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Running title: Ubiquitylation of SRβ by Pirh2

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ABSTRACT

Pirh2 is a RING finger type ubiquitin ligase which ubiquitylates various proteins including p53, $p27^{Kip1}$, HDAC1, and ε-COP. In this study, we identified signal recognition particle receptor β subunit (SRβ), an integral membrane protein of the endoplasmic reticulum (ER), as a novel Pirh2-interacting protein by yeast two-hybrid screening. We confirmed that Pirh2 interacted with SRβ in mammalian cells. An immunofluorescent staining revealed that Pirh2 colocalized with SRβ in the ER. Pirh2 poly-ubiquitylated SRβ in an intact RING finger domain-dependent manner *in vivo* and *in vitro*. Unexpectedly, different from other Pirh2 substrates, neither overexpression of Pirh2 nor depletion of cellular Pirh2 affected SRβ protein stability. Pirh2 preferentially utilized lysine residues 6 and 29 of the ubiquitin to mediate the formation of polyubiquitin chains on SRβ. These results suggest that Pirh2 may regulate SRβ function by mediating poly-ubiquitylation of SRβ without affecting the stability of SRβ protein per se.

Ubiquitylation is a versatile post-translational modification mechanism used by eukaryotic cells (10). It is catalyzed through a pathway involving three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). E3 is the most diverse, and it determines substrate specificity and the rate of ubiquitin conjugation. Substrates can be modified with monoubiquitin or with a polyubiquitin chain that is linked through lysines present within ubiquitin itself. The most common polyubiquitylation is linked through lysine 48 of ubiquitin which serves as a signal for the rapid degradation of substrate by the proteasome-dependent pathway (2). However, recent studies have revealed roles other than proteolysis for polyubiquitylation (17). While lysine 48- and lysine 29-linked polyubiquitin chains mediate proteasome-dependent degradation, lysine 63-linked polyubiquitin chains are a signal for endocytosis, IκB kinase activation, ribosome modification, and DNA repair (4, 7, 11, 20, 21). Although it has been reported that lysine 6-linked polyubiquitin chains are deubiquitylated by the 26 S proteasome and are not thought to be a proteasomal degradation signal (15), detailed biological function

of lysine 6-linked polyubiquitin is not well understood.

A RING-H2 type E3 ubiquitin ligase Pirh2 (p53-inducible protein with RING-H2 domain), also known as androgen receptor N-terminal-interacting protein (ARNIP) (1), was identified as a p53-inducible and -interacting protein which promotes ubiquitylation and degradation of p53 (12). Other studies have shown that Pirh2 interacts with various molecules including histone acetylase TIP60 (Tat-interactive protein of 60 kDa) (18), measles virus phosphoprotein (3), N-terminal kinase-like protein-binding protein 1 (NTKL-BP1) (22), and calmodulin (5). Recently, it has been reported that histone deacetylase 1 (HDAC1) and the ε-subunit of coatomer complex (ε-COP) were ubiquitylated and subsequently degraded by Pirh2 (13, 14). Moreover, we had found that Pirh2 promotes ubiquitin-dependent degradation of the CDK inhibitor $p27^{Kip1}$ at the G1/S phase of the cell cycle (9). These studies suggest that Pirh2 is involved in various cellular events by ubiquitylating and/or interacting with these molecules.

Secretory proteins are synthesized with an N-terminal hydrophobic signal sequence. As the signal sequence emerges from the ribosome, it is recognized

by the signal recognition particle (SRP) and subsequently targeting of the ribosome-nascent chain complex to the endoplasmic reticulum (ER) membrane occurs. At the membrane, SRP contacts the SRP receptor (SR), a heterodimer composed of SRα and SRβ. Contact between SRP and SRα leads to the transfer of the nascent chain from SRP into the lumen of the ER through the translocation channel formed by the Sec61p complex (6, 8).

In this study, we identified SRβ as a novel Pirh2-interacting protein by yeast two-hybrid screening. We found that Pirh2 promoted ubiquitylation of SRβ without affecting the stability of SRβ. Our findings suggest that Pirh2 may control the synthesis of secretory protein by the functional regulation of SRβ via ubiquitylation.

MATERIALS AND METHODS

Yeast two-hybrid screening. The yeast two-hybrid screening was performed with the Matchmaker Two-Hybrid System 2 (Clontech, Mountain View, CA, USA). The full-length rat Pirh2 was cloned into the yeast two-hybrid vector pAS2-1 and the plasmid was transformed into the yeast followed by screening a pACT2 rat brain cDNA library (Clontech) according to the manufacturer's instructions.

Cell culture, reagents, and antibodies. All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. MG132 (Peptide institute, Osaka, Japan) and cycloheximide (Wako, Osaka, Japan) were purchased. Polyclonal antibody against Pirh2 (Calbiochem, San Diego, CA, USA) was purchased. Monoclonal antibodies against FLAG-epitope (M2, Sigma, St. Louis, MO, USA), HA-epitope (12CA5, Roche, Basel, Switzerland), Xpress-epitope (Invitrogen, Carlsbad, CA, USA), α-tubulin (DM1A, Sigma), and β-actin (AC-15, Sigma) were used for immunoprecipitation and/or immunoblotting. Polyclonal antibody against SRβ

was raised in rabbit by standard procedures using the carboxy-terminus fragment of recombinant GST-hSRβ (residues 87-272) as antigens and purified by a Melon^{TM} Gel IgG Purification kit (PIERCE, Rockford, IL, USA).

Mammalian expression vectors and transfection. Human wild-type and RING finger mutant (C148A) Pirh2 expression vectors were constructed as described previously (9). Human SRβ cDNA was amplified by RT-PCR from HCT116 cell RNA and cloned into the pcDNA4-HisMax expression vector (Invitrogen). The resultant pcDNA4-HisMax-SRβ encodes 6×His and the Xpress epitope tag fused with the amino terminus of human SRβ. pCMV-2×FLAG-SRβ was constructed by ligating FLAG-SRβ cDNA into the pCMV-Tag2 expression vector (Stratagene, La Jolla, CA, USA). HEK293 and COS-1 cells were transfected by the calcium phosphate method. U2OS cells were transfected using FuGENE6 (Roche) according to the manufacturer's protocol. Small interfering RNA (siRNA) duplexes for Pirh2 were described previously (9). Non-silencing control siRNA was obtained from Qiagen (Valencia, CA, USA). The cells were transfected with

siRNA by using Oligofectamine (Invitrogen) according to manufacturer's protocol.

Immunoprecipitation and immunoblotting. Cells were lysed in immunoprecipitation lysis buffer (0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.6, and protease inhibitor mix). Immunoprecipitation and immunoblotting were performed as described previously (9).

Immunofluorescence. COS-1 cells were cotransfected with Xpress-Pirh2 and 2×FLAG-SRβ expression plasmids. After 48 h, the cells were fixed with 4% paraformaldehyde in PBS (-), permeabilized with 0.2% Triton X-100 in PBS (-), blocked with 10% normal goat serum, and treated with anti-FLAG antibody conjugated with AlexaFluor546 and anti-Xpress antibody conjugated with AlexaFluor488. The ER was stained using the ER labeling kit according to the manufacturer's protocol (Molecular Probe, Eugene, OR, USA). Fluorescence images were taken under fluorescence microscope system (Biozero, KEYENCE,

Tokyo, Japan).

In vitro ubiquitylation assay. GST-tagged FLAG-SRβ, Pirh2, and Pirh2 (C148A) proteins were expressed in *Escherichia Coli* BL21-CodonPlus (DE3) (Stratagene) and affinity-purified with glutathione-Sepharose 4B (GE Healthcare, Buckinghamshire, UK), and then the GST tag was removed by cleavage with PreScission protease (GE Healthcare). FLAG-SRβ protein was incubated with or without Pirh2 protein at 30°C for 60 min in a 20 μl ubiquitylation mixture supplemented with 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 2 mM DTT, 10 mM phosphocreatinine, 0.2 units/ml phosphocreatinine kinase, 5 mM adenosine-5'-triphosphate, 2 μl of GST-ubiquitin or His-ubiquitin, 250 μM MG132, protease inhibitor mix, E1 (120 ng, Boston Biochem, Cambridge, MA, USA), and UbcH5b (500 ng, Boston Biochem). After incubation, the reactants were subjected to immunoblotting with anti-FLAG antibody.

RESULTS

Identification of SRβ as a Pirh2-interacting protein

To identify novel interacting proteins with Pirh2, we performed yeast two-hybrid screening of a rat brain cDNA library using full-length Pirh2 as bait and found that Pirh2 interacted with signal recognition particle receptor β subunit (SRβ), a membrane protein of the endoplasmic reticulum (ER) essential for protein translocation across the ER membrane. To determine whether SRβ physically interacts with Pirh2 in mammalian cells, we coexpressed the 4×FLAG-Pirh2 and Xpress-SRβ in HEK293 cells. When extracts from transfected cells were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Xpress antibody, SRβ was only coprecipitated in the presence of transfected Pirh2 (Fig. 1A, IP: FLAG and IB: Xpress). In a reciprocal experiment using anti-Xpress antibody for immunoprecipitation, coprecipitated Pirh2 was observed in cells transfected with the Xpress SRβ plasmid but not in cells transfected with an empty vector (Fig. 1A, IP: Xpress and IB: FLAG). To further confirm this interaction, we examined whether transfected SRβ would associate with

endogenous Pirh2. HEK293 cells were transfected with 2×FLAG SRβ, and the cell extracts were subjected to immunoprecipitation with anti-FLAG antibody and immunoblotted with anti-Pirh2 antibody. As shown in Fig. 1B, endogenous Pirh2 was coprecipitated with SRβ only when 2×FLAG SRβ was transfected. Taken together, these results suggest that Pirh2 and SRβ physically interact with each other in mammalian cells. In order to examine whether Pirh2 colocalizes with SRβ in cells, we transfected 2×FLAG-SRβ with or without Xpress-Pirh2 into COS-1 cells and stained them immunocytochemically with fluorescent-conjugated antibodies. In this experiment, SRβ (Figs. 1C-E), Pirh2 (Figs. 1F-H), and ER (Figs. 1I-K) were stained red, green, and blue, respectively. In cells transfected with Pirh2 or SRβ alone, SRβ was localized in the ER (Figs. 1C, I, and L), whereas Pirh2 was distributed throughout both the nucleus and cytoplasm (Figs. 1G, J, and M). When both Pirh2 and SRβ were transfected into the same cell, Pirh2 was colocalized with SRβ in the ER (Figs. 1E, H, K, and N). These observations suggest that Pirh2 colocalizes with SRβ in the ER.

Pirh2 ubiquitylates SRβ in vivo as well as in vitro

To examine whether Pirh2 can promote ubiquitylation of SRβ *in vivo*, 2×FLAG-SRβ and HA-ubiquitin expression plasmids were cotransfected into HEK293 cells together with Xpress-Pirh2 or -Pirh2 RING mutant (in which residue cysteine 148 within the RING finger domain was replaced with alanine (C148A)) expression plasmid. The cell extracts were subjected to immunoblotting with anti-FLAG antibody. When HA-tagged ubiquitin was cotransfected together with SRβ, the amount of ubiquitylated SRβ protein was increased (Fig. 2A, left panel). Cotransfection with wild-type Pirh2 (wt) enhanced ubiquitylation of SRβ, which was not observed in the absence of Pirh2 or when cotransfected with the RING mutant (mt) Pirh2. To demonstrate that the high-molecular-mass species recognized by the anti-FLAG antibody is polyubiquitin-conjugated SRβ, SRβ was immunoprecipitated twice as described in legend for Fig. 2A. Then the immunoprecipitates were subjected to immunoblotting with anti-FLAG antibody for detection of SRβ as well as with anti-HA antibody for detection of the ubiquitin chain (Fig. 2A, right and middle

panels). In this experiment, anti-HA antibody detects only HA-ubiquitylated SRβ but not HA-ubiquitylation of the other coprecipitated proteins. Immunoblots with anti-FLAG and anti-HA antibodies gave ladders with similar mobility shifts identical to the molecular mass of a ubiquitin chain (note that $(Ub)_1$SRβ or $(Ub)_2$SRβ equally migrated in all panels of Fig. 2). These results indicated that Pirh2 promotes ubiquitylation of SRβ in a RING finger domain-dependent manner.

We then investigated whether Pirh2-induced ubiquitylation of SRβ can be reconstituted with purified components. Pirh2-mediated ubiquitylation of SRβ was detected only when all of the reaction components were present and depended on the existence of an intact RING finger domain of Pirh2 (Fig. 2B). In this *in vitro* ubiquitylation assay, the high-molecular-mass bands detected by anti-FLAG antibody disappeared when lysine free GST-ubiquitin was employed instead of wild-type GST-ubiquitin (Fig. 2C). This result indicated that SRβ ubiquitylation induced by Pirh2 directly consisted of a polymerization reaction of the ubiquitin chain but not monoubiquitylation of multiple lysines on SRβ.

Therefore, these observations strongly suggested that Pirh2 directly polyubiquitylated SRβ as a substrate for Pirh2.

Pirh2 is not involved in the regulation of SRβ protein stability

Polyubiquitin conjugation is a key signal which marks target proteins to the proteasome. We then examined whether Pirh2 can regulate the stability of SRβ in cells because Pirh2 facilitated the ubiquitylation of SRβ. To investigate the effects of expression of Pirh2 on SRβ stability, 2×FLAG-SRβ was transiently transfected into U2OS cells with or without Xpress-tagged wild-type Pirh2 or RING finger mutant (C148A) Pirh2. Unexpectedly, the steady-state level of SRβ was not affected in cells transfected with either wild-type or mutant Pirh2 compared with that in the absence of Pirh2 (Fig. 3A, lanes 1-3). Treatment with proteasome inhibitor, MG132 or *de novo* protein synthesis inhibitor, cycloheximide did not alter the SRβ expression level regardless of coexpression of Pirh2 or its mutant (Fig. 3A, lanes 4-9). Moreover, we analyzed the effect of Pirh2 expression on the half-life of SRβ protein by cycloheximide assay. Again,

coexpression of wild-type or mutant Pirh2 did not alter the stability of SRβ protein (Fig. 3B). These data suggested that SRβ was a stable protein and that Pirh2 did not induce the destabilization of SRβ protein. To further confirm whether or not Pirh2 regulated the stability of SRβ, we transfected with Pirh2 siRNA to deplete endogenous Pirh2 and assessed the expression level of endogenous SRβ protein. As shown in Fig. 3C, Pirh2 siRNA but not control siRNA induced a decrease in intracellular Pirh2 in T98G cells. However, ablation of Pirh2 did not alter the expression of SRβ protein. Taken together, it appeared that Pirh2 did not regulate the amount of SRβ protein even though Pirh2 promoted ubiquitylation of SRβ.

Pirh2 cannot mediate the polyubiquitylation of SRβ when lysine residue 6 or 29 of ubiquitin is substituted with arginine

The formation of a polyubiquitin chain by linkage of one ubiquitin moiety to lysine 48 (K48) of the adjacent ubiquitin had been thought to be a signal marking the target proteins for proteolysis by the 26 S proteasome. However, recent studies

demonstrated that polyubiquitin chains can also be assembled via lysine residues of ubiquitin other than K48, and the resulting ubiquitin chains function in indistinct biological processes (4, 7, 11, 15, 17, 20, 21). To determine which lysine residues of ubiquitin are required for polyubiquitylation of SRβ by Pirh2, we performed an *in vitro* ubiquitylation assay using 6×His-tagged ubiquitin mutants in which the lysine residue at position 6, 11, 29, 48, or 63 was individually replaced with arginine (Fig. 4A). As shown in Fig. 4B, Pirh2 was unable to mediate polyubiquitylation of SRβ with a ubiquitin mutant K6R and K29R. These data suggested that SRβ polyubiquitylation mediated by Pirh2 was assembled via lysine residues 6 and/or 29 of the ubiquitin, consistent with the notion that Pirh2 did not regulate SRβ protein stability.

DISCUSSION

We reported here for the first time that Pirh2 interacts with and ubiquitylates SRβ essential for the protein translocation across the ER in the synthesis of secretory protein. Immunocytochemical studies revealed that Pirh2 colocalized with SRβ in the ER when the two expression plasmids were cotransfected into the same cells. On the other hand, Pirh2 was widely distributed throughout the cells when Pirh2 was transfected alone (Figs. 1C-Q). These data suggested that Pirh2 can bind to SRβ on the ER and may function as a ubiquitin ligase for SRβ.

Unexpectedly, different from the case of other Pirh2 substrates such as p53, HDAC1 and $p27^{Kip1}$, neither overexpression nor depletion of Pirh2 affected the expression level and stability of the SRβ protein. Our studies further suggested that Pirh2 preferentially utilized lysine residues 6 and 29 of the ubiquitin to mediate the polyubiquitylation of SRβ. The function of lysine 6-linked polyubiquitylation is yet to be determined. However, given that the lysine 6-linked polyubiquitin chain is not a proteasomal degradation signal, it is suggested that ubiquitylation of SRβ by Pirh2 does not serve exclusively as the degradation

signal for the proteasome. As discussed above, lysine 6-linked polyubiquitylation of SRβ mediated by Pirh2 may serve as a regulatory ubiquitylation for the SRP-SR complex in the synthesis of secretory proteins without affecting the stability of SRβ per se. In any case, further studies are required to determine which lysine residues Pirh2 preferentially utilizes in ubiquitylating SRβ *in vivo*.

Recently, it was reported that Pirh2 ubiquitylated ε-COP, a subunit of the COP I coatomer complex, which coats the Golgi-derived vesicles involved in protein transport from the Golgi apparatus to the ER and within the Golgi apparatus (16, 19), and consequently promoted the degradation of ε-COP (14). Overexpression of Pirh2 in a prostate cancer cell line caused downregulation of the secretion of prostate-specific antigen (PSA), a secretory protein in prostate epithelial cells (14). Therefore, Pirh2 may control not only secretion of PSA but also the secretory protein synthesis machinery itself by promoting ubiquitylation of SRβ.

Thus, it is probable that Pirh2 functions not only in cancer cell proliferation by promoting the degradation of p53, HDAC1 and $p27^{kip1}$ in a ubiquitin-proteasome

pathway, but functions also in the synthesis of secretory protein by the functional regulation of SRβ via interaction or ubiquitylation.

Acknowledgements

We thank Dr. Hirotoshi Kikuchi for helpful discussions and Kazutoshi Asano, Sayuri Suzuki, Tomomi Abe, and Daisuke Yamada for technical assistance. This work was supported in part by Grants-in-aid for Science Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan (M.K., K.K., T.H., and C.U.) and COE program of Hamamatsu University School of Medicine funded by the Ministry of Education, Science, Sports, Culture and Technology of Japan (M.K. and K.A.).

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FIGURE LEGENDS

Fig. 1 Pirh2 interacts and colocalizes with SRβ. A: Pirh2 associated with SRβ *in vivo*. HEK293 cells were cotransfected with 4×FLAG-Pirh2 and/or Xpress-SRβ expression plasmids. After 48 h, the transfected cells were lysed and the cell lysates were subjected to immunoprecipitation (IP) or immunoblotting (IB) with indicated antibodies. B: Exogenous SRβ interacted with endogenous Pirh2. HEK293 cells were transfected with 2×FLAG-SRβ expression plasmid and analyzed as described in (A). C-Q: Pirh2 colocalized with SRβ. COS-1 cells were transfected with 2×FLAG-SRβ and/or Xpress-Pirh2 expression plasmids. Cells were fixed for immunofluorescence staining with anti-FLAG (red), anti-Xpress (green), and anti-PDI (ER marker, blue) antibodies. Scale bars: 20 μm.

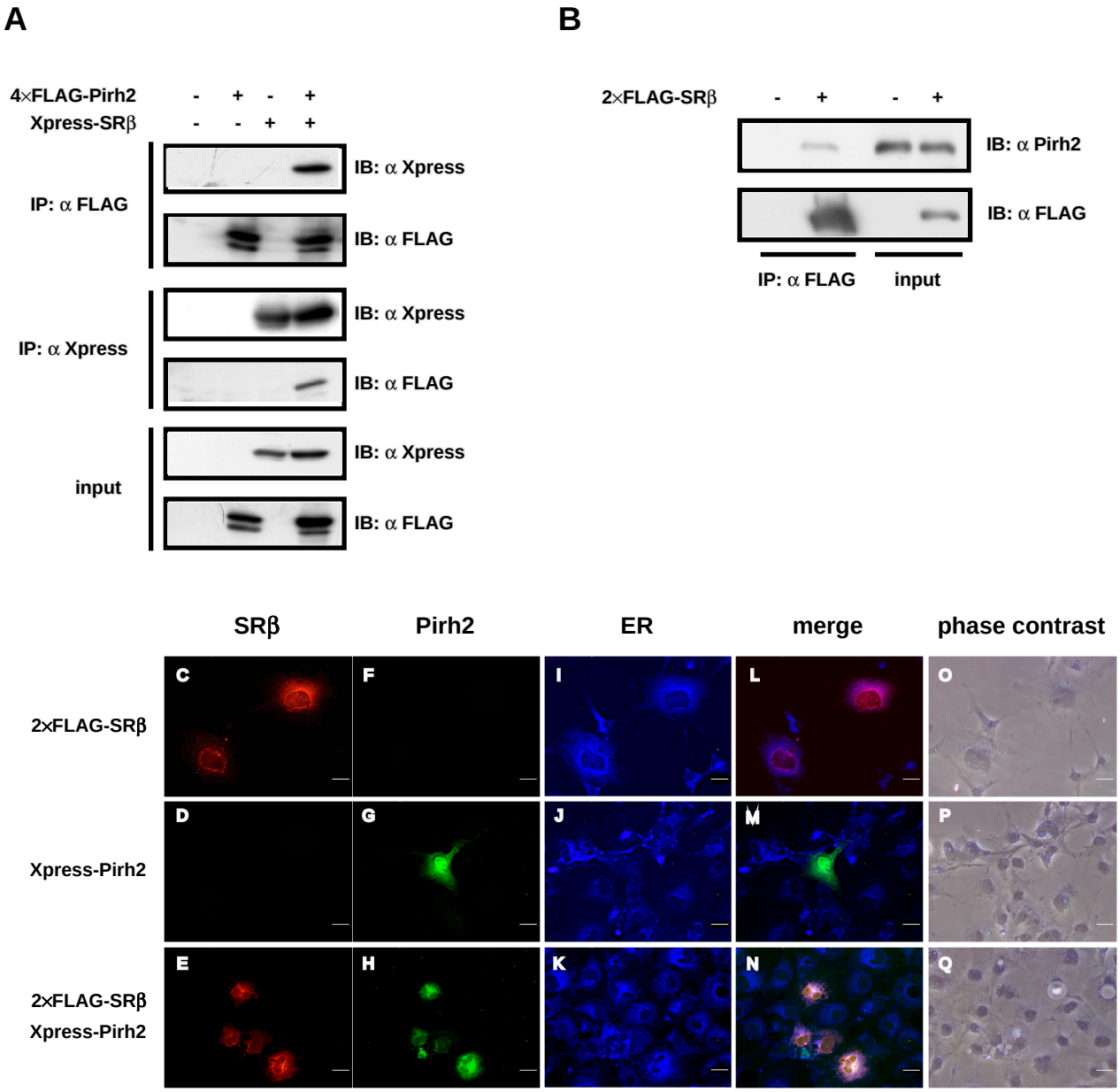

Figure 1
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Fig. 2 Pirh2 facilitates ubiquitylation of SRβ. A: Pirh2 mediated ubiquitylation of SRβ *in vivo*. HEK293 cells were cotransfected with 2×FLAG-SRβ and HA-ubiquitin expression plasmids with or without wild-type Pirh2 (wt) or RING finger mutant Pirh2 (mt) expression plasmid. At 40 h after transfection, cells were treated with 10 μM MG132 for 8 h. The cells were lysed, separated by SDS-PAGE, and immunoblotted (IB) with anti-FLAG and anti-Xpress antibodies (input, left panel). Alternatively, the cell lysates were immunoprecipitated (IP) with anti-FLAG antibody. The immunocomplex was denatured in Laemmli's sample buffer containing SDS and 2-mercaptoethanol to dissociate contaminant proteins associated with FLAG-SRβ. FLAG-SRβ was reimmunoprecipitated with anti-FLAG antibody (2×IP: αFLAG) and immunoblotted (IB) with anti-HA (middle panel) and anti-FLAG (right panel) antibodies. B: Pirh2 ubiquitylated SRβ *in vitro*. Purified FLAG-SRβ was incubated with GST-ubiquitin in the presence or absence of recombinant wild-type Pirh2 (wt) or RING finger mutant Pirh2 (mt) in the *in vitro* ubiquitylation buffer. The reactants were separated by SDS-PAGE

and detected with anti-FLAG antibody. Asterisk shows the bands derived from miss-cleaved GST-tagged FLAG-SRβ. C: Pirh2 induced poly-ubiquitylation but not multi-ubiquitylation of SRβ. Recombinant FLAG-SRβ was incubated with wild-type GST-ubiquitin (WT) or lysine free GST-ubiquitin (K (-)) together with recombinant Pirh2 in the *in vitro* ubiquitylation buffer. The reactants were analyzed as described in (B).

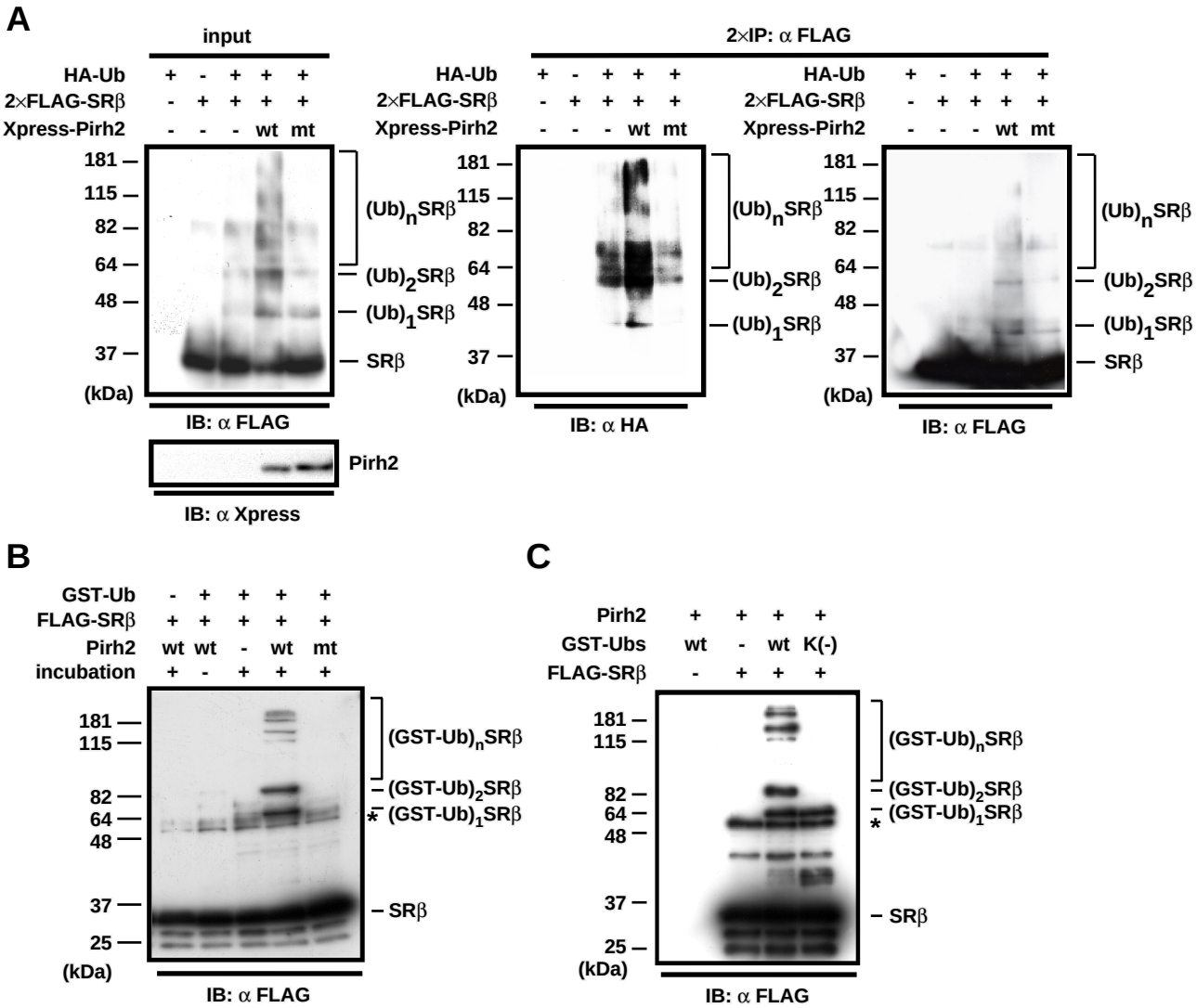

Figure 2
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Fig. 3 Pirh2 does not affect the stability of SRβ protein. A: U2OS cells were cotransfected with 2×FLAG-SRβ expression plasmid with or without Xpress-tagged wild-type Pirh2 (wt) or RING finger mutant Pirh2 (mt) expression plasmid. After 36 h, cells were treated with 10 μg/ml cycloheximide (CHX) or 10 μM MG132 for 12 h and then lysed and subjected to immunoblotting with anti-FLAG, anti-Xpress, and anti-α-tubulin antibodies. B: To evaluate effects of Pirh2 on the stability of SRβ, cells were transfected with indicated plasmids and harvested at indicated times after cycloheximide treatment. Then, they were lysed and subjected to immunoblotting with anti-FLAG, anti-Xpress, and anti-α-tubulin antibodies. C: Ablation of Pirh2 did not alter SRβ protein expression level. T98G cells were transfected with control siRNA or Pirh2 siRNA in triplicate. The cell lysates were subjected to immunoblotting with antibodies against Pirh2, SRβ, and β-actin antibodies.

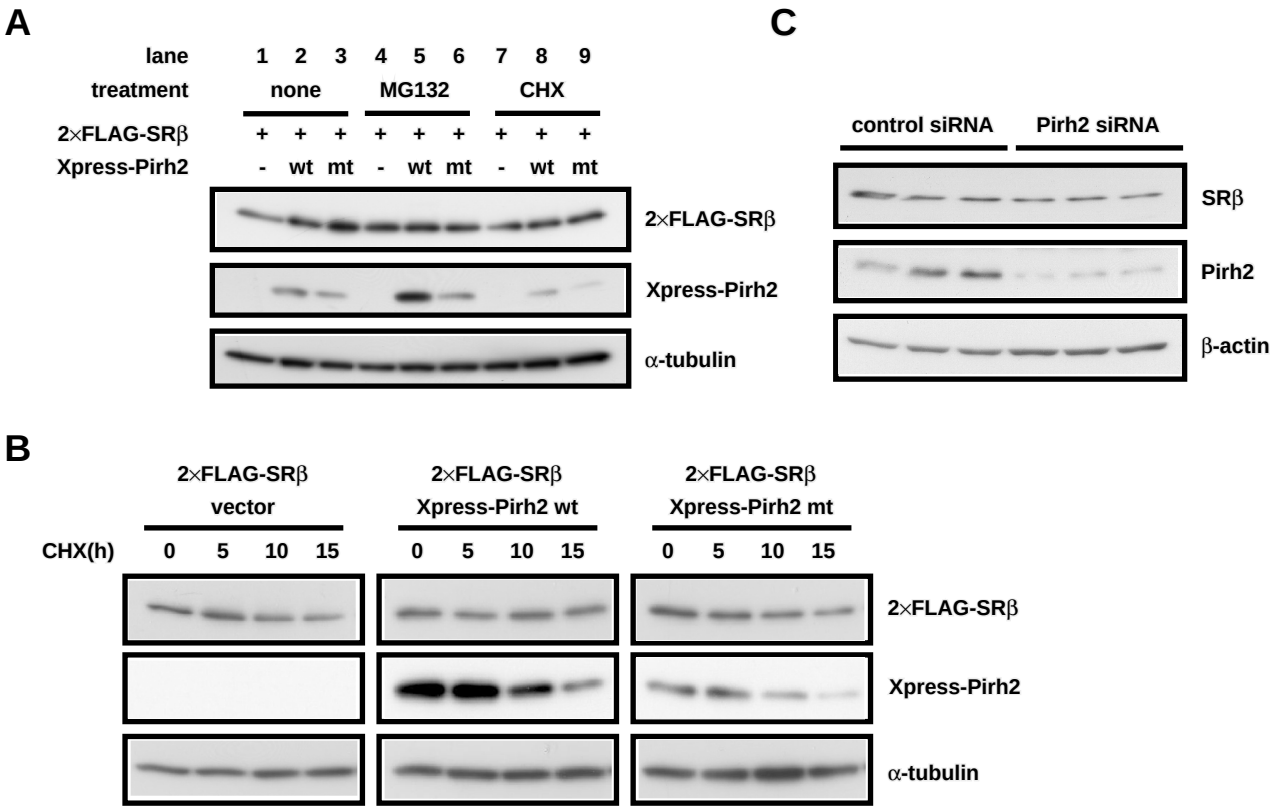

Figure 3
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Fig. 4 Pirh2 cannot utilize ubiquitin mutants in which lysine residue 6 or 29 is individually substituted for arginine in mediating the polyubiquitylation of SRβ. A: Schematic representation of His-tagged ubiquitin mutants in which the lysine residues at positions 6, 11, 29, 48, or 63 were individually substituted for arginine. B: *In vitro* ubiquitylation assay was performed with recombinant FLAG-SRβ and Pirh2 in the presence of wild-type ubiquitin (wt) or the ubiquitin mutants shown in (A). The reactants were analyzed as described in Fig. 2C. The amount of each ubiquitin mutant was confirmed by coomassie blue staining (bottom panel).

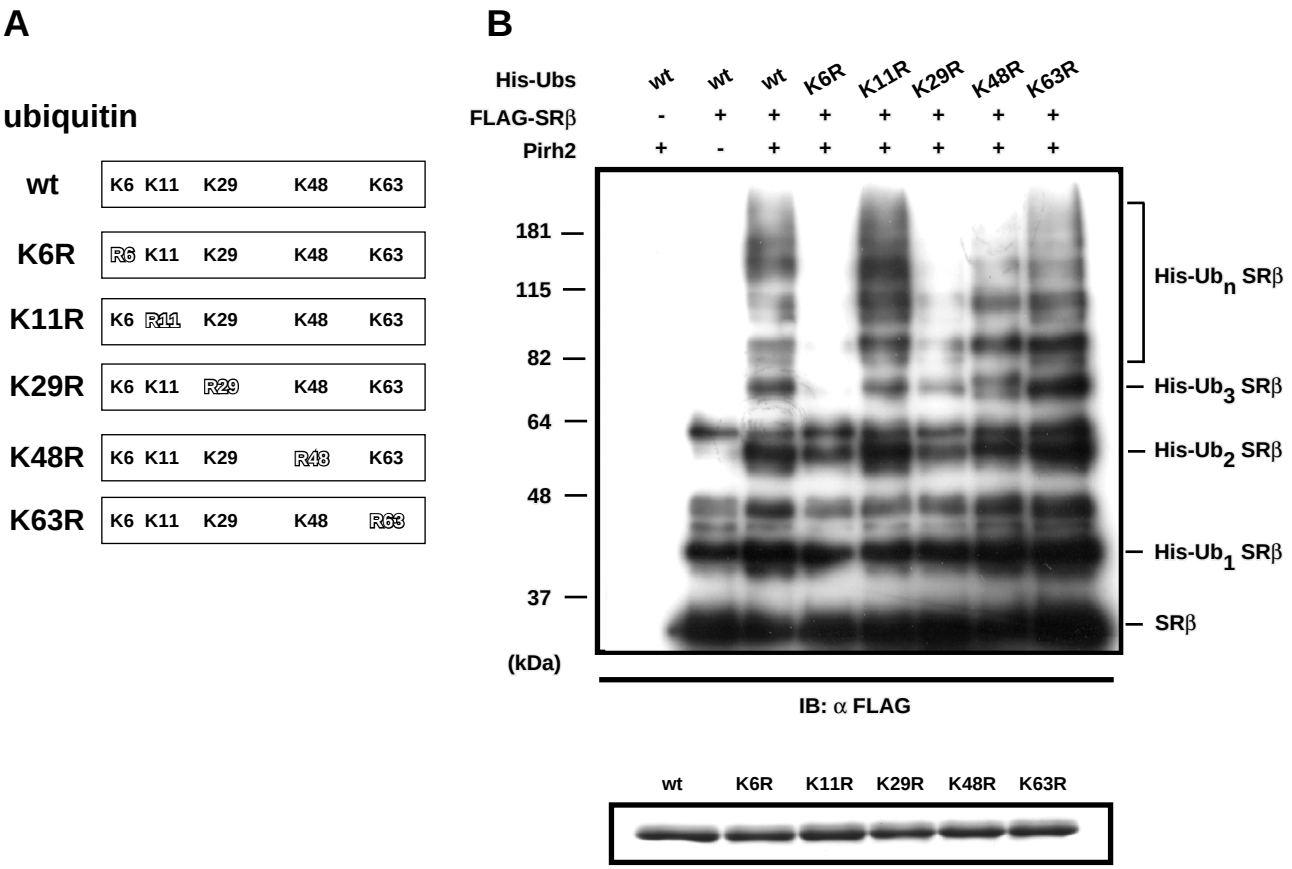

Figure 4
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