# Ubiquitin ligase SPSB4 diminishes cell repulsive responses mediated by EphB2

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**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.**

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## INTRODUCTION

Erythropoietin-producing human hepatocellular (Eph) receptor tyrosine kinases and their ligands, ephrins, guide cell migration in various processes during differentiation and development (Fagotto *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; Batlle and Wilkinson, 2012; Klein, 2012; Wilkinson, 2014). They also play crucial roles in cell invasion, contributing to tumor development (Chen, 2012; Kandouz, 2012; Gucciardo *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 (Batlle *et al.*, 2002). EphB2 and EphB3 restrict

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Abbreviations used: CBB, Coomassie brilliant blue; CD, cytoplasmic domain; CRL, Cullin-RING-ligase; CTF, C-terminal fragment; Cul, Cullin; DIC, differential interference contrast; ECS, Elongin B/C-Cullin 5-SOCS box protein; EGFP, enhanced green fluorescent protein; Eph, erythropoietin-producing human hepatocellular; ERK, extracellular signal-regulated kinase; GPI, glycosylphosphatidylinositol; HEK, human embryonic kidney; IB, immunoblot; IP, immunoprecipitate; LF, long fragment; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NTF, N-terminal fragment; RTK, receptor tyrosine kinase; SPSB, SPRY domain and SOCS box; TCGA, The Cancer Genome Atlas; TGF, transforming growth factor.

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 EphB2-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 elimination to sequester prolonged signal transduction. However, the responsible ubiquitin ligase targeting EphB2/CTF2 for the proteasomal degradation has not been identified.

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 attributed by ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). E3 is thought to be primarily responsible for substrate recognition (Skaar *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 BC box, which recruits an adaptor protein (Elongin B and C), and Cul5 box, which binds to Cullin 5 (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 SPSB-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 proteasomal 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; NOS2), resulting in its proteasomal degradation (Kuang *et al.*, 2010). iNOS 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 1, 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,

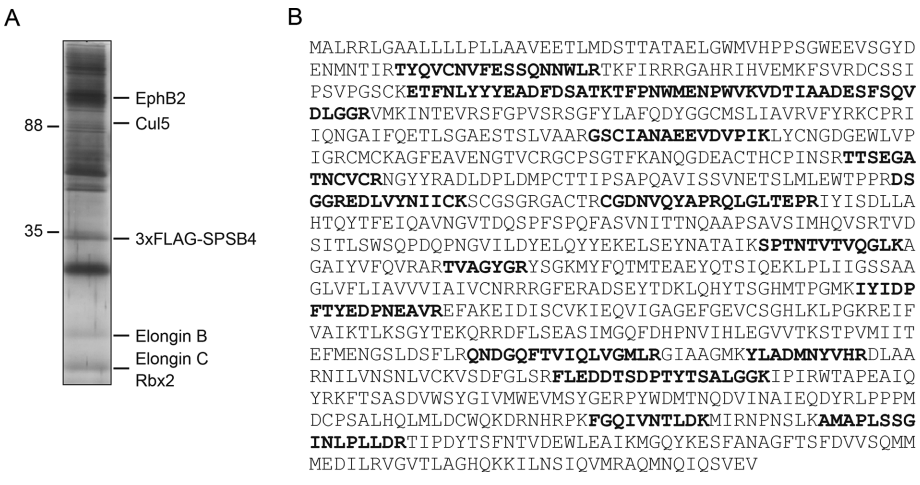


FIGURE 1: Identification of EphB2 as an SPSB4-interacting protein. (A) 3× FLAG-SPSB4 expressed in HEK293T cells was purified by using an anti-FLAG antibody and resolved by SDS–PAGE. SPSB4-interacting proteins were analyzed by mass spectrometry. (B) Identified peptides of EphB2 are indicated in boldface.

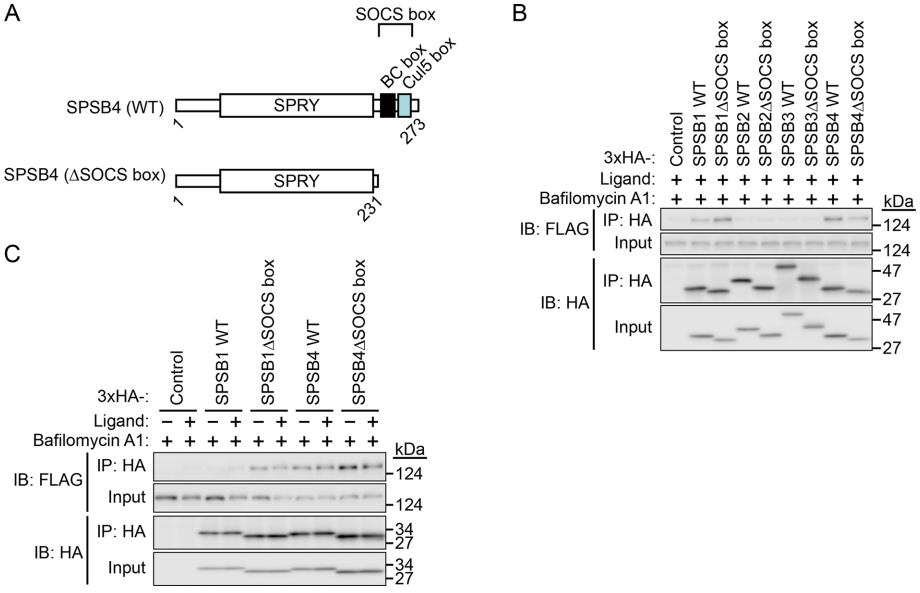


**FIGURE 2:** EphB2 interacts with SPSB1 and SPSB4. (A) Schematic representation of wild-type SPSB4 (WT) and SOCS box deletion mutant SPSB4 (ΔSOCS box). (B) 293T cells stably expressing C-terminally FLAG-tagged EphB2 (EphB2-FLAG) were transfected with empty plasmid or plasmid encoding 3× HA-SPSB1, SPSB2, SPSB3, or SPSB4 (WT or ΔSOCS 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 3× HA-SPSB1 or SPSB4. The experiment was performed as in A with or without EphB2 stimulation.

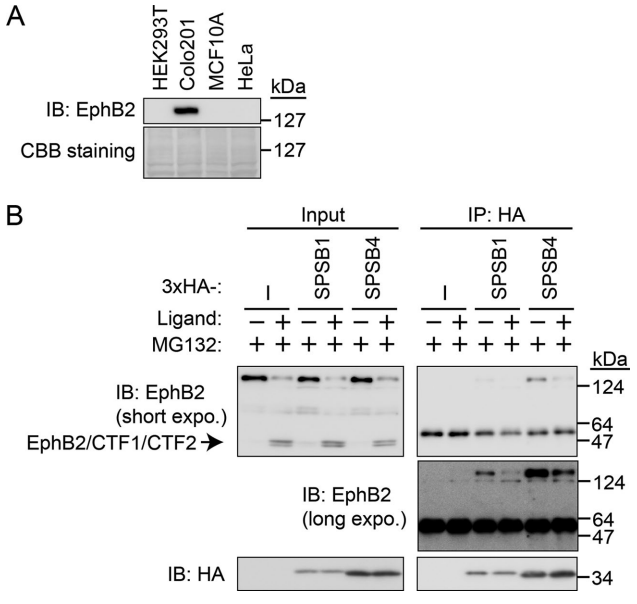


**FIGURE 3:** Endogenous EphB2 interacts with SPSB1 and SPSB4. (A) Expression of endogenous EphB2 in Colo201 cells. The cell lysates of HEK293T, Colo201, MCF10A, and HeLa cells were subjected to immunoblotting with anti-EphB2 antibody. Coomassie brilliant blue (CBB) staining is shown as a loading control. (B) The interaction between endogenous EphB2 and 3× HA-SPSB4 in Colo201 cells. Colo201 cells stably expressing 3× HA-SPSB1 or 3× HA-SPSB4, and control cells were cultured in the presence of MG132 (10 μM for 1 h), and then stimulated with the ligand (clustered ephrin-B2-Fc) for 7 h in the presence of MG132. The cell lysates were lysed, immunoprecipitated (IP) with anti-HA antibody, and immunoblotted (IB) with anti-HA or anti-EphB2 antibody (recognizes the C-terminus of EphB2).

SPSB2, or SPSB3 (3× HA-SPSB1, 3× HA-SPSB2, or 3× HA-SPSB3) as either wild type or SOCS box-deleted mutant. These mutants were expressed in HEK293T cells stably expressing C-terminally FLAG-tagged EphB2 (EphB2-FLAG; Figure 2B). To assess the physiological interaction between SPSB proteins and EphB2, cells were stimulated by ephrin-B2 in the presence of Bafilomycin A1 (inhibitor of endosomal acidification) to block EphB2 cleavage (Litterst *et al.*, 2007). Results showed that SPSB1 and SPSB4 interacted with EphB2 and that SOCS box was not involved in the interaction (Figure 2B). We next examined the effect of ligand stimulation on the interaction between SPSB1 or SPSB4 and EphB2 and found that ligand stimulation did not significantly affect this interaction (Figure 2C). The interaction between SPSB4 and EphB2 was stronger than that between SPSB1 and EphB2. Although there are conserved domains among SPSB proteins, SPSB1 and SPSB4 are considerably similar at the amino acid level among the SPSB family (Kleiber and Singh, 2009). Thus, both proteins seemed to be able to interact with EphB2 when overexpressed. Then, we compared the 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 3× HA-SPSB1, 3× 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 3× HA-SPSB1 and 3× 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 full-length EphB2 is relatively more stable than that between EphB2/CTF1 and CTF2. Altogether, these findings showed that SPSB1 and SPSB4 interact with EphB2.

### 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

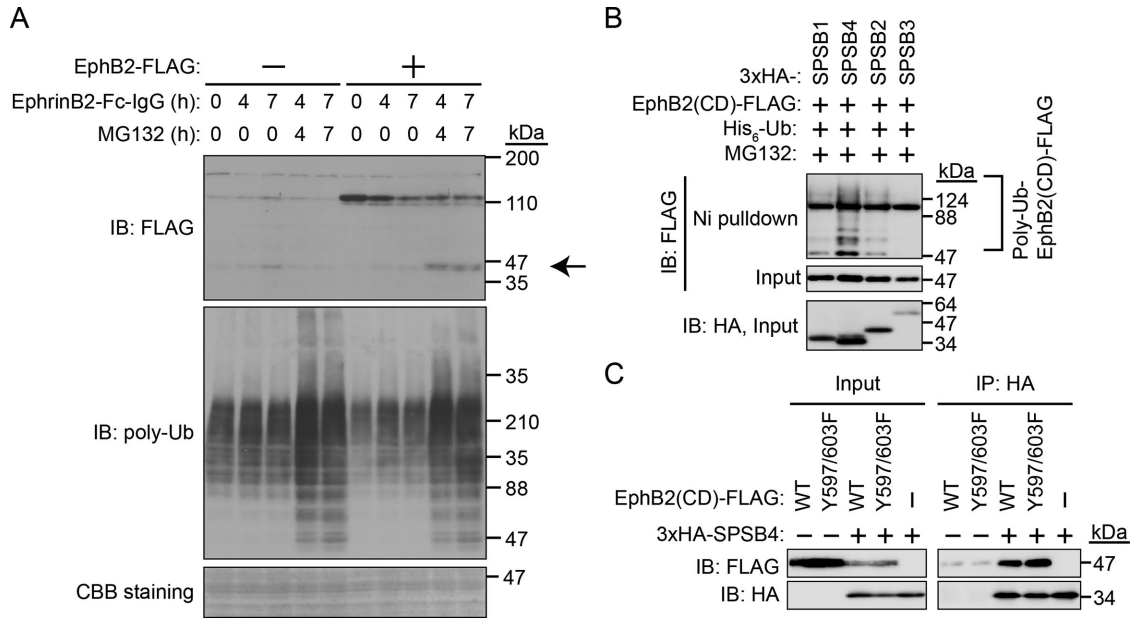


**FIGURE 4:** Degradation of EphB2/CTF1 and/or CTF2 by the ubiquitin-proteasome pathway. (A) Accumulation of EphB2/CTF1 and/or CTF2 after EphB2 stimulation and incubation with the proteasome inhibitor, MG132. Control or EphB2-FLAG expressing HEK293T cells were stimulated with the ligand (clustered ephrin-B2-Fc) for 4 or 7 h in the presence or absence of MG132. The lysates were subjected to Western blotting with antibodies against FLAG or polyubiquitin (FK2). Coomassie brilliant blue (CBB) staining is shown as a loading control. Arrow denotes EphB2/CTF1 and/or CTF2. (B) Polyubiquitination of EphB2-cytoplasmic domain (CD) in vivo. HEK293T cells were transfected with plasmids encoding 3× HA-SPSB1, 3× HA-SPSB2, 3× HA-SPSB3, or 3× HA-SPSB4 with mouse EphB2(CD)-FLAG and $His_6$-ubiquitin (Ub). MG132 (2 μM for 14 h) was used to detect polyubiquitination. Cell lysates were subjected to Ni-agarose pull down to purify proteins modified by $His_6$-ubiquitin, followed by immunoblot analysis with an anti-HA or FLAG antibody. (C) EphB2 kinase activity-independent interaction with SPSB4. HEK293T cells were transfected with plasmids encoding 3× HA-SPSB4 with or without mouse EphB2(CD)-FLAG (wild-type or Y597/603F). After 2 d, 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.

induced the degradation of full-length EphB2-FLAG, and it was not stabilized by MG132. In contrast, EphB2/CTF1 and/or CTF2 accumulated upon ligand stimulation in the presence of MG132. Then, we examined the ubiquitination of EphB2 cytoplasmic domain by utilizing $His_6$-tagged ubiquitin (Figure 4B). HEK293T cells were transfected with plasmid encoding mouse EphB2 cytoplasmic domain (CD; 571-987 amino acids), 3× HA-SPSB protein, and $His_6$-tagged ubiquitin. The cells were cultured in the presence of MG132 and lysed in the presence of 8 M urea to disrupt protein–protein interactions. $His_6$-tagged ubiquitin was pulled down using Ni-agarose beads. After SDS–PAGE and immunoblotting, nitrocellulose membrane was blotted by anti-FLAG antibody to detect covalently ubiquitin-modified EphB2(CD)-FLAG, but not EphB2(CD)-interacting proteins. Although the expression levels of SPSB proteins were not similar, SPSB4 increased polyubiquitination of EphB2(CD) (Figure 4B). We next examined whether the kinase activity of EphB2 is involved in the interaction with SPSB4. Phosphorylation of Tyr597 and Tyr603 of murine EphB2 is required for the kinase activity of EphB2, and mutation of these tyrosine residues to phenylalanine blocks kinase activity (Holland *et al.*, 1997; Zisch *et al.*, 1998; Wybenga-Groot *et al.*, 2001). We expressed either wild-type or Y597/603F double mutant EphB2(CD) in HEK293T cells with or without SPSB4. 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-FLAG or anti-HA antibody (Figure 4C). SPSB4 was found to interact 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

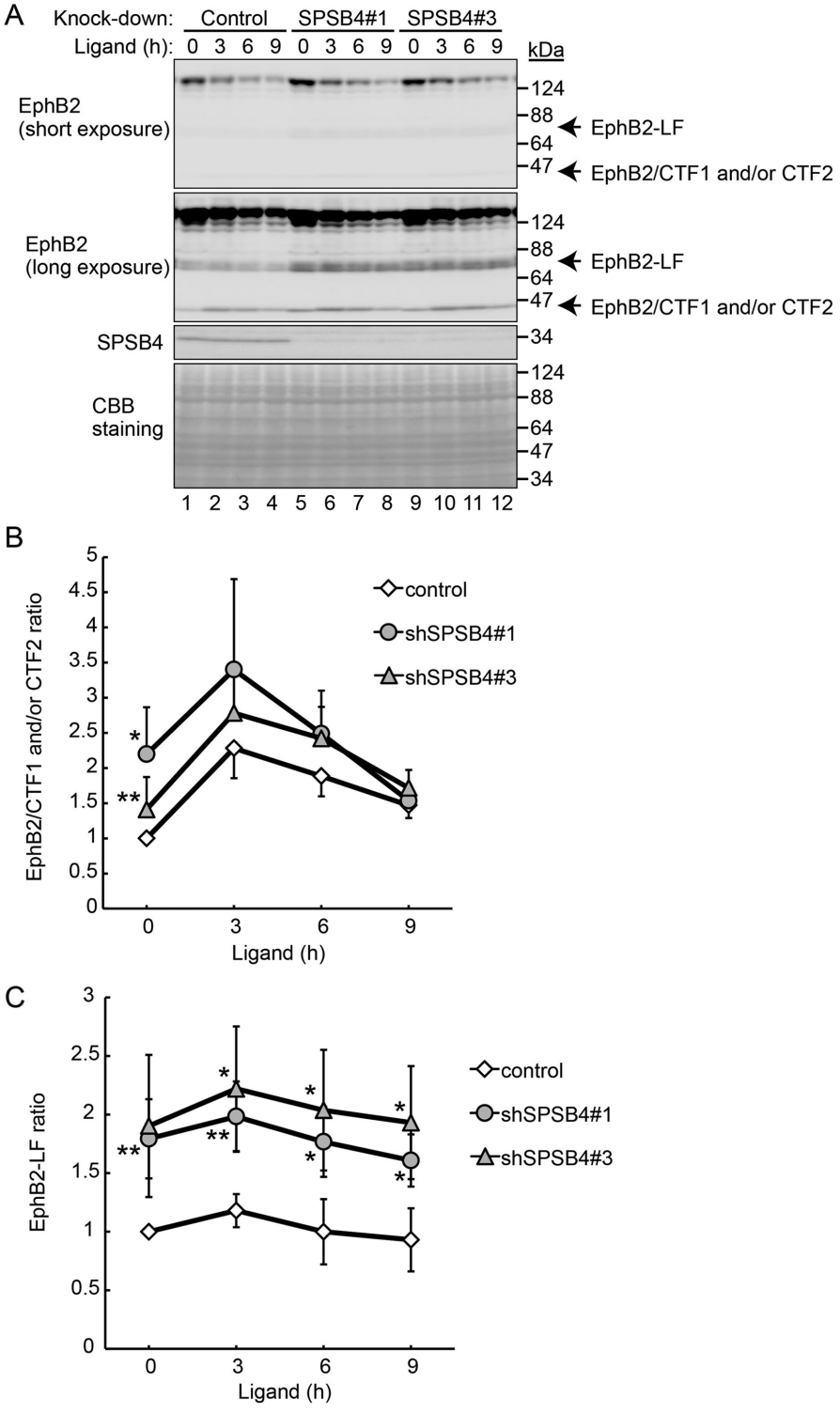


FIGURE 5: Accumulation of EphB2/LF and CTF1 and/or CTF2 after SPSB4 knockdown in Colo201 cells. (A) Control or two independent SPSB4-knocked down Colo201 cell lines (#1 and #3) were stimulated with the ligand (clustered ephrin-B2-Fc) for 3, 6, or 9 h. The cell lysates were lysed and immunoblotted with an anti-EphB2 (recognizes the C-terminus of EphB2) or SPSB4 antibody. CBB staining is shown as a loading control. (B) Quantification of EphB2/CTF1 and/or CTF2. The signals of EphB2/CTF1 and/or CTF2 shown in A were quantified. Control sample without ligand stimulation was set as 1. * and ** indicate $p < 0.03$ and $p < 0.01$, respectively. Data represent the mean ± SD of three independent experiments. (C) Quantification of EphB2-LF. The signals of EphB2-LF shown in A were quantified. Control sample without ligand stimulation was set as 1. * and ** indicate $p < 0.03$ and $p < 0.01$, respectively. Data represent the mean ± SD of three independent experiments.

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-$His_6$ 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 ($2 \times 10^5$) and Colo201 cells ($2 \times 10^5$) 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 than when cocultured with control HEK293T cells (Figure 6B). It should be noted that a similar number of Colo201 cells remained intermingled with ephrin-B2-expressing or control 293T cells (Figure 6, B and C). In contrast, SPSB4–knocked down Colo201 cells (#1 or #3) formed relatively bigger colonies than control Colo201 cells when cultured with ephrin-B2-expressing 293T cells. Furthermore, less SPSB4–knocked down Colo201 cells remained intermingled with ephrin-B2-expressing 293T cells, indicating that cell repulsive signals in SPSB4–knocked down Colo201 cells were greater than that in control Colo201 cells (Figure 6, B and C). 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

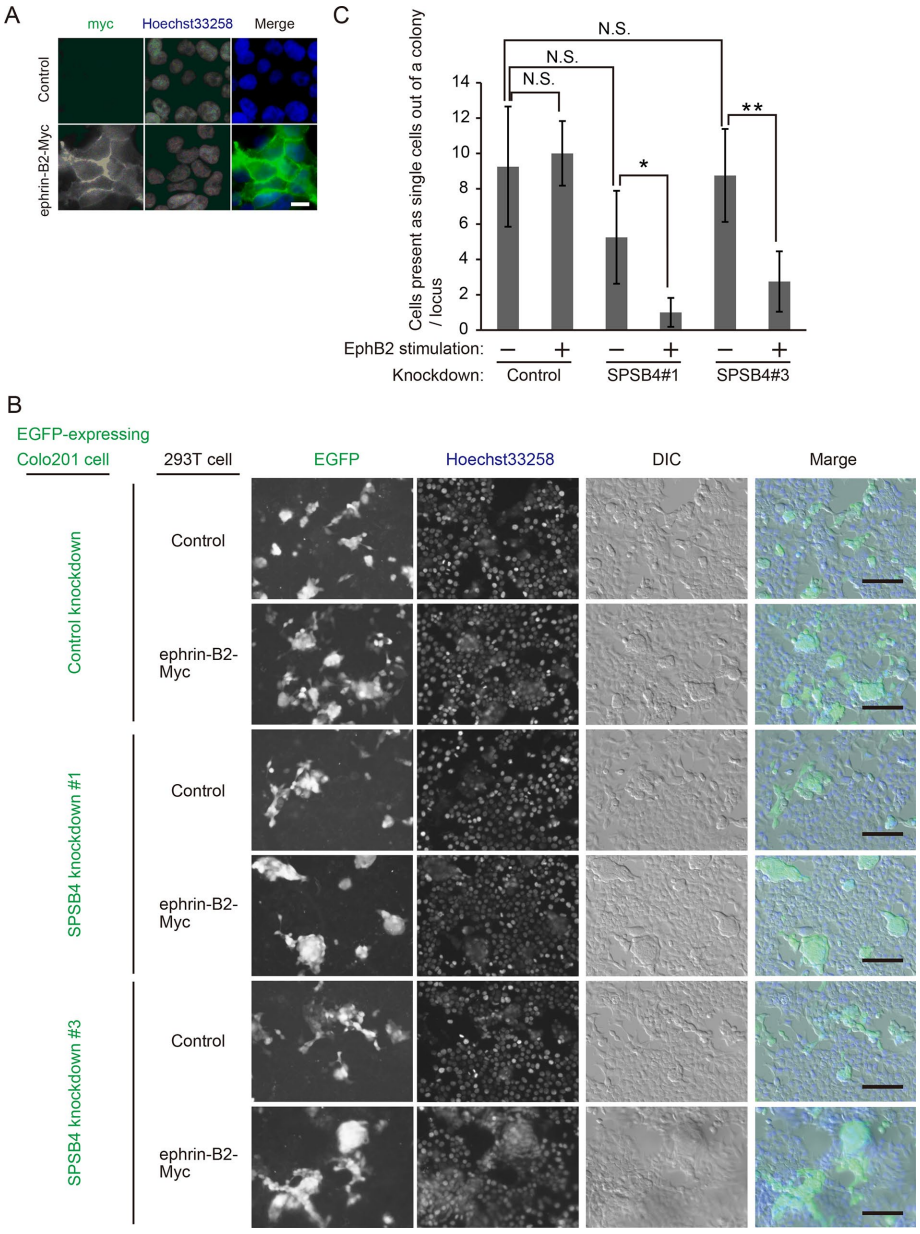


**FIGURE 6:** Effect of SPSB4 knockdown on Colo201 segregation. (A) Establishment of HEK293T cells stably expressing ephrin-B2-Myc. HEK293T cells were infected with control retroviruses or viruses encoding ephrin-B2-Myc and selected with puromycin (1 μg/ml) for 1 wk. The cells were fixed and immunostained 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.

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 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 transendocytosis (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 mediate 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

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 (Poliakov *et al.*, 2008). FGFR1 prevents cell segregation, repulsion, and collapse mediated by EphB2 activation (Poliakov *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 (Poliakov *et al.*, 2008). Thus, transcriptional targets of FGFR1 may prevent the feedback loop that promotes EphB2 activation and cell repulsion (Poliakov *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 (Poliakov *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 phosphatidylinositol-3-kinase (PI3K) and Akt-mediated GSK-3β inhibition (Jiang *et al.*, 2015). Thus, it is possible that EphB2 cleaved products, especially EphB2/CTF2, 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_025106), 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′-CCAGGCATGAAGATCTTTATAGACCCTTTCACC-3′ and 5′-GGTGAAAGGGTCTATAAAGATCTTCATGCCTGG-3′; and mouse EphB2(Y603F), 5′-ATAGACCCTTTCACCTTTGAAGATCCTAATGAGG-3′ and 5′-CCTCATTAGGATCTTCAAAGGTGAAAGGGTCTAT-3′.

### Antibodies

Antibodies against FLAG (1 μg/ml; M2; Sigma-Aldrich, St Louis, MO), HA (1 μg/ml; 12CA5; Sigma-Aldrich), $His_6$ (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

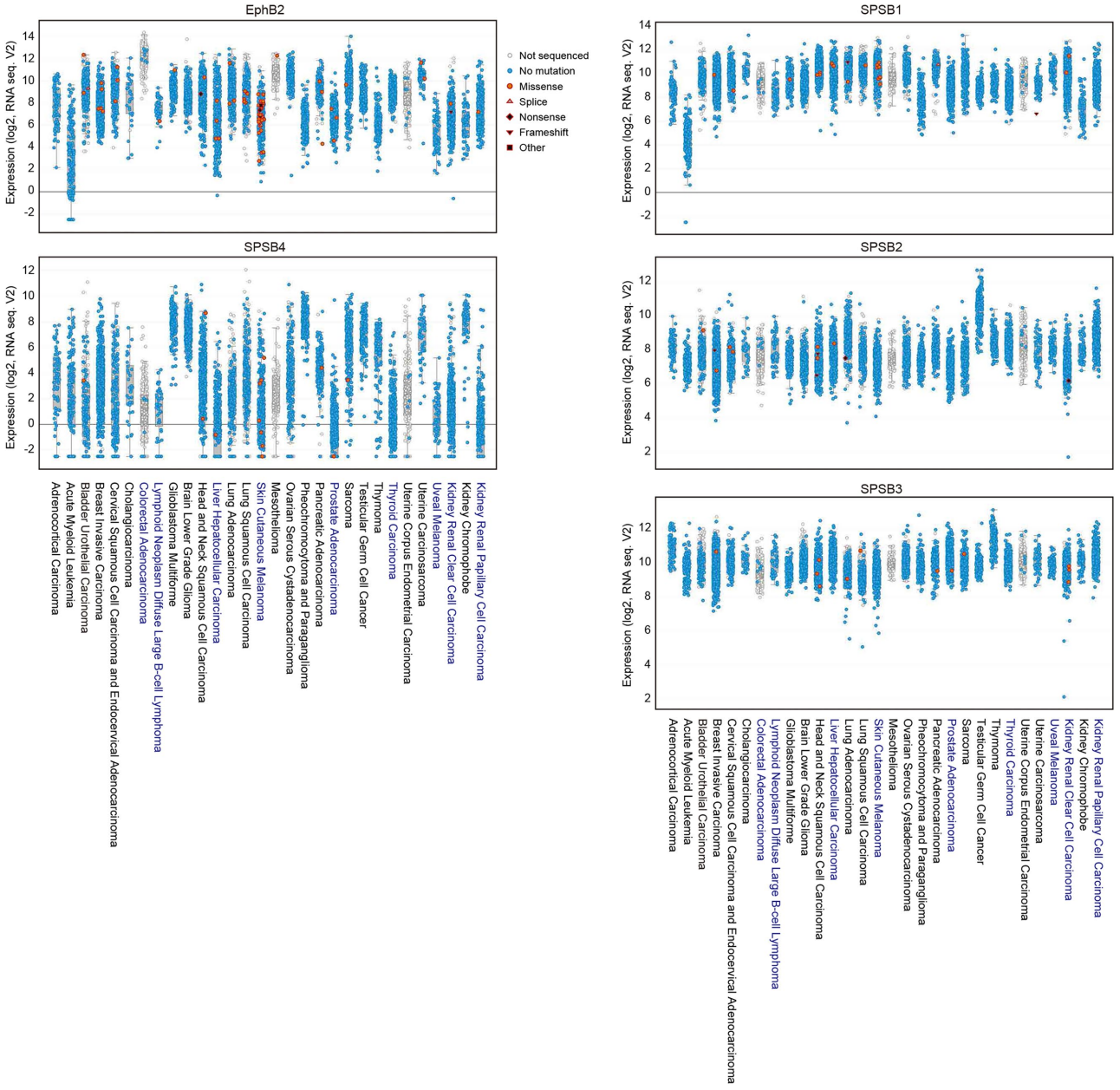


FIGURE 7: The expression of EphB2, SPSB1, SPSB2, SPSB3, and SPSB4 in cancers. The relative expression levels of EphB2, SPSB1, SPSB2, SPSB3, and SPSB4 in several cancers are shown. The cancers in which the expression of SPSB4 is relatively lower are colored in blue.

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 Bioscience (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 (μg):PEI (μg) = 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 μg/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

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 by 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.*, 2004).

### 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′-GAGCCTCAAGTCAGTGGAGGT-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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