Regulation of Hook1-mediated endosomal sorting of clathrin-independent cargo by γ-taxilin

Satoru Higashi[1], Tomohiko Makiyama[1, 2], Hiroshi Sakane[1, 3], Satoru Nogami[1, 4], and Hiromichi Shirataki[1, *]

[1]Department of Molecular and Cell Biology, Graduate School of Medicine, Dokkyo Medical University, 880 Kitakobayashi, Mibu-machi, Tochigi 321-0293, Japan
[2]Present address: Division of Biological Chemistry, Department of Pharmaceutical Sciences, Showa University School of Pharmacy, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan
[3]Present address: Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan
[4]Present address: Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

*****Corresponding author: Hiromichi Shirataki
Department of Molecular and Cell Biology, Graduate School of Medicine, Dokkyo Medical University, 880 Kitakobayashi, Mibu-machi, Tochigi 321-0293, Japan.
Tel: +81-282-87-2140, Fax: +81-282-86-3533, Email: hiro-sh@dokkyomed.ac.jp

Running title: γ-Taxilin controls CIE cargo sorting

Keywords: Clathrin-independent endocytosis, Endosomal sorting, Hook1, Recycling endosome, Taxilin

Summary statement

In clathrin-independent endocytosis, γ-taxilin negatively regulates the sorting of Hook1-mediated cargo proteins into recycling endosomes by interfering with the interactions between Hook1 and the cargo proteins.

Abstract

In clathrin-independent endocytosis, Hook1, a microtubule- and cargo-tethering protein, participates in sorting of cargo proteins such as CD98 and CD147 into recycling endosomes. However, the molecular mechanism that regulates Hook1-mediated endosomal sorting is not fully understood. Here, we found that γ-taxilin is a novel regulator of Hook1-mediated endosomal sorting. γ-Taxilin depletion promoted both CD98-positive tubular formation and CD98 recycling. Conversely, overexpression of γ-taxilin inhibited the CD98-positive tubular formation. Depletion of Hook1, or Rab10 or Rab22a (which are both involved in Hook1-mediated endosomal sorting), attenuated the effect of γ-taxilin depletion on the CD98-positive tubular formation. γ-Taxilin depletion promoted CD147-mediated spreading of HeLa cells, suggesting that γ-taxilin may be a pivotal player in various cellular functions in which Hook1-mediated cargo proteins are involved. γ-Taxilin bound to the *C*-terminal region of Hook1 and inhibited its interaction with CD98; the latter interaction is necessary for sorting CD98. We suggest that γ-taxilin negatively regulates the sorting of Hook1-mediated cargo proteins into recycling endosomes by interfering with the interactions between Hook1 and the cargo proteins.

Introduction

Endocytosis is involved in the maintenance of cellular homeostasis and the regulation of fundamental cellular functions including nutrient uptake, cell adhesion, cell migration, cell polarity, cytokinesis, and receptor signaling (Grant and Donaldson, 2009). Endocytosis is regulated by a series of trafficking processes including the internalization, sorting, degradation, and recycling of macromolecules. On the basis of the requirement for the coat protein clathrin for vesicle formation and internalization, endocytosis is classified into clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) (Grant and Donaldson, 2009). CME has been studied extensively and is involved in the selective internalization of plasma membrane proteins such as transferrin and low-density lipoprotein receptor, both of which have distinct cytoplasmic sorting sequences that are recognized by adaptor proteins of the clathrin coat. After internalization, CME cargo proteins undergo a sorting process leading to transport to lysosomes for degradation, the *trans* Golgi network, or recycling endosomes (REs) for return to the cell surface. CIE, by contrast, had been studied as a non-selective bulk endocytic entry mechanism, and until 20 years ago, CIE cargo proteins did not appear to have a mechanism for selective internalization. However, increasing evidence has shown that CIE has a variety of entry mechanisms including caveolar endocytosis, RhoA-associated endocytosis, Cdc42-associated endocytosis, Arf6-associated endocytosis, and flotillin-associated endocytosis (Mayor et al., 2014). Moreover, it has been revealed in parallel with the growing number of known CIE cargo proteins that CIE cargo proteins are involved in a variety of vital physiological processes, including immune surveillance, cell migration, metastasis, and cell signaling. As the trafficking itineraries of endocytosed CIE cargo proteins, i.e., transport to

lysosomes for degradation or REs for return to the cell surface, are thought to participate in regulation of the activities of the CIE cargo proteins, the molecular mechanisms of the endocytic trafficking processes of CIE after internalization have been studied extensively over the past decade.

The list of CIE cargo proteins identified so far is diverse, and includes major histocompatibility complex class I (MHC-I), glycosylphosphatidylinositol anchored proteins (GPI-APs; CD55 and CD59), proteins involved in interactions with the extracellular matrix (CD44, CD98, CD147, E-cadherin, and β1-integrin), ion channels (Mucolipin2 and Kir3.4), and nutrient transporters (CD98 and Lat1) (Grant and Donaldson, 2009; Mayor et al., 2014). It has been revealed that there are at least two endocytic trafficking pathways for CIE cargo proteins after internalization (Maldonado-Báez et al., 2013a,b). CIE cargo protein-loaded vesicles first mature into or fuse with Rab5-positive early endosomes (EEs). Then, a group of CIE cargo proteins (MHC-I and CD55) is transported to Rab5- and early endosome-associated antigen 1 (EEA1)-positive EEs, followed by co-localization with transferrin, a CME cargo protein. From there, the CIE cargo proteins are transported to late endosomes (LEs)/lysosomes for degradation, or to REs for recycling back to the plasma membrane. Another group of CIE cargo proteins (CD44, CD98, and CD147) is directly sorted into REs and recycled back to the plasma membrane in a Hook1- and Rab22a-dependent manner.

In mammals, the Hook family is comprised of Hook1, Hook2, and Hook3. Hook1 was initially identified in *Drosophila melanogaster* as a pivotal player in the delivery of internalized proteins to LEs (Krämer and Phistry, 1996; Sunio et al., 1999). Hook1 has a long coiled-coil region and interacts with microtubules and organelle membranes through its *N*-terminal and *C*-terminal regions, respectively, to facilitate the attachment

of organelles to microtubules (Krämer and Phistry, 1999). Loss of Hook1 function in mice results in ectopic positioning of microtubular structures within the spermatid and causes an abnormal spermatozoon head shape phenotype (Mendoza-Lujambio et al., 2002). Hook2 is localized at centrosomes through its interaction with CEP110, and participates in ciliogenesis (Baron Gaillard et al., 2011; Szebenyi et al., 2007). Hook3 participates in localization of the Golgi apparatus through its interaction with microtubules (Walenta et al., 2001). It has been revealed that Hook1 plays a role as a microtubule- and cargo-tethering factor in the recycling of some CIE cargo proteins (CD44, CD98, and CD147), but not of MHC-I or CD55 (Maldonado-Báez et al., 2013a,b). Hook1 interacts with the cytoplasmic sequence of CD98 and CD147 through its *C*-terminal domain and facilitates their recycling in coordination with Rab22a and the microtubule network (Maldonado-Báez et al., 2013a).

The Rab family, a member of the Ras small G protein superfamily, includes at least 70 members in mammals and is implicated in intracellular vesicle trafficking, such as exocytosis, endocytosis, and transcytosis (Zhen and Stenmark, 2015). A group of Rab family members including Rab5, Rab8a, Rab10, Rab11, Rab21, Rab22a, and Rab35 is implicated in endocytic trafficking processes of CIE cargo proteins (Del Olmo et al., 2019; Dutta and Donaldson, 2015; Etoh and Fukuda, 2019; Grant and Donaldson, 2009; Rahajeng et al., 2012; Sharma et al., 2009; Solis et al., 2013; Weigert et al., 2004). Together with Hook1 and microtubules, Rab22a functions in recycling to the plasma membrane of CIE cargo proteins, including CD44, CD98, and CD147. It has been shown that while REs appear as both vesicular and tubular endosomes, the tubular formation of REs is crucial for the recycling back to the plasma membrane of CIE cargo

proteins, and that Rab22a mediates the formation of tubular REs in coordination with Hook1 and the microtubule network (Maldonado-Báez et al., 2013a).

The taxilin family is composed of at least three members, α-, β-, and γ-taxilin, which share a *C*-terminal long coiled-coil region (Nogami et al., 2004). In mammals, α- and γ-taxilin are ubiquitously expressed (Horii et al., 2014; Nogami et al., 2003), while β-taxilin is specifically expressed in skeletal muscle and heart (Sakane et al., 2016). Extensive studies by our group and others over the past decade have uncovered the function of the taxilin family members as follows. α-Taxilin was initially identified as a binding partner of the syntaxin family that participates in intracellular vesicle trafficking. It is also involved in the recycling pathway of the transferrin receptor (TfnR) through interaction with sorting nexin 4 (Sakane et al., 2014). Moreover, α-taxilin participates in intracellular trafficking of hepatitis B virus DNA-containing particles (Hoffmann et al., 2013). Interestingly, α-taxilin is specifically expressed in proliferating cells of the gastrointestinal tract (Horii et al., 2014) and overexpressed in various tumor types such as hepatocellular carcinoma, renal cell carcinoma, and glioblastoma (Mashidori et al., 2011; Oba-Shinjo et al., 2005; Ohtomo et al., 2010), suggesting that α-taxilin might be involved in cell proliferation. β-Taxilin participates in differentiation of C2C12 myoblasts into myotubes by suppressing the function of dysbindin (Sakane et al., 2016). γ-Taxilin is localized to the centrosome during interphase and participates in Nek2A-mediated centrosome disjunction as a negative regulator (Makiyama et al., 2018). Moreover, γ-taxilin (also known as FIAT) is implicated in hypoxia-induced endoplasmic reticulum stress responses (Hotokezaka et al., 2015) and interacts with activating transcription factor 4 (ATF4) to repress its transcriptional activity in osteoblasts (Yu et al., 2005, Yu et al., 2008).

The function of α-taxilin in intracellular vesicle trafficking prompted us to examine whether γ-taxilin is also involved in processes of intracellular vesicle trafficking. In the present study, we identify Hook1 as a novel binding partner of γ-taxilin. Depletion of γ-taxilin prompted tubular formation of REs harboring CD98 or CD147, Hook1-mediated cargo proteins, leading to enhancement of the recycling of these cargo proteins back to the plasma membrane. Moreover, depletion of γ-taxilin prompted CD147-mediated spreading of HeLa cells, probably by enhancing Hook1-mediated recycling of CD147 back to the plasma membrane. Conversely, overexpression of γ-taxilin inhibited the tubular formation of CD98-positive REs through interaction with the *C*-terminal region of Hook1 competitively with CD98. We propose that γ-taxilin negatively regulates the sorting of Hook1-mediated CIE cargo proteins into REs by interfering with the interactions between Hook1 and CIE cargo proteins.

Results

γ-Taxilin interacts with Hook1 and Hook2

As α-taxilin interacts with sorting nexin 4 and participates in the recycling pathway of TfnR (Sakane et al., 2014), we postulated that γ-taxilin might also be involved in intracellular vesicle traffic. To test this possibility, we first attempted to identify γ-taxilin-interacting molecules by use of yeast two-hybrid screening using full-length γ-taxilin as bait and found that Hook1, a microtubule- and cargo-tethering protein, was a potential binding partner of γ-taxilin. Hook1 interacted with γ-taxilin but not α- or β-taxilin (Fig. 1A). When we examined the interactions between taxilin family members and other Hook family proteins, only γ-taxilin interacted with Hook2, and none of the taxilins interacted with Hook3 (Fig. 1A). Consistently, when human influenza hemagglutinin (HA)-tagged α-, β-, or γ-taxilin and myc-tagged Hook1, Hook2, or Hook3 were co-expressed in HeLa cells and the cell lysates were immunoprecipitated with anti-HA or anti-myc antibodies, Myc-Hook1 and -Hook2 were co-immunoprecipitated with HA-γ-taxilin and *vice versa* (Fig. 1B). Although it is possible that the interaction of γ-taxilin with Hook2 may provide a clue to the role of γ-taxilin in centrosomes, we focused here on the interaction of γ-taxilin with Hook1 and proceeded with examinations to reveal whether γ-taxilin is implicated in Hook1-related intracellular vesicle trafficking. We first attempted to detect the interaction between endogenous γ-taxilin and Hook1 using an immunoprecipitation assay and found that endogenous Hook1 was co-immunoprecipitated with endogenous γ-taxilin (Fig. 1C). Immunocytochemical analysis revealed that endogenous γ-taxilin and Hook1 showed a similar staining pattern (Fig 1D). Since both of available anti-γ-taxilin and anti-Hook1 antibodies were unfortunately produced in rabbit, we examined whether GFP-γ-taxilin

and myc-Hook1 are co-localized in HeLa cells. Immunocytochemical analysis showed that the staining patterns of GFP-γ-taxilin and myc-Hook1 were similar to those of the corresponding endogenous proteins and GFP-γ-taxilin was co-localized with myc-Hook1 (Fig. 1E).

γ-Taxilin depletion promotes the tubular formation of Hook1-mediated cargo proteins

Hook1 was originally discovered in *Drosophila melanogaster* as a microtubule- and cargo-tethering protein for the delivery of plasma membrane receptors to late endosomal compartments, and increasing evidence highlights a role of Hook1 in the sorting of a subset of CIE cargo proteins (e.g., CD98, CD147) into REs in mammals (Maldonado-Báez et al., 2013a). We examined by use of small interfering RNA (siRNA) whether γ-taxilin is involved in the Hook1-mediated sorting of CIE cargo proteins into REs. The efficiency of γ-taxilin depletion was first assessed by immunoblotting. In γ-taxilin siRNA-treated cells, γ-taxilin was depleted below the detection level (Fig. 2A), whereas other proteins including Hook1, CD98, CD147, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were not affected (Fig. S1). To label the Hook1-mediated CIE system, siRNA-treated cells were incubated with an anti-CD98 antibody at 37°C for 1 h to allow internalization of the antibody-bound CD98, and internalized CD98 was specifically detected by immunostaining as described in Materials and Methods (Fig. 2B). Internalized CD98 was found in discrete punctate structures in control cells but also found in tubular structures in about 35% of these cells (Fig. 2C). There was no difference in the expression levels of γ-taxilin between cells with or without CD98-positive tubular structures (data not shown). γ-Taxilin depletion

caused an ~1.8-fold increase in the percentage of cells exhibiting CD98-positive tubular structures (Fig. 2C). As described previously, these tubular structures are not an artifact of immunostaining (Weigert et al., 2004), and they were also visualized by time-lapse imaging of living cells (see below). A similar result was obtained using another γ-taxilin siRNA. Moreover, when siRNA rescue experiments were performed using cells expressing siRNA-resistant γ-taxilin, the effect of γ-taxilin siRNA treatment on the formation of CD98-positive tubular structures was attenuated in pEGFPC1-γ-taxilin-mt-transfected cells but not pEGFPC1-γ-taxilin-transfected cells (Fig 2. E,F). GFP-γ-taxilin was expressed in pEGFPC1-γ-taxilin-mt-transfected cells but not pEGFPC1-γ-taxilin-transfected cells (Fig. 2D). Together, the effect of γ-taxilin depletion on the formation of CD98-positive tubular structures is not due to an off-target effect of siRNA treatment. These results suggest that γ-taxilin is involved in the Hook1-mediated sorting of CIE cargo protein CD98 into REs. We thus examined by use of siRNA whether γ-taxilin also affects the Hook1-mediated sorting of another CIE cargo protein into REs. When a similar experiment to that described above was performed using anti-CD147 antibody instead of anti-CD98 antibody, γ-taxilin depletion caused an ~1.8-fold increase in the percentage of cells exhibiting CD147-positive tubular structures (Fig. 2G,H).

It has been revealed that besides the CIE cargo proteins that are sorted into REs mediated by Hook1, another subset of CIE cargo proteins (e.g., MHC-I, CD55) are sorted into REs independently of Hook1 (Maldonado-Báez et al., 2013a). We performed similar experiments to those described above to determine whether γ-taxilin is involved in the sorting of MHC-I and CD55 into REs. γ-Taxilin depletion did not affect the percentage of cells exhibiting MHC-I- or CD55-positive tubular structures (Fig 2. G,H).

Taken together, we suggest that γ-taxilin specifically participates in the Hook1-mediated tubular formation of CIE cargo proteins.

The effect of γ-taxilin depletion on Hook1-mediated tubular formation is attenuated by co-depletion of Hook1, Rab10, or Rab22a

To validate the involvement of γ-taxilin in Hook1-mediated sorting of CIE cargo proteins into REs, we examined whether Hook1 depletion affects the effect of γ-taxilin depletion on the formation of CD98-positive tubular structures. In Hook1 siRNA-treated cells, Hook1 was depleted below the detection level, whereas γ-taxilin and GAPDH were not affected (Fig. 3A). γ-Taxilin depletion did not affect the expression level of Hook1 (Fig. 3A). Hook1 depletion alone significantly decreased the percentage of cells exhibiting CD98-positive tubular structures (Fig. 3B,C). Simultaneous depletion of Hook1 and γ-taxilin significantly decreased the percentage of cells exhibiting CD98-positive tubular structures, to a similar level to that observed on Hook1 depletion alone, while γ-taxilin depletion increased the percentage of those cells (Fig. 3B,C).

It has been revealed that Rab22a participates in Hook1-mediated sorting of CIE cargo proteins into REs, and shown that Rab22a depletion decreases the percentage of cells exhibiting CD98-positive tubular structures (Maldonado-Báez et al., 2013a). To further test the possibility that γ-taxilin participates in Hook1-mediated sorting of CIE cargo proteins into REs, we examined whether Rab22a depletion affects the effect of γ-taxilin depletion on the formation of CD98-positive tubular structures. In Rab22a siRNA-treated cells, Rab22a was depleted below the detection level, whereas γ-taxilin and GAPDH were not affected (Fig. 3A). γ-Taxilin depletion did not affect the

expression level of Rab22a (Fig. 3A). Rab22a depletion alone significantly decreased the percentage of cells exhibiting CD98-positive tubular structures (Fig. 3B,C). Simultaneous depletion of Rab22a and γ-taxilin significantly decreased the percentage of cells exhibiting CD98-positive tubular structures, to a similar level to that observed on Rab22a depletion alone (Fig. 3B,C).

Moreover, it has recently been shown that Rab10 is involved in the tubular formation of MICAL-L1-positve REs (MICAL-L1 is an RE marker), and that overexpressed Rab10 co-localizes with CD147 at tubular REs (Etoh and Fukuda, 2019). We speculated that Rab10 is also involved in Hook1-mediated sorting of CIE cargo proteins into REs and performed similar experiments to those described above but using Rab10 siRNA instead of Rab22a siRNA. In Rab10 siRNA-treated cells, Rab10 was depleted below the detection level, whereas γ-taxilin and GAPDH were not affected (Fig. 3A). γ-Taxilin depletion did not affect expression level of Rab10 (Fig. 3A). Rab10 depletion alone significantly decreased the percentage of cells exhibiting CD98-positive tubular structures (Fig. 3B,C). Simultaneous depletion of Rab10 and γ-taxilin significantly decreased the percentage of cells exhibiting CD98-positive tubular structures, to a similar level observed on Rab10 depletion alone (Fig. 3B,C). Taken together, we suggest that γ-taxilin functionally interacts with Hook1 in the Hook1-mediated tubular formation of CIE cargo proteins.

γ-Taxilin depletion promotes the biogenesis of CD98-positive tubules

On the basis of the results above, γ-taxilin might regulate the biogenesis of CD98-positive tubules. To test the possibility, we first prepared an Alexa Fluor 488-labelled anti-CD98 antibody and cells treated with control or γ-taxilin siRNA were

incubated with the labelled anti-CD98 antibody at 37°C for 1 h to allow internalization of antibody-bound CD98. After washing out antibody remaining on the cell surface, the labelled antibody-bound CD98 was observed. At the start of observation (time 0:00), internalized CD98 prominently appeared in discrete punctate structures, but CD98-positive tubules were also observed in each treated cell. These observations are consistent with the immunostaining pattern of internalized CD98 shown in Fig. 2B. To analyze the formation of CD98-positive tubules, time-lapse images were captured, at 10-s intervals over 10 min (Fig. 4A and Movie 1,2). We measured the length of newly formed CD98-positive tubules during the period of observation; these tubules were categorized into five groups by their maximum length (<5 μm, 5–10 μm, 11–15 μm, 16–20 μm, and >20 μm). In our experimental conditions, γ-taxilin depletion resulted in an ~1.9-fold increase in the average number of newly formed CD98-positive tubules per cell (Fig. 4B), and the number of newly formed CD98-positive tubules >20 μm long significantly increased in γ-taxilin-depleted cells compared with control cells (Fig. 4C). In addition, we examined whether γ-taxilin depletion affects the frequency of CD98-positive tubule collapse. There was no significant difference in the frequency of CD98-positive tubule collapse between control and γ-taxilin-depleted cells (data not shown). These results indicate that γ-taxilin regulates the number and length of CD98-positive tubules, suggesting that γ-taxilin may inhibit the biogenesis of Hook1-mediated cargo protein-positive tubules.

γ-Taxilin depletion promotes the recycling of CD98 to the plasma membrane

Our results so far cannot eliminate the possibility that γ-taxilin might be indirectly involved in the Hook1-mediated biogenesis of tubular structures by affecting the

302 internalization of CIE cargo proteins. Thus, we next examined by use of siRNA whether
303 γ-taxilin affects the internalization of CD98. To label CD98 on the cell surface, cells
304 treated with control or γ-taxilin siRNA were incubated with anti-CD98 antibody for 1 h
305 at 4°C. After washing out unbound antibody, the cells were further incubated for
306 various time periods at 37°C to allow internalization of the antibody-bound CD98. To
307 measure the intensity of antibody-bound CD98 on the cell surface before its
308 internalization (time zero), cells were fixed and stained without permeabilization. There
309 was no difference in the intensity of antibody-bound CD98 on the cell surface between
310 control and γ-taxilin siRNA-treated cells (Fig. S2A,B). Next, to measure the intensity of
311 internalized antibody-bound CD98, cells were fixed and stained with permeabilization.
312 The intensity of internalized antibody-bound CD98 increased in a time-dependent
313 manner and was not altered in γ-taxilin-depleted cells compared with control cells (Fig.
314 S2A,C). This result indicates that γ-taxilin is not involved in the internalization of CD98,
315 suggesting that γ-taxilin directly affects the Hook1-mediated biogenesis of tubular
316 structures.
317 Taken together with the report that increased Hook1-mediated biogenesis of tubular
318 structures results in increased recycling of Hook1-mediated cargo proteins to the plasma
319 membrane (Maldonado-Báez et al., 2013a), our results raise the possibility that γ-taxilin
320 may affect the recycling of Hook1-mediated cargo proteins to the plasma membrane.
321 Thus, we examined by use of siRNA whether γ-taxilin affects the recycling of CD98 to
322 the plasma membrane. Cells treated with control or γ-taxilin siRNA were incubated
323 with anti-CD98 antibody for 1 h at 37°C to allow internalization of the antibody-bound
324 CD98. After washing out antibody remaining on the cell surface, the cells were further
325 incubated for various time periods at 37°C to allow recycling of the internalized

antibody-bound CD98 to the plasma membrane. As antibody-bound CD98 recycled back to the plasma membrane remains on the cell surface, the cells were fixed and stained without permeabilization to detect it. To detect antibody-bound CD98 in intracellular compartments, cells were fixed and stained with permeabilization after washing out antibody recycled back to the cell surface. The intensity of internalized antibody-bound CD98 before its recycling to the plasma membrane (time zero) was not altered in γ-taxilin siRNA-treated cells compared with control cells (Fig. S3A). Then, the intensity of internalized antibody-bound CD98 at time zero was set to 1.0 for control and γ-taxilin-depleted cells respectively, and the intensities of antibody-bound CD98 were determined at various time periods relative to that of internalized antibody-bound CD98 at time zero. The intensities of antibody-bound CD98 remaining in the intracellular compartments and antibody-bound CD98 recycled back to the plasma membrane are shown in Fig. 5E,F, respectively. In γ-taxilin-depleted cells, the intensity of antibody-bound CD98 remaining in the intracellular compartments significantly decreased in a time-dependent manner compared with control cells (Fig. 5A,E). Conversely, in γ-taxilin-depleted cells, the intensity of antibody-bound CD98 recycled back to the plasma membrane significantly increased in a time-dependent manner compared with control cells (Fig. 5B,F). Taken together with the evidence that the increase in the intensity of antibody-bound CD98 recycled back to the plasma membrane was proportional to the decrease in the intensity of antibody-bound CD98 remaining in the intracellular compartments, these results indicate that γ-taxilin depletion promotes the recycling of CD98 to the plasma membrane.

We also examined whether γ-taxilin affects the recycling of MHC-I, which is sorted into REs independently of Hook1, to the plasma membrane. γ-Taxilin depletion did not

affect the recycling of MHC-I to the plasma membrane (Figs. 5C,D,G,H, S3B), suggesting that γ-taxilin specifically participates in the recycling of Hook1-mediated cargo proteins to the plasma membrane.

Finally, to determine whether γ-taxilin participates in the recycling of CME cargo proteins to the plasma membrane, we examined the effect of γ-taxilin depletion on the recycling of endocytosed TfnR to the plasma membrane. After serum starvation for 30 min at 37°C, cells treated with control or γ-taxilin siRNA were incubated with Alexa Fluor 594-labeled transferrin (Tfn-594) for 1 h at 37°C. After washing out cell surface Tfn-594, the cells were further incubated for various time periods at 37°C to monitor recycling of Tfn-594 to the plasma membrane (Fig. S4A). The intensity of Tfn-594 at time zero was not affected by γ-taxilin depletion (Fig. S4B). The intensity of Tfn-594 at time zero was set to 1.0 for control and γ-taxilin-depleted cells respectively, and the intensity of Tfn-594 at various time periods relative to that at time zero is shown in Fig. S4C. γ-Taxilin depletion did not affect the recycling of Tfn-594 to the plasma membrane (Fig. S4C), suggesting that γ-taxilin is not involved in the recycling of CME cargo proteins to the plasma membrane.

γ-Taxilin depletion promotes HeLa cell spreading mediated by CD147

The recycling of CD98 and CD147 to the plasma membrane participates in the activation of β1-integrin/focal adhesion kinase (FAK) signaling mediated by cell spreading in human hepatocellular carcinoma SMMC-7721 cells (Wu et al., 2015; Wu et al., 2016). We examined by use of siRNA whether γ-taxilin is involved in the activation of β1-integrin/FAK signaling mediated by spreading of HeLa cells. The phosphorylation of FAK on Tyr397, which is known as an initial step in the activation

of β1-integrin/FAK signaling mediated by cell spreading (Huveneers and Danen, 2009), was assessed by immunoblotting. γ-Taxilin depletion did not affect the basal FAK phosphorylation level in HeLa cells (Fig. S5A,B). However, when cells were detached from culture dishes followed by replating on glass coverslips to allow spreading on them, FAK phosphorylation levels were significantly increased in a time-dependent manner in γ-taxilin-depleted cells compared with control cells (Fig. 6A,B). Taken together with the result that γ-taxilin depletion promotes the recycling of CD98 to the plasma membrane, these results suggest that γ-taxilin depletion may enhance the activation of β1-integrin/FAK signaling mediated by cell spreading through recycling of CD98 to the plasma membrane.

It has been revealed that the recycling of CIE cargo proteins to the plasma membrane is required for spreading of HeLa cells and that Hook1 depletion inhibits cell spreading (Maldonado-Báez et al., 2013a). As γ-taxilin depletion enhanced the activation of β1-integrin/FAK signaling mediated by cell spreading, it is possible that γ-taxilin may participate in cell spreading through the recycling of CIE cargo proteins to the plasma membrane. We thus examined by use of siRNA whether γ-taxilin is involved in spreading of HeLa cells (Fig. 6C,D). To assess cell spreading, cell area was measured. The average cell area was significantly increased in a time-dependent manner in γ-taxilin-depleted cells compared with control cells (Fig. 6E). Consistently, the average cell area at each time point was significantly lower for Hook1-depleted cells than control cells (Fig. 6E). The effect of γ-taxilin depletion on cell spreading was attenuated by simultaneous depletion of Hook1 (Fig. 6E). These results suggest that γ-taxilin is involved in spreading of HeLa cells by affecting the recycling of CIE cargo proteins that are sorted by Hook1.

We examined by use of siRNA which CIE cargo proteins are involved in spreading of HeLa cells. CD147 depletion significantly inhibited cell spreading, while CD98 depletion hardly affected cell spreading (Figs. 6E, S6). Next, we examined by use of siRNA whether CD147 is involved in the γ-taxilin-mediated cell spreading. The effect of γ-taxilin depletion on cell spreading was attenuated by simultaneous depletion of CD147 (Fig. 6E). In the above experiments, the treatment of cells with siRNA(s) induced depletion of the corresponding (target) proteins to below the detection level, but hardly affected the expression levels of untargeted proteins (Fig. 6C). Unfortunately, we could not assess the recycling of CD147 to the plasma membrane because anti-CD147 antibody on the cell surface could not be removed by acid washing, but it is possible that γ-taxilin depletion promotes the recycling to the plasma membrane of not only CD98 but also CD147. Our results suggest that γ-taxilin depletion promotes spreading of HeLa cells by enhancing the Hook1-mediated recycling of CD147 to the plasma membrane, probably leading to the activation of β1-integrin/FAK signaling.

γ-Taxilin binds to the *C*-terminal region of Hook1 competitively with CD98 and CD147

To test the possibility that γ-taxilin inhibits the Hook1-mediated biogenesis of tubular structures, we examined whether overexpression of γ-taxilin inhibits the formation of CD98-positive tubular structures. Overexpression of GFP-γ-taxilin, but not GFP, caused a significant decrease in the percentage of cells exhibiting CD98-positive tubular structures compared with control cells (Fig. 7A,B). CD98-positive tubular structures were prominently formed in GFP overexpressing cells rather than GFP-γ-taxilin overexpressing cells (Fig. 7A). The result raises the issue whether overexpression of

γ-taxilin affects the subcellular localization of CD98 or not. It has been reported that Hook1 depletion exhibiting the effect on the formation of CD98-positive tubular structures similar to that of γ-taxilin overexpression redirects CD98 to the EEA1-positive endosomes from the tubules associated with recycling (Maldonado-Báez et al., 2013a). Then, we examined whether overexpression of γ-taxilin enhanced the co-localization of CD98 with EEA1-positive endosomes. CD98 was hardly co-localized with EEA1-positive endosomes in control cells (Fig. S7A,C). Overexpression of GFP-γ-taxilin, but not GFP, caused a significant increase in the co-localization of CD98 with EEA1-positive endosomes (Fig. S7A,C). When a similar experiment was performed using anti-CD147 antibody instead of anti-CD98 antibody, a similar result was obtained (Fig. S7B,C). Additionally, we examined using subcellular fractionation whether overexpression of γ-taxilin affects the subcellular localization of Hook1 or not. Hook1 was almost present in the cytosol fraction in control cells. Overexpression of either GFP or GFP-γ-taxilin did not affect the subcellular localization of Hook1 (Fig. S7D). These results prompted us to clarify how γ-taxilin affects the function of Hook1 in the formation of CD98-positive tubular structures. Hook1 has domain organization: *N*-terminal microtubule-binding, central coiled-coil clusters, and *C*-terminal organelle-binding (Lee et al., 2018). First, we examined which region of Hook1 is necessary for its interaction with γ-taxilin. When various myc-tagged Hook1 truncation mutants were co-expressed with HA-γ-taxilin in HeLa cells and the cell lysates were immunoprecipitated with an anti-myc antibody, HA-γ-taxilin was immunoprecipitated with myc-Hook1 (residues 1–728), myc-Hook1 (residues 167–728), and myc-Hook1 (residues 486–728), but not myc-Hook1 (residues 1–166) or myc-Hook1 (residues 1–485) (Fig. 7C,D). Consistent with the above result, when we performed a yeast

446 two-hybrid screen using full length γ-taxilin as bait, we captured a 603-nucleotide-long
447 cDNA fragment coding for the *C*-terminal region of Hook1 (residues 528–728).
448 Next, we examined which region of Hook1 is necessary for its interaction with CD98,
449 and whether CD98 interacts with γ-taxilin. When myc-Hook1 (residues 1–728) and
450 myc-Hook1 (residues 486–728) were respectively co-expressed with Flag-CD98 in
451 HeLa cells and the cell lysates were immunoprecipitated with an anti-Flag antibody,
452 both were immunoprecipitated with Flag-CD98 (Fig. 7E). When HA-γ-taxilin was
453 co-expressed with Flag-CD98 in HeLa cells and the cell lysates were
454 immunoprecipitated with an anti-Flag antibody, we were unable to detect HA-γ-taxilin
455 co-immunoprecipitated with Flag-CD98 (Fig. 7F). These results indicate that γ-taxilin
456 and CD98 bind to the same region of Hook1, suggesting the possibility that they
457 competitively bind to Hook1.
458 Finally, to validate this possibility, we examined whether γ-taxilin interferes with the
459 interaction between Hook1 and CD98 or CD147. When Flag-CD98 and myc-Hook1
460 were co-expressed with or without HA-γ-taxilin in HeLa cells and the cell lysates were
461 immunoprecipitated with anti-Flag antibody, the amount of myc-Hook1
462 co-immunoprecipitated with Flag-CD98 was significantly reduced in the cells
463 expressing HA-γ-taxilin compared with control cells (Fig. 7G). When a similar
464 experiment was performed using pcDNA3-Flag-CD147 instead of pcDNA3-Flag-CD98,
465 a similar result was obtained (Fig. S8). To further strengthen the possibility, we
466 performed the competitive binding experiment using various amounts of
467 pcDNA3-HA-γ-taxilin. The expression levels of HA-γ-taxilin were increased in a
468 dose-dependent manner but the expression levels of either Flag-CD98 or myc-Hook1
469 were not changed (Fig. 7H). The amounts of myc-Hook1 co-immunoprecipitated with

Flag-CD98 were decreased in antiparallel with increasing expression levels of HA-γ-taxilin (Fig. 7H). Together, these results suggest that γ-taxilin interferes in the interaction of Hook1 with CD98 in the step where Hook1 recognizes and subsequently sorts CD98 to REs, leading to inhibition of the recycling of CD98 from sorting endosomes (SEs) to REs.

Discussion

γ-Taxilin depletion enhanced the recycling to the plasma membrane of CD98 but not MHC-I or TfnR, a CME cargo protein. Because it is practically difficult to detect only CD147 recycled back to the plasma membrane, we failed to show the effect of γ-taxilin depletion on recycling of CD147 to the plasma membrane, but it is possible that γ-taxilin regulates the recycling of CD147 as well as CD98. In human hepatocellular carcinoma SMMC-7721 cells, both CD98 and CD147 participate in cell spreading through the activation of β1-integrin/FAK signaling (Wu et al., 2015; Wu et al., 2016). However, in HeLa cells, as examined here, CD147 depletion significantly inhibited cell spreading, but CD98 depletion hardly inhibited spreading, suggesting that CD147 but not CD98 is involved in spreading of HeLa cells. γ-Taxilin depletion prompted cell spreading, and the effects of γ-taxilin depletion on cell spreading were attenuated by co-depletion of CD147 or Hook1. Taken together with the result that γ-taxilin depletion promoted cell adhesion-dependent phosphorylation of FAK on Tyr397 downstream of integrin signaling, it is possible that γ-taxilin participates in cell spreading through the regulation of Hook1-mediated sorting of CD147 from SEs into REs, probably affecting the activity of CD147-associated integrin.

Increasing evidence has shown that distinct cytoplasmic sequences of cargo proteins are involved not only in their internalization into cells but also in their sorting in endosomes. It has been revealed that SNX27 associated with SNX-BAR, retromer, and WASP complex is involved in endosomal recycling of CME cargo proteins harboring PDZ-interacting sequence such as β2 adrenergic receptor, MCT1, and ATP7A (Lauffer et al., 2010; Steinberg et al., 2013; Temkin et al., 2011). Subsequently, it has been shown that the same SNX27 complex is also involved in endosomal recycling of CIE

499 cargo proteins such as GLUT1 and CD147 (Steinberg et al., 2013) and that other
500 distinct cytoplasmic sequences of CIE cargo proteins than PDZ-interacting sequence are
501 involved in such sorting (Maldonado-Báez et al., 2013a). CIE cargo proteins entering
502 cells through vesicular structures associated with Arf6 are at first transported to
503 Rab5-positive EEs. From the endosomes, a group of CIE cargo proteins commonly
504 harboring clusters of acidic amino acids in their cytoplasmic tail (CD44, CD147, and
505 CD98) are directly transported to REs for recycling back to the plasma membrane, thus
506 avoiding degradation in lysosomes, while another group of CIE cargo proteins not
507 harboring the acidic clusters (MHC-I, CD55, and Tac) is transported to Rab5- and
508 EEA1-positive endosomes via a default route, followed by transport to LEs/lysosomes
509 for degradation, or to REs for recycling back to the plasma membrane
510 (Maldonado-Báez et al., 2013a). It has been revealed that the binding of Hook1 through
511 its *C*-terminal region to the acidic clusters of CD147 is involved in the sorting of
512 CD147 from Rab5-positive EEs into REs (Maldonado-Báez et al., 2013a). Our present
513 results strengthen the importance of the binding of Hook1 through its *C*-terminal region
514 to the acidic clusters of CD98 and CD147 in the sorting of endocytosed these cargo
515 proteins into REs.
516 We propose that, at least, γ-taxilin works at the initiation step where Hook1 recognizes
517 and directly sorts CIE cargo proteins from SEs into REs (Fig. 8). Two other proteins
518 that interact with the *C*-terminal region of Hook1, the Vps18 subunit of the homotypic
519 vesicular protein sorting (HOPS) complex and AKT-interacting protein (AKTIP), an E2
520 ubiquitin-conjugating enzyme also known as fused toes (FTS), have been identified
521 (Richardson et al., 2004; Xu et al., 2008). However, our present findings provide the
522 first evidence that a Hook1-interacting protein plays a role in sorting of CIE cargo

proteins into REs. We do not know whether γ-taxilin affects the interaction of Hook1 with Vps18 or AKTIP in the sorting step from EEs to LEs/lysosomes. Further studies are necessary to evaluate the possibility that γ-taxilin is involved in trafficking of endocytosed cargo proteins from EEs to LEs/lysosomes.

Rab proteins are key regulators in various steps of the endocytic pathway (Zhen and Stenmark, 2015) and increasing evidence has shown that several Rab proteins participate in RE dynamics. Rab10, Rab11, and Rab22a are involved in the formation of KIF13A-positive tubular REs; KIF13A is an RE marker (Delevoye et al., 2014; Etoh and Fukuda, 2019; Patel et al., 2021). Rab10 and Rab35 participate in the formation of MICAL-L1-positive tubular structures (Etoh and Fukuda, 2019; Rahajeng et al., 2012). It has been revealed that Rab22a is involved in sorting of CD98 and CD147 from SEs into REs (Maldonado-Báez et al., 2013a). Overexpression of Rab22a restores the formation of CD147-positive tubular structures in cells expressing a dominant-negative mutant of Hook1 in which the formation of CD147-positive tubular structures is decreased. Conversely, Hook1 overexpression restores the formation of CD147-positive tubular structures in cells expressing a dominant-negative mutant of Rab22a in which the formation of CD147-positive tubular structures is decreased (Maldonado-Báez et al., 2013a). On the basis of the reciprocal rescue of their respective dominant-negative phenotypes, it has been proposed that Rab22a works co-operatively with Hook1 in the same step of recycling of CIE cargo proteins back to the plasma membrane (Maldonado-Báez et al., 2013a). Our present result that simultaneous depletion of Rab22a and γ-taxilin attenuated the effect of γ-taxilin depletion on the formation of CD98-positive tubular structures supports our proposal that γ-taxilin negatively regulates the Hook1-mediated sorting of endocytosed CIE cargo proteins into REs. The

formation of MHC-I-positive tubular structures in cells expressing dominant-negative and dominant-active mutants of Rab22a is inhibited and enhanced, respectively, and both mutants inhibit recycling of MHC-I to the plasma membrane (Weigert et al., 2004). As in addition to larger MHC-I-positive tubular structures, MHC-I-positive vesicles at the cell periphery are observed in cells expressing the dominant-active mutant of Rab22a, it is thought that Rab22a cycling between GTP-bound active and GDP-bound inactive states is required for recycling of MHC-I from SEs to the plasma membrane (Weigert et al., 2004). CD98-positive peripheral vesicles similar to the MHC-I-positive peripheral vesicles in cells expressing the dominant-active mutant of Rab22a were not observed in γ-taxilin-depleted cells. Therefore, it is unlikely that γ-taxilin depletion inhibits the fusion of Hook1-mediated CIE cargo protein-loaded vesicles with the plasma membrane. On the other hand, our present result that simultaneous depletion of Rab10 with γ-taxilin attenuates the effect of γ-taxilin depletion on the formation of CD98-positive tubular structures raises the possibility that Rab10 is also involved in the formation and maintenance of Hook1-assocaited CIE cargo protein-positive tubular structures.

Nakamura and colleagues have shown that γ-taxilin is involved in hypoxia-induced endoplasmic reticulum (ER) stress responses in a GSK-3β-dependent manner (Hotokezaka et al., 2015). We do not know whether the ER stress responses induced by depletion of γ-taxilin indirectly participate in the regulation of the Hook1-mediated endocytic pathway, but it seems unlikely as γ-taxilin depletion was reported to induce ER stress responses followed by apoptosis in HeLaS3 cells 72–96 h after transfection but all our experiments were performed at earlier timepoints. Taken together with the evidence that CD147, a subunit of γ-secretase, is involved in the production of

571 amyloid-β related to the pathogenesis of Alzheimer's disease (AD) (Zhou et al., 2005),
572 the finding that the expression level of γ-taxilin is decreased not only in mouse brain
573 slices cultured in hypoxic conditions but also in the brain of patients with AD
574 (Hotokezaka et al., 2015) suggests that γ-taxilin might be implicated in the pathology of
575 AD by affecting the recycling of CD147 to the plasma membrane as well as its
576 involvement in ER stress responses. St-Arnaud and colleagues have shown that γ-taxilin
577 (also known as FIAT) present in the nucleus inhibits the transcriptional activity of
578 acting transcription factor 4 (ATF4), inducing the differentiation of osteoblasts (Yu et
579 al., 2005; Yu et al., 2008). We have shown that γ-taxilin localized to centrosomes
580 temporally regulates centrosome disjunction in an Nek2A-dependent manner
581 (Makiyama et al., 2018). Moreover, our finding suggesting the interaction of γ-taxilin
582 with Hook2, which is localized to centrosomes, implies an unraveled function of
583 γ-taxilin in centrosomes. Thus, accumulating evidence strongly supports that γ-taxilin
584 has diverse functions, and it is important to comprehensively reveal the relationships
585 between these functions.
586 CD98 and CD147, which are directly sorted from SEs into REs by Hook1, are
587 implicated in various physiological and pathological processes. CD147 is involved in
588 lymphocyte responsiveness, spermatogenesis, implantation, fertilization, and
589 neurological functions, and pathology including rheumatoid arthritis, atherosclerosis,
590 and tumor metastasis (Muramatsu, 2016). CD98 and CD147 on the cell surface
591 associate with and regulate the activities of various proteins such as transporters,
592 integrins, and matrix metalloproteinases (Muramatsu, 2016). On the basis of our present
593 findings suggesting that γ-taxilin may modulate the number of CD98 and CD147
594 molecules residing on the cell surface by regulating their recycling back from SEs to the

595 plasma membrane, it is possible that γ-taxilin is involved in these CD98- and
596 CD147-related physiological and pathological processes. In our current model (Fig. 8),
597 the molecular mechanism by which the interaction between γ-taxilin and Hook1 is
598 regulated is not clear. However, since it has been reported that Arf6 is involved in
599 HGF-induced cell spreading (Hongu et al., 2015) and that Arf6 participates in endocytic
600 recycling of CD147 (Qi et al., 2019), it is possible that the interaction between γ-taxilin
601 and Hook1 might be regulated by the Arf6-related pathway. Furthermore,
602 phosphorylation on several predicted phosphorylation sites of γ-taxilin, which are
603 reported by PhosphoSitePlus (http://www.phosphosite.org), might be involved in the
604 above putative regulation pathway.
605 In conclusion, our study uncovers a novel molecular mechanism underlying the
606 regulation of CIE, which at least sheds light on the mode of activation of CD98 and
607 CD147. Taken together with our previous reports (Sakane et al., 2014; Sakane et al.,
608 2016), our present findings at least suggest that taxilin family members may be
609 commonly involved in the sorting of various endocytosed cargo proteins on SEs.

Materials and Methods

Antibodies

An anti-γ-taxilin antibody (80 ng/mL for western blotting and 1 μg/20 μl beads for immunoprecipitation) was prepared as described previously (Nogami et al., 2004). An anti-Hook1 antibody (ab151756, 1:1000 for western blotting and 1:100 for immunocytochemistry) was purchased from Abcam. Anti-CD55 (311302, 1:100 for antibody uptake assay), anti-CD98 (315602, 1:100 for antibody uptake assay, recycling assay, and internalization assay), anti-CD147 (306202, 1:100 for antibody uptake assay), and anti-MHC-I (311402, 1:100 for antibody uptake assay and recycling assay) antibodies were purchased from BioLegend. Anti-CD98 (15193-1-AP, 1:5000 for western blotting) and anti-Rab22a (12125-1-AP, 1:1000 for western blotting) antibodies were purchased from Proteintech. Anti-CD147 (34-5600, 1:125 for western blotting) and anti-TfnR (13-6800, 1:1000 for western blotting) antibodies were purchased from Invitrogen. Anti-EEA1 (2411, 1:100 for immunocytochemistry), anti-FAK (3285, 1:1000 for western blotting), anti-pFAK (Tyr397) (3283, 1:1000 for western blotting), and anti-Rab10 (8127, 1:1000 for western blotting) antibodies were purchased from Cell Signaling Technology. Anti-α-Tubulin (T9026, 1:5000 for western blotting) and anti-γ-taxilin (HPA000841, 1:200 for immunocytochemistry) antibodies were purchased from Sigma-Aldrich. Anti-GAPDH (M171-3, 1:4000 for western blotting), anti-HA (561, 1:1000 for western blotting), anti-myc (562, 1:1000 for western blotting), anti-Flag (M185, 1:10000 for western blotting) antibodies, mouse IgG2a (M076-3), and rabbit IgG (PM035) were purchased from MBL. Horseradish peroxidase- and Alexa Fluor-conjugated secondary antibodies were purchased from GE Healthcare and Thermo Fisher Scientific, respectively.

634

635 **DNA constructs**

636 pGBKT7-α-, β-, and γ-taxilin and pEGFPC1-γ-taxilin were constructed as described
637 previously (Makiyama et al., 2018; Sakane et al., 2016). Full-length human α-, β-, and
638 γ-taxilin cDNA fragments were amplified from pGBKT7-α- and β-taxilin, and
639 pEGFPC1-γ-taxilin, respectively, by PCR. The full-length human α- and β-taxilin
640 cDNA fragments were introduced into the *Eco*RI and *BamH*I site of pcDNA3-HA using
641 an In-FusionHD Cloning Kit (Clontech, Takara Bio). The full-length human γ-taxilin
642 cDNA fragment was introduced into the *Eco*RV site of pcDNA3-HA using the same Kit.
643 For generating siRNA-resistant γ-taxilin expression plasmid (pEGFPC1-γ-taxilin-mt),
644 five silent substitutions were introduced in the region complementary to γ-taxilin #1
645 siRNA using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio) according to the
646 manufacturer's protocol. Full-length cDNA fragments encoding human Hook1, 2, and 3,
647 human CD98, and human CD147 were amplified by PCR from HeLa cDNA. The
648 full-length human Hook1 and 2 cDNA fragments were introduced into the *Eco*RI and
649 *Xho*I sites of vectors pGADT7 and pcDNA3-myc. The full-length human Hook3 cDNA
650 fragment was introduced into the *Eco*RI and *Cla*I sites of pGADT7 and the *Eco*RI and
651 *Xho*I sites of pcDNA3-myc. pcDNA3-myc-Hook1 (residues 1–485),
652 pcDNA3-myc-Hook1 (residues 1–166), pcDNA3-myc-Hook1 (residues 167–728), and
653 pcDNA3-myc-Hook1 (residues 486–728) were constructed from pcDNA3-myc-Hook1
654 using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio) according to the
655 manufacturer's protocol. The full-length human CD98 and CD147 cDNA fragments
656 were introduced into the *Bam*HI and *Eco*RI sites of pcDNA3-*C*-terminal Flag. The PCR
657 products and the structures of all plasmids were confirmed by DNA sequencing.

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose (Wako) with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (referred to hereafter as complete DMEM) in a 5% CO_2 incubator at 37°C. Transfection of plasmid DNA and siRNA into cells was performed using Lipofectamine 2000 and Lipofectamine RNAiMAX (Thermo Fisher Scientific), respectively, according to the manufacturer's protocols. Lipofectamine 2000- and Lipofectamine RNAiMAX-treated cells were, respectively, used for experiments 24 and 48 h post-transfection. For siRNA rescue experiments, γ-taxilin siRNA-treated cells were transfected with pEGFPC1-γ-taxilin-mt or pEGFPC1-γ-taxilin at 24 h after treatment with γ-taxilin siRNA. After 24 h of incubation time, cells were processed for the experiments. Silencer Select negative control siRNA (4390843) and Silencer Select siRNAs against γ-taxilin#1 (ID s31509), γ-taxilin#2 (ID s31511), Hook1 (ID s28011), CD98 (ID s12943), CD147 (ID s2099), Rab10 (ID s21390), and Rab22a (ID s32992) were purchased from Thermo Fisher Scientific.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed using the Matchmaker Gold Yeast Two-Hybrid System (Takara Bio) according to the manufacturer's protocol. pGBKT7-γ-taxilin was introduced into yeast strain Y2H Gold, and the transformant was mated with yeast strain Y187 harboring a human normalized cDNA library (Clontech, Takara Bio). Library plasmids from positive clones were analyzed by transformation tests and DNA sequencing. BLAST searches were conducted using the NCBI online service. To examine the interaction of Hook1, Hook2, or Hook3 with α-, β-, or -γ-taxilin, a pGADT7-based vector harboring a Hook1, Hook2, or Hook3 cDNA

682 fragment and a pGBKT7-based vector harboring an α-, β-, or γ-taxilin cDNA fragment
683 were introduced into strain Y2H Gold. Equal amounts of the transformants were spotted
684 onto synthetic medium lacking leucine, tryptophan, and histidine with 10 mM
685 3-amino-1, 2, 4-triazole (Tokyo Kasei Kogyo) (−LWH plates) to examine the
686 interaction between co-expressed proteins, or onto synthetic medium lacking leucine
687 and tryptophan (−LW plates) to examine plasmid maintenance, and then incubated for 3
688 days at 30°C.
689
690 **Western blotting**
691 Cells were lysed in lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1%
692 Nonidet P-40, 10% glycerol, and protease inhibitor cocktail (Roche Diagnostics)].
693 Protein concentration was determined using a DC Protein Assay Kit (Bio-Rad). Cell
694 lysates were subjected to SDS-PAGE followed by western blotting. Immunoreactive
695 bands were detected using the ECL Prime Western Blotting Detection Reagent (GE
696 Healthcare), Clarity Western ECL substrate (Bio-Rad), or SuperSignal West Atto
697 Ultimate Sensitivity Substrate (Thermo Fisher Scientific) and captured using an
698 Amersham Imager 600 (Amersham). Band intensities were measured using FIJI/ImageJ
699 software (NIH).
700
701 **Immunoprecipitation**
702 Transfected HeLa cells were lysed in immunoprecipitation buffer (20 mM
703 HEPES-NaOH, pH 7.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and protease
704 inhibitor cocktail) and then 250 μl of the cell lysate (0.3 mg protein) was
705 immunoprecipitated with 2 μg of mouse IgG2a, rabbit IgG, or anti-HA, anti-myc, or

anti-flag antibody overnight at 4°C. The immunoprecipitates were collected with 20 μl of protein G-Sepharose (GE Healthcare), washed five times with lysis buffer, and used for western blotting. The secondary antibody used for western blot analysis was TidyBlot Western Blot Detection Reagent (Bio-Rad). For analysis of endogenous protein interactions, HeLa cells were lysed in immunoprecipitation buffer and then 1 ml of the cell lysate (1 mg protein) was immunoprecipitated with 1 μg of rabbit IgG or anti-γ-taxilin antibody overnight at 4°C. The following steps were performed as described above and immunoreactive bands were detected using SuperSignal West Atto Ultimate Sensitivity Substrate (Thermo Fisher Scientific).

Immunocytochemistry

Cells grown on glass coverslips were fixed in PBS containing 2% PFA for 10 min and stained with primary antibody in PBS containing 0.5% BSA and 0.2% saponin for 1 h at room temperature. After washing three times with PBS, the cells were treated with the secondary antibody in PBS containing 0.5% BSA and 0.2% saponin for 1 h at room temperature. Cells were observed using a confocal laser scanning microscope (LSM 710, Carl Zeiss).

Antibody uptake assay

Cells grown on glass coverslips were incubated with primary antibodies against CIE cargo proteins (CD55, CD98, CD147, or MHC-I) for 1 h at 37°C to allow internalization of the antibody-bound CIE cargo proteins. The following procedures were performed at room temperature. In the case of CD98 and MHC-I, cells were treated with low pH solution (0.5% acetic acid, 0.5 M NaCl) for 30 s to remove

surface-bound antibody before fixation with 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min. In the case of CD55 and CD147, cells were treated with unlabeled anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 1 h to block surface-bound antibodies after the same fixation. Internalized antibodies were detected by incubating cells with Alexa Fluor-conjugated secondary antibody in PBS containing 5% bovine serum albumin (BSA) and 0.1% saponin for 1 h. Cells were observed using a confocal laser scanning microscope (LSM 710, Carl Zeiss). Cells containing at least one tubule >5 μm long were counted as exhibiting tubular structures.

Time-lapse imaging

A CD98 antibody was labelled for fluorescence detection using an Alexa Fluor 488 Antibody Labeling Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Cells grown on glass-bottomed dishes were incubated with the Alexa Fluor 488-labelled anti-CD98 antibody for 1 h at 37°C. After washing twice with complete DMEM, the cells were further incubated in complete DMEM to analyze internalized Alexa Fluor 488-labelled anti-CD98 antibody by live-cell imaging using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss). During live-cell imaging, cells were maintained in a 5% CO_2 stage top incubator with an objective heater (Tokai Hit) at 37°C. Time-lapse images were captured at 10-s intervals over 10 min. Because CD98-positive tubules are highly dynamic structures that frequently extend, retract, and branch, CD98-positive tubules newly formed during the period of observation, but not pre-formed CD98-positive tubules, were analyzed. When the newly formed CD98-positive tubules branched, the branched CD98-positive tubule was analyzed as another newly formed CD98-positive tubule. The number and length of newly formed

CD98-positive tubules during the period of observation were counted and manually measured, respectively. The number and the length of newly formed tubules were evaluated independently by three of the authors. Newly formed CD98-positive tubules were categorized into five groups (<5 μm, 5–10 μm, 11–15 μm, 16–20 μm, and >20 μm) by the maximum length of the newly formed CD98-positive tubule.

Internalization assay

Cells grown on glass coverslips were incubated with anti-CD98 antibody in DMEM containing 0.5% BSA for 1 h at 4°C. The cells were washed twice with PBS, followed by two rinses with DMEM. Then, the cells were further incubated for various time periods in complete DMEM at 37°C. In the case of detection of surface-bound anti-CD98 antibody at time zero, cells were fixed and stained without permeabilization. In the case of detection of internalized anti-CD98 antibody, the cells were treated with the low pH solution to remove surface-bound anti-CD98 antibody. Then, the cells were fixed, permeabilized, and stained. Cells were observed using a confocal laser scanning microscope (LSM 710, Carl Zeiss). The signal intensities of intracellular and surface anti-CD98 per μm^2 were measured using ZEN (blue edition) software.

Recycling assay

In the case of CD98 and MHC-I, cells grown on glass coverslips were incubated with anti-CD98 or anti-MHC-I antibody for 1 h at 37°C. After incubation, the cells were treated with the low pH solution to remove surface-bound antibodies, followed by two rinses with PBS and two rinses with complete DMEM. The cells were further incubated in complete DMEM for various time periods at 37°C. The following procedures were

778 performed at room temperature. In the case of detection of intracellular antibodies, cells
779 were treated with the low pH solution to remove antibody recycled back to the surface
780 membrane. Then, the cells were fixed in PBS containing 2% PFA for 10 min and
781 stained with Alexa Fluor-conjugated secondary antibody in PBS containing 5% BSA
782 and 0.1% saponin. In the case of detection of antibodies recycled back to the surface
783 membrane, cells were fixed and stained without permeabilization. In the case of TfnR,
784 cells grown on glass coverslips were serum-starved for 30 min at 37°C and then
785 incubated with Tfn-594 (Thermo Fisher Scientific) for 1 h at 37°C. Then, the cells were
786 treated with the low pH solution to remove cell surface Tfn-594, followed by two rinses
787 with PBS and two rinses with complete DMEM. The cells were further incubated for
788 various time periods at 37°C in complete DMEM, subsequently washed twice with PBS,
789 and fixed at room temperature. Cells were observed using a confocal laser scanning
790 microscope (LSM 710, Carl Zeiss). The signal intensities of intracellular and cell
791 surface antibodies and intracellular Tfn-594 per μm^2 were measured
792 using ZEN (blue edition) software.
793
794 **FAK signaling analysis**
795 For analysis of basal phosphorylation of FAK on Tyr397, cells were lysed in lysis
796 buffer containing phosphatase inhibitor cocktail (524625, Merck). For analysis of cell
797 adhesion-dependent phosphorylation of FAK on Tyr397, cells were trypsinized,
798 neutralized by addition of PBS containing 0.25% soybean trypsin inhibitor, washed
799 twice with DMEM by centrifugation, and resuspended in DMEM. After incubation for
800 15 min at 37°C, the suspended cells were seeded on culture dishes and further cultured
801 in complete DMEM for various time periods at 37°C. Replated cells were lysed in lysis

802 buffer containing phosphatase inhibitor cocktail. Cell lysates were processed for
803 western blotting as described above.
804
805 **Cell spreading assay**
806 Cells were trypsinized, washed once with complete DMEM by centrifugation to
807 neutralize the trypsin, and resuspended in complete DMEM. The suspended cells were
808 seeded on glass coverslips and cultured in complete DMEM for various time periods at
809 37°C. The cells were fixed in PBS containing 2% PFA for 10 min and stained with
810 Alexa Fluor 594-conjugated phalloidin (Thermo Fisher Scientific). Cells were observed
811 using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss). The cell
812 area was measured with FIJI/ImageJ software (NIH).
813
814 **Subcellular fractionation**
815 Subcellular fractionation was performed using the SF PTS Kit (7510-11400, GL
816 Sciences) according to the manufacturer's protocol.
817
818 **Statistical analysis**
819 All statistical analyses were performed using IBM SPSS Statistics version 26 software.
820 $P < 0.05$ was considered statistically significant.
821

Acknowledgements

We thank T. Namatame for DNA sequencing and Ms. M. Ooshima and A. Akima for technical assistance and secretarial assistance. We thank James Allen, DPhil, from Edanz Group (https://en-author-services.edanz.com/ac) for editing a draft of this manuscript.

Competing interests

The authors declare that they have no conflicts of interest with regard to the content of this article.

Author contributions

Conceptualization: S.H., H.Shirataki.; Methodology: S.H., T.M.; Formal analysis: S.H., T.M., H.Shirataki; Investigation: S.H.; Resources: S.H., T.M., H.Sakane., S.N., H.Shirataki.; Writing - original draft: S.H., H.Shirataki.; Writing - review & editing: S.H., S.N., H.Shirataki.; Visualization: S.H.; Supervision: H.Shirataki.; Project administration: H.Shirataki.; Funding acquisition: S.H.

Funding

This work was supported in part by JSPS KAKENHI (Grant Number: 19K17045 to S.H.).

References

Baron Gaillard, C. L., Pallesi-Pocachard, E., Massey-Harroche, D., Richard, F., Arsanto, J. P., Chauvin, J. P., Lecine, P., Krämer, H., Borg, J. P. and Le Bivic, A. (2011). Hook2 is involved in the morphogenesis of the primary cilium. *Mol. Biol. Cell* **22**, 4549-62.

Del Olmo, T., Lauzier, A., Normandin, C., Larcher, R., Lecours, M., Jean, D., Lessard, L., Steinberg, F., Boisvert, F. M. and Jean, S. (2019). APEX2-mediated RAB proximity labeling identifies a role for RAB21 in clathrin-independent cargo sorting. *EMBO Rep.* **20**, e47192

Delevoye, C., Miserey-Lenkei, S., Montagnac, G., Gilles-Marsens, F., Paul-Gilloteaux, P., Giordano, F., Waharte, F., Marks, M. S., Goud, B. and Raposo, G. (2014). Recycling endosome tubule morphogenesis from sorting endosomes requires the kinesin motor KIF13A. *Cell Rep.* **6**, 445-54.

Dutta, D. and Donaldson, J. G. (2015). Sorting of Clathrin-Independent Cargo Proteins Depends on Rab35 Delivered by Clathrin-Mediated Endocytosis. *Traffic* **16**, 994-1009.

Etoh, K. and Fukuda, M. (2019). Rab10 regulates tubular endosome formation through KIF13A and KIF13B motors. *J. Cell Sci.* **132**.

Grant, B. D. and Donaldson, J. G. (2009). Pathways and mechanisms of endocytic recycling. *Nat. Rev. Mol. Cell Biol.* **10**, 597-608.

Hoffmann, J., Boehm, C., Himmelsbach, K., Donnerhak, C., Roettger, H., Weiss, T. S., Ploen, D. and Hildt, E. (2013). Identification of α-taxilin as an essential factor for the life cycle of hepatitis B virus. *J. Hepatol.* **59**, 934-41.

Hongu, T., Funakoshi, Y., Fukuhara, S., Suzuki, T., Sakimoto, S., Takakura, N., Ema, M., Takahashi, S., Itoh, S., Kato, M. et al. (2015). Arf6 regulates tumour angiogenesis and growth through HGF-induced endothelial β1 integrin recycling. *Nat Commun* **6**, 7925.

Horii, Y., Sakane, H., Nogami, S., Ohtomo, N., Tomiya, T. and Shirataki, H. (2014). Expression of α-taxilin in the murine gastrointestinal tract: potential implication in cell proliferation. *Histochem. Cell Biol.* **141**, 165-80.

Hotokezaka, Y., Katayama, I., van Leyen, K. and Nakamura, T. (2015). GSK-3β-dependent downregulation of γ-taxilin and αNAC merge to regulate ER stress responses. *Cell Death Dis.* **6**, e1719.

Huveneers, S. and Danen, E. H. (2009). Adhesion signaling - crosstalk between integrins, Src and Rho. *J. Cell Sci.* **122**, 1059-69.

Krämer, H. and Phistry, M. (1996). Mutations in the Drosophila hook gene inhibit endocytosis of the boss transmembrane ligand into multivesicular bodies. *J. Cell Biol.* **133**, 1205-15.

Krämer, H. and Phistry, M. (1999). Genetic analysis of hook, a gene required for endocytic trafficking in drosophila. *Genetics* **151**, 675-84.

Lauffer, B. E., Melero, C., Temkin, P., Lei, C., Hong, W., Kortemme, T. and von Zastrow, M. (2010). SNX27 mediates PDZ-directed sorting from endosomes to the plasma membrane. *J Cell Biol* **190**, 565-74.

Lee, I. G., Olenick, M. A., Boczkowska, M., Franzini-Armstrong, C., Holzbaur, E. L. F. and Dominguez, R. (2018). A conserved interaction of the dynein light intermediate chain with dynein-dynactin effectors necessary for processivity. *Nat. Commun.* **9**, 986.

Makiyama, T., Higashi, S., Sakane, H., Nogami, S. and Shirataki, H. (2018). γ-Taxilin temporally regulates centrosome disjunction in a Nek2A-dependent manner. *Exp. Cell Res.* **362**, 412-423.

Maldonado-Báez, L., Cole, N. B., Krämer, H. and Donaldson, J. G. (2013a). Microtubule-dependent endosomal sorting of clathrin-independent cargo by Hook1. *J. Cell Biol.* **201**, 233-47.

Maldonado-Báez, L., Williamson, C. and Donaldson, J. G. (2013b). Clathrin-independent endocytosis: a cargo-centric view. *Exp. Cell Res.* **319**, 2759-69.

Mashidori, T., Shirataki, H., Kamai, T., Nakamura, F. and Yoshida, K. (2011). Increased alpha-taxilin protein expression is associated with the metastatic and invasive potential of renal cell cancer. *Biomed. Res.* **32**, 103-10.

Mayor, S., Parton, R. G. and Donaldson, J. G. (2014). Clathrin-independent pathways of endocytosis. *Cold Spring Harb. Perspect Biol.* **6**, a016758.

Mendoza-Lujambio, I., Burfeind, P., Dixkens, C., Meinhardt, A., Hoyer-Fender, S., Engel, W. and Neesen, J. (2002). The Hook1 gene is non-functional in the abnormal spermatozoon head shape (azh) mutant mouse. *Hum. Mol. Genet.* **11**, 1647-58.

Muramatsu, T. (2016). Basigin (CD147), a multifunctional transmembrane glycoprotein with various binding partners. *J. Biochem.* **159**, 481-90.

Nogami, S., Satoh, S., Nakano, M., Shimizu, H., Fukushima, H., Maruyama, A., Terano, A. and Shirataki, H. (2003). Taxilin; a novel syntaxin-binding protein that is involved in Ca^{2+}-dependent exocytosis in neuroendocrine cells. *Genes Cells* **8**, 17-28.

Nogami, S., Satoh, S., Tanaka-Nakadate, S., Yoshida, K., Nakano, M., Terano, A. and Shirataki, H. (2004). Identification and characterization of taxilin isoforms. *Biochem. Biophys. Res. Commun.* **319**, 936-43.

Oba-Shinjo, S. M., Bengtson, M. H., Winnischofer, S. M., Colin, C., Vedoy, C. G., de Mendonça, Z., Marie, S. K. and Sogayar, M. C. (2005). Identification of novel differentially expressed genes in human astrocytomas by cDNA representational difference analysis. *Brain Res. Mol. Brain Res.* **140**, 25-33.

Ohtomo, N., Tomiya, T., Tanoue, Y., Inoue, Y., Nishikawa, T., Ikeda, H., Seyama, Y., Kokudo, N., Shibahara, J., Fukayama, M. et al. (2010). Expression of α-taxilin in hepatocellular carcinoma correlates with growth activity and malignant potential of the tumor. *Int. J. Oncol.* **37**, 1417-23.

Patel, N. M., Siva, M. S. A., Kumari, R., Shewale, D. J., Rai, A., Ritt, M., Sharma, P., Setty, S. R. G., Sivaramakrishnan, S. and Soppina, V. (2021). KIF13A motors are regulated by Rab22A to function as weak dimers inside the cell. *Sci. Adv.* **7**, eabd2054.

Qi, S., Su, L., Li, J., Zhang, C., Ma, Z., Liu, G., Zhang, Q., Jia, G., Piao, Y. and Zhang, S. (2019). Arf6-driven endocytic recycling of CD147 determines HCC malignant phenotypes. *J Exp Clin Cancer Res* **38**, 471.

Rahajeng, J., Giridharan, S. S., Cai, B., Naslavsky, N. and Caplan, S. (2012). MICAL-L1 is a tubular endosomal membrane hub that connects Rab35 and Arf6 with Rab8a. *Traffic* **13**, 82-93.

Richardson, S. C., Winistorfer, S. C., Poupon, V., Luzio, J. P. and Piper, R. C. (2004). Mammalian late vacuole protein sorting orthologues participate in early endosomal fusion and interact with the cytoskeleton. *Mol. Biol. Cell* **15**, 1197-210.

Sakane, H., Horii, Y., Nogami, S., Kawano, Y., Kaneko-Kawano, T. and Shirataki, H. (2014). α-Taxilin interacts with sorting nexin 4 and participates in the recycling pathway of transferrin receptor. *PLoS One* **9**, e93509.

Sakane, H., Makiyama, T., Nogami, S., Horii, Y., Akasaki, K. and Shirataki, H. (2016). β-Taxilin participates in differentiation of C2C12 myoblasts into myotubes. *Exp. Cell Res.* **345**, 230-8.

Sharma, M., Giridharan, S. S., Rahajeng, J., Naslavsky, N. and Caplan, S. (2009). MICAL-L1 links EHD1 to tubular recycling endosomes and regulates receptor recycling. *Mol. Biol. Cell* **20**, 5181-94.

Solis, G. P., Hülsbusch, N., Radon, Y., Katanaev, V. L., Plattner, H. and Stuermer, C. A. (2013). Reggies/flotillins interact with Rab11a and SNX4 at the tubulovesicular recycling compartment and function in transferrin receptor and E-cadherin trafficking. *Mol. Biol. Cell* **24**, 2689-702.

Steinberg, F., Gallon, M., Winfield, M., Thomas, E. C., Bell, A. J., Heesom, K. J., Tavaré, J. M. and Cullen, P. J. (2013). A global analysis of SNX27-retromer assembly and cargo specificity reveals a function in glucose and metal ion transport. *Nat Cell Biol* **15**, 461-71.

Sunio, A., Metcalf, A. B. and Krämer, H. (1999). Genetic dissection of endocytic trafficking in Drosophila using a horseradish peroxidase-bride of sevenless chimera: hook is required for normal maturation of multivesicular endosomes. *Mol. Biol. Cell* **10**, 847-59.

Szebenyi, G., Hall, B., Yu, R., Hashim, A. I. and Krämer, H. (2007). Hook2 localizes to the centrosome, binds directly to centriolin/CEP110 and contributes to centrosomal function. *Traffic* **8**, 32-46.

Temkin, P., Lauffer, B., Jäger, S., Cimermancic, P., Krogan, N. J. and von Zastrow, M. (2011). SNX27 mediates retromer tubule entry and endosome-to-plasma membrane trafficking of signalling receptors. *Nat Cell Biol* **13**, 715-21.

Walenta, J. H., Didier, A. J., Liu, X. and Krämer, H. (2001). The Golgi-associated hook3 protein is a member of a novel family of microtubule-binding proteins. *J. Cell Biol.* **152**, 923-34.

Weigert, R., Yeung, A. C., Li, J. and Donaldson, J. G. (2004). Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. *Mol. Biol. Cell* **15**, 3758-70.

Wu, B., Wang, Y., Yang, X. M., Xu, B. Q., Feng, F., Wang, B., Liang, Q., Li, Y., Zhou, Y., Jiang, J. L. et al. (2015). Basigin-mediated redistribution of CD98 promotes cell spreading and tumorigenicity in hepatocellular carcinoma. *J. Exp. Clin. Cancer Res.* **34**, 110.

Wu, B., Zhou, Y., Wang, Y., Yang, X. M., Liu, Z. Y., Li, J. H., Feng, F., Chen, Z. N. and Jiang, J. L. (2016). Dominant Suppression of β1 Integrin by Ectopic CD98-ICD Inhibits Hepatocellular Carcinoma Progression. *Int. J. Mol. Sci.* **17**, 1882.

Xu, L., Sowa, M. E., Chen, J., Li, X., Gygi, S. P. and Harper, J. W. (2008). An FTS/Hook/p107(FHIP) complex interacts with and promotes endosomal clustering by the homotypic vacuolar protein sorting complex. *Mol. Biol. Cell* **19**, 5059-71.

Yu, V. W., Ambartsoumian, G., Verlinden, L., Moir, J. M., Prud'homme, J., Gauthier, C., Roughley, P. J. and St-Arnaud, R. (2005). FIAT represses ATF4-mediated transcription to regulate bone mass in transgenic mice. *J. Cell Biol.* **169**, 591-601.

Yu, V. W., Gauthier, C. and St-Arnaud, R. (2008). FIAT represses bone matrix mineralization by interacting with ATF4 through its second leucine zipper. *J. Cell Biochem.* **105**, 859-65.

Zhen, Y. and Stenmark, H. (2015). Cellular functions of Rab GTPases at a glance. *J. Cell Sci.* **128**, 3171-6.

Zhou, S., Zhou, H., Walian, P. J. and Jap, B. K. (2005). CD147 is a regulatory subunit of the gamma-secretase complex in Alzheimer's disease amyloid beta-peptide production. *Proc. Natl. Acad. Sci. U S A* **102**, 7499-504.

990 **Figure legends**

991 **Fig. 1. γ-Taxilin interacts with Hook1 and Hook2 but not Hook3.**

992 (A) Yeast two-hybrid assay. pGBKT7 harboring a cDNA fragment encoding α-, β-, or
993 γ-taxilin, and pGADT7 harboring a cDNA fragment encoding Hook1, Hook2, or Hook3
994 were co-introduced into yeast reporter strain Y2H Gold. Equal amounts of the indicated
995 transformants were spotted onto synthetic defined medium (SD) −LW (non-selective) or
996 SD −LWH with 3-amino-1, 2, 4-triazole (selective) plates.
997 (B,C) Co-immunoprecipitation assay. Cell lysates of HeLa cells co-transfected with
998 HA-tagged α-, β-, or γ-taxilin and myc-tagged Hook1, Hook2, or Hook3 in B or not in
999 C were immunoprecipitated with the indicated antibodies. The immunoprecipitate was
1000 subjected to SDS-PAGE followed by western blotting with indicated antibodies for
1001 detection of corresponding proteins (n = 3, each experiment). The amount of cell lysates
1002 used for western blotting were 2.5% (B) or 1.25% (C) of those used for IP.
1003 (D,E) Immunocytochemistry. Hela cells were immunostained with an anti-γ-taxilin or
1004 anti-Hook1 antibody in C. Hela cells expressing GFP-γ-taxilin (green) and myc-Hook1
1005 were immunostained with an anti-Hook1 antibody (red) in D. Scale bars: 10 μm.
1006

1007 **Fig. 2. γ-Taxilin depletion promotes the tubular formation of Hook1-mediated**
1008 **cargo proteins.**
1009 (A) Depletion of γ-taxilin by small interfering RNA (siRNA). HeLa cells were
1010 transfected with control (Ctrl) or γ-taxilin (γ-Taxilin #1 or #2) siRNA. Cell lysates were
1011 subjected to SDS-PAGE followed by western blotting with the indicated antibodies.

(B) Formation of CD98-positive tubular structures in the indicated siRNA-treated cells. Magnified views of the boxed areas in the upper panels are shown in the lower panels. Scale bars: 20 μm.

(C) Quantitative analysis of the formation of CD98-positive tubular structures in B. The percentages of cells exhibiting CD98-positive tubular structures are shown as the mean ± s.e.m. (n = 3; >100 cells were analyzed in each experiment). ***, $P < 0.001$ by two-tailed Student's *t*-test.

(D) siRNA rescue experiments. γ-Taxilin siRNA-treated cells were transfected with the indicated plasmids at 24 h after treatment with γ-taxilin siRNA. Cell lysates were subjected to SDS-PAGE followed by western blotting with the indicated antibodies.

(E) Formation of CD98-positive tubular structures in cells treated with the indicated siRNA and plasmids. Scale bars: 20 μm.

(F) Quantitative analysis of the formation of CD98-positive tubular structures in E. The percentages of cells exhibiting CD98-positive tubular structures are shown as the mean ± s.e.m. (n = 3; >100 cells were analyzed in each experiment). ***, $P < 0.001$; n.s., not significant by one-way analysis of variance (ANOVA) with *post-hoc* Tukey's multiple comparison test.

(G) Formation of CD147-, MHC-I-, or CD55-positive tubular structures in the indicated siRNA-treated cells. Scale bars: 20 μm.

(H) Quantitative analyses of the formation of CD147-, MHC-I-, or CD55-positive tubular structures in D. The percentages of cells exhibiting CD147-, MHC-I-, or CD55-positive tubular structures are shown as the mean ± s.e.m. (n = 3; >100 cells were analyzed in each experiment). ***, $P < 0.001$; n.s., not significant by two-tailed Student's *t*-test.

Fig. 3. The effect of γ-taxilin depletion on the formation of CD98-positive tubular structures is attenuated by co-depletion of Hook1, Rab10, or Rab22a.

(A) Co-depletion of γ-taxilin and Hook1, Rab10, or Rab22a by siRNA. HeLa cells were transfected with control (Ctrl) or γ-taxilin (γ-Taxilin #1) siRNA and Hook1, Rab10, or Rab22a siRNA. Cell lysates were subjected to SDS-PAGE followed by western blotting with the indicated antibodies.

(B) Formation of CD98-positive tubular structures in the indicated siRNA-treated cells. Scale bars: 20 μm.

(C) Quantitative analysis of the formation of CD98-positive tubular structures in B. The percentages of cells exhibiting CD98-positive tubular structures are shown as the mean ± s.e.m. (n = 3; >100 cells were analyzed in each experiment). ***, $P < 0.001$ by one-way ANOVA with *post-hoc* Tukey's multiple comparison test.

Fig. 4. γ-Taxilin depletion promotes the biogenesis of CD98-positive tubules.

(A) Dynamics of CD98-positive tubules in the indicated siRNA-treated cells. Time-lapse images were captured at 10-s intervals over 10 min. Arrows indicate newly formed CD98-positive tubules. Scale bars: 10 μm.

(B) Quantitative analysis of the number of newly formed CD98-positive tubules in A. The number of CD98-positive tubules newly formed during the period of observation is shown as the mean ± s.e.m. (n = 3; >10 cells were analyzed in each experiment). *, $P < 0.05$ by two-tailed Student's t-test.

(C) Histogram analysis of the length of newly formed CD98-positive tubules in A. The length of each CD98-positive tubule newly formed during the period of observation was

measured. Newly formed CD98-positive tubules were categorized into groups by maximum length (<5 μm, 5–10 μm, 11–15 μm, 16–20 μm, and >20 μm). The percentages of the categorized tubules per cell are shown as the mean ± s.e.m. (n = 3; >10 cells were analyzed in each experiment). **, $P < 0.01$ by two-tailed Student's t-test.

Fig. 5. γ-Taxilin depletion promotes recycling of CD98 but not MHC-I.

(A–D) Recycling of CD98 and MHC-I in the indicated siRNA-treated cells. Scale bars: 20 μm.

(E–H) Quantitative analyses of recycling of CD98 (E,F) and MHC-I (G,H) in A and B, and C and D, respectively. Each antibody signal was measured as signal intensity per μm^2. In E and G, results shown are the mean ± s.e.m. of the ratio of the intracellular antibody signal at each time point to that at time zero. In F and H, results shown are the mean ± s.e.m. of the ratio of the cell surface antibody signal at each time point to the intracellular antibody signal at time zero measured in experiments described in E and G (n = 3; >40 cells were analyzed in each experiment). *, $P < 0.05$ by two-tailed Student's t-test.

Fig. 6. γ-Taxilin depletion promotes cell spreading mediated by CD147.

(A) Cell adhesion-dependent focal adhesion kinase (FAK)-Tyr 397 phosphorylation. HeLa cells treated with the indicated siRNA were trypsinized and suspended in serum-free Dulbecco's modified Eagle's medium (DMEM) for 15 min. The suspended cells (Sus) were lysed or replated and then incubated for the indicated time periods. Cell lysates were subjected to SDS-PAGE followed by western blotting with the indicated antibodies.

(B) Quantitative analysis of FAK-Tyr 397 phosphorylation in A. The results shown are the mean ± SD of the ratio of pFAK to FAK relative to that with the Ctrl siRNA at time 15 min (n = 3). *, P < 0.05 by two-tailed Student's t-test.

(C) Co-depletion of γ-taxilin and Hook1 or CD147 by siRNA. Cells were transfected with control (Ctrl) or γ-taxilin (γ-Taxilin #1) siRNA and Hook1 or CD147 siRNA. Cell lysates were subjected to SDS-PAGE followed by western blotting with the indicated antibodies.

(D) Cell spreading assay. siRNA-treated HeLa cells were trypsinized and resuspended. The suspended cells were replated, incubated for the indicated time periods, and fixed. Cells were visualized using Alexa Fluor 594-labeled phalloidin. Scale bars: 40 μm.

(E) Quantitative analysis of cell spreading in D. The area of cell spreading at each time point was measured. Data are expressed as the mean ± s.e.m. (n = 3; >100 cells were analyzed in each experiment). **, P < 0.01; ***, P < 0.001 by one-way ANOVA with *post-hoc* Tukey's multiple comparison test.

Fig. 7. γ-Taxilin binds to the *C*-terminal region of Hook1 competitively with CD98.

(A) Formation of CD98-positive tubular structures in GFP- or GFP-γ-taxilin-expressing cells. Magnified views of the boxed areas in the panels of CD98 are shown in the right panels. Scale bars: 20 μm.

(B) Quantitative analysis of the formation of CD98-positive tubular structures in A. Results shown are the mean ± s.e.m. of the percentages of cells exhibiting CD98-positive tubular structures (n = 3; >100 cells were analyzed in each experiment). ***, P < 0.001; n.s., not significant by one-way ANOVA with *post-hoc* Tukey's multiple comparison test.

(C) Construction of myc-tagged Hook1 mutants used in D. MBD: microtubule-binding domain, CC: coiled-coil, OBD: organelle-binding domain.
(D-H) Co-immunoprecipitation assay. Cells were co-transfected with the indicated plasmids. Cell lysates were immunoprecipitated with the indicated antibodies. The immunoprecipitate was subjected to SDS-PAGE followed by western blotting with the indicated antibodies for detection of corresponding proteins (n=3, each experiment). The amount of cell lysates used for western blotting were 2.5% of those used for IP.

Fig. 8. The proposed role of γ-taxilin in recycling of clathrin-independent cargo. γ-Taxilin functions, at least, in the step where Hook1 recognizes and subsequently sorts Hook1-mediated cargo proteins such as CD98 and CD147 from sorting endosomes to recycling endosomes, forming tubular structures. γ-Taxilin interferes with the interaction between Hook1 and Hook1-mediated cargo proteins, leading to inhibition of the recycling of these cargo proteins back to the plasma membrane. ERC: endocytic recycling compartment, PM: plasma membrane, RE: recycling endosome, SE: sorting endosome.