB2-dependent cell repulsive responses.

\textbf{INTRODUCTION} Erythropoietin-producing human hepatocellular (Eph) receptor tyrosine kinases and their ligands, ephrins, guide cell migration in various processes during differentiation and development (Fagotto \textit{et al.}, 2014; Perez White and Getsios, 2014; Wilkinson, 2014; Park and Lee, 2015). Eph receptors and ephrins play pivotal roles in morphogenesis, in which they establish and maintain the organization of cell types or regional domains within tissues (Pasquale, 2005; Battle and Wilkinson, 2012; Klein, 2012; Wilkinson, 2014). They also play crucial roles in cell invasion, contributing to tumor development (Chen, 2012; Kandouz, 2012; Gucicciardo \textit{et al.}, 2014). In contrast, extensive studies have indicated opposite tumor-promoting and tumor-suppressing effects, even though the same Eph receptor in the same type of cancer was studied (Pasquale, 2008; Noberini and Pasquale, 2009).

There are nine EphA receptors, which bind to five glycosylphosphatidylinositol-anchored ephrin-A ligands, and five EphB receptors, which bind to three transmembrane ephrin-B ligands (Pasquale, 2005). EphB receptors of the intestine have been well studied. EphB2 and EphB3 receptors and ephrin-B1 and ephrin-B2 ligands are expressed in complementary gradients along the crypt-villus axis and in colorectal cancer under the control of the β-catenin/Tcf pathway, which up-regulates EphB and down-regulates ephrin-B expression (Battle \textit{et al.}, 2002). EphB2 and EphB3 restrict...
cell intermingling and allocate cell populations within the intestinal epithelium (Batlle et al., 2002). EphB- and ephrin-B-mediated repulsive responses prevent unnatural positioning of the proliferating progenitor cells located near the bottom of the crypts into the more differentiated intestinal lumen.

When human embryonic kidney (HEK) cells stably expressing EphB2 are cocultured with cells stably expressing ephrin-B1, EphB2-expressing cells are segregated from ephrin-B1 cells (Poliakov et al., 2008). Mechanistically, ephrin-B1-activated EphB2 activates mitogen-activated protein kinase (MAPK), which is required for EphB-mediated cell repulsion. Activation of MAPK, in turn, activates EphB2, suggesting that a positive feedback loop mediated by MAPK promotes EphB2 activation (Poliakov et al., 2008). After activation by ephrin-B2, the ectodomain of EphB2 is cleaved by matrix metalloproteinases (MMPs) such as MMP-2/MMP-9, producing an EphB2/N-terminal fragment (NTF) and a C-terminal long fragment (EphB2-LF; Lin et al., 2008). EphB2-LF is further cleaved by MMPs, producing an EphB2/C-terminal fragment (CTF1; Litterst et al., 2007; Lin et al., 2008). The remaining plasma membrane-associated EphB2/CTF1 is further cleaved by the presenilin-dependent γ-secretase activity after EphB2 residue 569 in the transmembrane domain, releasing an intracellular peptide, EphB2/CTF2, that contains the whole cytoplasmic domain of EphB2 (Litterst et al., 2007).

Inhibition of MMP-2/MMP-9 or cleavage-resistant mutations in the ectodomain of EphB2 prevents EphB2-mediated cell repulsion, and blocks ephrin-B2–induced growth cone withdrawal in cultured hippocampal neurons (Lin et al., 2008). EphB2/CTF2 functions in signal transduction and protein phosphorylation. EphB2/CTF2 presents tyrosine kinase activity and phosphorylates downstream proteins such as N-methyl-D-aspartate receptor (NMDAR) subunits in primary neuronal cultures to increase the cell surface expression of NMDAR (Xu et al., 2009). EphB2/CTF2 is degraded by the proteasome (Litterst et al., 2007), suggesting the importance of EphB2/CTF2 for the process of EphB2-dependent cell repulsion.

The ubiquitin-proteasome system regulates various cellular processes, including cell-cycle progression, gene transcription, and signal transduction through the degradation of ubiquitinated proteins by proteasome (Liu et al., 2015b). Covalent attachment of ubiquitin to the substrate is achieved by ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). It is thought to be primarily responsible for substrate recognition (Skaer et al., 2014). The ECS (Elongin B/C-Cullin 5-SOCS box) protein family is a member of the largest RING finger E3 superfamily, the Cullin-RING-ligases (CRLs; Okumura et al., 2012). SOCS box consists of BH box, which recruits an adaptor protein (Elongin B and C), and Cul5 box, which binds to Cul5 S (Cul5). Cul5 is a scaffold protein and assembles multiple proteins into complexes, which include a small RING finger protein (Rbx2), Elongin B and C, and a substrate targeting protein (SOCS box protein; Kile et al., 2002; Kamura et al., 2004; Okumura et al., 2012). The SPRY domain and SOCS box-containing proteins, SPSB1, SPSB2, SPSB3, and SPSB4 (also known as SSB-1 to SSB-4), are characterized by a central SPRY domain and a C-terminal SOCS box, suggesting that the SOCS-containing protein complex may function as an ubiquitin ligase (Nicholson and Hilton, 1998; Okumura et al., 2012). In fact, SPSB1 ubiquitinates transforming growth factor-β (TGF-β) type II receptor (TβRII) and promotes prosenologal degradation to maintain TβRII at a low level (Liu et al., 2015a). More importantly, SPSB1 knockdown results in enhanced TGF-β signaling, migration, and invasion of tumor cells (Liu et al., 2015a). SPSB2 ubiquitinates inducible nitric oxide (NO) synthase (iNOS; NO2), resulting in its proteasomal degradation (Kuang et al., 2010). NO2 plays a crucial role in macrophage bactericidal and tumoricidal activities (Bogdan, 2015; Vannini et al., 2015), and SPSB2-deficient macrophages showed prolonged iNOS expression, increased NO production, and enhanced killing of Leishmania major parasites (Kuang et al., 2010). In contrast, SPSB1 and SPSB4 have a greater effect on iNOS regulation than SPSB2 (Nishiya et al., 2011). Thus, SPSB proteins are a component of the ubiquitin ligase complex.

Here, we identified EphB2 as a substrate of SPSB4 for proteasomal degradation. SPSB4 increases ubiquitination of EphB2/CTF2, and SPSB4 knockdown stabilizes EphB2-LF, but not full-length EphB2. Importantly, SPSB4 knockdown enhances cellular repulsive responses mediated by EphB2. Altogether, SPSB4 plays a crucial role in cellular repulsive responses through the degradation of the cytoplasmic domain of EphB2.

RESULTS

SPSB4 binds to EphB2

To identify novel substrates targeted by SPSB4, 3× FLAG-tagged SPSB4 was expressed and purified from 293T cell lysates, and potential SPSB4-interacting proteins were analyzed by mass spectrometry (Figure 1A). In addition to molecules expected to interact with SOCS box, such as Cul5, Elongin B, Elongin C, and Rbx2, EphB2 was identified as a SPSB4-interacting protein from an excised SDS–PAGE gel band of ∼120 kDa (Figure 1A, A and B). As shown in Figure 1A, several bands overlapped and some proteins were identified from the same gel band (data not shown). Because similar experiments were performed using other E3s and EphB2 was identified by SPSB4 pull down, but not by pull down with other E3s, the interaction between SPSB4 and EphB2 was examined further. To confirm the interaction between SPSB4 and EphB2, N-terminally 3× HA-tagged wild-type or SOCS box-deleted mutant SPSB4 was constructed (Figure 2A). We also constructed 3× HA-tagged SPSB1,
FIGURE 2: EphB2 interacts with SPSB1 and SPSB4. (A) Schematic representation of wild-type SPSB4 (WT) and SOCS box deletion mutant SPSB4 (∆SOCS box). (B) 293T cells stably expressing C-terminally FLAG-tagged EphB2 (EphB2-FLAG) were transfected with empty plasmid or plasmid encoding 3x HA-SPSB1, 3x HA-SPSB2, 3x HA-SPSB3, or SPSB4 (WT or ∆SOCS box). After 2 d, transfected cells were cultured in the presence of Bafilomycin A1 (0.5 μM) for 1 h, and then stimulated with the ligand (clustered ephrin-B2-Fc). The cells were lysed, and lysates were immunoprecipitated (IP) with an anti-HA antibody and immunoblotted (IB) with an anti-HA or anti-FLAG antibody. (C) Stimulation-independent interaction between EphB2 and 3x HA-SPSB1 or SPSB4. The experiment was performed as in A with or without EphB2 stimulation.

FIGURE 3: Endogenous EphB2 interacts with SPSB1 and SPSB4. (A) Expression of endogenous EphB2 in Colo201 cells. The cell lysates of HEK293T, Colo201, MCF10A, and HeLa cells were subjected to immunoblotting with anti-EphB2 antibody. Coomassie brilliant blue (CBB) staining is shown as a loading control. (B) The interaction between endogenous EphB2 and 3x HA-SPSB4 was analyzed by immunoprecipitation (IP) with anti-HA antibody, and immunoblotted (IB) with anti-HA antibody. (C) Difference in EphB2 interaction between SPSB1 or SPSB4 and endogenous EphB2. Colo201 is a colorectal tumor cell line that expresses relatively high levels of EphB2 mRNA (Jubb et al., 2005). However, to the best of our knowledge, EphB2 protein level has not been examined. We first examined whether Colo201 cells expressed EphB2 protein at a detectable level by Western blotting (Figure 3A). As expected, EphB2 was detected in Colo201, but not in the other examined cell lines such as HEK293T (human embryonic kidney), MCF10A (human mammary gland), or HeLa (human cervix) cell lines. Colo201 cell lines expressing 3x HA-SPSB1, 3x HA-SPSB4, or control cells were established and stimulated with ephrin-B2 in the presence of the proteasome inhibitor, MG132, to stabilize EphB2/CTF1 and CTF2 (Figure 3B). As reported previously (Litterst et al., 2007), ligand stimulation cleaved EphB2 and produced EphB2/CTF1 and CTF2, with a molecular weight of ~50 kDa. The cell lysates were subjected to immunoprecipitation with an anti-HA antibody, and the resulting immunoprecipitates were subjected to SDS–PAGE and immunoblotting with an anti-EphB2 or anti-HA antibody (Figure 3B). Because the expression levels of 3x HA-SPSB1 and 3x HA-SPSB4 were not similar in Colo201 cells, it was not clear whether SPSB4 binding to full-length EphB2 was stronger than that of SPSB1. In contrast, the interactions between EphB2/CTF1 or CTF2 and SPSB1 or SPSB4 were under the detectable level, which suggests that the affinity of SPSB1 or SPSB4 with EphB2 was relative more than that of SPSB1.

Polyubiquitination of EphB2 cytoplasmic domain by SPSB4

We confirmed previous findings that EphB2/CTF2 is degraded by the ubiquitin-proteasomal pathway (Litterst et al., 2007) by utilizing HEK293T cells stably expressing EphB2-FLAG and ephrin-B2 stimulation in the presence of MG132 (Figure 4A). Ligand stimulation
with both wild-type and mutant EphB2(CD), indicating that the kinase activity of EphB2 is not involved in the interaction with SPSB4. This result corroborates the results depicted in Figure 3B, which show the ligand stimulation–independent interaction between EphB2 and SPSB4. Altogether, our data suggested that SPSB4 is a major ubiquitin ligase targeting EphB2/CTF1 and/or CTF2 for proteasomal degradation.

Stabilization of EphB2-LF and EphB2/CTF1 and/or CTF2

We next utilized Colo201 cells to examine the physiological regulation of EphB2. SPSB4 knockdown Colo201 cell lines (#1 and #3, each targeting different sequences of SPSB4) as well as control knockdown cells were stimulated with ephrin-B2, and cells were harvested every 3 h up to 9 h (Figure 5A). SPSB4 knockdown did not affect ligand-dependent degradation of full-length EphB2. Thus, SPSB4 does not affect the cleavage of EphB2 by ligand stimulation. As reported previously, full-length EphB2 was cleaved by ligand stimulation and produced EphB2/CTF1 and/or CTF2 of around 45 kDa (Figure 5A; Litterst et al., 2007). Notably, we detected EphB2/LF and EphB2/CTF1 and/or CTF2 without ligand stimulation, which may suggest basal activation of EphB2 in Colo201 cells (Figure 5A, lanes 1, 5, and 9). As expected, SPSB4 knockdown resulted in an increase in EphB2/LF and EphB2/CTF1 and/or CTF2 (Figure 5, A–C). However, the increase in EphB2/CTF1 and/or CTF2 was significant only in the absence of exogenous ligand stimulation, and it was not significant after ligand stimulation (Figure 5B). Most
importantly, a longer cleaved product, EphB2-LF (estimated by molecular weight; Lin et al., 2008) significantly accumulated after SPSB4 knockdown in all situations examined (Figure 5C). The half-life of EphB2-CTF1 and/or CTF2 was ∼3–4 h, and that of EphB2-LF was hard to determine. These data suggest that SPSB4 degrades EphB2-LF and EphB2/CTF1 and/or CTF2, and that another ubiquitin ligase, including SPSB1, or other degradation pathways could degrade these fragments.

Effect of SPSB4 on cell segregation and repulsion

The above data prompted us to assess the biological significance of SPSB4 in cell segregation and repulsion in vivo. Activation of EphB2 by ephrin-B2 contributes to cell repulsion, and EphB2-expressing cells are segregated from ephrin-B2-expressing cells (Poliakov et al., 2008). To examine the biological significance of SPSB4, HEK293T cells stably expressing C-terminal Myc-tagged ephrin-B2 (ephrin-B2-Myc) or control cells were established (Figure 6A). As expected, ephrin-B2-Myc-His6 mainly localized to the plasma membrane. In contrast, enhanced green fluorescent protein (EGFP) was stably expressed in the control or SPSB4–knocked down Colo201 cells to distinguish Colo201 cells from ephrin-B2-expressing 293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B). HEK293T cells (2 × 10^5) and Colo201 cells (2 × 10^5) were plated in a six-well culture plate and incubated for 3 d. As expected, some control Colo201 cells formed relatively bigger colonies when cocultured with ephrin-B2-expressing HEK293T cells (Figure 6B).

Altogether, these results suggested that SPSB4 negatively regulates cell repulsion mediated by EphB2 and ephrin-B2.

DISCUSSION

In the present study, we identified EphB2 as a novel substrate of the ubiquitin ligase SPSB4. Because EphB2 activation by its
γ-secretase activity, releasing an intracellular peptide EphB2/CTF2 that contains the whole CD of EphB2 (Litterst et al., 2007). Inhibition of EphB2 cleavage prevents EphB2-mediated cell repulsion and blocks ephrin-B2–induced growth cone withdrawal in cultured hippocampal neurons (Lin et al., 2008). EphB2/CTF2 presents tyrosine kinase activity and phosphorylates downstream proteins such as NMDAR subunits in primary neuronal cultures to increase the cell surface expression of NMDAR (Xu et al., 2009). Importantly, EphB2/CTF2 is degraded by the proteasome (Litterst et al., 2007), indicating that the ubiquitin ligase targeting EphB2/CTF2 plays a crucial role in sequestering prolonged signal transduction during cell repulsion. Because SPSB4 knockdown increased the expression of EphB2-LF and EphB2/CTF1 and/or CTF2 (Figure 5), and enhanced cell repulsion (Figure 6), SPSB4 may contribute to normal cell positioning and synapse formation by regulating the protein amount of EphB2 cleaved products. Recently, it was reported that the actin-regulating pathway is required for EphB2-stimulated contact repulsion, and Rac-specific guanine nucleotide exchange factor Tiam2 was identified as a key molecule for both EphB2 and ephrinB1 transendoctysis (Gaitanos et al., 2016). Extensive studies have demonstrated opposite tumor-promoting and tumor-suppressing effects of EphB2 (Pasquale, 2008; Noberini and Pasquale, 2009; Chen, 2012; Kandouz, 2012; Gucciardo et al., 2014). EphB2 is strongly expressed in tumor cell lines (Jubb et al., 2005; Chukkapalli et al., 2014) and suppresses cancer progression (Batlle et al., 2005; Senior et al., 2010; Chukkapalli et al., 2014), indicating its tumor-suppressive function. In contrast, glioma migration and invasion are promoted by EphB2 activation (Nakada et al., 2004). These reports suggest diverse and complex functions of EphB2 in different cell types and the surrounding environment. Reverse signal transduction mediated by the ligand, ephrin, has been demonstrated (Pasquale, 2010; Park and Lee, 2015), indicating the complexity of ephrins and EphB2-mediated signal transduction. In fact, ephrin-B1 complexes with adjacent claudin 1 or claudin 4 via the extracellular domains of these proteins, and ephrin-B1 mediates the cell–cell adhesion of epithelial and cancer cells via a novel Eph receptor-independent mechanism (Tanaka et al., 2005). Furthermore, the C-terminus of ephrin-B1 regulates the exocytosis of matrix metalloproteinase-8 (MMP-8), which is a protease of ephrin-B1, in response to the interaction with EphB2, and the expression of ephrin-B1 promotes the invasion of cancer cells in vivo (Tanaka et al., 2007).

Although whether SPSB4 is inactivated or down-regulated in these tumors remains unclear, the delay in the removal of EphB2 ligand induces EphB2 cleavage within the ectodomain by MMPs such as MMP-2/MMP-9 and produces N-terminal EphB2/NTF and C-terminal EphB2-LF (Lin et al., 2008), the regulation of these cleaved products is important for appropriate signal transduction. EphB2-LF is further cleaved by MMPs and produces EphB2/CTF1 (Litterst et al., 2007; Lin et al., 2008). The remaining plasma membrane-associated EphB2/CTF1 is further cleaved by the presenilin-dependent FIGURE 6: Effect of SPSB4 knockdown on Colo201 segregation. (A) Establishment of HEK293T cells stably expressing ephrin-B2-Myc. HEK293T cells were infected with control retroviruses or viruses encoding ephrin-B2-Myc and selected with puromycin (1 μg/ml) for 1 wk. The cells were fixed and immunostained with an anti-Myc antibody. Scale bar, 10 μm. The nucleus was stained with Hoechst 33258. (B) Segregation of Colo201 cells from ephrin-B2-expressing HEK293T cells. Control or two independent SPSB4-knocked down Colo201 cell lines (#1 and #3) stably expressing EGFP were cocultured with ephrin-B2-expressing or control HEK293T cells for 3 d. The cells were fixed and Colo201 cells were monitored by visualizing the EGFP signal. Scale bar, 100 μm. The nucleus was stained with Hoechst 33258. DIC, differential interference contrast. (C) Quantification of the independent single Colo201 cell numbers. Colo201 cells present as single cells out of a colony were counted in four independent loci. * and ** indicate p < 0.03 and p < 0.01, respectively. N.S., not significant. Data represent the mean ± SD of four independent loci.
cleaved products (Figure 5) should affect tumor development or tumor suppression depending on the tissue type. EphB2 functions as a tumor suppressor by abrogating Ras activity and, consequently, the extracellular signal-regulated kinase (ERK) MAPK pathway (Elowe et al., 2001). Furthermore, suppression of the Ras-ERK-MAPK pathway is important for EphB2-mediated neurite retraction (Elowe et al., 2001). In contrast, if FGFR1 is not activated, EphB2 activates the MAPK pathway, which, in turn, promotes EphB2 activation in a positive feedback loop (Poljakov et al., 2008). FGFR1 prevents cell segregation, repulsion, and collapse mediated by EphB2 activation (Poljakov et al., 2008). Mechanistically, FGFR1 activation induces the expression of feedback antagonists of the MAPK pathway, including Sprouty genes (Masoumi-Moghaddam et al., 2014), which could inhibit EphB2-induced MAPK activation. Sprouty genes inhibit the MAPK pathway downstream from EphB2 and decrease cell repulsion and segregation (Poljakov et al., 2008). Thus, transcriptional targets of FGFR1 may prevent the feedback loop that promotes EphB2 activation and cell repulsion (Poljakov et al., 2008). Because we detected no difference in terms of ERK activation by SPsb4 knockdown in Colo201 cells (data not shown), the enhanced cell repulsion by SPsb4 knockdown might be induced by other signal pathways.

Contact of Eph receptors with ephrins is involved in the guidance of migrating cells and axons; Eph receptor activation leads to repulsion responses and inhibits entry into ligand-expressing populations (Poljakov et al., 2008; Pasquale, 2010; Wilkinson, 2014), and axon outgrowth (Santiago and Erickson, 2002). Although the exact molecular mechanisms causing these cellular responses are unknown, EphB1 senses ligand density; low density of ephrin-B1 promotes cell adhesion, while high density of ligand induces cell repulsion (Huynh-Do et al., 1999). Therefore, EphB2 may also mediate various signal transductions depending on the degree of receptor activation. In fact, the abundance of monomers, dimers, and multimers of EphB2 determines the strength of the cellular response (Schaupp et al., 2014). Monomers and dimers are essentially inactive, and multimers lead to a physiological response (Schaupp et al., 2014). The C-terminal PDZ (postsynaptic density-95/disks large/zona occludens-1)-binding motif and sterile α motif domain of EphB2 negatively regulate ephrin-B2-induced clustering by an unknown mechanism (Schaupp et al., 2014). Therefore, SPsb4 could be a candidate to regulate the oligomerization of EphB2.

Myosin 1b interacts with EphB2 and links plasma membrane and cytoskeleton (Prosperi et al., 2015). Myosin 1b regulates the redistribution of myosin II in actomyosin fibers and the formation of filopodia at the interface of ephrinB1 and EphB2 cells, which contributes to cell repulsion (Prosperi et al., 2015). Therefore, it would be interesting to examine whether EphB2 cleaved products could still interact with myosin 1b and whether SPsb4 regulates the interaction between EphB2 and myosin 1b as well as clustering of EphB2 in the future. Abnormal hyperphosphorylation of microtubule-associated protein tau is an early pathological marker of Alzheimer’s disease (AD), and glycogen synthase kinase-3β (GSK-3β) is a crucial tau kinase (Hoshi et al., 1996; Khan and Bloom, 2016). Activation of EphB2 receptor dephosphorylates tau through phosphorylase-kinase-3-kinase (PI3K) and Akt-mediated GSK-3β inhibition (Jiang et al., 2015). Thus, it is possible that EphB2 cleaved products, especially EphB2-LF, contribute to the dephosphorylation of tau and prevent AD progression. If so, increased expression of SPsb4 should worsen AD pathogenesis by down-regulating EphB2 cleaved products. SPsb4 mRNA expression is ubiquitous, and SPsb4 is expressed in embryonic stem cells, neuroepithelial cells, astrocytes, fibroblasts, epithelial cells, smooth muscle cells, hematopoietic stem cells, erythroblasts, macrophages, B-cells, T-cells, etc. (the complete list is available at BioGPS, biogps.org). Therefore, SPsb4 might have a role in neuronal cells, and it is important to examine SPsb4 expression level in AD patients in future studies.

We next compared the expression profiles of EphB2, SPsb1, SPsb2, SPsb3, and SPsb4 in several cancers by utilizing the Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov/) and cBioPortal (http://www.cbioportal.org/). The expression patterns of these genes, except SPsb4, were relatively similar in the cancers examined (Figure 7). In contrast, the expression of SPsb4 was relatively low in colorectal adenocarcinoma, lymphoid neoplasm diffuse large B-cell lymphoma, liver hepatocellular carcinoma, skin cutaneous melanoma, prostate adenocarcinoma, thyroid carcinoma, uveal melanoma, kidney renal clear cell carcinoma, and kidney renal papillary cell carcinoma. Therefore, EphB2 cytoplasmic fragments might persist in these cancers for much longer than in other cancers.

This study presents some limitations. Although we identified that SPsb4 down-regulates EphB2 cytoplasmic fragments, the data are based on in vitro experiments utilizing cancer cell lines; it is important to study the role of SPsb4 on the degradation of these fragments in vivo in the future.

In conclusion, our results demonstrate that SPsb4 interacts with EphB2 and increases polyubiquitination of the CD of EphB2. SPsb4 knockdown increases EphB2 cleaved products, especially EphB2-LF, and enhances cell repulsive responses. These results suggest that SPsb4 regulates cell repulsive responses through the degradation of EphB2. Future studies are warranted to investigate the function and activity of EphB2 cleaved products, especially EphB2-LF, to further our understanding of EphB2 tumor-promoting and tumor-suppressing activities. SPsb4 deregulation may prevent appropriate elimination of EphB2 cleaved products and might contribute to abnormal cell positioning, tumorigenesis, and AD, among others. In fact, SPsb4 mRNA expression is increased in astrocytoma, glioblastoma, and oligodendroglioma (Sun et al., 2006). Therefore, it would be important to investigate the activity of SPsb4 in these tumors. Altogether, our data suggest SPsb4 as a promising target for the development of new therapeutics to treat cancer or AD.

MATERIALS AND METHODS
Plasmid construction
Mouse EphB2 (NM_001290753), human ephrin-B2 (NM_004093), human SPsb1 (NM_025116), human SPsb2 (NM_032641), human SPsb3 (NM_080861), and human SPsb4 (NM_080862) were introduced into pcDNA3, pCI-neo, or pMX-puro. Point mutations were introduced by PCR, using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic oligonucleotides used were as follows: mouse EphB2(Y597F), 5′-CCAGGCGATGAA-GATCTTTATAGGCTTCCAC-3′ and 5′-GGTGAAAGGGCTCAT-AAAAGATCTTACGTGCGG-3′; and mouse EphB2(Y603F), 5′-ATAAG-ACCCCTTACCCCTTGAAGATCCTAATGAGG-3′ and 5′-CCTCATT-AGGATCCTTAAAGGTGAAGGCTT-3′.

Antibodies
Antibodies against FLAG (1 μg/mL; M2; Sigma-Aldrich, St Louis, MO), HA (1 μg/mL; 12CA5; Sigma-Aldrich), His6 (1 μg/mL; MAB050; R&D Systems, Minneapolis, MN), EphB2 (recognizes the extracellular region; 1 μg/mL; AF467; R&D Systems), EphB2 (recognizes the C-terminal region; 1 μg/mL; 37-1700; Invitrogen, Carlsbad, CA) were used. The rabbit anti-SPsb4 antibody was generated by using recombinant human SPsb4, which was purified from Escherichia coli by using Ni-agarose beads (149-07984; Wako Pure
MCF10A cells were provided by Chin Ha Chung (Seoul National University, Korea). Colo201 cells were provided by Reiji Kannagi (Aichi Cancer Center and Aichi Medical University, Japan). HEK293T cells were transfected with the expression plasmid using polyethylenimine (PEI; MW-25K; Polyscience, Warrington, PA), plasmid DNA (\( \mu g \)) : PEI (\( \mu g \)) = 1:3. Retroviral infections were performed as described previously (Okumura et al., 2016). Briefly, cells were incubated in retrovirus-containing culture medium for 2 d and selected by using puromycin (1 \( \mu g/ml \)) for 1 wk.

**Chemical Industries, Osaka, Japan.** Anti-SPSB4 antibody was further purified by recombinant ASB7.

**Reagents**
Cycloheximide and Hoechst 33258 were purchased from Sigma-Aldrich. Protein A sepharose was purchased from GE Healthcare Bio-Science (Piscataway, NJ) and MG132 from Peptide Institute (Osaka, Japan). Bafilomycin A1 was purchased from Wako Pure Chemical Industries.

**Cell culture and transfection**
HEK293T and HeLa cell lines were purchased from the American Type Culture Collection (Manassas, VA). HEK293T and HeLa cells were cultured as described previously (Okumura et al., 2016). MCF10A cells were provided by Chin Ha Chung (Seoul National University, Korea). Colo201 cells were provided by Reiji Kannagi (Aichi Cancer Center and Aichi Medical University, Japan). HEK293T cells were transfected with the expression plasmid using polyethylenimine (PEI; MW-25K; Polyscience, Warrington, PA), plasmid DNA (\( \mu g \)) : PEI (\( \mu g \)) = 1:3. Retroviral infections were performed as described previously (Okumura et al., 2016). Briefly, cells were incubated in retrovirus-containing culture medium for 2 d and selected by using puromycin (1 \( \mu g/ml \)) for 1 wk.

**Stimulation of EphB2**
Preclustered oligomers of ephrin-B2-Fc were generated as reported previously (Lin et al., 2008). In brief, baculoviruses encoding a chimera protein consisting of human ephrin-B2 extracellular domain and

**FIGURE 7:** The expression of EphB2, SPSB1, SPSB2, SPSB3, and SPSB4 in cancers. The relative expression levels of EphB2, SPSB1, SPSB2, SPSB3, and SPSB4 in several cancers are shown. The cancers in which the expression of SPSB4 is relatively lower are colored in blue.
the Fc portion of human immunoglobulin G1 (IgG1) were infected to SF21 cells. Three days after infection, chimera protein was purified from the culture medium by using Ni-agarose beads (149-07984; Wako Pure Chemical Industries). Chimera protein (5 μg) and goat anti-human IgG-Fc fragment antibody (2.5 μg; A80-104A; Bethyl Laboratories, Montgomery, TX) were incubated in DMEM without serum (100 μl) overnight at 4°C. Colo201 cells in six-well culture plates with 1 ml of complete culture medium were stimulated by the addition of 100 μl of preclustered ephrin-B2-Fc solution.

Immunoprecipitation and immunoblot analyses

Immunoprecipitation (IP) and immunoblot (IB) analyses were performed as reported previously (Okumura et al., 2016).

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and extensively washed with PBS. The cells were then incubated overnight at 4°C with anti-Myc antibody in PBS (1 μg/ml) containing 0.1% bovine serum albumin (BSA) and 0.1% Triton X-100. Cells were washed three times with PBS, followed by incubation with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen; 1:2000 dilution) in PBS containing 0.1% BSA and 0.1% Triton X-100 for 1 h at room temperature in the dark. The cells were further incubated with Hoechst 33258 (0.1 μg/ml) in PBS for 1 min followed by extensive washing with PBS and then photographed using a Zeiss Axio Observer Z1 microscope (Carl Zeiss, Göttingen, Germany).

Isolation and identification of ASB7-interacting proteins

The substrates of SPSB4 ubiquitin ligase were identified as described previously (Kamura et al., 2016).

Knockdown

Nonspecific control knockdown (Ryther et al., 2004) and SPSB4 knockdown were performed as described previously (Okumura et al., 2016). The target sequences for SPSB4#1 and SPSB4#3 were 5′-GCTACATCAACGGCCTTGACC-3′ and 5′-GAGCCTCAAGTCA-GTGGAGGT-3′, respectively.

Statistical analysis

The statistical significance of differences between groups was determined by one-way analysis of variance. P < 0.05 was considered statistically significant.

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