（千葉大学学位申請論文審査用）

# Identification of novel target proteins of E3 ubiquitin ligase, Smurf1 in the ubiquitin-proteasome pathway

2008年1月

千葉大学大学院自然科学研究科

生物資源応用科学専攻生物資源機能学

山口　圭

## Contents

# Chapter1.

## Regulation of hPEM-2, a GEF for Cdc42, by Smurf1 via a novel combination of protein interaction modules.

## Summary

Smurf1, a member of HECT-type E3 ubiquitin ligases, regulates cell polarity and protrusive activity by inducing ubiquitination and subsequent proteasomal degradation of small GTPase RhoA. I report here that hPEM-2, a guanine nucleotide exchange factor (GEF) for small GTPase Cdc42, is a novel target of Smurf1. Pulse-chase labeling and an ubiquitination experiment using MG132, a proteasomal inhibitor, indicated that Smurf1 induced proteasomal degradation of hPEM-2 in cells. GST pull-down assays with heterologously expressed firefly luciferase-fusion proteins that included partial sequences of hPEM-2 revealed that part of the PH domain (residues 318-343) of hPEM-2 was sufficient for binding to Smurf1. In contrast, the hPEM-2 binding domain in Smurf1 was mapped to the C2 domain. Although it is reported that the binding activities of some C2 domains to target proteins are regulated by $Ca^{2+}$, Smurf1 interacted with hPEM-2 in a $Ca^{2+}$-independent manner. My discovery that hPEM-2 is, in addition to RhoA, a target protein of Smurf1 suggests that Smurf1 plays a crucial role in the spatiotemporal regulation of Rho GTPase family members.

## Introduction

Ubiquitin-dependent protein degradation plays an essential role in a number of key biological processes including signal transduction, cell cycle progression and transcriptional regulation (Hershko and Ciechanover, 1998). Protein ubiquitination requires three steps through ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). There are two main classes of E3 proteins, defined by either a zinc-binding RING finger adaptor domain or a HECT catalytic domain. The RING finger E3 ligases appear to function as docking proteins that recruit target proteins to a multicomponent complex incorporating an E2 enzyme (Pickart, 2001). On the other hand, HECT E3 ligases are characterized by the ability to form a thiolester intermediate with activated ubiquitin, and directly transfer ubiquitin to the target proteins (Hershko and Ciechanover, 1998; Laney and Hochstrasser, 1999).

Smurf1, a member of the HECT-containing E3s, was originally identified as an E3 that induces ubiquitination and degradation of bone morphogenetic protein-specific Smad1 and Smad5 in the TGF-$\beta$ signaling pathway (Zhu et al., 1999). It is composed of a protein kinase C conserve 2 (C2) domain at the N terminus, two WW domains in the middle, and a HECT domain at the C terminus. The WW domains on Smurf1 bind to PPXY motif, a small proline-rich sequence, and different WW domains possess different target protein specificity. Both Smad1 and Smad5 interact with Smurf1 via the PPXY-WW mediated interaction.

The Rho family of small GTPases (RhoA, Rac1 and Cdc42) organize the architecture of the actin cytoskeleton to adapt the cellular morphology to constraints imposed by cellular programs of differentiation, division, and migration (Etienne-Manneville and Hall, 2002; Burridge and Wennerberg, 2004). They switch between an inactive state via GTPase activating proteins (GAPs) and an active state via guanine nucleotide exchange factors (GEFs). In neurons, it was observed that Rac1 and Cdc42 induce, while RhoA inhibits neurite outgrowth (Luo, 2000). However, little is known of the regulating factors involved in their coordinated activities.

It has also been demonstrated that Smurf1 targets RhoA for ubiquitination and subsequent proteasomal degradation in HEK293T cells (Wang et al., 2003; Zhang et al., 2004). Interestingly, ubiquitination of RhoA by Smurf1 disrupts fibroblast polarity, leading to activation of Rac1 and Cdc42, and formation of protrusions at the leading

edge, while Smurf1 does not directly interact with Rac1and Cdc42. Moreover, by a similar mechanism, it has been shown that Smurf1 promotes neurite outgrowth in Neuro2a cells (Bryan et al., 2005). Thus, one might expect that Smurf1 largely generates an intracellular asymmetry of Rho family GTPase activity; however, details of the events are still unclear. I hypothesized that Smurf1 interacts with GEFs of Rac1 and Cdc42 in addition to RhoA, because GEFs specifically activate individual members of Rho family within particular spatial and temporal contexts, and induce actin cytoskeleton reorganization (Rossman et al., 2005). Additionally, it was recently shown that FWD1/$\beta$-TrCP, a member of the RING finger E3 ligases, targets FGD1, a GEF that activates Cdc42 (Hayakawa et al., 2005). It was found that a mutant FGD1 that fails to interact with FWD1/$\beta$-TrCP led to sustained Cdc42 activation.

In the present study, I attempted to identify the GEFs that are ubiquitinated by Smurf1 and consequently found hPEM-2, GEF for Cdc42, using an ubiquitination experiment. I also demonstrated that Smurf1 binds the PH domain of hPEM-2 via its C2 domain in a $Ca^{2+}$-independent manner and induces proteasomal degradation of hPEM-2.

## Materials and methods

### Construction of expression clones

To create N-terminal FLAG and Myc tag mammalian expression vectors (pcDNAnFLAG-DEST and pcDNAnMyc-DEST), double-stranded oligonucleotides including FLAG or Myc tag sequences were cloned into *Hind* III site of pcDNA-DEST47 Δ GFP that was constructed by ligation of 1460 bp *Pst* I fragment of pcDNA-DEST53 (Invitrogen) with a 5772 bp *Pst* I fragment of pcDNA-DEST47 (Invitrogen). Smurf1 and hPEM-2 were derived from sequences with GenBank accession numbers of AB046845 (KIAA1625) and AB007884 (KIAA0424), respectively. Tag-fused Smurf1 and hPEM-2 were constructed using pcDNAnMyc-DEST and pcDNAnFLAG-DEST by the Gateway LR recombination reaction (Invitrogen). GeneEditor System (Promega) was used to generate a catalytically inactive form of Smurf1 (Smurf1CA), in which the cysteine at position 725 was replaced with alanine and a deletion mutant of PPXY motif on hPEM-2 (hPEM-2 Δ PPSYPPP) and, where the PPSYPPP sequence between amino-acid residues 465-471 was deleted. N-terminal Halo-tagged hPEM-2 was created using pHT2 vector (Promega). GST-fusion proteins, Smurf1CA and hPEM-2, were subcloned into a pGEX-6PDES (Hara et al., 2003) vector. Construction of His tagged Luc2 which was derived from pGL4.10 (luc2) vector (Promega) and hPEM2 were performed by amplifying the reading frame by PCR, followed by cloning into the *Sma* I/*Eco*R I sites of pET-47b(+) (TaKaRa). N-and C-terminal deletion mutants of hPEM-2 were amplified by PCR and cloned into pcDNAnFLAG-DEST. The Luc2 coding region was cloned into pcDNAnFLAG-DEST for FLAG-tagged Luc2 fusion proteins, and part of hPEM-2 and Smurf1 sequences amplified by PCR were inserted at the C-terminus of Luc2. The HA-Ub construct was kindly provided from Dr. M. Treier (Treier et al., 1994).

### Cell Culture and Transfection

HEK293 cells were maintained in Dulbecco's modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% of Tet System Approved Fetal Bovine Serum (BD) and 100 x antibiotic-antimycotic liquid (Invitrogen). The HEK293 cells were transfected with various expression vectors with FuGENE 6 Transfesfection Reagent

(Roche Diagnostics) according to the manufacturer's recommendations. Total plasmid DNA in individual transfections was adjusted to equivalent amounts in all transfections with empty vectors. Transfected cells were cultured for 48 h and used for immunoprecipitation or *in vitro* pull down assays.

**Immunoprecipitation and Western Blotting**

Transfected cells were washed in PBS and incubated 20 min on ice with lysis buffer (50 mM Hepes, pH7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin A and 250 μM PMSF) just prior to use. Cell debris was removed by a microcentrifuge at 14,000 x *g* at 4 °C for 10 min, and the supernatant was used for immunoprecipitation and western blot analysis. For the immunoprecipitation assay, cell lysates were incubated with anti-FLAG-M2 (Sigma) for 2 h at 4 °C followed by incubation for an additional 1 h with protein G/A agarose suspension (Calbiochem). The agarose suspension was then washed twice with lysis buffer, three times with wash buffer1 (20 mM Hepes, pH7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), and then boiled in SDS sample buffer. In Western blot analysis, the precipitated and total proteins of cell lysates were separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% milk in TBST and subsequently probed with the different primary antibodies diluted in TBST. Antibody dilutions used were as follows: anti-HA (Roche), 1:2800 dilution; anti-FLAG-M2, 1:10,000; anti-Myc (Invitrogen), 1:5000; and anti-His (Wako), 1:1000. After incubation with the primary antibody, membranes were incubated with the appropriate anti-mouse horseradish peroxidase secondary antibodies and visualized using chemiluminescence (ECL plus) detection system (GE Healthcare). In the proteasome inhibitor experiment, transfected cells were treated with 10 μM MG-132 (Calbiochem) for 1 h prior to cell lysis.

**Quantitative Reverse Transcription (RT)-PCR Condition**

For real-time quantive PCR, total RNA was extracted from HEK293 cells transfected by RNeasy Mini (QIAGEN). The total RNA was reverse-transcribed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen). Quantitative PCR was performed by monitoring in real time the increase in fluorescence of SYBR Green on an

ABI PRISM 7500FAST Sequence Detection System (Applied Biosystems). Smurf1-specific primer was designed using Primer Express Software (Applied Biosystems). Sequences for Smurf1 were 5'-CAGACACGAACTGTCGCTTCA and 5'-TTTCGGTCGCATCTTCATTATCT.

**Pulse-Chase Experiments**

HaloTag Interchangeable Labeling Technology (Promega) was applied for pulse labeling of HaloTag-fused hPEM-2 protein. HEK293 cells were transfected with HaloTag-fused hPEM-2 or HaloTag as a control and the indicated expression plasmid, and cultured for 36 h. The HaloTag-fused hPEM-2 and HaloTag proteins were labeled for 10 min with 50 nM HaloTag TMR fluorescent ligand (Promega). The cells were then washed four times in normal medium and chased in normal medium supplemented with 500 nM HaloTag Biotin Ligand (Promega). At the indicated times, the cells were washed with PBS and lysed in SDS sample buffer. The TMR-labeled proteins were resolved by SDS-PAGE and visualized by fluorescent image analyzer FLA3000 (Fujifilm). Image Gauge software (Fujifilm) was used to quantify the labeled HaloTag-fused hPEM-2 presented at the indicated times.

**GST Pull-down Assay**

GST, GST-Smurf1 and GST-hPEM-2 were expressed and purified from *E. coli* strain Rosetta. Briefly, the culture was induced at $OD_{600}$ 0.5 with 0.5 mM IPTG for 3 h. The pellet was collected and resuspended in sonication buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.02% Tween-20), supplemented with protease inhibitors just prior to use. The suspension was then sonicated and the insoluble fraction was removed by spinning 20 min at 12,000 rpm at 4 °C. The remaining cleared supernatant was incubated with 50% slurry of glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) for 2 h at 4 °C and washed five times with ice cold wash buffer2 (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.02% Tween-20). About 2 μg of the GST fusion proteins immobilized on beads were incubated with cell lysates in lysis buffer. After 2 h at 4 °C, the binding mixtures were washed five times with wash buffer1, and then resuspended in SDS sample buffer. To test a direct protein-protein interaction, His-tagged proteins were expressed from *E. coli* strain BL21. The pellet was resuspended in lysis buffer followed by sonication. Lysates were then incubated

with the GST beads and washed as previously described. Additionally, FLAG-tagged proteins, translated *in vitro*, were expressed by using the $T_NT$ Quick Coupled Transcription/Translation Systems (Promega), and the reticulocyte lysates were mixed with the GST beads in lysis buffer as described previously. In the experiment for $Ca^{2+}$-independency, GST fusion proteins were incubated with the reticulocyte lysates expressing FLAG-tagged proteins with either 3.5 mM $CaCl_2$ or 5 mM EGTA.

## Results

### Smurf1 mediated ubiquitination and proteasomal degradation of hPEM2 *in vivo*

Through computer-assisted analysis, I found that hPEM-2, GEF of Cdc42, contains the sequence $^{465}$PPSYPPP, which matches the consensus sequence recognized by the WW domain on Smurf1. I wanted to test whether Smurf1 promoted ubiquitination of hPEM-2, thus, FLAG-hPEM-2 was immunoprecipitated from extract of transfected cells with anti-FLAG antibody, and the precipitates were subsequently used for Western blot analysis. Anti-HA antibody was used to detect conjugation of HA-Ub (Figure 1A, top), and anti-FLAG antibody to detect the co-precipitation levels (Figure 1A, bottom). The expression of Smurf1 was also confirmed by Western blot analysis using anti-Myc antibody (Figure 1A, middle). Although hPEM-2 exhibited a low level of polyubiquitination in the absence of transiently expressed Smurf1, the co-expression of hPEM-2 with Smurf1 led to a marked increase in the polyubiquitination of hPEM-2 (Figure 1A, top, lanes 3 and 4). Moreover, accumulation of ubiquitinated hPEM-2 was observed by treating the cells with MG132, an inhibitor of proteasomal activity, for 1 h prior to the preparation of cell lysates (Figure 1A, top, lanes 5 and 6). These results indirectly demonstrated that ubiquitinated hPEM-2 is degraded through proteasomes.

I constructed an expression plasmid for an inactive point mutant of Smurf1 (Smurf1CA) to confirm that ubiquitination of hPEM-2 requires the catalytic activity of the HECT domain in Smurf1. This was achieved by replacing the cysteine residue at position 725 that forms a thiolester bond with ubiquitin with alanine. As a result, Smurf1CA did not increase hPEM-2 ubiquitination and furthermore, even low level constitutive ubiquitination of hPEM-2 was blocked (Figure 1B).

I have tried RNAi-mediated depletion of endogenous Smurf1 to evaluate the essential role of Smurf1 for hPEM-2 because my experiments using cultured cells were designed as a model system using cells exogenously overexpressing Smurf1 and hPEM-2. As shown in Figure 2A, ubiquitination of transiently expressed hPEM-2 by endogenous ubiquitin ligase(s) could be diminished in HEK293 in the presence of Smurf1 siRNAs, which can reduce the expression level of endogenous Smurf1 (Smurf1 siRNA-1 is more effective than Smurf1 siRNA-2). Expression level of endogenous Smurf1 was estimated in the presence of Smurf1 siRNA-1, Smurf1 siRNA-2 or control siRNA by quantitative RT-PCR and Smurf1 specific primers (Figure 2B). I could show that the amount of

Smurf1 mRNA was reduced by the Smurf1 siRNAs, not control one. I also demonstrated that those Smurf1 siRNAs could reduce the expression level of Smurf1-GFP construct transiently-expressed in HEK293 (Figure 2C).

I further examined whether Smurf1-assisted ubiquitination of hPEM-2 induced subsequent degradation of the protein. The stability of hPEM-2 protein was analyzed by a pulse-chase experiment applied to a HaloTag-fused hPEM-2 protein, where the HaloTag portion can covalently bind a fluorescent ligand. The FLAG-hPEM-2 fused HaloTag protein with MW 33kDa was enhanced its polyubiqutination by Smurf1, as well as the simple FLAG-hPEM-2 in Figure 1 (Figure 3A). Therefore, I have successfully used the HaloTag-fused hPEM-2 protein for the pulse-chase labeling. And so in fact, the Halo-tagged hPEM-2 and HaloTag as a control were produced in HEK293 cells, labeled with HaloTag TMR fluorescent ligand and chased for 8 h (Figure 3B). Although the protein stability of HaloTag itself was not changed in the presence of wild-type Smurf1 (Smurf1WT) and Smurf1CA (Figure 3B, closed triangles and open circulars), HaloTag-hPEM-2 was degraded faster in the presence of Smurf1WT (Figure 3B, diamonds) not Smurf1CA (Figure 3B, squares). Thus, Smurf1 appeared to regulate the protein stability of hPEM-2. Overall, the results indicate that Smurf1 induces degradation of hPEM-2 through the ubiquitin-proteasome system.

**Smurf1 interacts with hPEM-2 directly but is not mediated by PY motif**

To check whether hPEM-2 ubiquitination by Smurf1 occurs through a PPXY-WW mediated interaction, I carried out an ubiquitination experiment using a deletion mutant of PPXY motif (hPEM-2 Δ PPSYPPP). As shown in Figure 4A, polyubiquitination of hPEM-2 Δ PPSYPPP was enhanced by Smurf1, as well as the positive control in wild-type hPEM-2. This result suggested that the PPXY motif of hPEM-2 was not required for Smurf1-mediated ubiquitination. Therefore, to elucidate the mechanism used by Smurf1 to target hPEM-2, I investigated the interaction *in vivo*. In these assays, Smurf1CA was utilized instead of wild type Smurf1 to avoid protein degradation and to stabilize the protein complex. Myc-tagged Smurf1CA was transiently co-expressed with FLAG-tagged hPEM-2 in HEK293 cells. The expression level of Smurf1 was determined by Western blot with anti-Myc antibody (Figure 4B, middle). Anti-FLAG antibody was used for immunoprecipitation of hPEM-2 from the cell lysates (Figure 4B, bottom), and precipitates were detected by Western blot using anti-Myc or anti-FLAG

antibodies (Figure 4B, top and bottom, respectively). Myc-Smurf1 was efficiently co-precipitated with FLAG-hPEM-2 when both proteins were co-expressed in HEK293 cells (Figure 4B, lane 3).

It has recently been reported that Smurf1 transfers ubiquitin molecules to certain target proteins by mediating adaptor proteins (Shearwin-Whyatt et al., 2006). Hence, I used *in vitro* GST pull-down assays to examine whether the complex formation between Smurf1 and hPEM-2 occurs through direct interaction. Indeed, glutathione bead-bound GST-Smurf1CA expressed and purified from *E. coli* Rosetta incubated with *E. coli* lysates expressed His-hPEM-2 (Figure 4C, bottom), where potential adaptor proteins do not exist. The binding activity of GST-Smurf1CA was detected for the His-hPEM-2, but not for luciferase protein (His-Luc2) as a negative control (Figure 4C, top). GST alone was also used as a negative control. These results indicate that Smurf1 and hPEM-2 can interact directly without any additional proteins.

**Determination of the Smurf1-binding site in hPEM-2**

The data described above indicate that Smurf1 interacts with hPEM-2 via a binding site other than the PPXY motif. To identify the hPEM-2 domain responsible for Smurf1-binding, I produced various deletion mutants of FLAG-Tagged hPEM-2 in HEK293 cells and subjected them to *in vitro* GST pull-down assays (Figure 5A). The expression levels of the transfected hPEM-2 deletion mutants were determined by Western blot analysis with anti-FLAG antibody (Figure 5B, bottom). The hPEM-2Full was efficiently recovered from lysates after incubation with GST-Smurf1CA, but not GST (Figure 5B, lanes 1 and 2). The hPEM-2 deletion mutants were also incubated with GST-Smurf1CA, co-purified with GST-Smurf1CA and detected with anti-FLAG antibody. The hPEM-2 Δ (C-terminus+PH) was not sufficient to bind to GST-Smurf1CA (Figure 5B, lane 5), while the other hPEM-2 mutants containing the PH domain region were able to bind. These results demonstrated that a region of hPEM-2 between amino acid residues 300 and 434 (PH domain region) is a candidate binding region for Smurf1.

Moreover, to narrow the search for the Smurf1 binding region on hPEM-2, I constructed various hPEM-2 C-terminal deletion mutants with more limited truncations in the region encompassing residues 300-434. The respective mutants from transfected HEK293 cells were used to analyze the binding activity to GST-Smurf1 as before.

Although GST-Smurf1CA bound the hPEM-2 mutants (1-409, 1-396, 1-375, 1-358, and 1-343), the 1-329 mutant slightly reduced binding activity and the 1-318 mutant markedly reduced binding activity to the GST-Smurf1 (Figure 5C). These results suggest that the region of hPEM-2 between amino acid residues 318 and 343 is required for binding to Smurf1.

I continued to focus our attention in this region, and hPEM-2 deletion mutants produced in rabbit reticulocyte lysates were used for binding assays with GST-Smurf1 as before. The results obtained for the hPEM-2 C-terminal deletion mutants (1-358, 1-343, 1-329, 1-318, and 1-299) were consistent with those for HEK293 lysates (Figure 4A, lanes 1-5). Conversely, GST-Smurf1CA was able to bind the 318-516, 330-516, and 335-516 N terminal deletion mutants of hPEM-2, while binding activity was eliminated by the 344-516 hPEM-2 mutant (Figure 6A, lanes 6-9).

As shown in Figure 6C, the region encompassing amino acid residues from 318 to 343 in the PH domain of hPEM-2 is important for binding to Smurf1. Therefore, I next confirmed whether this region is sufficient to bind to Smurf1 by using firefly luciferase (Luc2), a heterologous protein. FLAG-tagged Luc2 fusion proteins, including segments of hPEM-2 residues at C-terminus were produced in rabbit reticulocyte lysates and binding to GST-Smurf1 was analyzed as before. Although GST-Smurf1 failed to bind the Luc2 fusion protein containing hPEM-2 residues from 335 to 343 (hPEM-2 *335-343*), it did bind hPEM-2 *318-343* (Figure 6B, lane 2 and 3). In addition, GST-Smurf1 also bound hPEM-2 *304-343*, hPEM-2 *287-343*, and hPEM-2 *267-343*, which have additional N-terminal regions relative to hPEM-2 *318-343* (Figure 6B, lanes 4-6). In particular, hPEM-2 *267-343* was readily recovered by GST-Smurf1CA (Figure 6B, lane 6). Binding to GST-Smurf1CA was markedly reduced by hPEM-2 *267-329*, which lacks the 14 C-terminal amino acid residues found in hPEM-2 267-343, and deletion of another 12 amino acids (hPEM-2 *267-317*) abolished binding (Figure 6B, lanes 7 and 8). Moreover, hPEM-2 *241-317*, which had 27 amino acids added to the N terminal residues relative to hPEM-2 *267-317*, rarely bound to the GST-Smurf1 (Figure 6B, lane 9). Based on these results, I conclude that residues 318-343 (extreme N-terminus of the PH domain) of hPEM-2 are essential for interaction with Smurf1, and that the neighboring regions potentiate binding activity.

**The hPEM-2 ubiquitination by Smurf1 requires C2 domain of Smurf1**

To delineate the hPEM-2-binding domain in Smurf1, a set of FLAG-tagged Luc2 fusion proteins containing C2, WW, and HECT domains of Smurf1 (Figure 7A, schematic diagram) were synthesized in rabbit reticulocyte lysates and used for *in vitro* GST pull-down assays as before. GST-hPEM-2 bound the FLAG-Luc2 containing the C2 domain (Figure 7A, lane 4), whereas it failed to bind the FLAG-Luc2 containing the two WW or HECT domains (Figure 7A, lanes 6 and 8) and GST alone did not bind to any of the FLAG-Luc2-Smurf1 mutants.

Accordingly, it was expected that Δ C2 Smurf1 would not enhance the polyubiquitination of hPEM-2 in cultured cells, because Δ C2 Smurf1 fails to interact with hPEM-2. Indeed, no increase in hPEM-2 polyubiquitination was observed in HEK293 cells with co-expressed hPEM-2 and Δ C2 Smurf1 (Figure 7B, lane 3), as well as the negative control in the absence of Smurf1 (Figure 7B, lane 1). These results strongly suggest that Smurf1 associates with hPEM-2 through its C2 domain and enhances ubiquitination of hPEM-2.

It was reported that many C2 domains interact with a variety of phospholipids and proteins in a $Ca^{2+}$-dependent manner (Nalefski and Falke, 1996). I therefore tested whether $Ca^{2+}$ is essential for the binding of Smurf1 to hPEM-2 using GST-hPEM-2 purified from *E. coli* and FLAG-Luc2 fusion proteins synthesized in rabbit reticulocyte lysates via the *in vitro* pull-down assays. EGTA, a chelating agent of calcium, was added to the reaction solution at a final concentration of 5 mM to completely eliminate calcium ions from the reaction. Conversely, calcium ions were added to the reaction solution as $CaCl_2$ at a final concentration of 3.5 mM. Binding activity was observed in combination with GST-hPEM-2 and FLAG-Luc2-Smurf1C2, but not with GST or FLAG-Luc2, regardless of the absence or presence of calcium ions (Figure 7C).

## Discussion

In this study, I identified hPEM-2, a GEF for small GTPase Cdc42 (Reid et al., 1999), as a novel target protein for Smurf1. This means that Cdc42 can be indirectly regulated by Smurf1 through hPEM2 degradation. This finding could shed light on the spatiotemporal regulation of the activities of the Rho family of small GTPases by Smurf1, since Smurf1 also controls spatial distribution of RhoA in cells through ubiquitin-mediated degradation (Wang et al., 2003). Moreover, it suggests the existence of signaling cross-talk in Rho family GTPases with qualitative alteration by GEFs and GAPs, and with variability in quantity through ubiquitin-mediated degradation.

I performed *in vivo* ubiquitination assays to examine whether Smurf1 exhibits E3 ligase activity for hPEM-2. In the absence of ectopically expressed Smurf1, low level constitutive ubiquitination of hPEM-2 was detected. Co-expression of Smurf1 with hPEM-2 markedly enhanced ubiquitin conjugation to hPEM-2, whereas Smurf1CA, a ligase-inactive mutant with an amino acid substitution in the conserved cysteine in the HECT domain, abolished such an effect. In fact, Smurf1CA also reduced constitutive ubiquitination of hPEM-2 and might act as a dominant negative mutant, competing with endogenous E3 ligases for hPEM-2. Additionally, accumulation of ubiquitinated hPEM-2 in the presence of MG132, an inhibitor of proteasomal activity, suggests that ubiquitinated hPEM-2 is targeted for proteasomal degradation. Because our experiments using cultured cells were designed as a model system using cells transiently overexpressing Smurf1 and hPEM-2, there is concern about the essential role of endogenous Smurf1 for hPEM-2. It was indirectly indicated that the endogenous Smurf1 targets hPEM-2 because the constitutive ubiquitination of transiently expressed hPEM-2 could be diminished in HEK293 cells in the presence of Smurf1 siRNAs, which can reduce the expression level of endogenous Smurf1. Moreover, I applied a pulse-chase labeling assay with fluorescence-labeled HaloTag-fusion hPEM-2 to elucidate whether Smurf1 induces proteolysis of hPEM-2 protein. The half-life of the HaloTag-hPEM-2 in the presence of Smurf1WT was much shorter than that in the presence of Smurf1CA. Actually, amount of HaloTag-hPEM-2 in the presence of Smurf1WT was considerably reduced at zero time point of the experiment compared with that in the presence of Smurf1CA, that means the reduction of steady-state level of hPEM-2 by Smurf1WT. These data directly demonstrate that Smurf1 regulates the

stability of hPEM-2 in cultured cells. Furthermore, my data suggests that Smurf1 promotes degradation of hPEM-2 through the ubiquitin-proteasome system. The GEF hPEM-2 is known as a human homologue of rat collybistin, which appears to be a gephyrin-binding partner and is an essential determinant of neuronal gephyrin clustering (Harvey et al., 2004). Smurf1 may be involved in neuronal gephyrin clustering through regulation of the stability of collybistin.

Smurf1 transfers ubiquitin molecules to Smad4 by mediating an adaptor protein, I-Smad, because Smad4 lacks a PY motif and does not directly bind to Smurf1 (Shearwin-Whyatt et al., 2006). Although hPEM-2 has a PY motif, this motif is not required for Smurf1 binding. To confirm whether Smurf1-induced hPEM-2 degradation results from direct interaction of those proteins, an *in vitro* GST pull-down assay was applied using GST-Smurf1CA and His-hPEM-2 synthesized in *E. coli,* where no possible intermediary proteins exist. Specific interaction of His-hPEM-2 with GST-Smurf1CA was observed, and this indicated direct interaction of both proteins. Next, I tried to expose the Smurf1-binding site in hPEM-2. Analysis of hPEM-2 deletion mutants and Luc2 fusion proteins containing hPEM-2 residues defined a 26-amino acid region (residues 318-343), corresponding to the extreme N-terminus of PH domain. The PH domain is a small protein module of about 120 amino acids residues and their three-dimensional folded structures are highly conserved, despite a low primary sequence similarity (Lemmon and Ferguson, 2000). The PH domain possesses multiple functions including the ability to bind inositol phosphates and various proteins. Thus, GEF proteins other than hPEM-2 may be a target of Smurf1, since each of the 60 plus GEF proteins possesses the PH domain and specifically activates individual members of Rho family within particular spatial and temporal contexts (Rossman et al., 2005). The various combinations of Smurf1 and GEFs might play a key role in the fine-tuning of Rho family GTPase activity.

On the other hand, a GST pull-down assay with Luc2 fusion proteins containing respective Smurf1 domains (C2, WW, and HECT) revealed that the C2 domain of Smurf1 is sufficient for the binding to hPEM-2. Moreover, ΔC2 Smurf1 did not enhance polyubiquitination of hPEM-2. Intriguingly, these results suggest that Smurf1 binds hPEM-2 via its C2 domain, but not via the WW domains, which appear to mediate substrate recognition. The C2 domain was originally identified in classical protein kinase C isoforms as a $Ca^{2+}$-dependent phospholipid binding domain (Nalefski

and Falke, 1996; Rizo and Sudhof, 1998). However, the C2 domain can also mediate protein-protein interaction and small molecule binding, in addition to the binding of $Ca^{2+}$ itself. For example, the second C2 domain of most synaptotagmin isoforms binds in a $Ca^{2+}$-independent manner with high affinity to clathrin –AP2, a protein complex involved in coated pit assembly (Zhang et al., 1994). Indeed, the binding of Smurf1 to hPEM-2 did not require $Ca^{2+}$. My finding is a valuable example showing the extended function of the C2 domain as a protein binding motif.

Thus, my studies showed that the interaction of Smurf1 with hPEM-2 occurs through the C2 domain and extreme N-terminus of the PH domain, but not the PPXY motif and the WW domain, despite the presence of a PPXY motif on hPEM-2. In contrast the interaction of Smurf1 with Smad1, Smad5 and Smad7 in the TGF-$\beta$ signaling pathway occurs via a WW-PPXY mediated association (Ebisawa et al., 2001). It is also reported that the C2 domain of Smurf1 is important for localization and the inhibitory effect of Smad7 in the TGF-$\beta$ signaling pathway (Suzuki et al., 2002). These results in addition to our finding strongly suggest that Smurf1 is an important factor that can regulate a variety of signaling cascades, using two WW domains and the C2 domain as protein- and membrane-targeting modules.

My study provides another approach for understanding the mechanisms regulating GTPase activity. In addition, further insight into the Rho signaling pathway may highlight the ubiquitin ligases as molecular switches that generate an intracellular asymmetry of temporal and spatial GTPase activity.

## References

**Bryan, B., Cai, Y., Wrighton, K., Wu, G., Feng, X.H., and Liu, M.** (2005). Ubiquitination of RhoA by Smurf1 promotes neurite outgrowth. FEBS Lett. *579*, 1015-1019.

**Burridge, K., and Wennerberg, K.** (2004). Rho and Rac take center stage. Cell *116*, 167-179.

**Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K.** (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J. Boil. Chem. *276*, 12477-12480.

**Etienne-Manneville, S., and Hall, A.** (2002). Rho GTPases in cell biology. Nature *420*, 629-635.

**Hara, Y., Shimada, K., Kohga, H., Ohara, O., and Koga, H.** (2003). High-throughput production of recombinant antigens for mouse KIAA proteins in Escherichia coli: computational allocation of possible antigenic regions, and construction of expression plasmids of glutathione-S-transferase-fused antigens by an *in vitro* recombination-assisted method. DNA Res. *10*, 129-136.

**Hayakawa, M., Kitagawa, H., Miyazawa, K. Kitagawa, M., and Kikugawa K.** (2005). The FWD1/beta-TrCP-mediated degradation pathway establishes a 'turning off switch' of a Cdc42 guanine nucleotide exchange factor, FGD1. Genes Cells *10*, 241-251.

**Hershko, A., and Ciechanover, A.** (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425-479.

**Harvey, K., Duguid, I.C., Alldred, M.J., Beatty, S.E., Ward, H., Keep, N.H., Lingenfelter, S.E., Pearce, B.R., Lundgren, J., Owen, M.J., Smart, T.G., Lüscher, B., Rees, M.I., and Harvey, R.J.** (2004). The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. J. Neurosci. *24*, 5816-5826.

**Laney, J.D., and Hochstrasser, M.** (1999). Substrate targeting in the ubiquitin system. Cell *97*, 427-430.

**Lemmon, M.A., and Ferguson, K.M.** (2000). Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem. J. *350*, 1-18.

**Luo, L.** (2000). Rho GTPases in neuronal morphogenesis. Nat. Rev. Neurosci. *1*,

173-180.

**Nalefski, E.A., and Falke, J.J.** (1996). The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. *5*, 2375-2390.

**Pickart, C.M.** (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503-533.

**Reid, T., Bathoon, A., Ahmadian, M.R., and Collard, J.G.** (1999). Identification and characterization of hPEM-2, a guanine nucleotide exchange factor specific for Cdc42. J. Biol. Chem. *47*, 33587-33593.

**Rizo, J., and Sudhof, T.C.** (1998). C2-domains, structure and function of a universal $Ca^{2+}$-binding domain. J. Biol. Chem. *273*, 15879-15882.

**Rossman, K.L., Der, C.J., and Sondek, J.** (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat. Rev. Mol. Cell. Biol. *6*, 167-180.

**Shearwin-Whyatt, L., Dalton, H.E., Foot, N., and Kumar, S.** (2006). Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. Bioessays *28*, 617-628.

**Suzuki, C., Murakami, G., Fukuchi, M., Shimanuki, T., Shikauchi, Y., Imamura, T., and Miyazono, K.** (2002). Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. J. Biol. Chem. *277*, 39919-39925.

**Treier, M., Staszewski, L.M., and Bohmann, D.** (1994). Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the delta domain. Cell *78*, 787-798.

**Wang, H.R., Zhang, Y., Ozdamar, B., Ogunjimi, A.A., Alexandrova, E., Thomsen, G.H., and Wrana, J.L.** (2003). Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. Science *302*, 1775-1779.

**Zhang, J.Z., Davletov, B.A., Südhof, T.C., and Anderson, R.G.** (1994). Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. Cell *78*, 751-760.

**Zhang, Y., Wang, H.R., and Wrana, J.L.** (2004). Smurf1: a link between cell polarity and ubiquitination. Cell Cycle *3*, 391-392.

**Zhu, H., Kavsak, P., Abdollah, S., Wrana, J.L., and Thomsen, G.H.** (1999). A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature *400*, 687-693.

## Figure legends

**Figure 1** Smurf1 mediates ubiquitination of hPEM-2.

(A and B) Ubiquitination of hPEM-2 in HEK293 cells. At 48 h after co-transfection with the indicated expression plasmids (FLAG-tagged hPEM-2, Myc-tagged Smurf1 WT/CA and HA tagged-Ub), FLAG-hPEM-2 proteins were immunoprecipitated (IP) with anti-FLAG antibody from the cell lysates. The ubiquitinated hPEM-2 was detected by Western blot analysis (Blot) with anti-HA antibody (top), and appeared as high molecular weight ladders, indicated by brackets to the right of the top panel. The expression levels of Myc-Smurf1 and the co-precipitation levels of FLAG-hPEM-2 were determined with anti-Myc antibody (middle) and anti-FLAG antibody (bottom), respectively. When the cells were treated with proteasomal inhibitor MG132 (10 μM), the reagent was added to cells for 1 h prior to cell lysis. WT, wild type; CA, catalytically inactive mutant.

**Figure 2** Effect of endogenous Smurf1 on ubiqutination of hPEM-2 in HEK293 cells.

(A) Effect of Smurf1 siRNA on ubiquitination of ectopically-expressed hPEM-2. At 35 h after co-transfection with expression plasmids (FLAG-tagged hPEM-2 and HA tagged-Ub) and one of the siRNAs (control siRNA, Smurf1 siRNA-1 or Smurf1 siRNA-2), the cells were treated with proteasomal inhibitor MG132 (5 μM or 10 μM) for 1.5 h prior to cell lysis. FLAG-hPEM-2 proteins were immunoprecipitated (IP) with anti-FLAG antibody from the cell lysates and the ubiquitinated hPEM-2 was detected by Western blot analysis (Blot) with anti-HA antibody (top). The co-precipitation levels of FLAG-hPEM-2 were determined with anti-FLAG antibody (bottom). (B) Reduction of the endogenous Smurf1 mRNA by Smurf1 siRNAs. Amounts of Smurf1 mRNA were estimated by qRT-PCR (ABI 7500FAST) using total RNAs extracted from cells transfected as described in (A). (C) Decrease of the Smurf1-GFP proteins transiently expressed in HEK293 cells by Smurf1 siRNA. Cells transfected with Smurf1-GFP expression plasmid together with the siRNA indicated were imaged by fluorescence microscopy 40 h after the transfection.

**Figure 3** Smurf1 mediates proteasomal degradation of hPEM-2.

(A) Ubiquitination of HaloTag-fused hPEM-2 in HEK293 cells. HEK293 cells were

transfected with the indicated expression plasmids. The HaloTag-FLAG-hPEM-2 proteins were immunoprecipitated (IP) with anti-FLAG antibody from the cell lysates. The ubiquitinated HaloTag-hPEM-2 was detected by Western blot analysis (Blot) with anti-HA antibody (top). The expression levels of Myc-Smurf and the co-precipitation levels of FLAG-hPEM-2 were determined with anti-Myc antibody (middle) and anti-FLAG antibody (bottom), respectively. (B) The effect of Smurf1 on hPEM-2 protein stability. HEK293 cells were transfected with either Smurf1WT (diamonds), or Smurf1CA with HaloTag-fused hPEM-2 (squares). After 36 h of cell culture, the Halo-hPEM-2 proteins were labeled for 10 min with HaloTag TMR ligand, and then chased for indicated times after removal of unreacted HaloTag TMR ligand by washing cells and blocking with HaloTag biotin ligand. The TMR-labeled Halo-hPEM-2 was quantified by fluoroimaging and the level was plotted relative to the amount present at time 0. Similarly, the stability of HaloTag protein was also analyzed as a control. WT, wild type; CA, catalytically inactive mutant.

**Figure 4** hPEM-2 is ubiqutinated by and directly interacts with Smurf1 through a region other than PPXY motif.

(A) Ubiquitination of hPEM-2 lacking PPXY motif ( Δ PPSYPPP) in HEK293 cells. HEK293 cells were transfected with the indicated expression plasmids. The hPEM-2WT and hPEM-2 Δ PPSYPPP proteins were immunoprecipitated (IP) with anti-FLAG antibody from the cell lysates. The ubiquitinated hPEM-2WT and hPEM-2 Δ PPSYPPP was detected by Western blot analysis (Blot) with anti-HA antibody (top). The expression levels of Myc-Smurf and the co-precipitation levels of FLAG-hPEM-2 were determined with anti-Myc antibody (middle) and anti-FLAG antibody (bottom), respectively. (B) Smurf1co-precipitates with hPEM-2 transiently co-expressed in mammalian cells. HEK293 cells were transfected with FLAG-tagged hPEM-2 and Myc- tagged Smurf1CA. The cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, followed by western blot analysis (Blot) with anti-Myc antibody (top). The expression level of Myc-Smurf and the co-precipitation level of FLAG-hPEM-2 were determined by anti-Myc antibody (middle) and anti-FLAG antibody (bottom), respectively. (C) hPEM-2 directly interacts with Smurf1 without requiring an intermediary protein. His-tagged hPEM-2 and Luciferase protein (His-Luc2) were expressed in E.coli. The lysates of His-proteins (bottom) were

incubated with purified GST-Smurf1CA in the *in vitro* GST-pull down assay. GST alone also served as a negative control. The bound His-tagged proteins were eluted from the beads and detected by Western blot analysis with anti-His antibody (top).

**Figure 5** Identification of the Smurf1-binding region in hPEM-2.

(A) A schematic diagram to illustrate various hPEM-2 deletion mutants. (B) HEK293 cells were transfected with the FLAG-tagged hPEM-2 deletion mutants and the cell lysates were incubated with the GST-Smurf1CA. The precipitates eluted from the beads were analyzed by Western blot analysis (Blot) with anti-FLAG antibody (top). The expression levels of the hPEM-2 deletion mutants were analyzed by anti-FLAG using whole cell lysates (bottom). (C) hPEM-2 various C-terminal deletion mutants were transfected into HEK293 cells. The hPEM-2 mutant proteins binding to GST-Smurf1CA from cell lysates (top) and total mutant proteins in cell lysates (bottom) were detected by anti-FLAG antibody, according to the method in B.

**Figure 6** Detailed analysis of the Smurf1-binding sequences in hPEM-2.

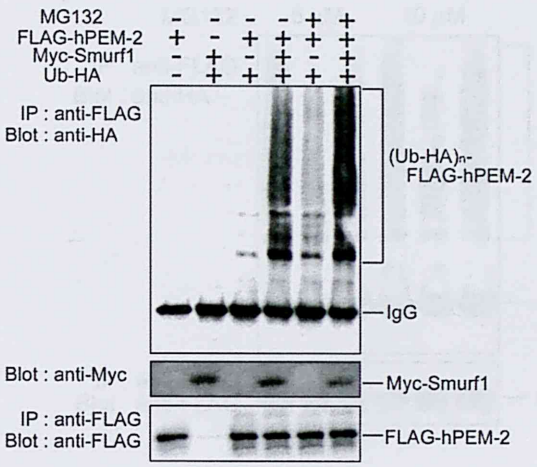
(A) FLAG-tagged different C-terminal and N-terminal mutants of hPEM-2 were expressed in rabbit reticulocyte lysates. The lysates were incubated with the GST-Smurf1, and bound proteins were detected by Western blot analysis with anti-FLAG antibody (top). The bottom panel demonstrates the level of the FLAG-hPEM-2 truncation mutants in total lysates. (B) Rabbit reticulocyte extracts expressed FLAG-tagged Luc2 fusion proteins including different amino acid residues of hPEM-2 at C-terminus. The lysates were analyzed for binding to GST-Smurf1 as in A. (C) A schematic representation of hPEM-2 deletion mutants and the binding activities to Smurf1CA, along with the amino acid sequences surrounding the Smurf1-binding region.

**Figure 7** C2 domain of Smurf1 is responsible for interaction with and ubiquitination of hPEM-2 in a $Ca^{2+}$-independent manner.

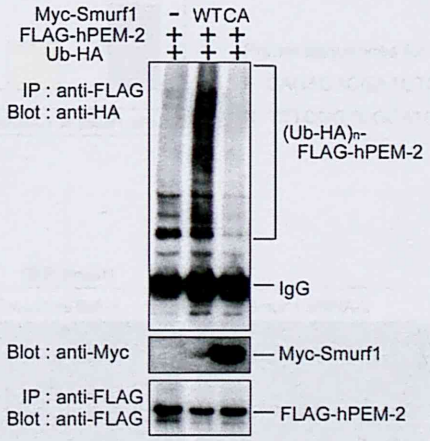
(A) Determination of the hPEM-2-Binding domain in Smurf1. Top: A schematic diagram to illustrate Luc2 fusion proteins containing C2, WW, and HECT domain of Smurf1, respectively. Bottom: Pull down assays were performed using the GST-hPEM-2 and the FLAG-tagged Luc2 fusion proteins synthesized in rabbit

reticulocyte lysates. The bound FLAG-Luc2 fusion proteins were detected by western blot analysis with anti-FLAG antibody (top). The expression of FLAG-Luc2 fusion proteins was detected by western blot with anti- FLAG antibody (bottom). (B) Ubiquitination of hPEM-2 by C2 domain deletion mutant of Smurf1 (Smurf1 Δ C2) in HEK293 cells. The cell lysates transfected with FLAG-tagged hPEM-2, HA-tagged ubiquitin and Myc-tagged Smurf1WT or Myc-tagged Smurf1 Δ C2 were used for *in vivo* ubiquitination assays, according to the procedure described in Figure 1. (C) $Ca^{2+}$-independent interaction of C2 domain of Smurf1 with hPEM-2. The GST-hPEM-2 was incubated with rabbit reticulocyte lysates expressing FLAG-tagged Luc2-Smurf1C2 containing either 5 mM EGTA, no additions, or 3.5 mM $CaCl_2$ in the *in vitro* GST-pull down assay.

A

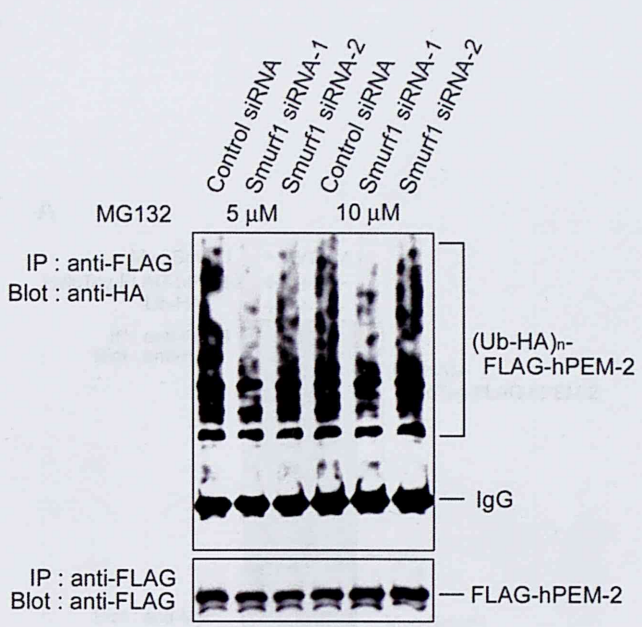



B



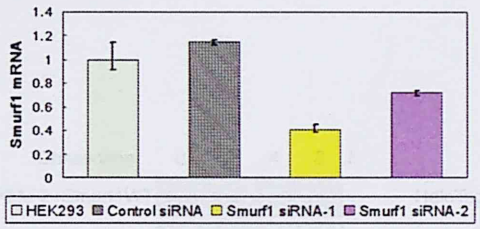

Chapter1 fig. 1

A



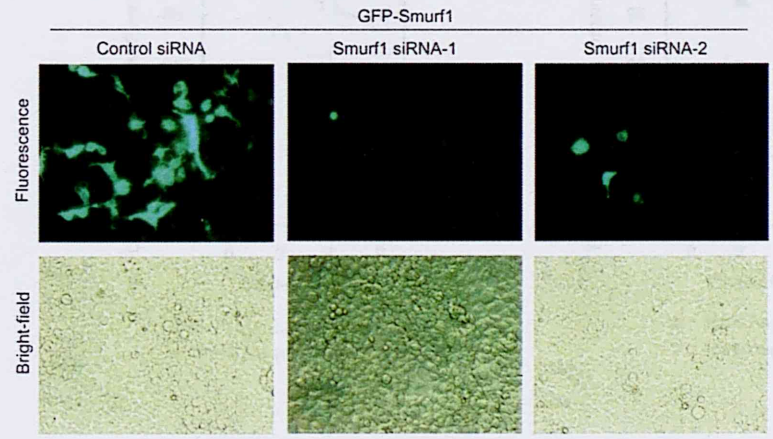

Control siRNA: Stealth RNAi Negative Control Med#2(Invitrogen)
Smurf1 siRNA-1: Smurf1 Validated Stealth Duplex#1(Invitrogen)
Smurf1 siRNA-2: Smurf1 Validated Stealth Duplex#2(Invitrogen)

B



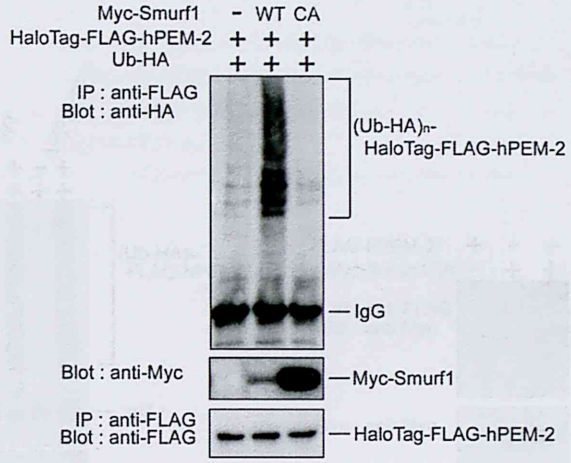

Primer sequences for Smurf1
F: CAGACACGAACTGTCGCTTCA
R: TTTCGGTCGCATCTTCATTATCT

C

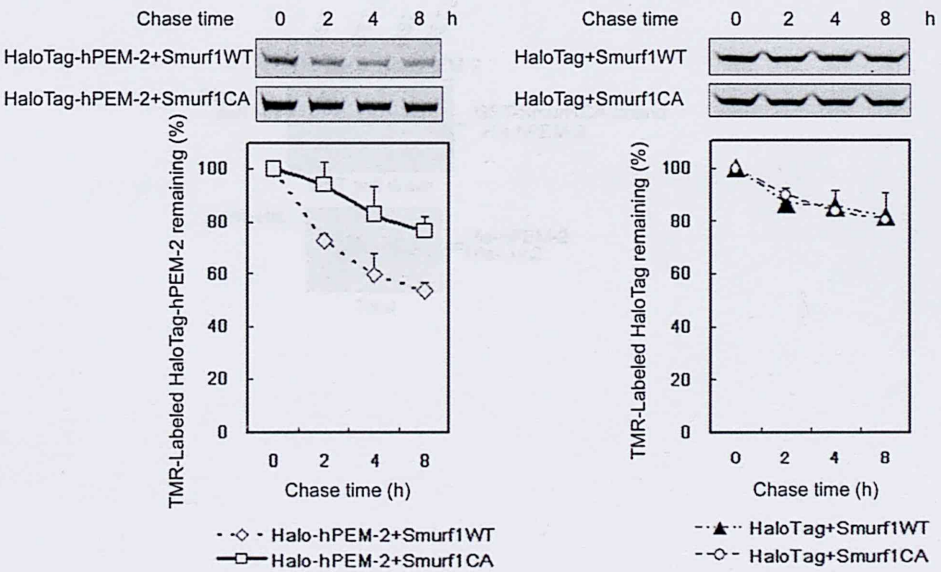



Chapter1 fig. 2

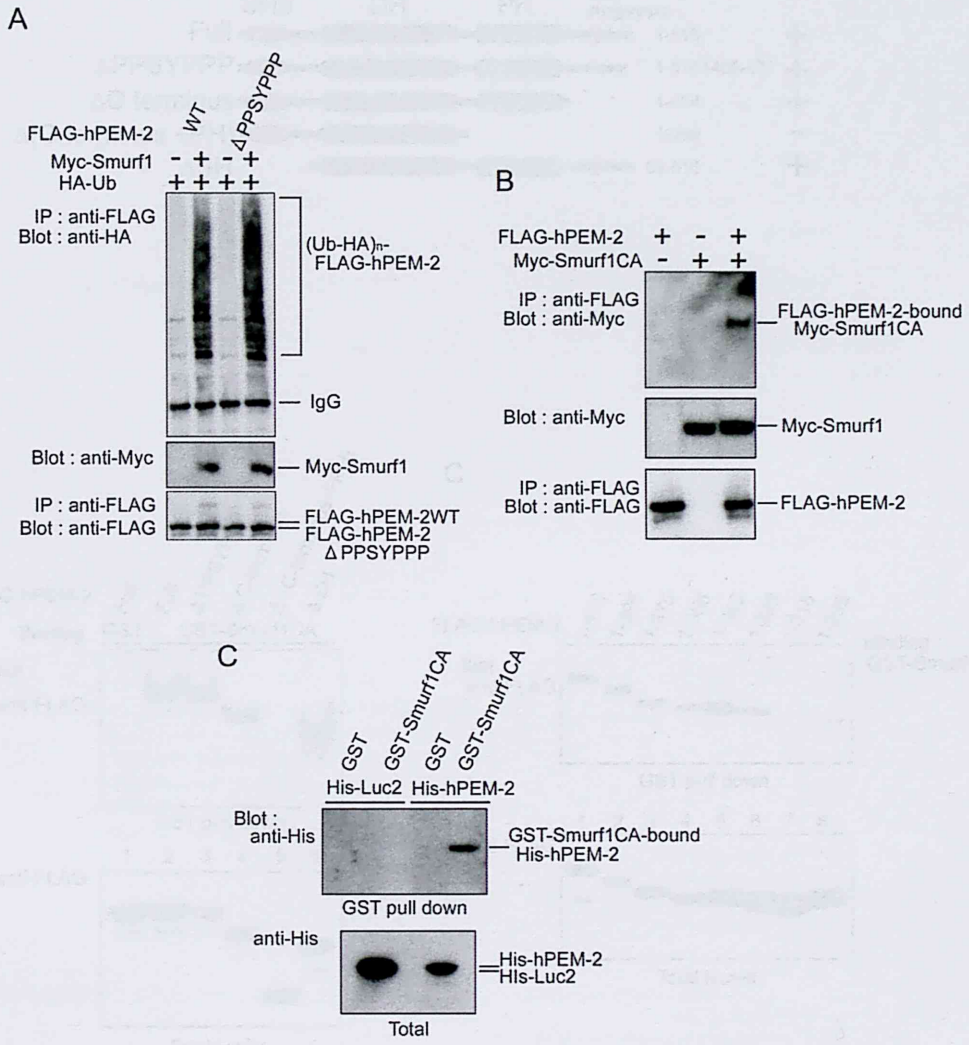
A




B

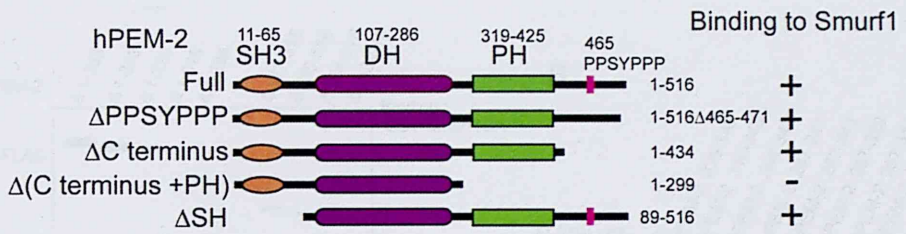



Chapter1 fig. 3

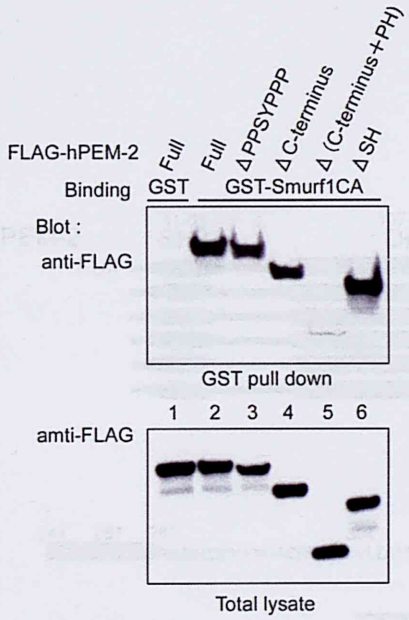



Chapter1 fig. 4

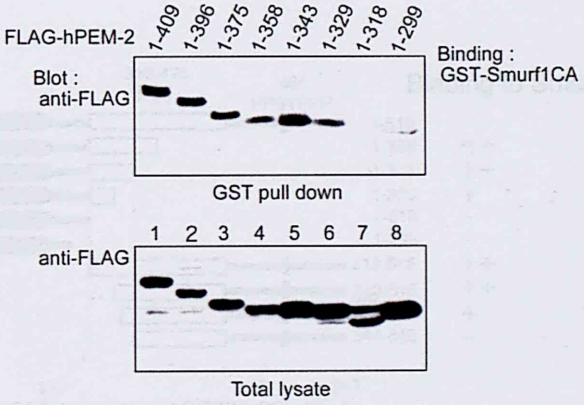
A



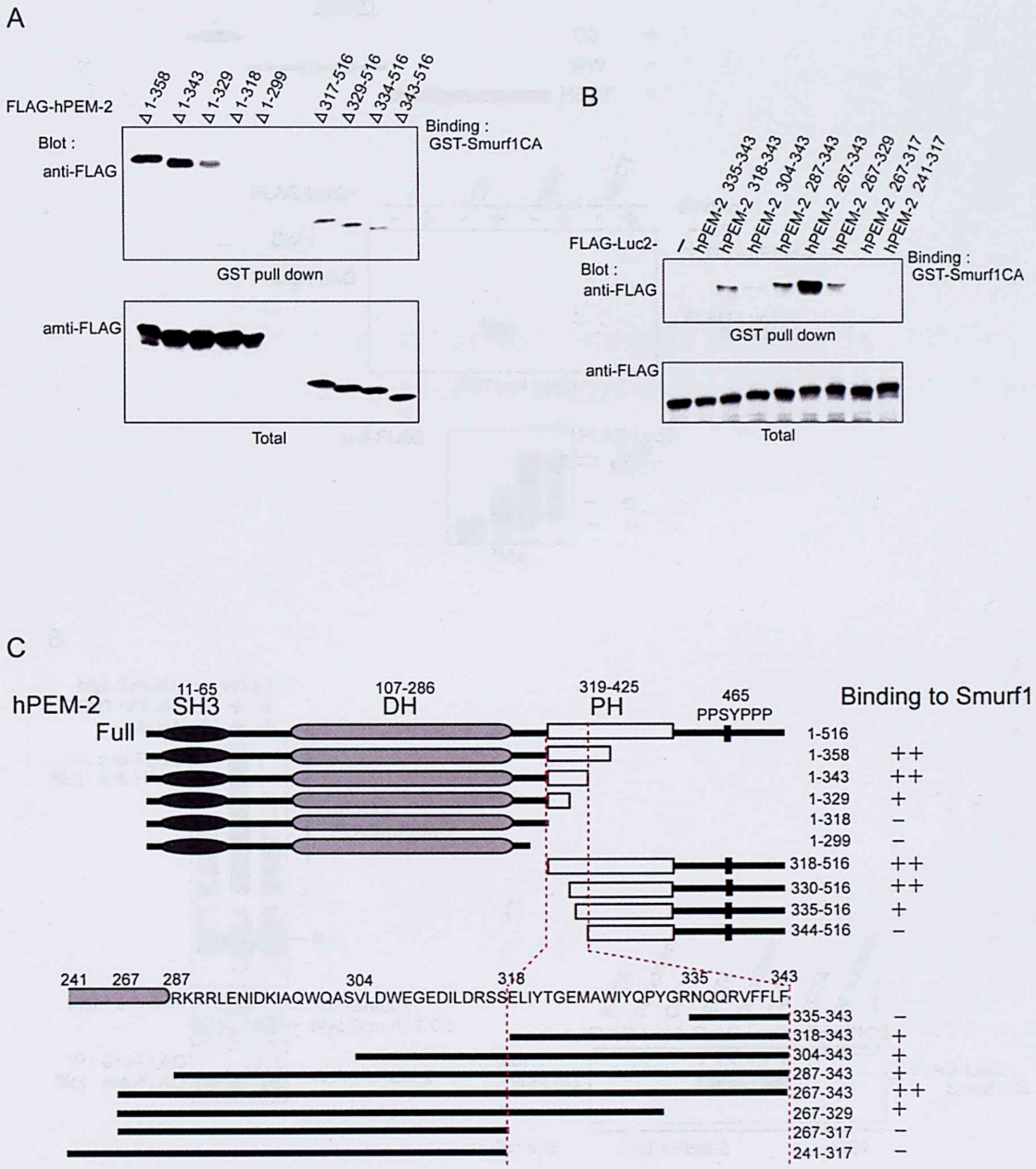

B



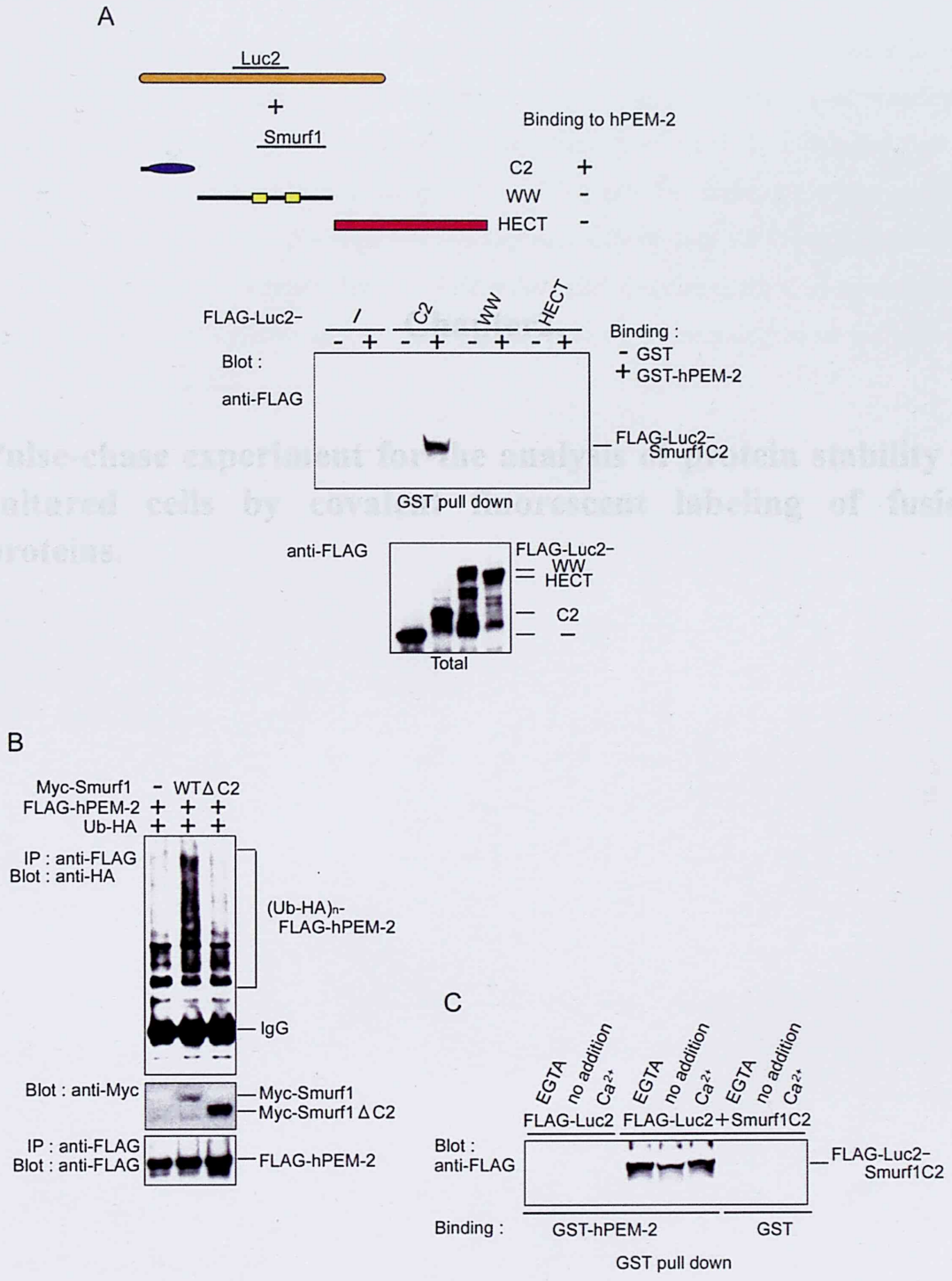

C




Chapter1 fig. 5




Chapter1 fig. 6




Chapter1 fig. 7

# Chapter2.

## Pulse-chase experiment for the analysis of protein stability in cultured cells by covalent fluorescent labeling of fusion proteins.

## Summary

I applied HaloTag® labeling technology, in which a HaloTag-fusion protein can covalently bind to a single chemical fluorescent ligand, for pulse labeling of proteins in cultured mammalian cells. HaloTag-fusion proteins thus specifically labeled can be chased and observed after separation of cell lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by quantification of the labeled proteins by a fluorescent image analyzer. Herein, I demonstrated that the method is available for analysis of the intracellular protein stability affected by protein-degradation signals or an E3 ubiquitin ligase.

## Introduction

Pulse-chase experiments by metabolic labeling of cultured cells are used for time course profiling of newly synthesized proteins such as intracellular transport, posttranslational modification and degradation (Zhao et al., 2007; Magee et al., 1995; Mosteller et al., 1980). Especially, [$^{35}$S]methionine has been often used as a radiolabeled amino acid of choice for labeling of proteins because [$^{35}$S]methionine is relatively stable with high specific activity and has no modification by transamination and interconversion in cells (Bonifacino 1999). However, development of more convenient procedure is required since the conventional method is rather time-consuming and needs a specific antibody against the protein of interest to isolate the protein from the labeled cell extract for the observation.

Recently, several technologies for labeling of certain fusion proteins *in vivo* were reported and those are based on covalent bond with small molecules harboring a functional moiety such as fluorophores, affinity molecules and anchoring materials (Gronemeyer et al., 2005; Keppler et al., 2003; Los and Wood, 2007). HaloTag is a 33 kDa mutant haloalkane dehalogenase which can rapidly form a stable covalent bond with various ligands composed of aliphatic chloride containing a labeling moiety. It is possible to adapt fluorescent-labeled HaloTag-fusion proteins to pulse-chase experiment of the protein because HaloTag ligand has a high rate of cell permeability and a rapid labeling activity in living cells. Moreover, the labeling is specific for HaloTag and the resultant labeled proteins can be observed without any purification after separation of cell lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) followed by quantification of the labeled proteins by a fluorescent image analyzer.

## Determination of materials and methods

I first optimized the amount of HaloTag blocking ligands for a pulse-chase experiment to prevent a continuous-labeling of the target proteins. HEK293 cells were plated in MICROTEST$^{TM}$ Tissue Culture Plate, 96 well (BD, 4.5 x $10^4$ cells/well) with Dulbecco's Modified Eagle Medium (Invitrogen,) supplemented with 10% Fetal Bovine Serum (BD) and 100 x Antibiotic-Antimycotic liquid (Invitrogen) and incubated for 24 h at 37°C with 5% $CO_2$. The cells were transfected with HaloTag expression plasmid using FuGENE6 Transfection Reagent (Roche Diagnostics) and cultured for 36 h. The HaloTag proteins were labeled for 10 min with the growth medium containing HaloTag tetramethylrhodamine (TMR) ligands (Pulse) and washed out free TMR ligands by a 4-times rinse with 100 ml of the growth medium. 50 nM TMR ligand was used for the labeling because an excess amount of TMR ligands results in increment of the residual free ligands in cells and culture medium. The cells were continuously incubated in the growth medium including a different amount of HaloTag blocking ligands for the indicated time period (Chase) except for the sample at zero time point. The blocking ligand was prepared by masking of the functional group of 20 mM HaloTag Succinimidyl Ester (O4) Ligand (Promega) with 100 mM Tris-HCl (pH 8.0) for 60 min at 25°C. The cells were washed with PBS and dissolved in 40 μl of 2 x Laemmli's SDS sample buffer at the indicated time point of the chase in Figure 1. TMR labeled-proteins were separated by SDS-PAGE after boiling for 5 min at 95°C and amounts of the labeled proteins were measured as fluorescence intensities of the TMR by FLA3000 and MultiGauge software (Fujifilm). Although total amounts of labeled- and unlabeled-HaloTag proteins detected by Western blot analysis using anti-HaloTag antibody (Promega) were slightly increased in cells during the time of the experiment (Figure 1, upper right), the detectable pulse labeled HaloTag are gradually decreased at the each time indicated depending on the increment of the amount of the blocking ligands (Figure 1, upper left). The fluorescent intensities of TMR bound to HaloTag at the indicated time are plotted relative to the amount present at zero time point (Figure 1, lower). The results of similar experiments using other four HaloTag-fusion proteins indicated that 5 μM blocking ligand is sufficient to prevent an excess continuous-labeling in our experimental condition for 50 nM TMR ligand labeling (data not shown).

## Result and Discussion

In general, tag proteins may have an inhibitory effect of potential protein function on tag-fusion proteins. Subcellular localization of proteins is important for protein function. Thus, I examined whether or not the HaloTag protein can be localized in an expected subcellular compartment upon fusion of localization signals in COS-7 cells. To do this, pFC8A (C-terminal HaloTag vector, Promega) expression clones for the HaloTag protein containing signal sequences such as membane-localized signal (Memb), nuclear localization signal (NLS), endplasmic reticulum targeting sequence (ER), Golgi-localized signal (Golgi) and mitochondrial targeting sequence (Mito) were prepared by the members of Laboratory in Kazusa DNA Research Institute. These sequences were all place at the N-terminal region of HaloTag protein. Eighteen hours after the transfection of these constructs into COS-7 cells, the HaloTag-fusion proteins were labeled with TMR ligand and observed with a fluorescent microscope system. Figure 2A shows that all fused proteins were detected in an expected subcellular compartment, indicating that HaloTag protein does not inhibit the function of these subcellular localization signals. Next, I directly evaluated whether HaloTag-fusion proteins were co-localized with the same green fluorescent protein (GFP)-fusion proteins when both fusions were expressed in the same cells. C-terminal HaloTag- and Monster Green® Fluorescent protein (MGFP, Promega)-fusion protein expression clones were prepared by the Flexi® cloning system (Promega) using Flexi ORF clones (http://www.kazusa.or.jp/kop/). After the co-transfection of HEK293 cells with the same ORF clone fuse to the two different fusion tags, HaloTag-fusion proteins was labeled with TMR ligand and then the fluorescence images were obtained for TMR-(red) and MGFP-labeled (green) protein for 6 ORFs. Examples of the cellular localization of the fusion proteins are shown in Figure 2B. Among them, subcellular locations of Glucocorticoid modulatory element-binding protein 2 (GMEB-2/KIAA1269), Phosphatidylserine synthase 1 (PTDSS1/KIAA0024), Membrane-bound transcription factor site-1 protease (MBTPS1/KIAA0091) and Transmembrane protein 127 (TMEM127/KIBB2508) are reported in the UniProt Knowledgebase as nucleus/cytoplasm, membrane, endoplasmic reticulum/Golgi apparatus, and membrane, respectively (http://ca.expasy.org/sprot/). In contrast, the subcellular location of Mesoderm development candidate 2 (MESDC2/KIAA0081) is

not mentioned and Secernin-1 (SCRN1/KIAA0193) is predicted to exist in cytoplasm by similarity in the database. The subcellular localizations of 6 HaloTag-fusion proteins analyzed here were the same as with those for MGFP-fusion proteins. These results indicated that HaloTag-fusion proteins could be used as an equivalant alternative for subcellular localization analysis in place of a conventional autofluorescent protein and do not also inhibit these potential subcellular localizations. Therefore, I have successfully used the HaloTag-fusion proteins for pulse-chase labeling.

This application will rather be suitable for experiments to elucidate the differences of degradation states of certain protein in different conditions, though the results cannot properly reflect the intrinsic half-life of the corresponding native protein. Therefore, I applied this method for comparing the degradation rates of HaloTags with or without C-terminal protein-degradation signals (Figure 3A). The DNA fragments containing PEST or CL1 sequence which are known as protein-degradation signals were obtained from pGL4.19 [luc2CP/Neo] (Promega) (Rogers et al., 1986; Gilon et al., 1998) by PCR and inserted into a derivative of pHT2 vector (Promega) for construction of HaloTag-PEST and HaloTag-CL1 expression clones. The pulse-chase labeling was done in the condition as described above and the cells were incubated in the growth medium including 5 μM blocking ligands for indicating period. Increasing degradation rate was observed in HaloTag-fusion proteins appended PEST or CL1 sequence (half-lives of less than 2h) in comparison with HaloTag (a half-life of approximately 6h) (Figure 3A). As a result of rapid degradation, the steady-state levels of TMR-labeled HaloTag-PEST and TMR-labeled HaloTag-CL1 were considerably lower than that of HaloTag at the time point zero (17% and 9% relative to the amount of TMR-labeled HaloTag, respectively), even though they are driven by a strong cytomegalovirus enhancer/promoter. I could also demonstrate that the degradation of C-terminal HaloTag-fused Smad1 (SMAD1-HaloTag), a target protein of E3 ubiquitin ligase, Smurf1, was induced in the presence of the co-expressed Myc-tagged Smurf1 in HEK293 cells (Fig. 2B). Targeted ubiquitination and degradation of ectopically-expressed Smad1 by co-expressed Flag-tagged hSmurf1 have been originally reported by a pulse-chase labeling experiment using [$^{35}$S]methionine and immunoprecipitation with an anti-Smad1 polyclonal antibody (Zhu et al., 1999). In spite of the fact that the HaloTag technology pulse-labels a part of steady-state level of HaloTag-fusion proteins and only newly synthesized proteins are labeled by

[$^{35}$S]methionine, induced degradation of labeled Smad1 by Smurf1 was observed in the both methods.

In conclusion, the pulse-chase labeling technique using HaloTag technology based on a non-radioisotopic direct labeling of target protein has superiority over conventional methods from several points of view such as ease, accuracy and safety. This is the first report describing a method elucidating protein degradation rate in cultured cells using a recent protein labeling technology.

## References

**Bonifacino, J. S.** (1999). Metabolic labeling with amino acids. Current Protocols in Protein Science Vol. 1, pp. 3.7.1-3.7.10, Hoboken, New Jersey.

**Gilon, T., Chomsky, O., and Kulka, R. G.** (1998). Degradation signals for ubiquitin system proteolysis in *Saccharomyces cerevisiae*. EMBO J. *17*, 2759-2766.

**Gronemeyer, T., Godin, G., and Johnsson, K.** (2005). Adding value to fusion proteins through covalent labeling. Curr. Opin. Biotechnol. *16*, 453-458.

**Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnsson, K.** (2003). A general method for the covalent labeling of fusion proteins with small molecules *in vivo*. Nat. Biotechnol. *21*, 86-89.

**Liu, R. Y., and Li, X.** (1993). Preparation of metabolically labeled protein samples for quantitative analysis in SDS-polyacrylamide gel electrophoresis. Analytical Biochemistry *214*, 340-343.

**Los, V. G., and Wood, K.** (2007). In: Taylor, D. L., Haskins, J. R., Giuliano, K. (eds) Methods in Molecular Biology Vol. 356. Human Press, Totowa, pp195-208.

**Magee, A. I., Wootton, J., and de Bony, J.** (1995). Detecting radiolabeled lipid-modified proteins in polyacrylamide gels. Methods Enzymol. *250*, 330-336.

**Mosteller, R. D., Goldstein, R. V., and Nishimoto, K. R.** (1980). Metabolism of individual proteins in exponentially growing Escherichia coli. J. Biol. Chem. *255*, 2524-2532.

**Rogers, S., Wells, R., and Rechsteiner, M.** (1986). Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. Science *234*, 364-368.

**Zhao, Z., Li, X., Hao, J., Winston, J. H., and Weinman, S. A.** (2007). The ClC-3 chloride transport protein traffics through the plasma membrane via interaction of an N-terminal dileucine cluster with clathrin. J Biol. Chem. *282*, 29022-29031.

**Zhu, H., Kavsak, P., Abdollah, S., Wrena, J. L., and Thomsen, G. H.** (1999). A Smad ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature *400*, 687-693.

## Figure legends

**Figure 1.** Blocking of constitutive labeling of HaloTag protein with HaloTag blocking ligand.

HaloTag proteins produced in HEK293 cells were pulse-labeled by 50 nM TMR ligand and chased at the indicated times with or without HaloTag blocking ligands (0, 500 nM, 5 μM or 50 μM). Fluorescent images of TMR ligand covalently bound to HaloTag were recorded with FLA3000 after SDS-PAGE of cell lysates (upper) and the fluorescent intensities are plotted relative to the amount present at zero time point (lower).

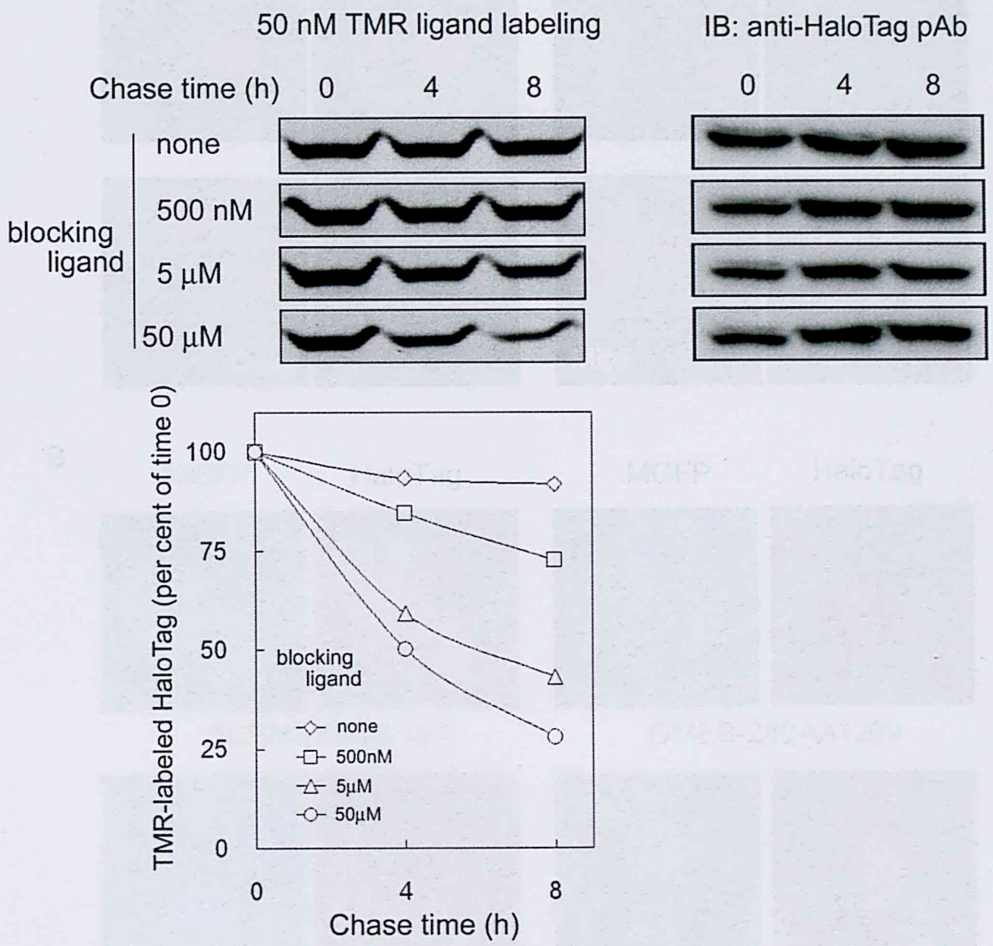
**Figure 2. Subcellular localization of HaloTag-fusion proteins in cultured cells.**

(A) Subcellular localization of transiently-expressed HaloTag proteins with various signal sequences. COS-7 cells were transfected with pFC8A expression clones for HaloTag proteins containing signal sequences such as plasma-membrane-localized signal (Memb), nuclear localization signal (NLS), ER targeting sequence (ER), Golgi-localized signal (Golgi) and mitochondria-localized signal (Mito) and HaloTag pHT2 vector which expresses HaloTag ORF (HaloTag). HaloTag-fusion proteins were labeled with medium containing 1 mM HaloTag TMR ligand (red) for 15 min, washed and incubated for 30 min. Nuclei were stained with Hoechst33342 (blue). Those were observed by BioZERO fluorescent microscope (TMR). Photos labeled with Hoechst33342 and TMR ligand are merged (TMR + Hoechst) (B) Comparison of subcellular localizations between HaloTag- and MGFP-fusion proteins. HEK293 cells were simultaneously transfected with HaloTag-fusion (red) and MGFP-fusion protein (green) expression clones. The HaloTag-fusion proteins (red) and nuclei (blue) were observed as described above except that the final incubation before the observation was 24 h. Photos labeled with Hoechst33342 are merged with those of MGFP- or HaloTag-fusion proteins (MGFP or HaloTag). Gene symbols and alias names are indicated on the downside of the photos.

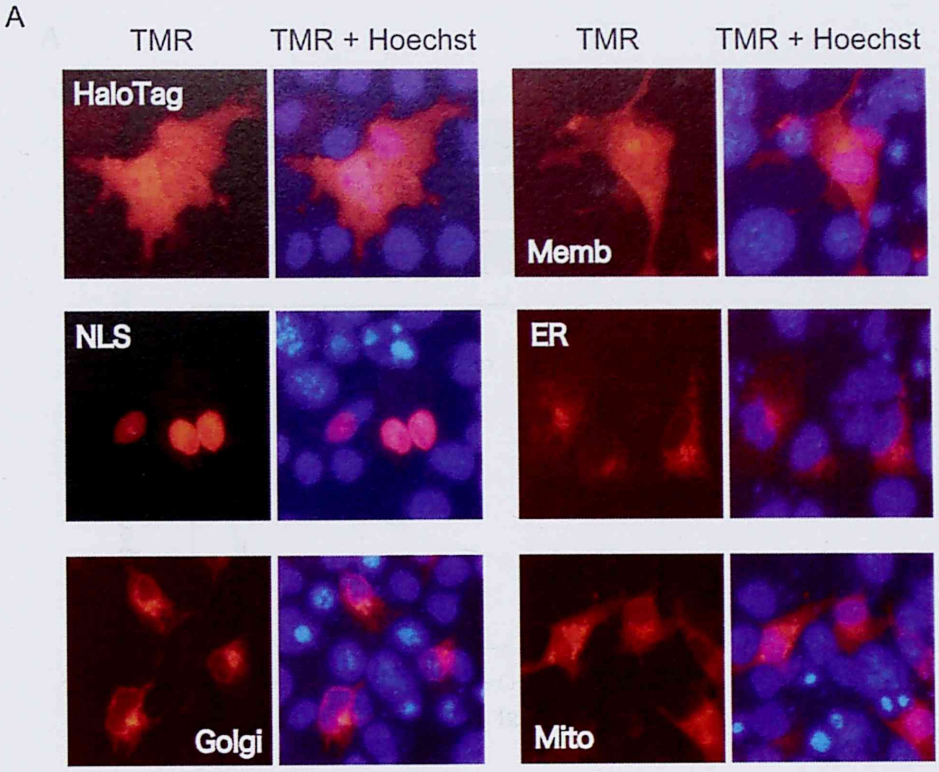
**Figure 3.** Pulse-chase labeling experiments of HaloTag-fusion proteins affected by protein-degradation signals and an E3 ubiquitin ligase.

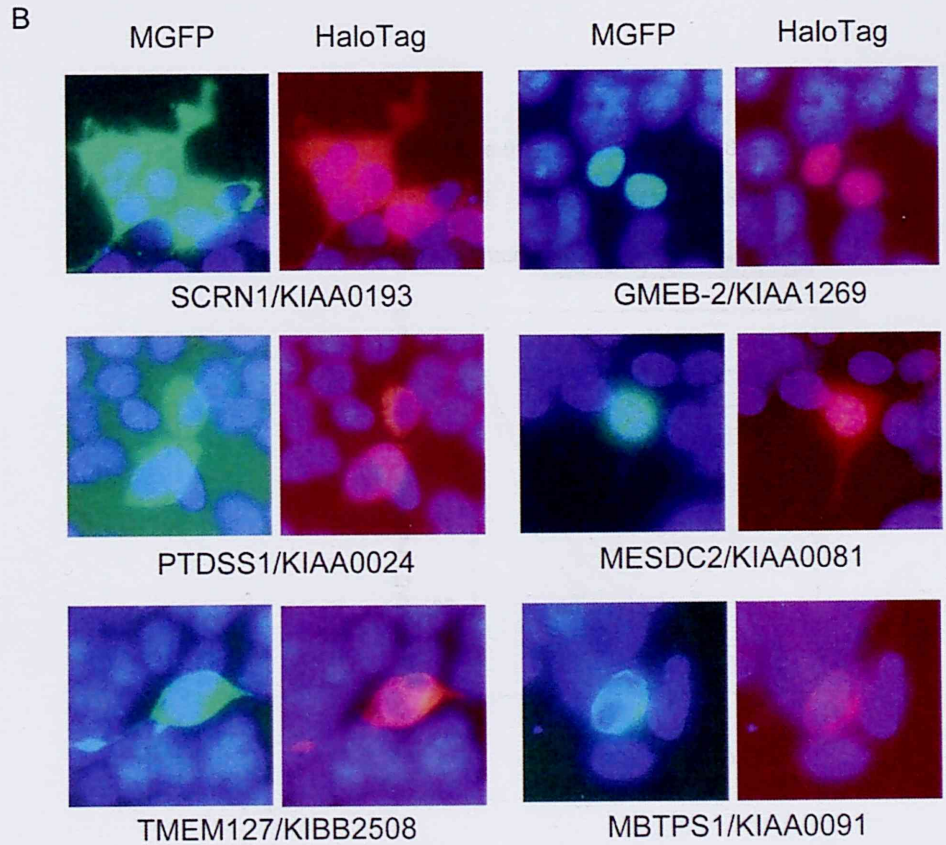
(A) HaloTag, HaloTag-PEST or HaloTag-CL1 produced in HEK293 cells were

pulse-labeled by 50 nM TMR ligand and chased at the indicated times with 5 μM blocking ligand. (B) C-terminal HaloTag-fused Smad1 was expressed in HEK293 with or without Myc-tagged Smurf1 expression clone. SMAD1-HaloTag proteins were pulse-labeled by 50 nM TMR ligand and chased at the indicated times with 5 μM blocking ligand. In the both experiments, fluorescent images of TMR were obtained with FLA3000 after SDS-PAGE of cell lysates (upper in A and B) and the fluorescent intensities are plotted relative to the amount present at zero time point as an average of two independent experiments (lower in A and B).



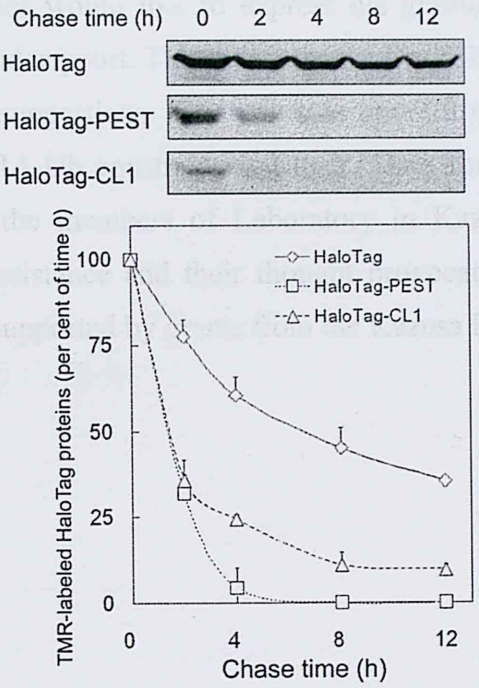

Chapter2 fig. 1



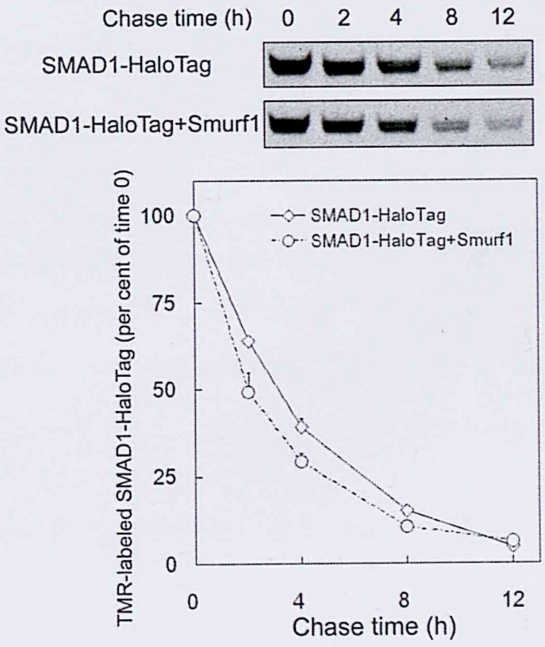




Chapter2 fig. 2

A




B




Chapter2 fig. 3

## Acknowledgements

The author would like to express his gratitude to Prof. A. Ando for his constant guidance and support. The author thanks Dr. T. Nagase for truly helpful discussions and invaluable suggestions. The author is grateful to M. Treier for providing the plasmid pMT123, HA-Ub construct, and to Y. Hara for providing pGEX-6PDES. The author thanks all the members of Laboratory in Kazusa DNA Research Institute for their technical assistance and their thought provocative discussions during the study. This study was supported by grants from the Kazusa DNA Research Institute.

## 投稿論文

Yamaguchi, K., Ohara, O., Ando, A., and Nagase, T.

Smurf1 directly targets hPEM-2, a GEF for Cdc42, via a novel combination of protein interaction modules in the ubiquitin-proteasome pathway.

Biological Chemistry; accepted, December, 2007

## 掲載受理の通知

From: uhartl@biochem.mpg.de

Date: December 19, 2007 12:01:31 AM JST

To: nagase@kazusa.or.jp

Cc: biol.chem.editorial@degruyter.de

Subject: Your Submission to Biological Chemistry (BIOLCHEM-07-268R1)

Ref.: MS no. BIOLCHEM-07-268R1

Kei Yamaguchi; Osamu Ohara, Ph.D; Akikazu Ando, Ph.D; Takahiro Nagase, Ph.D

Smurf1 directly targets hPEM-2, a GEF for Cdc42, via a novel combination of protein interaction modules in the ubiquitin-proteasome pathway

Dear Dr. Nagase,

Thank you very much for submitting your revised manuscript. I am pleased to inform you that your paper has now been accepted for publication in Biological Chemistry. In case of any problems with the preparation of your manuscript for printing, our Production Office will contact you directly.

Thank you very much for publishing your work in Biological Chemistry.

Yours sincerely,

F. Ulrich Hartl

Executive Editor Biological Chemistry

**Smurf1 directly targets hPEM-2, a GEF for Cdc42, via a novel combination of protein interaction modules in the ubiquitin-proteasome pathway**

**Kei Yamaguchi[1], Osamu Ohara[2, 3], Akikazu Ando[1], and Takahiro Nagase[2]***

[1]Graduate School of Science and Technology, Chiba University, 648 Matsudo, Matsudo, Chiba 271-8510, Japan

[2]Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan

[3]Laboratory of Immunogenomics, RIKEN Yokohama Institute, 1-7-22 Suehiro, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

*Corresponding author

e-mail: nagase@kazusa.or.jp

short title: Ubiquitination of hPEM-2 by Smurf1

**Abstract**

Smurf1, a member of HECT-type E3 ubiquitin ligases, regulates cell polarity and protrusive activity by inducing ubiquitination and subsequent proteasomal degradation of small GTPase RhoA. We report here that hPEM-2, a guanine nucleotide exchange factor (GEF) for small GTPase Cdc42, is a novel target of Smurf1. Pulse-chase labeling and an ubiquitination experiment using MG132, a proteasomal inhibitor, indicated that Smurf1 induced proteasomal degradation of hPEM-2 in cells. GST pull-down assays with heterologously expressed firefly luciferase-fusion proteins that included partial sequences of hPEM-2 revealed that part of the PH domain (residues 318-343) of hPEM-2 was sufficient for binding to Smurf1. In contrast, the hPEM-2 binding domain in Smurf1 was mapped to the C2 domain. Although it is reported that the binding activities of some C2 domains to target proteins are regulated by $Ca^{2+}$, Smurf1 interacted with hPEM-2 in a $Ca^{2+}$-independent manner. Our discovery that hPEM-2 is, in addition to RhoA, a target protein of Smurf1 suggests that Smurf1 plays a crucial role in the spatiotemporal regulation of Rho GTPase family members.

**Keywords:** C2 domain; E3 ubiquitin ligase; guanine nucleotide exchange factor; HECT domain; PH domain; Rho signaling pathway.

**Introduction**

Ubiquitin-dependent protein degradation plays an essential role in a number of key biological processes including signal transduction, cell cycle progression and transcriptional regulation (Hershko and Ciechanover, 1998). Protein ubiquitination requires three steps through ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s). There are two main classes of E3 proteins, defined by either a zinc-binding RING finger adaptor domain or a HECT catalytic domain. The RING finger E3 ligases appear to function as docking proteins that recruit target proteins to a multicomponent complex incorporatingan E2 enzyme (Pickart, 2001). On the other hand, HECT E3 ligases are characterized by the ability to form a thiolester intermediate with activated ubiquitin, and directly transfer ubiquitin to the target proteins (Hershko and Ciechanover, 1998; Laney and Hochstrasser, 1999).

Smurf1, a member of the HECT-containing E3s, was originally identified as an E3 that induces ubiquitination and degradation of bone morphogenetic protein-specific Smad1 and Smad5 in the TGF-$\beta$ signaling pathway (Zhu et al., 1999). It is composed of a protein kinase C conserve 2 (C2) domain at the N-terminus, two WW domains in the middle, and a HECT domain at the C-terminus. The WW domains on Smurf1 bind to PPXY motif, a small proline-rich sequence, and different WW domains possess different target protein specificity. Both Smad1 and Smad5 interact with Smurf1 via the PPXY-WW mediated interaction.

The Rho family of small GTPases (RhoA, Rac1 and Cdc42) organize the architecture of the actin cytoskeleton to adapt the cellular morphology to constraints imposed by cellular programs of differentiation, division, and migration (Etienne-Manneville and Hall, 2002; Burridge and Wennerberg, 2004). They switch between an inactive state via GTPase activating proteins (GAPs) and an active state via guanine nucleotide exchange factors (GEFs). In neurons, it was observed that Rac1 and Cdc42 induce, while RhoA inhibits neurite outgrowth (Luo, 2000). However, little is known of the regulating factors involved in their coordinated activities.

It has also been demonstrated that Smurf1 targets RhoA for ubiquitination and subsequent proteasomal degradation in HEK293T cells (Wang et al., 2003; Zhang et al., 2004). Interestingly, ubiquitination of RhoA by Smurf1 disrupts fibroblast polarity, leading to activation of Rac1 and Cdc42, and formation of protrusions at the leading edge, while Smurf1 does not directly interact with Rac1and Cdc42. Moreover, by a similar mechanism, it has been shown that Smurf1 promotes neurite outgrowth in Neuro2a cells (Bryan et al., 2005). Thus, one might expect that Smurf1 largely generates an intracellular asymmetry of Rho family GTPase activity. However, details of the events are still unclear. We hypothesized that Smurf1 interacts with GEFs of Rac1 and Cdc42 in addition to RhoA, because GEFs specifically activate individual members of Rho family within particular spatial and temporal contexts, and induce actin cytoskeleton reorganization (Rossman et al., 2005). Additionally, it was recently shown that FWD1/$\beta$-TrCP, a member of the RING finger E3 ligases, targets FGD1, a GEF that activates Cdc42 (Hayakawa et al., 2005). It was found that a mutant FGD1 that fails to interact with FWD1/$\beta$-TrCP led to sustained Cdc42 activation.

In the present study, we attempted to identify the GEFs that are ubiquitinated by Smurf1 and consequently found hPEM-2, GEF for Cdc42, using an ubiquitination experiment. We also demonstrated that Smurf1 binds the PH domain of hPEM-2 via its C2 domain in a $Ca^{2+}$-independent manner and induces proteasomal degradation of hPEM-2.

**Results**

**Smurf1 mediated ubiquitination and proteasomal degradation of hPEM2 *in vivo***

Through computer-assisted analysis, we found that hPEM-2, GEF of Cdc42, contains the sequence $^{465}$PPSYPPP, which matches the consensus sequence recognized by the WW domain on Smurf1. We wanted to test whether Smurf1 promoted ubiquitination of hPEM-2, thus, FLAG-hPEM-2 was immunoprecipitated from extract of transfected cells with anti-FLAG antibody, and the precipitates were subsequently used for Western blot analysis. Anti-HA antibody was used to detect conjugation of HA-Ub (Figure 1A, top), and anti-FLAG antibody to detect the co-precipitation levels (Figure 1A, bottom). The expression of Smurf1 was also confirmed by Western blot analysis using anti-Myc antibody (Figure 1A, middle). Although hPEM-2 exhibited a low level of polyubiquitination in the absence of transiently expressed Smurf1, the co-expression of hPEM-2 with Smurf1 led to a marked increase in the polyubiquitination of hPEM-2 (Figure 1A, top, lanes 3 and 4). Moreover, accumulation of ubiquitinated hPEM-2 was observed by treating the cells with MG132, an inhibitor of proteasomal activity, for 1 h prior to the preparation of cell lysates (Figure 1A, top, lanes 5 and 6). These results indirectly demonstrated that ubiquitinated hPEM-2 is degraded through proteasomes.

We constructed an expression plasmid for an inactive point mutant of Smurf1 (Smurf1CA) to confirm that ubiquitination of hPEM-2 requires the catalytic activity of the HECT domain in Smurf1. This was achieved by replacing the cysteine residue at position 725 that forms a thiolester bond with ubiquitin with alanine. As a result, Smurf1CA did not increase hPEM-2 ubiquitination and furthermore, even low level constitutive ubiquitination of hPEM-2 was blocked (Figure 1B).

We further examined whether Smurf1-assisted ubiquitination of hPEM-2 induced subsequent degradation of the protein. The stability of hPEM-2 protein was analyzed by a pulse-chase experiment applied to a HaloTag-fused hPEM-2 protein, where the HaloTag portion can covalently bind a fluorescent ligand. To achieve this, the Halo-tagged hPEM-2 and HaloTag as a control were produced in HEK293 cells, labeled with HaloTag TMR fluorescent ligand and chased for 8 h (Figure 1C). Although the protein stability of HaloTag itself was not

changed in the presence of wild-type Smurf1 (Smurf1WT) and Smurf1CA (Figure 1C, closed triangles and open circulars), HaloTag-hPEM-2 was degraded faster in the presence of Smurf1WT (Figure 1C, diamonds) not Smurf1CA (Figure 1C, squares). Moreover, amount of HaloTag-hPEM-2 in the presence of Smurf1WT has already reduced to approximately 50% at zero time point (36h after the co-transfection) of the pulse chase labeling compared with that in the presence of Smurf1CA. Thus, Smurf1 appeared to regulate the protein stability of hPEM-2. Overall, the results indicate that Smurf1 induces degradation of hPEM-2 through the ubiquitin-proteasome system.

**Smurf1 interacts with hPEM-2 directly but is not mediated by PY motif**

To check whether hPEM-2 ubiquitination by Smurf1 occurs through a PPXY-WW mediated interaction, we carried out an ubiquitination experiment using a deletion mutant of PPXY motif (hPEM-2 Δ PPSYPPP). As shown in Figure 2A, polyubiquitination of hPEM-2 Δ PPSYPPP was enhanced by Smurf1, as well as the positive control in wild-type hPEM-2. This result suggested that the PPXY motif of hPEM-2 was not required for Smurf1-mediated ubiquitination. Therefore, to elucidate the mechanism used by Smurf1 to target hPEM-2, we investigated the interaction *in vivo*. In these assays, Smurf1CA was utilized instead of wild type Smurf1 to avoid protein degradation and to stabilize the protein complex. Myc-tagged Smurf1CA was transiently co-expressed with FLAG-tagged hPEM-2 in HEK293 cells. The expression level of Smurf1 was determined by Western blot with anti-Myc antibody (Figure 2B, middle). Anti-FLAG antibody was used for immunoprecipitation of hPEM-2 from the cell lysates (Figure 2B, bottom), and precipitates were detected by Western blot using anti-Myc or anti-FLAG antibodies (Figure 2B, top and bottom, respectively). Myc-Smurf1 was efficiently co-precipitated with FLAG-hPEM-2 when both proteins were co-expressed in HEK293 cells (Figure 2B, lane 3).

It has recently been reported that Smurf1 transfers ubiquitin molecules to certain target proteins by mediating adaptor proteins (Shearwin-Whyatt et al., 2006). Hence, we used *in vitro* GST pull-down assays to examine whether the complex formation between Smurf1 and hPEM-2 occurs through direct interaction. Indeed, glutathione bead-bound GST-Smurf1CA expressed and purified from *E. coli* Rosetta incubated with *E. coli* lysates expressed His-hPEM-2 (Figure 2C, bottom), where potential adaptor proteins do not exist. The binding activity of GST-Smurf1CA was detected for the His-hPEM-2, but not for luciferase protein (His-Luc2) as a negative control (Figure 2C, top). GST alone was also used as a negative control. These results indicate that Smurf1 and hPEM-2 can interact directly without any additional proteins.

**Determination of the Smurf1-binding site in hPEM-2**

The data described above indicate that Smurf1 interacts with hPEM-2 via a binding site other than the PPXY motif. To identify the hPEM-2 domain responsible for Smurf1-binding, we produced various deletion mutants of FLAG-Tagged hPEM-2 in HEK293 cells and subjected them to *in vitro* GST pull-down assays (Figure 3A). The expression levels of the transfected hPEM-2 deletion mutants were determined by Western blot analysis with anti-FLAG antibody (Figure 3B, bottom). The hPEM-2Full was efficiently recovered from lysates after incubation with GST-Smurf1CA, but not GST (Figure 3B, lanes 1 and 2). The hPEM-2 deletion mutants were also incubated with GST-Smurf1CA, co-purified with GST-Smurf1CA and detected with anti-FLAG antibody. The hPEM-2 Δ (C terminus+PH) was not sufficient to bind to GST-Smurf1CA (Figure 3B, lane 5), while the other hPEM-2 mutants containing the PH domain region were able to bind. These results demonstrated that a region of hPEM-2 between amino acid residues 300 and 434 (PH domain region) is a candidate binding region for Smurf1.

Moreover, to narrow the search for the Smurf1 binding region on hPEM-2, we constructed various hPEM-2 C-terminal deletion mutants with more limited truncations in the region encompassing residues 300-434. The respective mutants from transfected HEK293 cells were used to analyze the binding activity to GST-Smurf1 as before. Although GST-Smurf1CA bound the hPEM-2 mutants (1-409, 1-396, 1-375, 1-358, and 1-343), the 1-329 mutant slightly reduced binding activity and the 1-318 mutant markedly reduced binding activity to the GST-Smurf1 (Figure 3C). These results suggest that the region of hPEM-2 between amino acid residues 318 and 343 is required for binding to Smurf1.

We continued to focus our attention in this region, and hPEM-2 deletion mutants produced in rabbit reticulocyte lysates were used for binding assays with GST-Smurf1 as before. The results obtained for the hPEM-2 C-terminal deletion mutants (1-358, 1-343, 1-329, 1-318, and 1-299) were consistent with those for HEK293 lysates (Figure 4A, lanes 1-5). Conversely, GST-Smurf1CA was able to bind the 318-516, 330-516, and 335-516 N-terminal deletion mutants of hPEM-2, while binding activity was eliminated by the 344-516 hPEM-2 mutant (Figure 4A, lanes 6-9).

As shown in Figure 4C, the region encompassing amino acid residues from 318 to 343 in the PH domain of hPEM-2 is important for binding to Smurf1. Therefore, we next confirmed whether this region is sufficient to bind to Smurf1 by using firefly luciferase (Luc2), a heterologous protein. FLAG-tagged Luc2 fusion proteins, including segments of hPEM-2 residues at C-terminus were produced in rabbit reticulocyte lysates and binding to GST-Smurf1 was analyzed as before. Although GST-Smurf1 failed to bind the Luc2 fusion protein containing hPEM-2 residues from 335 to 343 (hPEM-2 *335-343*), it did bind hPEM-2 *318-343* (Figure 4B, lane 2 and 3). In addition, GST-Smurf1 also bound hPEM-2 *304-343*, hPEM-2 *287-343*, and hPEM-2 *267-343*, which have additional N-terminal regions relative to hPEM-2 *318-343* (Figure 4B, lanes 4-6). In particular, hPEM-2 *267-343* was readily recovered by GST-Smurf1CA (Figure 4B, lane 6). Binding to GST-Smurf1CA was markedly reduced by hPEM-2 *267-329*, which lacks the 14 C-terminal amino acid residues found in hPEM-2 267-343, and deletion of another 12 amino acids (hPEM-2 *267-317*) abolished binding (Figure 4B, lanes 7 and 8). Moreover, hPEM-2 *241-317*, which had 27 amino acids added to the N-terminal residues relative to hPEM-2 *267-317*, rarely bound to the GST-Smurf1 (Figure 4B, lane 9). Based on these results, we conclude that residues 318-343 (extreme N-terminus of the PH domain) of hPEM-2 are essential for interaction with Smurf1, and that the neighboring regions potentiate binding activity.

**The hPEM-2 ubiquitination by Smurf1 requires C2 domain of Smurf1**

To delineate the hPEM-2-binding domain in Smurf1, a set of FLAG-tagged Luc2 fusion proteins containing C2, WW, and HECT domains of Smurf1 (Figure 5A, schematic diagram) were synthesized in rabbit reticulocyte lysates and used for *in vitro* GST pull-down assays as before. GST-hPEM-2 bound the FLAG-Luc2 containing the C2 domain (Figure 5A, lane 4), whereas it failed to bind the FLAG-Luc2 containing the two WW or HECT domains (Figure 5A, lanes 6 and 8) and GST alone did not bind to any of the FLAG-Luc2-Smurf1 mutants.

Accordingly, it was expected that ΔC2 Smurf1 would not enhance the polyubiquitination of hPEM-2 in cultured cells, because ΔC2 Smurf1 fails to interact with hPEM-2. Indeed, no increase in hPEM-2 polyubiquitination was observed in HEK293 cells with co-expressed

hPEM-2 and ΔC2 Smurf1 (Figure 5B, lane 3), as well as the negative control in the absence of Smurf1 (Figure 5B, lane 1). These results strongly suggest that Smurf1 associates with hPEM-2 through its C2 domain and enhances ubiquitination of hPEM-2.

It was reported that many C2 domains interact with a variety of phospholipids and proteins in a $Ca^{2+}$-dependent manner (Nalefski and Falke, 1996). We therefore tested whether $Ca^{2+}$ is essential for the binding of Smurf1 to hPEM-2 using GST-hPEM-2 purified from *E. coli* and FLAG-Luc2 fusion proteins synthesized in rabbit reticulocyte lysates via the *in vitro* pull-down assays. EGTA, a chelating agent of calcium, was added to the reaction solution at a final concentration of 5 mM to completely eliminate calcium ions from the reaction. Conversely, calcium ions were added to the reaction solution as $CaCl_2$ at a final concentration of 3.5 mM. Binding activity was observed in combination with GST-hPEM-2 and FLAG-Luc2-Smurf1C2, but not with GST or FLAG-Luc2, regardless of the absence or presence of calcium ions (Figure 5C).

**Discussion**

In this study, we identified hPEM-2, a GEF for small GTPase Cdc42 (Reid et al., 1999), as a novel target protein for Smurf1. This means that Cdc42 can be indirectly regulated by Smurf1 through hPEM2 degradation. This finding could shed light on the spatiotemporal regulation of the activities of the Rho family of small GTPases by Smurf1, since Smurf1 also controls spatial distribution of RhoA in cells through ubiquitin-mediated degradation (Wang et al., 2003). Moreover, it suggests the existence of signaling cross-talk in Rho family GTPases with qualitative alteration by GEFs and GAPs, and with variability in quantity through ubiquitin-mediated degradation.

We performed *in vivo* ubiquitination assays to examine whether Smurf1 exhibits E3 ligase activity for hPEM-2. In the absence of ectopically expressed Smurf1, low level constitutive ubiquitination of hPEM-2 was detected. Co-expression of Smurf1 with hPEM-2 markedly enhanced ubiquitin conjugation to hPEM-2, whereas Smurf1CA, a ligase-inactive mutant with an amino acid substitution in the conserved cysteine in the HECT domain, abolished such an effect. In fact, Smurf1CA also reduced constitutive ubiquitination of hPEM-2 and might act as a dominant negative mutant, competing with endogenous E3 ligases for hPEM-2. Additionally, accumulation of ubiquitinated hPEM-2 in the presence of MG132, an inhibitor of proteasomal activity, suggests that ubiquitinated hPEM-2 is targeted for proteasomal degradation. Because our experiments using cultured cells were designed as a model system using cells transiently overexpressing Smurf1 and hPEM-2, there is concern about the essential role of endogenous Smurf1 for hPEM-2. According to our preliminary data, it was indirectly indicated that the endogenous Smurf1 targets hPEM-2 because the constitutive ubiquitination of transiently expressed hPEM-2 could be diminished in HEK293 cells in the presence of Smurf1 siRNAs, which can reduce the expression level of endogenous Smurf1 (data not shown). Moreover, we applied a pulse-chase labeling assay with fluorescence-labeled HaloTag-fusion hPEM-2 to elucidate whether Smurf1 induces proteolysis of hPEM-2 protein. The half-life of the HaloTag-hPEM-2 in the presence of Smurf1WT was much shorter than that in the presence of Smurf1CA. Actually, amount of HaloTag-hPEM-2 in the presence of Smurf1WT was considerably reduced at zero time point of the experiment compared with that in the presence of Smurf1CA, that means the reduction of steady-state level of hPEM-2 by Smurf1WT. These data directly demonstrate that Smurf1 regulates the stability of hPEM-2 in cultured cells. Furthermore, our data suggests that Smurf1 promotes degradation of hPEM-2 through the ubiquitin-proteasome system. The GEF hPEM-2 is known as a human homologue of rat collybistin, which appears to be a gephyrin-binding partner and is an essential determinant of neuronal gephyrin clustering (Harvey et al., 2004). Smurf1 may be involved in neuronal gephyrin clustering through regulation of the stability of collybistin.

Smurf1 transfers ubiquitin molecules to Smad4 by mediating an adaptor protein, I-Smad, because Smad4 lacks a PY motif and does not directly bind to Smurf1 (Shearwin-Whyatt et al., 2006). Although hPEM-2 has a PY motif, this motif is not required for Smurf1 binding. To confirm whether Smurf1-induced hPEM-2 degradation results from direct interaction of those proteins, an *in vitro* GST pull-down assay was applied using GST-Smurf1CA and His-hPEM-2 synthesized in *E. coli*, where no possible intermediary proteins exist. Specific interaction of His-hPEM-2 with GST-Smurf1CA was observed, and this indicated direct interaction of both proteins. Next, we tried to expose the Smurf1-binding site in hPEM-2. Analysis of hPEM-2 deletion mutants and Luc2 fusion proteins containing hPEM-2 residues defined a 26-amino acid region (residues 318-343), corresponding to the extreme N-terminus of PH domain. The PH domain is a small protein module of about 120 amino acids residues and their three-dimensional folded structures are highly conserved, despite a low primary sequence similarity (Lemmon and Ferguson, 2000). The PH domain possesses multiple functions including the ability to bind inositol phosphates and various proteins. Thus, GEF proteins other than hPEM-2 may be a target of Smurf1, since each of the 60 plus GEF proteins possesses the PH domain and specifically activates individual members of Rho family within particular spatial and temporal contexts (Rossman et al., 2005). The various combinations of Smurf1 and GEFs might play a key role in the fine-tuning of Rho family GTPase activity.

On the other hand, a GST pull-down assay with Luc2 fusion proteins containing respective Smurf1 domains (C2, WW, and HECT) revealed that the C2 domain of Smurf1 is sufficient for the binding to hPEM-2. Moreover, ΔC2 Smurf1 did not enhance polyubiquitination of hPEM-2. Intriguingly, these results suggest that Smurf1 binds hPEM-2 via its C2 domain, but not via the WW domains, which appear to mediate substrate recognition. The C2 domain was originally identified in classical protein kinase C isoforms as a $Ca^{2+}$-dependent phospholipid binding domain (Nalefski and Falke, 1996; Rizo and Sudhof, 1998). However, the C2 domain can also mediate protein-protein interaction and small molecule binding, in addition to the binding of $Ca^{2+}$ itself. For example, the second C2 domain of most synaptotagmin isoforms binds in a $Ca^{2+}$-independent manner with high affinity to clathrin –AP2, a protein complex involved in coated pit assembly (Zhang et al., 1994). Indeed, the binding of Smurf1 to hPEM-2 did not require $Ca^{2+}$. Our finding is a valuable example showing the extended function of the C2 domain as a protein binding motif.

Thus, our studies showed that the interaction of Smurf1 with hPEM-2 occurs through the C2 domain and extreme N-terminus of the PH domain, but not the PPXY motif and the WW domain, despite the presence of a PPXY motif on hPEM-2. In contrast the interaction of Smurf1 with Smad1, Smad5 and Smad7 in the TGF-$\beta$ signaling pathway occurs via a WW-PPXY mediated association (Ebisawa et al., 2001). It is also reported that the C2 domain of Smurf1 is important for localization and the inhibitory effect of Smad7 in the TGF-$\beta$ signaling pathway (Suzuki et al., 2002). These results in addition to our finding strongly suggest that Smurf1 is an important factor that can regulate a variety of signaling cascades, using two WW domains and the C2 domain as protein- and membrane-targeting modules.

Our study provides another approach for understanding the mechanisms regulating GTPase activity. In addition, further insight into the Rho signaling pathway may highlight the ubiquitin ligases as molecular switches that generate an intracellular asymmetry of temporal and

spatial GTPase activity.

**Materials and methods**

**Construction of expression clones**

To create N-terminal FLAG and Myc tag mammalian expression vectors (pcDNAnFLAG-DEST and pcDNAnMyc-DEST), double-stranded oligonucleotides including FLAG or Myc tag sequences were cloned into *Hind* III site of pcDNA-DEST47 Δ GFP that was constructed by ligation of 1460 bp *Pst* I fragment of pcDNA-DEST53 (Invitrogen, Carlsbad, CA USA) with a 5772 bp *Pst* I fragment of pcDNA-DEST47 (Invitrogen). Smurf1 and hPEM-2 were derived from sequences with GenBank accession numbers of AB046845 (KIAA1625) and AB007884 (KIAA0424), respectively. Tag-fused Smurf1 and hPEM-2 were constructed using pcDNAnMyc-DEST and pcDNAnFLAG-DEST by the Gateway LR recombination reaction (Invitrogen). GeneEditor System (Promega, Madison, WI USA) was used to generate a catalytically inactive form of Smurf1 (Smurf1CA), in which the cysteine at position 725 was replaced with alanine and a deletion mutant of PPXY motif on hPEM-2 (hPEM-2 Δ PPSYPPP) and, where the PPSYPPP sequence between amino-acid residues 465-471 was deleted. N-terminal Halo-tagged hPEM-2 was created using pHT2 vector (Promega). GST-fusion proteins, Smurf1CA and hPEM-2, were subcloned into a pGEX-6PDES (Hara et al., 2003) vector. Construction of His tagged Luc2 which was derived from pGL4.10 (luc2) vector (Promega) and hPEM2 were performed by amplifying the reading frame by PCR, followed by cloning into the *Sma* I/*Eco*R I sites of pET-47b(+) (TaKaRa, Siga, Japan). N-and C-terminal deletion mutants of hPEM-2 were amplified by PCR and cloned into pcDNAnFLAG-DEST. The Luc2 coding region was cloned into pcDNAnFLAG-DEST for FLAG-tagged Luc2 fusion proteins, and part of hPEM-2 and Smurf1 sequences amplified by PCR were inserted at the C-terminus of Luc2. The HA-Ub construct was kindly provided from Dr. M. Treier (Treier et al., 1994).

**Cell Culture and Transfection**

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotic-antimycotic (Invitrogen). The HEK293 cells were transfected with various expression vectors with FuGENE 6 (Roche Diagnostics, Indianapolis, IN USA) according to the manufacturer's recommendations. Total plasmid DNA in individual transfections was adjusted to equivalent amounts in all transfections with empty vectors. Transfected cells were cultured for 48 h and used for immunoprecipitation or *in vitro* pull down assays.

**Immunoprecipitation and Western Blotting**

Transfected cells were washed in PBS and incubated 20 min on ice with lysis buffer (50 mM Hepes, pH7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml pepstatin A and 250 μM PMSF) just prior to use. Cell debris was removed by a microcentrifuge at 14 000 × *g* at 4°C for 10 min, and the supernatant was used for immunoprecipitation and western blot analysis. For the immunoprecipitation assay, cell lysates were incubated with anti-FLAG-M2 (Sigma, St. Louis, MO USA) for 2 h at 4°C followed by incubation for an additional 1 h with protein G/A agarose suspension (Calbiochem, San Diego, CA USA). The agarose suspension was then washed twice with lysis buffer, three times with wash buffer1 (20 mM Hepes, pH7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), and then boiled in SDS sample buffer. In Western blot analysis, the precipitated and total proteins of cell lysates were separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% milk in TBST and subsequently probed with the different primary antibodies diluted in TBST. Antibody dilutions used were as follows: anti-HA (Roche), 1:2800 dilution; anti-FLAG-M2, 1:10 000; anti-Myc (Invitrogen), 1:5000; and anti-His (Wako, Osaka, Japan), 1:1000. After incubation with the primary antibody, membranes were incubated with the appropriate anti-mouse horseradish peroxidase secondary antibodies and visualized using chemiluminescence (ECL plus) detection system (GE Healthcare, Buckinghamshire, UK). In the proteasome inhibitor experiment, transfected cells were treated with 10 μM MG-132 (Calbiochem) for 1 h prior to cell lysis.

**Pulse-Chase Experiments**

HaloTag Interchangeable Labeling Technology (Promega) was applied for pulse labeling of HaloTag-fused hPEM-2 protein. HEK293 cells were transfected with HaloTag-fused hPEM-2 or HaloTag as a control and the indicated expression plasmid, and cultured for 36 h. The HaloTag-fused hPEM-2 and HaloTag proteins were labeled for 10 min with 50 nM HaloTag TMR fluorescent ligand (Promega). The cells were then washed four times in normal medium and chased in normal medium supplemented with 500 nM HaloTag Biotin Ligand (Promega). At the indicated times, the cells were washed with PBS and lysed in SDS sample buffer. The TMR-labeled proteins were resolved by SDS-PAGE and visualized by fluorescent image analyzer FLA3000 (Fujifilm, Tokyo, Japan). Image Gauge software (Fujifilm) was used to quantify the labeled proteins presented at the indicated times.

**GST Pull-down Assay**

GST, GST-Smurf1 and GST-hPEM-2 were expressed and purified from *E. coli* strain Rosetta. Briefly, the culture was induced at $OD_{600}$ 0.5 with 0.5 mM IPTG for 3 h. The pellet was collected and resuspended in sonication buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.02% Tween-20), supplemented with protease inhibitors just prior to use. The suspension was then sonicated and the insoluble fraction was removed by spinning 20 min at 12,000 rpm at 4 °C. The remaining cleared supernatant was incubated with 50% slurry of glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) for 2 h at 4 °C and washed five times with ice cold wash buffer2 (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.02% Tween-20). About 2 μg of the GST fusion proteins immobilized on beads were incubated with cell lysates in lysis buffer. After 2 h at 4 °C, the binding mixtures were washed five times with wash buffer1, and then resuspended in SDS sample buffer. To test a direct protein-protein interaction, His-tagged proteins were expressed from *E. coli* strain BL21. The pellet was resuspended in lysis buffer followed by sonication. Lysates were then incubated with the GST beads and washed as previously described. Additionally, FLAG-tagged proteins, translated *in vitro*, were expressed by using the $T_NT$ Quick Coupled Transcription/Translation Systems (Promega), and the reticulocyte lysates were mixed with the GST beads in lysis buffer as described previously. In the experiment for $Ca^{2+}$-independency, GST fusion proteins were incubated with the reticulocyte lysates expressing FLAG-tagged proteins with either 3.5 mM $CaCl_2$ or 5 mM EGTA.

**Acknowledgements**

We are grateful to M. Treier for providing the plasmid pMT123, HA-Ub construct, and to Y. Hara for providing pGEX-6PDES. We thank K.

Shimada, N. Okazaki, S. Inoue and H. Koga for discussion and comments on the work. We also thank K. Ozawa, K. Yamada, K. Sumi, T. Kato Y. Shirai Nobue Kashima and Yasuhide Itokawa for their technical assistance. This work was supported by grants from the Kazusa DNA Research Institute.

**References**

Bryan, B., Cai, Y., Wrighton, K., Wu, G., Feng, X.H., and Liu, M. (2005). Ubiquitination of RhoA by Smurf1 promotes neurite outgrowth. FEBS Lett. *579*, 1015-1019.

Burridge, K., and Wennerberg, K. (2004). Rho and Rac take center stage. Cell *116*, 167-179.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J. Boil. Chem. *276*, 12477-12480.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature *420*, 629-635.

Hara, Y., Shimada, K., Kohga, H., Ohara, O., and Koga, H. (2003). High-throughput production of recombinant antigens for mouse KIAA proteins in Escherichia coli: computational allocation of possible antigenic regions, and construction of expression plasmids of glutathione-S-transferase-fused antigens by an *in vitro* recombination-assisted method. DNA Res. *10*, 129-136.

Hayakawa, M., Kitagawa, H., Miyazawa, K. Kitagawa, M., and Kikugawa K. (2005). The FWD1/beta-TrCP-mediated degradation pathway establishes a 'turning off switch' of a Cdc42 guanine nucleotide exchange factor, FGD1. Genes Cells *10*, 241-251.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425-479.

Harvey, K., Duguid, I.C., Alldred, M.J., Beatty, S.E., Ward, H., Keep, N.H., Lingenfelter, S.E., Pearce, B.R., Lundgren, J., Owen, M.J., Smart, T.G., Lüscher, B., Rees, M.I., and Harvey, R.J. (2004). The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. J. Neurosci. *24*, 5816-5826.

Laney, J.D., and Hochstrasser, M. (1999). Substrate targeting in the ubiquitin system. Cell *97*, 427-430.

Lemmon, M.A., and Ferguson, K.M. (2000). Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem. J. *350*, 1-18.

Luo, L. (2000). Rho GTPases in neuronal morphogenesis. Nat. Rev. Neurosci. *1*, 173-180.

Nalefski, E.A., and Falke, J.J. (1996). The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. *5*, 2375-2390.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503-533.

Reid, T., Bathoon, A., Ahmadian, M.R., and Collard, J.G. (1999). Identification and characterization of hPEM-2, a guanine nucleotide exchange factor specific for Cdc42. J. Biol. Chem. *47*, 33587-33593.

Rizo, J., and Sudhof, T.C. (1998). C2-domains, structure and function of a universal $Ca^{2+}$-binding domain. J. Biol. Chem. *273*, 15879-15882.

Rossman, K.L., Der, C.J., and Sondek, J. (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat. Rev. Mol. Cell. Biol. *6*, 167-180.

Shearwin-Whyatt, L., Dalton, H.E., Foot, N., and Kumar, S. (2006). Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. Bioessays *28*, 617-628.

Suzuki, C., Murakami, G., Fukuchi, M., Shimanuki, T., Shikauchi, Y., Imamura, T., and Miyazono, K. (2002). Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. J. Biol. Chem. *277*, 39919-39925.

Treier, M., Staszewski, L.M., and Bohmann, D. (1994). Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the delta domain. Cell *78*, 787-798.

Wang, H.R., Zhang, Y., Ozdamar, B., Ogunjimi, A.A., Alexandrova, E., Thomsen, G.H., and Wrana, J.L. (2003). Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. Science *302*, 1775-1779.

Zhang, J.Z., Davletov, B.A., Südhof, T.C., and Anderson, R.G. (1994). Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. Cell *78*, 751-760.

Zhang, Y., Wang, H.R., and Wrana, J.L. (2004). Smurf1: a link between cell polarity and ubiquitination. Cell Cycle *3*, 391-392.

Zhu, H., Kavsak, P., Abdollah, S., Wrana, J.L., and Thomsen, G.H. (1999). A SMAD ubiquitin ligase targets the BMP pathway and affects

embryonic pattern formation. Nature *400*, 687-693.

**Figure legends**

**Figure 1** Smurf1 mediates ubiquitination and proteasomal degradation of hPEM-2.

(A and B) Ubiquitination of hPEM-2 in HEK293 cells. At 48 h after co-transfection with the indicated expression plasmids (FLAG-tagged hPEM-2, Myc-tagged Smurf1WT/CA and HA tagged-Ub), FLAG-hPEM-2 proteins were immunoprecipitated (IP) with anti-FLAG antibody from the cell lysates. The ubiquitinated hPEM-2 was detected by Western blot analysis (Blot) with anti-HA antibody (top), and appeared as high molecular weight ladders, indicated by brackets to the right of the top panel. The expression levels of Myc-Smurf1 and the co-precipitation levels of FLAG-hPEM-2 were determined with anti-Myc antibody (middle) and anti-FLAG antibody (bottom), respectively. When the cells were treated with proteasomal inhibitor MG132 (10 μM), the reagent was added to cells for 1 h prior to cell lysis. (C) The effect of Smurf1 on hPEM-2 protein stability. HEK293 cells were transfected with either Smurf1WT (diamonds), or Smurf1CA with HaloTag-fused hPEM-2 (squares). After 36 h of cell culture, the Halo-hPEM-2 proteins were labeled for 10 min with HaloTag TMR ligand, and then chased for indicated times after removal of unreacted HaloTag TMR ligand by washing cells and blocking with HaloTag biotin ligand. The TMR-labeled Halo-hPEM-2 was quantified by fluoroimaging and the level was plotted relative to the amount present at time 0. Similarly, the stability of HaloTag protein was also analyzed as a control. WT, wild type; CA, catalytically inactive mutant.

**Figure 2** hPEM-2 is ubiqutinated by and directly interacts with Smurf1 through a region other than PPXY motif.

(A) Ubiquitination of hPEM-2 lacking PPXY motif ( Δ PPSYPPP) in HEK293 cells. HEK293 cells were transfected with the indicated expression plasmids. The hPEM-2WT and hPEM-2 Δ PPSYPPP proteins were immunoprecipitated (IP) with anti-FLAG antibody from the cell lysates. The ubiquitinated hPEM-2WT and hPEM-2 Δ PPSYPPP was detected by Western blot analysis (Blot) with anti-HA antibody (top). The expression levels of Myc-Smurf and the co-precipitation levels of FLAG-hPEM-2 were determined with anti-Myc antibody (middle) and anti-FLAG antibody (bottom), respectively. (B) Smurf1co-precipitates with hPEM-2 transiently co-expressed in mammalian cells. HEK293 cells were transfected with FLAG-tagged hPEM-2 and Myc- tagged Smurf1CA. The cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, followed by western blot analysis (Blot) with anti-Myc antibody (top). The expression level of Myc-Smurf and the co-precipitation level of FLAG-hPEM-2 were determined by anti-Myc antibody (middle) and anti-FLAG antibody (bottom), respectively. (C) hPEM-2 directly interacts with Smurf1 without requiring an intermediary protein. His-tagged hPEM-2 and Luciferase protein (His-Luc2) were expressed in *E.coli*. The lysates of His-proteins (bottom) were incubated with purified GST-Smurf1CA in the *in vitro* GST-pull down assay. GST alone also served as a negative control. The bound His-tagged proteins were eluted from the beads and detected by Western blot analysis with anti-His antibody (top).

**Figure 3** Identification of the Smurf1-binding region in hPEM-2.

(A) A schematic diagram to illustrate various hPEM-2 deletion mutants. (B) HEK293 cells were transfected with the FLAG-tagged hPEM-2 deletion mutants and the cell lysates were incubated with the GST-Smurf1CA. The precipitates eluted from the beads were analyzed by Western blot analysis (Blot) with anti-FLAG antibody (top). The expression levels of the hPEM-2 deletion mutants were analyzed by anti-FLAG using whole cell lysates (bottom). (C) hPEM-2 various C-terminal deletion mutants were transfected into HEK293 cells. The hPEM-2 mutant proteins binding to GST-Smurf1CA from cell lysates (top) and total mutant proteins in cell lysates (bottom) were detected by anti-FLAG antibody, according to the method in B.

**Figure 4** Detailed analysis of the Smurf1-binding sequences in hPEM-2.

(A) FLAG-tagged different C-terminal and N-terminal mutants of hPEM-2 were expressed in rabbit reticulocyte lysates. The lysates were incubated with the GST-Smurf1, and bound proteins were detected by Western blot analysis with anti-FLAG antibody (top). The bottom panel demonstrates the level of the FLAG-hPEM-2 truncation mutants in total lysates. (B) Rabbit reticulocyte extracts expressed FLAG-tagged Luc2 fusion proteins including different amino acid residues of hPEM-2 at C-terminus. The lysates were analyzed for binding to GST-Smurf1 as in A. (C) A schematic representation of hPEM-2 deletion mutants and the binding activities to Smurf1CA, along with the amino acid sequences surrounding the Smurf1-binding region.

**Figure 5** C2 domain of Smurf1 is responsible for interaction with and ubiquitination of hPEM-2 in a $Ca^{2+}$-independent manner.

(A) Determination of the hPEM-2-Binding domain in Smurf1. Top: A schematic diagram to illustrate Luc2 fusion proteins containing C2, WW, and HECT domain of Smurf1, respectively. Bottom: Pull down assays were performed using the GST-hPEM-2 and the FLAG-tagged Luc2 fusion proteins synthesized in rabbit reticulocyte lysates. The bound FLAG-Luc2 fusion proteins were detected by western blot analysis with anti-FLAG antibody (top). The expression of FLAG-Luc2 fusion proteins was detected by western blot with anti- FLAG antibody (bottom). (B) Ubiquitination of hPEM-2 by C2 domain deletion mutant of Smurf1 (Smurf1 Δ C2) in HEK293 cells. The cell lysates transfected with FLAG-tagged hPEM-2, HA-tagged ubiquitin and Myc-tagged Smurf1WT or Myc-tagged Smurf1 Δ C2 were used for *in vivo* ubiquitination assays, according to the procedure described in Figure 1. (C) $Ca^{2+}$-independent interaction of C2 domain of Smurf1 with hPEM-2. The GST-hPEM-2 was incubated with rabbit reticulocyte lysates expressing FLAG-tagged Luc2-Smurf1C2 containing either 5 mM EGTA, no additions, or 3.5 mM $CaCl_2$ in the *in vitro* GST-pull down assay.

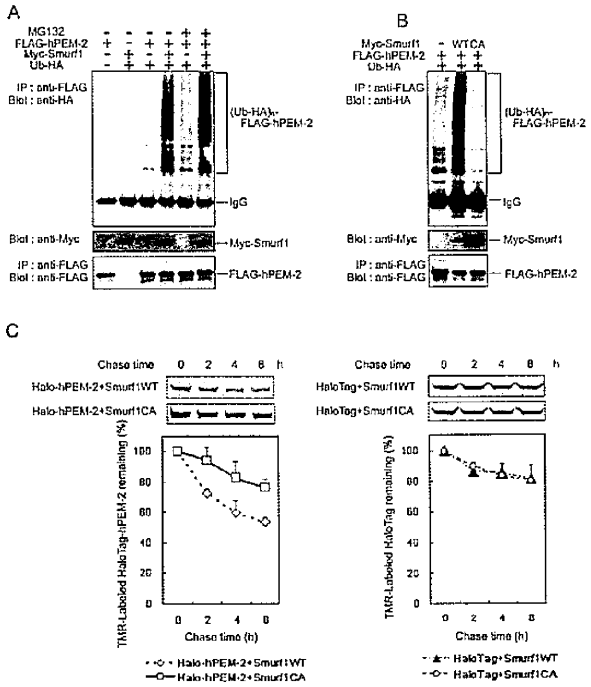
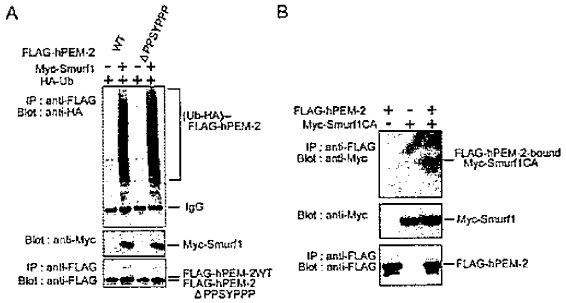



Figure 1. Kei Yamaguchi



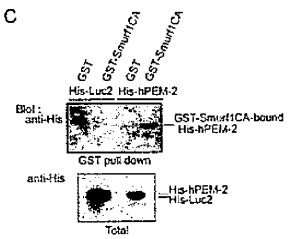




Figure 2. Kei Yamaguchi

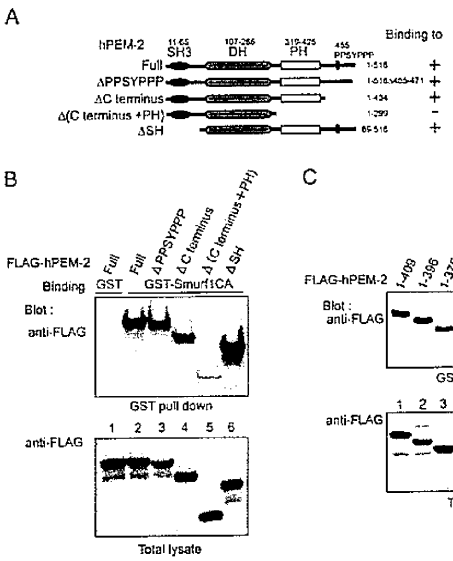



Figure 3. Kei Yamaguchi

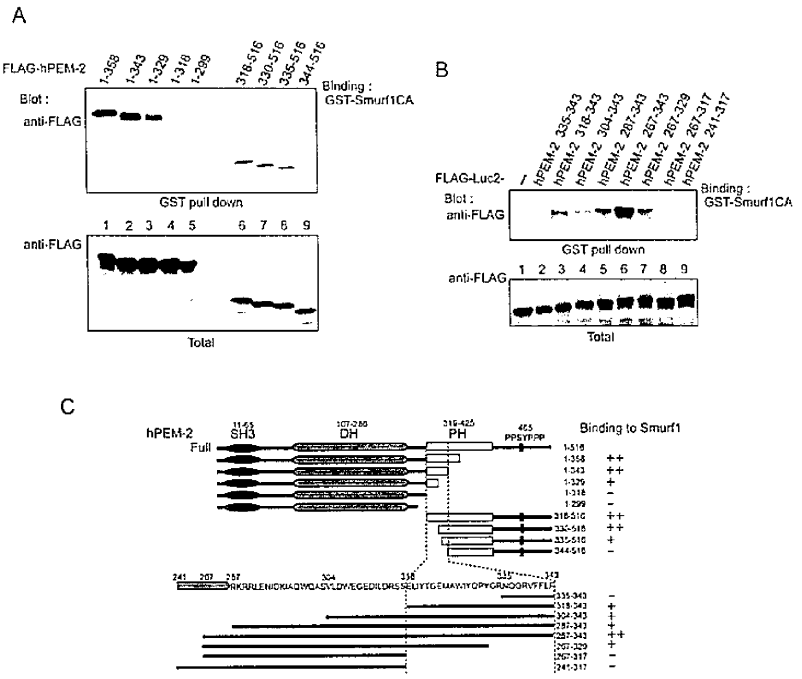



Figure 4. Kei Yamaguchi

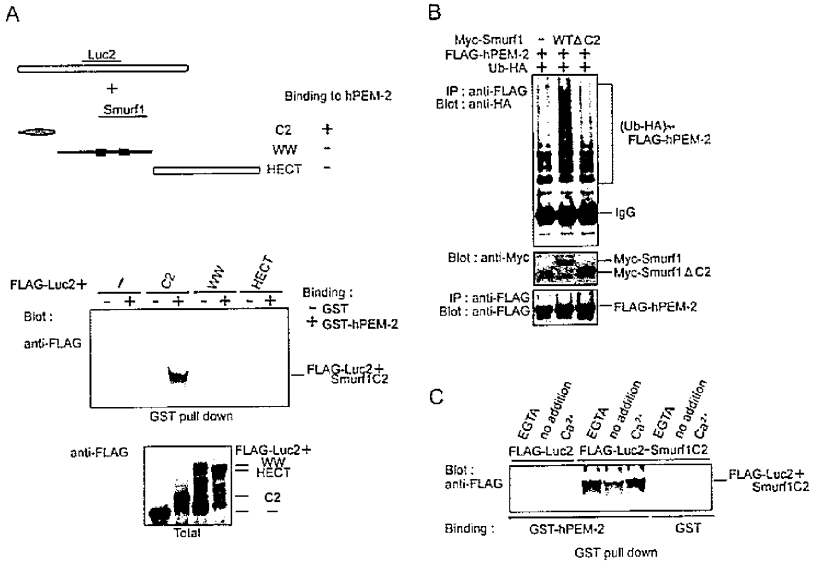



Figure 5. Kei Yamaguchi