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FBXO15 regulates P-glycoprotein/ABCB1 expression through the ubiquitin–proteasome pathway in cancer cells

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Expression of P-glycoprotein (P-gp)/ABCB1 on cancer cell surfaces is a critical determinant of anticancer drug resistance. Regulators of P-gp expression and function are key molecules controlling drug resistance. Here we report the mechanism underlying the ubiquitin–proteasome pathway-mediated degradation of P-gp. The proteasome inhibitor MG132 increased the P-gp level, enhanced its ubiquitination, and delayed the disappearance of the ubiquitinated P-gp. To search for regulators of P-gp ubiquitination, MALDI–time of flight mass spectrometry analyses were carried out, and 22 candidates were identified as P-gp binding partners. Among them, FBXO15/Fbx15 is known as an F-box protein in the ubiquitin E3 ligase complex, Skp1-Cullin1-FBXO15 (SCF^{Fbx15}); therefore, we further studied the involvement of FBXO15 on P-gp degradation. Coprecipitation assays revealed that FBXO15 bound to P-gp. We screened ubiquitin-conjugating enzyme E2s that bind to FBXO15 and P-gp; Ube2r1/Cdc34/Ubc3 was found to be a binding partner. Exogenous FBXO15 expression enhanced P-gp ubiquitination, but FBXO15 knockdown suppressed it. FBXO15 knockdown increased P-gp expression without affecting its mRNA level. Ube2r1 knockdown decreased P-gp ubiquitination, and simultaneous knockdown of Ube2r1 with FBXO15 further suppressed the ubiquitination. Ube2r1 knockdown increased P-gp expression, suggesting that Ube2r1 is a partner of FBXO15 in P-gp ubiquitination. FBXO15 knockdown enhanced vincristine resistance and lowered intracellular levels of rhodamine 123. These data suggest that FBXO15 and Ube2r1 regulate P-gp expression through the ubiquitin–proteasome pathway. (*Cancer Sci* 2013; 104: 694–702)

The ABC transporter is a critical determinant of the multidrug resistant phenotype in cancer cells.[1–3] P-glycoprotein (P-gp)/ABCB1 is an ABC transporter encoded by the *MDR1* gene. It consists of symmetric structures, with each half harboring an ATP-binding site and a six-transmembrane domain.[1–3] P-gp transports various structurally unrelated anticancer drugs, including anthracyclines, *vinca* alkaloids, and taxanes, from inside cells to outside using ATP hydrolysis energy, resulting in a reduction in their cytotoxic effects.[1–3]

P-gp is expressed on the cell surface after several modifications and then works as an efflux pump. Several transcription factors, including c-Jun,[4] the vitamin D receptor,[5] FOXO3a[6] and nuclear factor-κB,[7] are involved in the transcription of *MDR1* mRNA. Heat shock factor 1 and heat shock protein 27 have been also reported to suppress P-gp expression through inhibition of the accumulation of nuclear factor-κB and transcriptionally active mutant p53.[8] After translation, P-gp undergoes phosphorylation and glycosylation.[9–11] Thus, the transcription and modification of P-gp during its biosynthesis are well understood, whereas the regulation of P-gp degradation is poorly understood.

In our previous study, inhibition of MAPK signaling by specific inhibitors and siRNAs was shown to decrease P-gp levels by promoting its degradation and to enhance sensitivity to paclitaxel, an anticancer drug and substrate of P-gp.[12] These results led us to investigate how P-gp degradation is regulated. Here we show that Ube2r1/Cdc34/Ubc3, which is a ubiquitin-conjugating enzyme E2, and FBXO15/FBX15, which is an F-box protein and part of the Skp1-Cullin1-FBXO15 (SCF^{Fbx15}) ubiquitin E3 ligase complex,[13–15] regulate P-gp expression through the ubiquitin–proteasome pathway.

Materials and Methods

Cells. Human colorectal cancer HCT-15 and SW620 cells were obtained from the National Cancer Institute (Bethesda, MD, USA). The SW620-14 cells were isolated from SW620 cells as described previously.[12] The HT1080/3HisMDR and 293/MDR cells were established from human fibrosarcoma HT1080 and human embryonic kidney HEK293 cells by transduction with the Ha3HisMDR or HaMDR retroviruses, respectively, as described previously.[12] All cell lines were maintained in DMEM supplemented with 7% FBS and kanamycin (50 μg/mL) at 37°C in 5% CO_2.

Small interfering RNAs, plasmids, and transfection. Control scramble and *ABCB1*-targeting siRNAs were purchased from Qiagen (Hilden, Germany). *FBXO15*-targeting and *Cdc34*-targeting siRNAs were from Thermo Scientific Dharmacon (Lafayette, CO, USA).

Ubiquitin, *ubiquitin-conjugating enzyme E2*, *FBXO15*, carboxy-terminal fragment of *MDR1*, and *GFP* cDNAs were generated by standard PCR methods and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) or pFLAG-CMV-2 (Sigma-Aldrich, St. Louis, MO, USA) vectors.

For the transfection of plasmids and siRNAs, FuGENE HD transfection reagent (Promega, Madison, WI, USA) and Lipofectamine 2000 transfection reagent (Invitrogen), respectively, were used according to the manufacturers' instructions.

Western blotting, immunoprecipitation, and MALDI-TOF MS analyses. Western blotting was carried out as described previously[12] using the following antibodies: anti-MDR1+3 mouse mAb (C219; Invitrogen); peroxidase-conjugated anti-FLAG M2 mAb (Sigma-Aldrich); peroxidase-conjugated anti-HA rat mAb (3F10; Roche Applied Science, Penzberg, Germany); anti-GAPDH mouse mAb (6C5; Merck Millipore, Billerica, MA, USA); anti-ubiquitin mouse mAb (1B3; Medical and Biological Laboratories, Nagoya, Japan); and anti-UBC3 rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA). For the immunoprecipitation of FLAG- or HA-tagged proteins, lysates were incubated with anti-FLAG or anti-HA affinity gels

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(Sigma-Aldrich), respectively, for 2 h or overnight at 4°C. For the immunoprecipitation of P-gp, precleared cell lysates were incubated with anti-MDR rabbit polyclonal antibodies (4007, which had been purified in our laboratory; H-241, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at 4°C with rocking and then incubated with protein A–sepharose (GE Healthcare, Little Chalfont, UK) for 16 h at 4°C. Immunoprecipitants were solubilized with 2× Laemmli sample buffer by boiling for 10 min at 70°C and subjected to SDS-PAGE. After SDS-PAGE of the immunoprecipitants, gels were stained with Coomassie Brilliant Blue. Each protein band was separated and digested with trypsin. Digested peptides were purified using ZipTip pipette tips (Merck Millipore) after elution with acetonitrile and analyzed using a MALDI-TOF MS system (AXIMA-CFR plus, Shimadzu, Kyoto, Japan).

Semiquantitative RT-PCR. Total RNA was extracted using an RNeasy kit (Qiagen). The RT-PCR was carried out using an RNA LA PCR kit (Takara Bio, Otsu, Japan) as described previously.[12]

Flow cytometric analyses. For assessment of P-gp expression on the cell surface, 5×10^5 cells were harvested, washed twice with Hanks' buffer, and incubated with or without a biotinylated $F(ab')_2$ fragment of MRK16 followed by R-phycoerythrin-conjugated streptavidin (eBioscience, San Diego, CA, USA). After two washes, the cells were analyzed using a BD LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and data were analyzed using CellQuest software (Becton Dickinson). For the rhodamine 123 (R123) accumulation assay, 1×10^6 cells were incubated with 300 nmol/L R123 in the presence or absence of 10 μmol/L cyclosporine A (CsA) in DMEM for 45 min at 37°C in the dark. The reaction was stopped immediately by cooling tubes for 5 min on ice. Cells were washed once and analyzed using the BD LSRII flow cytometer.

Cell growth inhibition assay. Chemosensitivity to vincristine (VCR) was examined by a cell growth inhibition assay in which cell numbers were counted using a Coulter counter after culturing cells for 3 days. Briefly, the IC_{50} values of each cell line treated with VCR for 3 days were preliminarily determined. The siRNA-transfected cells were treated with or without VCR (IC_{50} concentrations) for 3 days. Relative cell numbers were calculated by dividing the numbers of cells treated with VCR by those of cells treated without the drug. Data were obtained from six independent experiments, and statistical analyses were carried out using Student's *t*-test. A *P*-value <0.01 was considered statistically significant.

Results

Inhibition of the proteasome upregulates P-gp expression. We first examined the effect of MG132 on P-gp expression in the

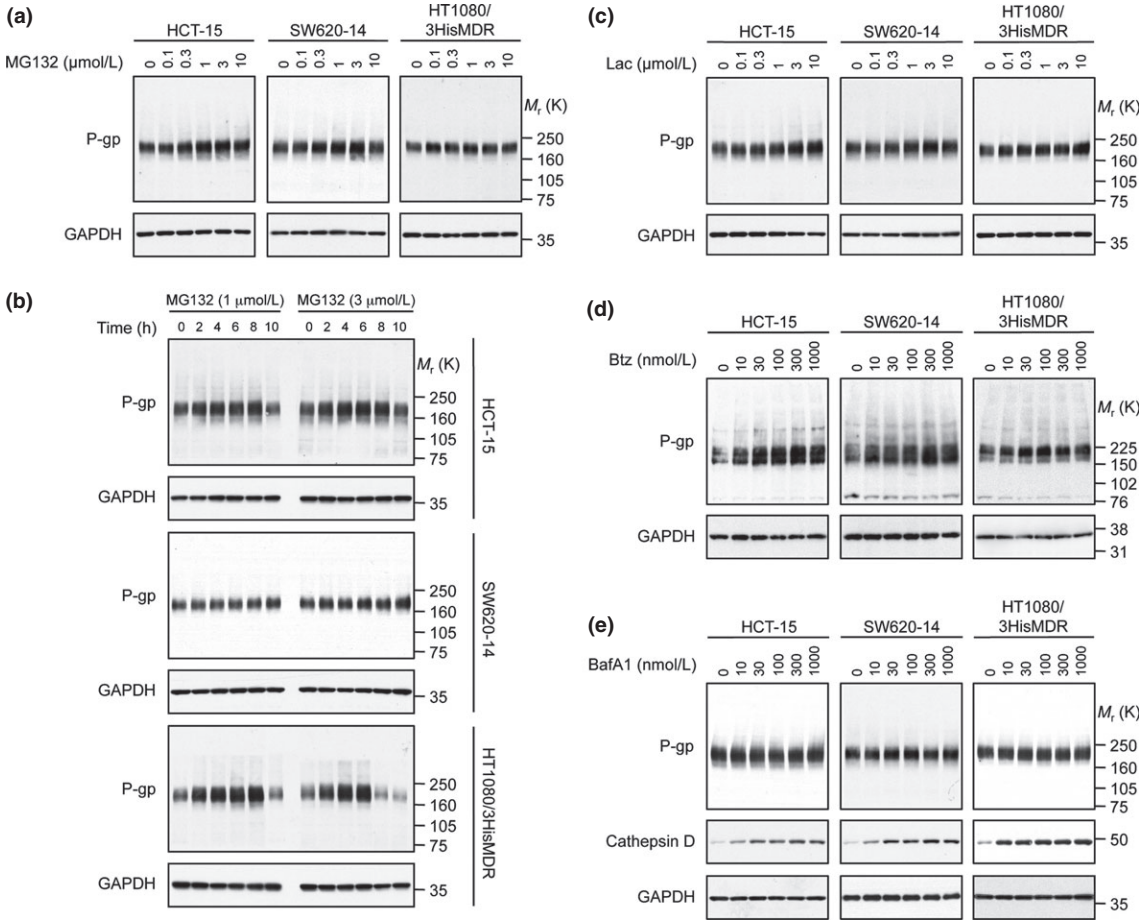

Fig. 1. Inhibition of the proteasome upregulates P-glycoprotein (P-gp) expression. (a, b) MG132 increases P-gp expression in a manner dependent on dose and time. HCT-15, SW620-14, and HT1080/3HisMDR cells were treated with increasing concentrations of MG132 for 6 h (a) or treated with 1 or 3 μmol/L MG132 for 0–10 h (b), and P-gp levels were analyzed by Western blotting. (c, d) Lactacystin and bortezomib also increase P-gp levels. HCT-15, SW620-14, and HT1080/3HisMDR cells were treated with increasing concentrations of lactacystin (Lac) (c) or bortezomib (Btz) (d) for 6 h, and Western blotting was carried out. (e) Bafilomycin A1 (BafA1) does not affect P-gp expression. HCT-15, SW620-14, and HT1080/3HisMDR cells were treated with the indicated concentrations of BafA1 for 6 h, and Western blotting was carried out. Expression of cathepsin D expression was monitored as a positive control of treatment with BafA1.

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human colorectal cancer cell lines HCT-15 and SW620-14, which express endogenous P-gp, and in HT1080/3HisMDR cells, which express exogenous P-gp. Treatment of cells with MG132 increased P-gp expression in a dose-dependent manner in all the cell lines tested (Fig. 1a). In the time-course experiment (Fig. 1b), treatment of HCT-15 cells with MG132 increased P-gp expression for up to 8 h, but the protein level was reduced at 10 h. MG132 slightly increased P-gp expression in SW620-14 cells until 10 h. Similar to HCT-15 cells, MG132 also increased P-gp expression in HT1080/3HisMDR cells for up to 8 and 6 h at concentrations of 1 and 3 μmol/L, respectively, then the protein level was reduced drastically. Long-time treatment of cells with MG132 caused apoptotic cleavage of PARP (p85 fragment) and activation of caspase 3 (Fig. S1),[16] suggesting that apoptotic proteases also degrade P-gp in such conditions. Treatment of cells with other proteasome inhibitors such as lactacystin and bortezomib for 6 h also increased P-gp expression in a dose-dependent manner (Fig. 1c,d). By contrast, bafilomycin A1, which inhibits autophagy-mediated protein degradation,[17] did not affect P-gp expression when cells were treated with increasing concentrations of the drug for 6 h (Fig. 1e). These data indicate that the ubiquitin–proteasome pathway is involved in P-gp degradation.

P-glycoprotein degraded by ubiquitin–proteasome pathway. Next, we carried out immunoprecipitation of P-gp followed by Western blotting analyses to confirm the ubiquitination of P-gp. When P-gp was immunoprecipitated using a specific antibody, polyubiquitinated P-gp was detected in the immunoblots with anti-MDR and anti-ubiquitin antibodies in the absence of MG132, and the presence of MG132 increased the level of ubiquitinated P-gp (Fig. 2a). Exogenous expression of ubiquitin increased the level of polyubiquitinated P-gp in HCT-15 cells (Fig. 2b) and HT1080/3HisMDR cells (Fig. 2c). To ascertain if ubiquitinated P-gp was degraded by the proteasome, HCT-15 cells were transfected with FLAG-tagged ubiquitin cDNA, and cells were treated with CHX combined with or without MG132 for up to 12 h. The rate of disappearance of ubiquitinated P-gp in the presence of CHX alone was faster than that in the presence of CHX plus MG132 (Fig. 2d), suggesting that ubiquitinated P-gp was degraded specifically by the proteasome.

FBXO15 binds to P-gp. To identify the molecules associated with the ubiquitin–proteasome pathway-mediated degradation of P-gp, we generated cDNA containing a FLAG-HA tandem-tagged 3′-terminal intracellular fragment of *MDR1* (3′-*MDR1* gene, 2989–3840 bp; C-ter P-gp, 997–1280 amino acids) (Fig. 3a). The C-ter P-gp was immunopurified from transfected HEK293 cell lysates using an anti-FLAG affinity gel followed by an anti-HA affinity gel. Immunoprecipitants were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue

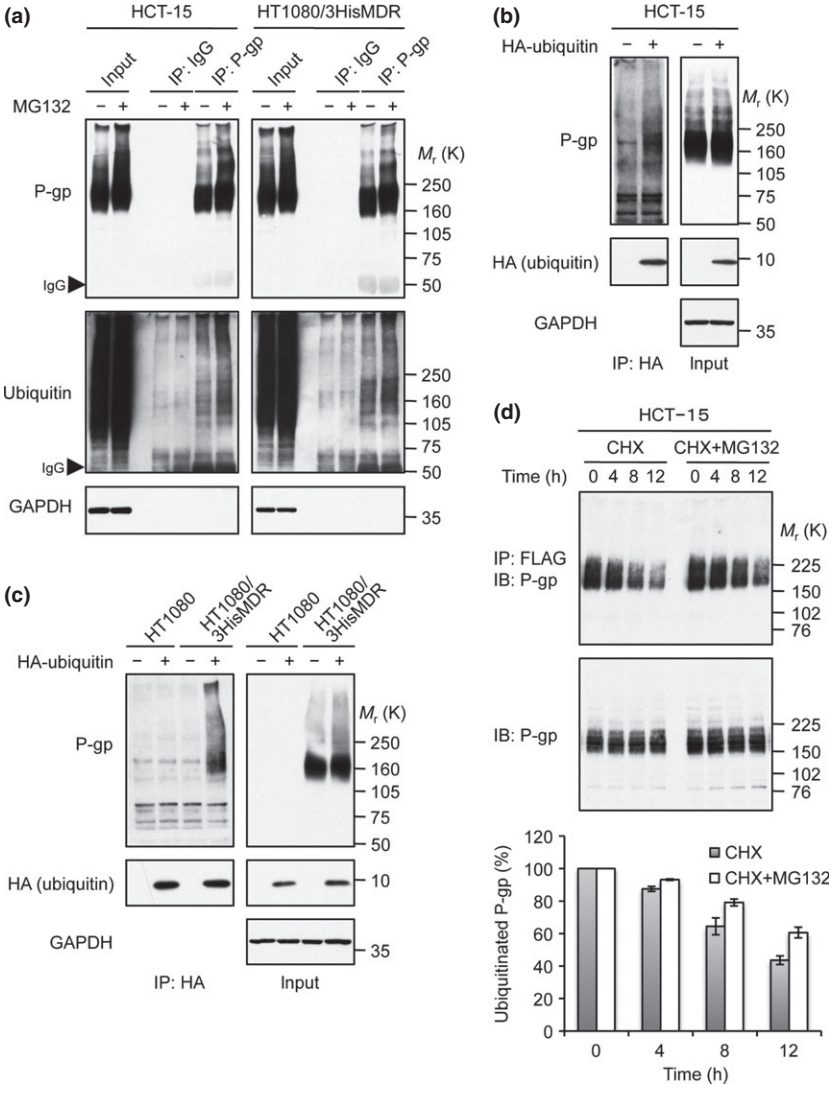

Fig. 2. P-glycoprotein (P-gp) is degraded by the ubiquitin–proteasome pathway. (a) MG132 increases the level of ubiquitinated P-gp. HCT-15 and HT1080/3HisMDR cells were treated with 10 μmol/L MG132 for 4 h then subjected to immunoprecipitation with IgG (IP: IgG) or an anti-MDR antibody (IP: P-gp) followed by Western blotting using anti-MDR (P-gp), anti-ubiquitin, and anti-GAPDH antibodies. (b, c) P-gp is coprecipitated with ubiquitin. HCT-15 (b), HT1080 and HT1080/3HisMDR (c) cells were transfected with HA-tagged ubiquitin. Twenty-four hours after transfection, cell lysates were subjected to immunoprecipitation using an anti-HA affinity gel (IP: HA) followed by Western blotting using anti-MDR (P-gp), anti-HA [HA (ubiquitin)], and anti-GAPDH antibodies. (d) Ubiquitinated P-gp is degraded by the proteasome. HCT-15 cells were transfected with FLAG-tagged ubiquitin for 24 h and treated with cycloheximide (CHX; 100 μg/mL) alone or combined with MG132 (3 μmol/L) for 0–12 h. Cell lysates were immunoprecipitated using an anti-FLAG affinity gel (IP: FLAG) and subjected to Western blotting using an anti-MDR antibody (IB: P-gp). Normalized quantification of immunoblots was carried out in two independent experiments. Lower graphic data are presented as the means ± standard error.

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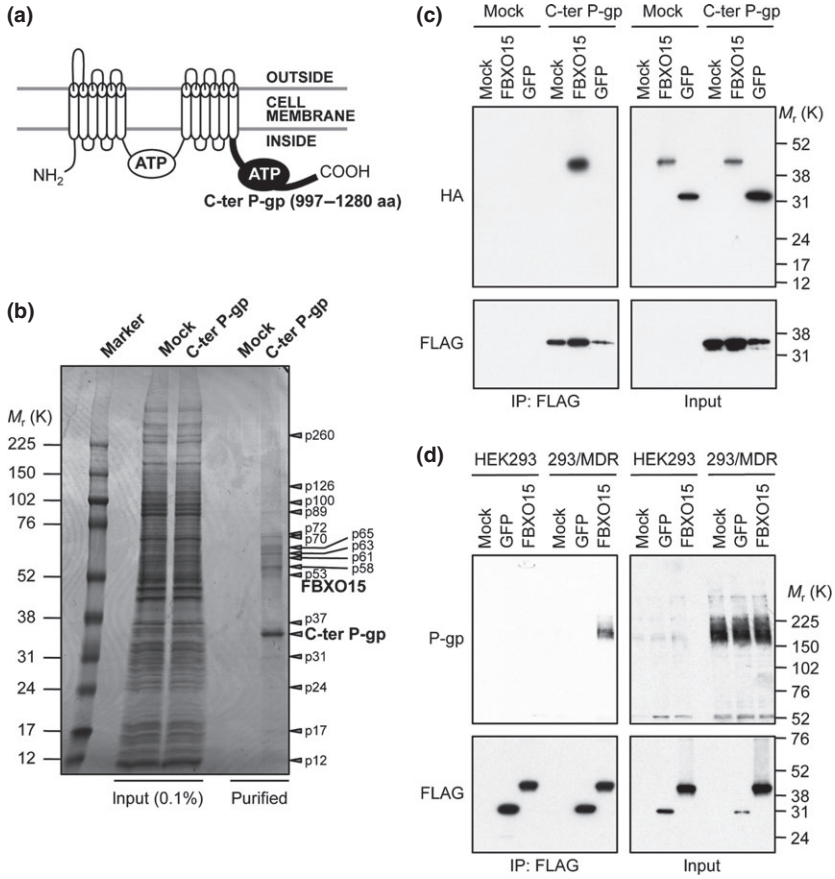

Fig. 3. FBXO15 binds to P-glycoprotein (P-gp). (a) Schematic depiction of P-gp. C-ter, C-terminal fragment. (b) FBXO15 is a P-gp binding partner. HEK293 cells were transfected with a FLAG-HA tandem-tagged empty vector (mock) or 3′-*MDR1* (C-ter P-gp, amino acids 997–1280) for 24 h. Twenty milligrams of each cell lysate was subjected to immunoprecipitation with an anti-FLAG affinity gel followed by an anti-HA affinity gel. Immunopurified proteins (purified) were subjected to SDS-PAGE and visualized by Coomassie Brilliant Blue staining. (c) FBXO15 binds to C-ter P-gp. HEK293 cells were cotransfected with FLAG-tagged empty vector (mock) or 3′-*MDR1* (C-ter P-gp) combined with HA-tagged empty vector (mock), *FBXO15* or *GFP* for 24 h. Cell lysates were subjected to immunoprecipitation with an anti-FLAG affinity gel (IP: FLAG) followed by Western blotting. (d) FBXO15 binds to full-length P-gp. HEK293 and 293/MDR cells were transfected with FLAG-tagged empty vector (mock), *FBXO15*, or *GFP* for 24 h. Immunoprecipitation and Western blotting were carried out as described in (c).

staining. As shown in Figure 3(b), 17 bands (including C-ter P-gp) were observed in the gel. Each protein band was separated, digested with trypsin and analyzed by MALDI-TOF MS after purification. Following two independent experiments, 22 candidates were identified by searching the MASCOT database (Table S1). Among these, three proteins, FBXO15, ubiquitin-specific peptidase 16, and calpain 7, are known to be associated with protein degradation.[13–15,18–20] Immunoprecipitation combined with Western blotting analyses of lysates from transfected HEK293 and 293/MDR cells revealed that P-gp was stably co-immunoprecipitated with FBXO15 but without ubiquitin-specific peptidase 16 and calpain 7 (data not shown). Therefore, we further investigated the interaction of FBXO15 with P-gp.

A coprecipitation assay in HEK293 cells cotransfected with HA-tagged *FBXO15* and FLAG-tagged 3′-*MDR1* cDNAs revealed that FBXO15 co-immunoprecipitated with C-ter P-gp (Fig. 3c). We also carried out a coprecipitation assay in HEK293 and 293/MDR cells transfected with FLAG-tagged *FBXO15* cDNA, and wild-type P-gp co-immunoprecipitated with FLAG-tagged FBXO15 (Fig. 3d), suggesting that FBXO15 binds to P-gp in cells.

Ube2r1 associates with P-gp. Because ubiquitin-conjugating enzyme E2s bind indirectly to target proteins through the action of ubiquitin E3 ligase (such as the multiprotein complex containing FBXO15) and ubiquitinate them,[21–23] we searched for E2s that were associated with P-gp. A coprecipitation assay of 23 E2s with FBXO15 was carried out in cotransfected HEK293 cells, and Ube2e2/UbcH8, Ube2g2/Ubc7, Ube2j1/Ubc6e, Ube2j2/Ubc6, and Ube2r1 co-immunoprecipitated with FBXO15 (Fig. S2). To confirm this finding, HEK293 cells were cotransfected with eight selected *E2* cDNAs, including *Ube2e2*, *Ube2g2*, *Ube2j1*, *Ube2j2*, and *Ube2r1*, combined with pcDNA3.1-3×HA plasmids encoding FBXO15 or empty plasmids (mock). Again, Ube2e2, Ube2g2, Ube2j1, Ube2j2, and Ube2r1 co-immunoprecipitated specifically with FBXO15 but the proteins did not coprecipitate from the lysates of 3×HA-tagged mock transfectants (Fig. 4a). We then carried out a coprecipitation assay of each protein with P-gp in 293/MDR cells. Ube2r1 co-immunoprecipitated with P-gp but the other E2s did not (Fig. 4b), suggesting that Ube2r1 is a P-gp binding partner. However, it was not identified in the proteomic analyses (Table S1).

The molecular mass of Ube2r1 (32 kDa) is similar to that of C-ter P-gp (35 kDa). Ube2r1 may not have been detected in the experiment due to overlapping the protein band on gels with that of C-ter P-gp. However, the coprecipitation assays clearly show that Ube2r1 binds to FBXO15 and P-gp and indicate that the ubiquitin E3 ligase complex including FBXO15 causes Ube2r1 to associate with P-gp.

FBXO15 and Ube2r1 mediate P-gp ubiquitination, and their knockdown upregulates P-gp expression. We carried out a ubiquitination assay in cultured cells to confirm the FBXO15-mediated ubiquitination of P-gp (Fig. 5a). HT1080, HT1080/3HisMDR, and HCT-15 cells were transfected with a non-silencing control or *FBXO15*-targeting siRNAs. Forty-eight hours after transfection, cells were transfected with a 3×HA-tagged empty vector or *FBXO15* cDNAs combined with FLAG-tagged empty vector or *ubiquitin* cDNAs for 24 h. Four hours before cell harvest, all cells were treated with MG132. As shown previously (Fig. 2b,c), P-gp co-immunoprecipitated with ubiquitin from HT1080/3HisMDR and HCT-15 cells transfected with FLAG-tagged *ubiquitin* cDNA alone, and co-transfection of cells with *FBXO15* and *ubiquitin* cDNAs increased the ubiquitination of P-gp in both cell lines. Preliminary transfection of cells with *FBXO15* siRNA clearly

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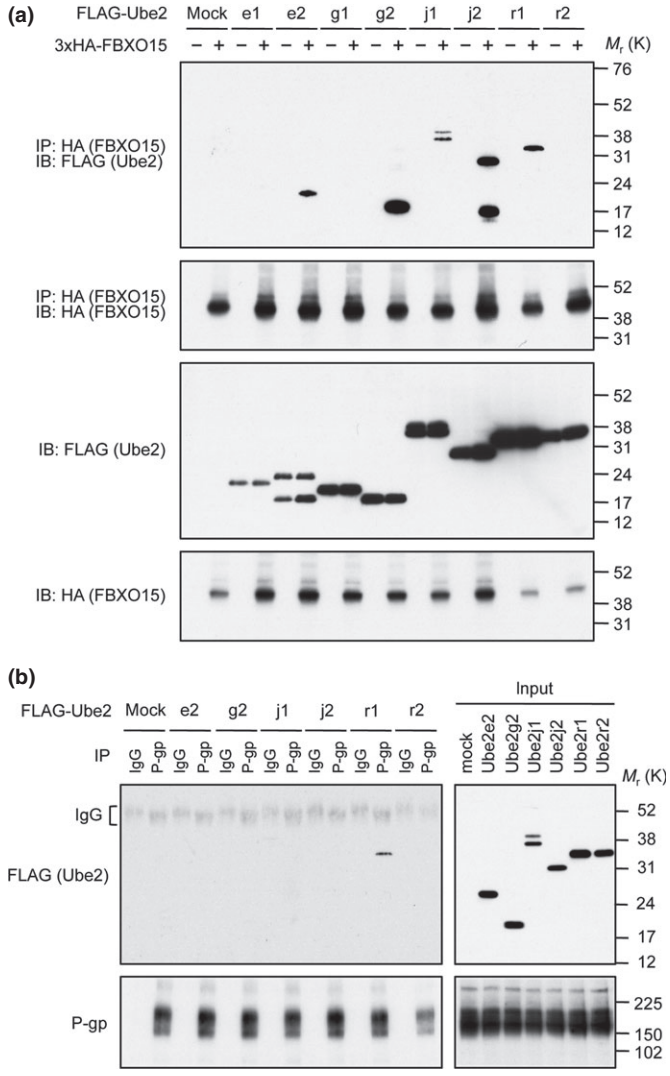

Fig. 4. Ubiquitin-conjugating enzyme E2 R1 (Ube2r1) associates with P-glycoprotein (P-gp). (a) Ube2e2, Ube2g2, Ube2j1, Ube2j2, and Ube2r1 bind to FBXO15. HEK293 cells were cotransfected with the indicated combinations of cDNAs for 24 h and treated with MG132 (10 μmol/L, 4 h) before cell harvest. Cell lysates were immunoprecipitated with an anti-HA antibody (IP: HA) and subjected to Western blotting using anti-FLAG (IB: FLAG) or anti-HA (IB: HA) antibodies. (b) Ube2r1 is associated with P-gp. 293/MDR cells were transfected with the indicated FLAG-tagged ubiquitin-conjugating enzyme E2 (FLAG-Ube2) plasmids for 24 h and treated with MG132 (10 μmol/L, 4 h) before cell harvest. Cell lysates were immunoprecipitated (IP) with IgG or an anti-MDR antibody (P-gp) and subjected to Western blotting using anti-FLAG or anti-MDR (P-gp) antibodies.

decreased the level of ubiquitination. These results suggest that FBXO15 acts as a part of the ubiquitin E3 ligase complex for P-gp.

To investigate the physiological role of FBXO15 expression in the control of P-gp levels, P-gp expression in *FBXO15* siRNA transfectants was examined. The level of P-gp expression in *FBXO15* siRNA transfectants was higher than that in control siRNA transfectants (Fig. 5b) without affecting the *ABCB1/MDR1* mRNA level (Fig. 5c). Similarly, cell-surface P-gp expression was also higher in *FBXO15* siRNA-transfectants than in control siRNA transfectants (Fig. 5d). These results suggest that P-gp function can be stimulated in *FBXO15* siRNA transfectants compared with control siRNA transfectants.

Again, a ubiquitination assay was carried out in *Ube2r1* siRNA transfected HCT-15 cells (Fig. 5e). Cells were transfected with non-silencing control, *Ube2r1*, and/or *FBXO15* siRNAs for 48 h. *Calpain 7* siRNA was also used as a constructive negative control. Cells were then transfected with FLAG-tagged empty vector or *ubiquitin* cDNAs for 24 h and treated with MG132 for 4 h before cell harvest. The ubiquitination level of P-gp in *Ube2r1* or *FBXO15* siRNA transfectants was lower than that in non-silencing control or *calpain 7* siRNA transfectants. Simultaneous knockdown of Ube2r1 with FBXO15 further reduced the ubiquitination level of P-gp compared with their separate knockdown. Next, Western blotting analyses were carried out to examine P-gp expression in *Ube2r1* siRNA transfectants for 72 h in the absence of proteasome inhibitors (Fig. 5f). The level of P-gp expression in *Ube2r1* siRNA transfectants was higher than that in control siRNA transfectants, and the results were similar to those in *FBXO15* siRNA transfectants. These data suggest that Ube2r1 promotes the ubiquitination and degradation of P-gp in cooperation with the ubiquitin E3 ligase complex including FBXO15.

FBXO15 knockdown enhances efflux activity of P-gp. A cell growth inhibition assay was carried out in siRNA transfectants treated with VCR. The IC_{50} values of cells treated with VCR for 3 days were preliminarily determined as approximately 100 nmol/L in HCT-15 and HT1080/3HisMDR cells, 10 nmol/L in SW620-14 cells, and 2 nmol/L in HT1080 cells. The siRNA transfectants were cultured in the presence of IC_{50} concentrations of VCR for 3 days and counted using a Coulter counter. *FBXO15* siRNA transfectants showed more significant resistance to VCR than control siRNA transfectants (Fig. 6a) with the exception of HT1080 cells, which did not express P-gp (Fig. 5a). Meanwhile, ABCB1 knockdown enhanced sensitivity to VCR only in P-gp-expressing cells. These results indicate that FBXO15 expression negatively regulates P-gp expression, resulting in chemosensitivity to anticancer drugs such as substrates of P-gp. Finally, an accumulation assay using R123, a fluorescent P-gp substrate, was carried out. The level of intracellular R123 in *FBXO15* siRNA transfectants was lower than that in control siRNA transfectants (Fig. 6b) and was inversely correlated with the level of cell-surface P-gp expression (Fig. 6b compared with Fig. 5d). The P-gp inhibitor CsA increased the level of R123 in *FBXO15* siRNA transfectants to the same levels seen in control siRNA transfectants treated with CsA, suggesting that the low R123 level in *FBXO15* siRNA transfectants is dependent on P-gp.

Discussion

Protein degradation is important for the maintenance of protein homeostasis in cells, and the ubiquitin–proteasome pathway plays a central role in protein degradation.[13,14,21–23] The conjugation of ubiquitin to a substrate is regulated by the continuous reaction between three enzymes: the ubiquitin-activating enzyme E1; the ubiquitin-conjugating enzyme E2; and the ubiquitin E3 ligase. Of these three enzymes, the E3 ligases are the most important components of ubiquitin conjugation because E3 ligases bind directly to the substrate proteins.

FBXO15 has been reported to be a part of the Skp1-Cullin1-FBXO15 (SCF^{Fbx15}) complex, a ubiquitin E3 ligase.[15] FBXO15 has been categorized as an F-box protein based on the results of this report and domain analyses,[13–15] but its substrates are still unknown. Our results reveal that FBXO15 is a negative regulator of P-gp through ubiquitin–proteasome pathway-mediated protein degradation. This is the first study to report P-gp to be a substrate of FBXO15. In the ubiquitination assay (Fig. 5a), polyubiquitinated FBXO15 was observed as ladder bands (Fig. S3). This result suggests that FBXO15

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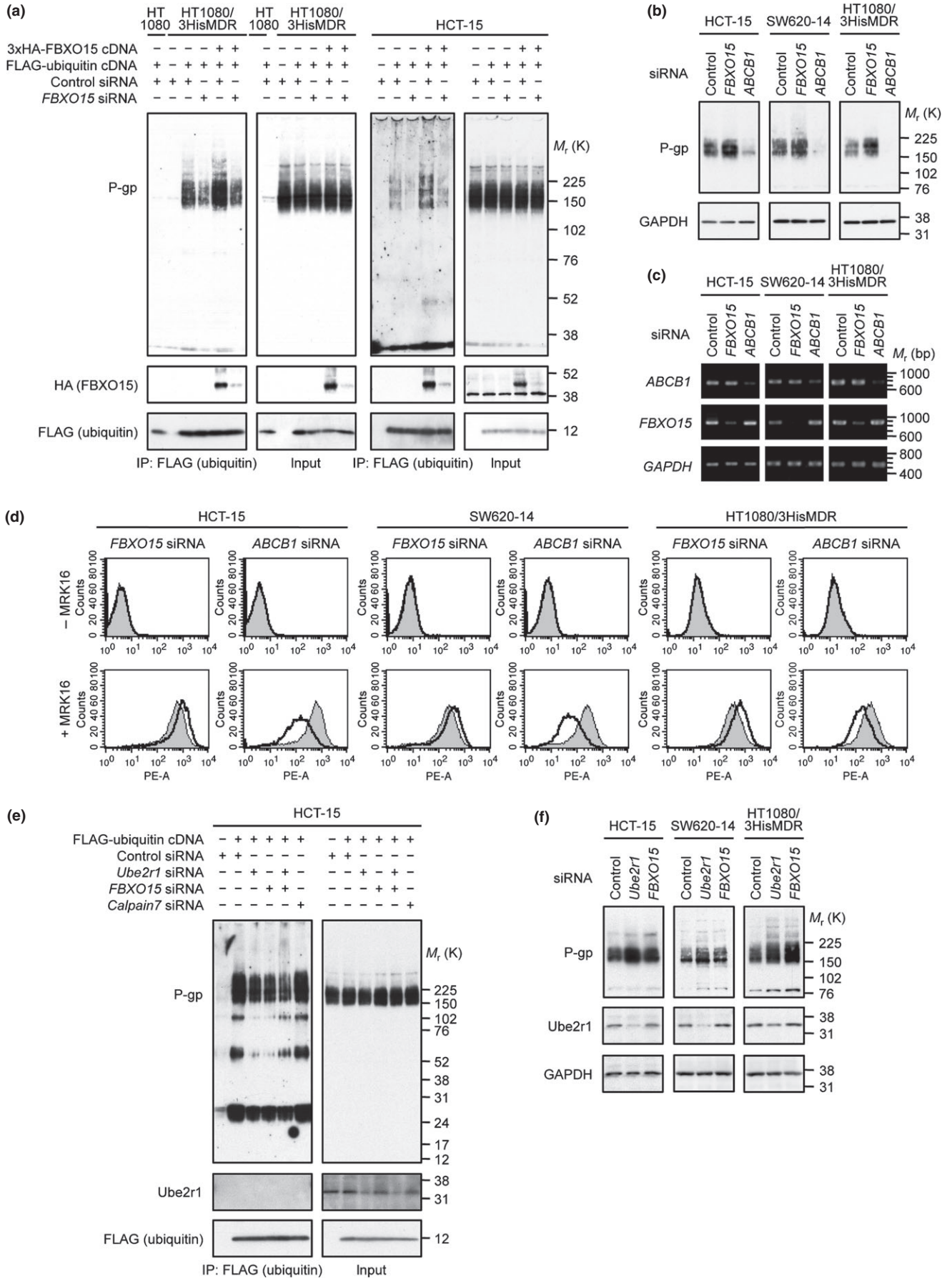

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Fig. 5. (On previous page) FBXO15 and ubiquitin-conjugating enzyme E2 R1 (Ube2r1) mediate ubiquitination of P-glycoprotein (P-gp), and their knockdown upregulates P-gp expression. (a) FBXO15 expression enhances the ubiquitination of P-gp. HT1080, HT1080/3HisMDR, and HCT-15 cells were transfected with non-silencing control or *FBXO15* siRNAs. Forty-eight hours after transfection, cells were cotransfected with FLAG-tagged ubiquitin (FLAG-ubiquitin) and/or 3×HA-tagged *FBXO15* (3xHA-FBXO15) cDNAs for 24 h and treated with MG132 (10 μmol/L, 4 h) before cell harvest. Cell lysates were immunoprecipitated with an anti-FLAG affinity gel (IP: FLAG [ubiquitin]) and subjected to Western blotting. (b) FBXO15 knockdown increases P-gp expression. HCT-15, SW620-14, and HT1080/3HisMDR cells were transfected with control, *FBXO15*, or *ABCB1* siRNAs for 72 h. Normalized quantification of immunoblots was carried out using the cell lysates. (c) FBXO15 knockdown does not affect *ABCB1* mRNA levels. HCT-15, SW620-14, and HT1080/3HisMDR cells were transfected with control, *FBXO15*, or *ABCB1* siRNAs for 24 h. The levels of each mRNA were analyzed by semiquantitative RT-PCR. (d) FBXO15 knockdown increases cell-surface P-gp expression. HCT-15, SW620-14, and HT1080/3HisMDR cells were transfected with control, *FBXO15* or *ABCB1* siRNAs for 72 h. Flow cytometric analyses were carried out. Bold line, *FBXO15* or *ABCB1* siRNA transfectants; gray-filled graph, control siRNA transfectants. (e) Ube2r1 knockdown decreases the ubiquitination of P-gp. HCT-15 cells were transfected with non-silencing control, *Ube2r1*, and/or *FBXO15* siRNAs for 48 h. *Calpain 7* siRNA was also used as a constructive negative control. Cells were transfected with FLAG-tagged ubiquitin (FLAG-ubiquitin) for 24 h then treated with MG132 (10 μmol/L, 4 h) before cell harvest. Cell lysates were immunoprecipitated with an anti-FLAG affinity gel (IP: FLAG [ubiquitin]) and subjected to Western blotting. (f) Ube2r1 knockdown increases P-gp expression. HCT-15, SW620-14, and HT1080/3HisMDR cells were transfected with control, *Ube2r1*, or *FBXO15* siRNAs for 72 h. Normalized quantification of immunoblots was carried out using the cell lysates.

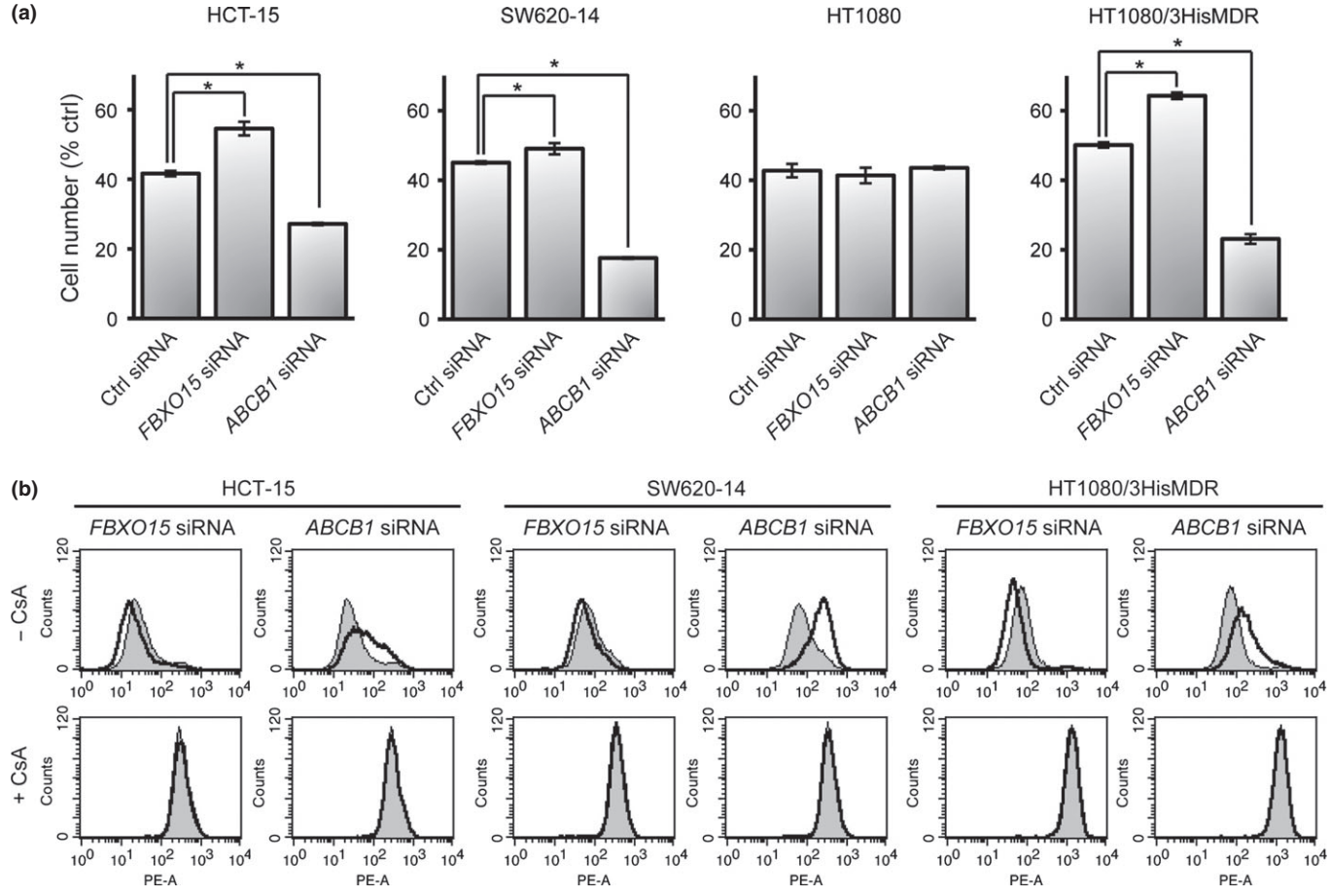

Fig. 6. FBXO15 knockdown enhances the efflux activity of P-glycoprotein (P-gp). (a) FBXO15 knockdown lowers sensitivity to vincristine (VCR). HCT-15, SW620-14, and HT1080/3HisMDR cells were transfected with control, *FBXO15*, or *ABCB1* siRNAs for 24 h. Cells were reseeded and treated with the IC_{50} concentrations of VCR for 3 days. Cell numbers were counted using a Coulter counter, and the numbers of cells treated with VCR relative to untreated cells were calculated. Error bars represent the SD from the mean ($n = 6$; $^*P < 0.002$, Student's *t*-test). (b) FBXO15 knockdown decreases the intracellular rhodamine 123 level. HCT-15, SW620-14, and HT1080/3HisMDR cells were transfected with control, *FBXO15*, or *ABCB1* siRNAs for 72 h. A rhodamine 123 accumulation assay in the presence or absence of cyclosporine A (CsA) was carried out. Bold line, *FBXO15* or *ABCB1* siRNA transfectants; gray-filled graph, control siRNA transfectants.

can also ubiquitinate itself and may promote its proteasomal degradation.

P-glycoprotein is a membrane protein harboring two 6-transmembrane domains and two large intracellular regions. In the present study, we used one of the intracellular regions as "bait" for the screening of P-gp binding proteins (Fig. 3a,b) and identified FBXO15 as a regulator in P-gp degradation. Further screening using the other intracellular regions would also be important to understand the mechanism of P-gp degradation.

FBXO15 bound to P-gp (Fig. 3b,c) and enhanced its ubiquitination (Fig. 5a); Ube2r1 coprecipitated with P-gp (Fig. 4b), and its knockdown decreased the ubiquitination of P-gp (Fig. 5e). Based on these results, we propose a ubiquitination model for P-gp (Fig. S4). The ubiquitin E3 ligase complex SCF^{Fbx15} would recognize P-gp and associate it with Ube2r1,

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which would ubiquitinate P-gp. After cycling these processes, the polyubiquitinated P-gp is degraded by the proteasome (Fig. 2d). Although the details remain to be elucidated, we successfully showed that Ube2r1 and FBXO15 mediate P-gp ubiquitination and that FBXO15 expression is a key determinant of P-gp levels.

A chlorine-selective anion channel, CFTR/ABCC7, is expressed in epithelial tissues. The most common mutation in CFTR, ΔF508, which results in deletion of phenylalanine at position 508, has been reported to cause cystic fibrosis.[24] The ΔF508-CFTR is a misfolded protein that is rapidly degraded by endoplasmic reticulum-associated degradation.[25] The Hsc70–CHIP complex is a ubiquitin E3 ligase for ΔF508-CFTR, and inactivation by overexpression of the dominant negative mutant CHIP accumulates ΔF508-CFTR.[26,27] SCF^{Fbx2}, which consists of Skp1, Cullin1, and Fbx2, also recognizes the sugar chain on ΔF508-CFTR.[28] SCF^{Fbx2} associates ΔF508-CFTR with Ube2d1/UbcH5a, resulting in the endoplasmic reticulum-associated degradation of ΔF508-CFTR after Ube2d1-mediated polyubiquitination.[25–28] Thus, expression of ubiquitin E3 ligase is a determinant of the level of target proteins, including ABC transporters. Meanwhile, mature CFTR protein levels on the plasma membrane are regulated by subapical vesicles delivering CFTR protein for either lysosomal degradation or recycling.[25] P-glycoprotein might be degraded in the same way as CFTR. Our results showed that FBXO15 knockdown upregulated cell-surface P-gp expression and enhanced its efflux activity (Fig. 5), suggesting that ubiquitin–proteasome system-mediated P-gp degradation could target mature P-gp on the plasma membrane.

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Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

ABC	ATP-binding cassette
CHIP	carboxyl terminus of Hsc70-interacting protein
CHX	cycloheximide
CsA	cyclosporine A
C-ter	C-terminal fragment
Hsc70	70-kDa heat shock cognate protein
MALDI-TOF MS	MALDI–time of flight mass spectrometry
P-gp	P-glycoprotein
R123	rhodamine 123
VCR	vincristine

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of cleaved poly(ADP-ribose) polymerase (PARP) and pro-caspase 3 (inactive form).

Fig. S2. Screening of ubiquitin-conjugating enzyme E2s bound to FBXO15.

Fig. S3. FBXO15 ubiquitinates itself.

Fig. S4. Schematic model of the ubiquitin–proteasomal degradation of P-glycoprotein.

Table S1. Results of MALDI–time of flight mass spectrometry analyses.

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