

# Caveolin-1 Interacts with Derlin-1 and Promotes Ubiquitination and Degradation of Cyclooxygenase-2 via Collaboration with p97 Complex\*

Received for publication, September 24, 2013. Published, JBC Papers in Press, October 2, 2013, DOI 10.1074/jbc.M113.521799

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**Background:** Caveolin-1 assists in COX-2 degradation through the proteasome pathway.

**Results:** Caveolin-1 enhances interactions among COX-2, Derlin-1, and the p97-Ufd1 complex and assists in COX-2 retrotranslocation and ubiquitination.

**Conclusion:** Caveolin-1 is a cofactor facilitating COX-2 degradation via a Derlin-1-p97 pathway.

**Significance:** Results represent a novel model for Derlin-1-mediated *N*-glycosylated protein degradation facilitated by caveolin-1.

Caveolin-1 (Cav-1) interacts with and mediates protein trafficking and various cellular functions. Derlin-1 is a candidate for the retrotranslocation channel of endoplasmic reticulum proteins. However, little is known about how Derlin-1 mediates glycosylated protein degradation. Here, we identified Cav-1 as a key player in Derlin-1- and p97-mediated cyclooxygenase 2 (COX-2) ubiquitination and degradation. Derlin-1 augmented the interaction of Cav-1 and COX-2 and mediated the degradation of COX-2 in a COX-2 C terminus-dependent manner. Suppression of Cav-1 decreased the ubiquitination of COX-2, and mutation of Asn-594 to Ala to disrupt *N*-glycosylation at the C terminus of COX-2 reduced the interaction of COX-2 with Cav-1 but not Derlin-1. Moreover, suppression of p97 increased the ubiquitination of COX-2 and up-regulated COX-2 but not COX-1. Cav-1 enhanced the interaction of p97 with Ufd1 and Derlin-1 and collaborated with p97 to interact with COX-2. Cav-1 may be a cofactor in the interaction of Derlin-1 and *N*-glycosylated COX-2 and may facilitate Derlin-1- and p97 complex-mediated COX-2 ubiquitination, retrotranslocation, and degradation.

folded and non-native proteins and is known as the ER quality control. Blocking the ERAD pathway may result in an unfolded protein response and ER stress, which is associated with the pathophysiologic features of a number of human diseases (3). Conserved from yeast to mammals, the ERAD pathway contains Derlin-1, a homologue of yeast Der1p, a candidate for the retrotranslocation channel mediating the initial translocation of the ER protein to the cytosol for ubiquitination (1, 4, 5). The p97 ATPase (also called valosin-containing protein or Cdc48 in yeast) and its associated cofactors, Ufd1 and Npl4, interact with valosin-containing protein-interacting membrane protein (VIMP), Derlin-1, and polyubiquitinated protein to facilitate protein retrotranslocation from the ER lumen to cytoplasm for degradation by 26S proteasome (6–10). Derlin-1 may mediate ER luminal nonglycosylated protein retrotranslocation and degradation (11); however, suppressed Derlin-1 expression is associated with accumulation of glycosylated proteins (2, 6, 7, 12, 13). Whether a cofactor interacts with Derlin-1 and p97 to facilitate glycoprotein retrotranslocation and degradation remains to be determined.

Caveolin-1 (Cav-1), the major structure protein of caveolae, specialized plasma membrane invaginations, is essential for caveolae formation. Caveolae participate in cellular functions including vesicular trafficking, cholesterol and lipid homeostasis, and signaling transduction. The caveolae structure is abolished in Cav-1-deleted mice (14). Cav-1 interacts with various proteins and regulates protein activity, phagocytosis, and protein transportation (14–16). Mutation and dysregulation of Cav-1 are implicated in tumor progression and Alzheimer disease (17–19). Deletion of Cav-1 also leads to various disease phenotypes in mice, including cardiomyopathy, diabetes, and pulmonary and inflammatory disorders (20–22). In addition, Cav-1 is suggested to mediate transforming growth factor  $\beta$ 1 receptor degradation via caveolae-mediated endocytosis (23), and overexpression of Cav-1 enhances inducible nitric oxide degradation in cancer cells (24). Cav-1 is also suggested to

Endoplasmic reticulum (ER)<sup>2</sup>-associated degradation (ERAD) is mediated by a protein complex of chaperones and associated factors that mediate the recognition, targeting, ubiquitination, and retrotranslocation of luminal substrates to the cytoplasm for proteasomal degradation (1, 2). This process eradicates mis-

\* This study was supported by National Science Council, Taiwan, Grants NSC 94-2320-B-001-038 and NSC 99-2628-B-001-013-MY3 and by the Institute of Biomedical Sciences, Academia Sinica.

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<sup>2</sup> The abbreviations used are: ER, endoplasmic reticulum; aa, amino acid(s); Cav-1, caveolin-1; ERAD, endoplasmic reticulum-associated degradation; PG, prostaglandin; VIMP, valosin-containing protein-interacting membrane protein.

facilitate cyclooxygenase 2 (COX-2) degradation *in vivo* and *in vitro* (25). These studies suggest that Cav-1 plays important roles in various cellular and physiological functions.

COX converts arachidonic acid to prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) and PGG<sub>2</sub> to PGH<sub>2</sub>. PGH<sub>2</sub> is converted to various prostanoids by a specific isomerase (26). Two COX isoforms (COX-1 and COX-2) have been identified. The COX-1 isoform is constitutively expressed in most mammalian cells, whereas COX-2 can be markedly induced by various stimuli, including proinflammatory and mitogenic factors. Prostanoids generated by COX mediate inflammation and cellular functions and are important mediators in regulating and maintaining physiological homeostasis (27). Dysregulation of COX-2 is implicated in a number of human diseases, such as cancer and cardiovascular diseases (28–30). Moreover, expression of COX-2 is controlled by both transcription and post-translational regulation (31). Although proteasome-mediated COX-2 degradation through ERAD has been suggested for some time (25, 32), the cellular machinery remains mostly unknown.

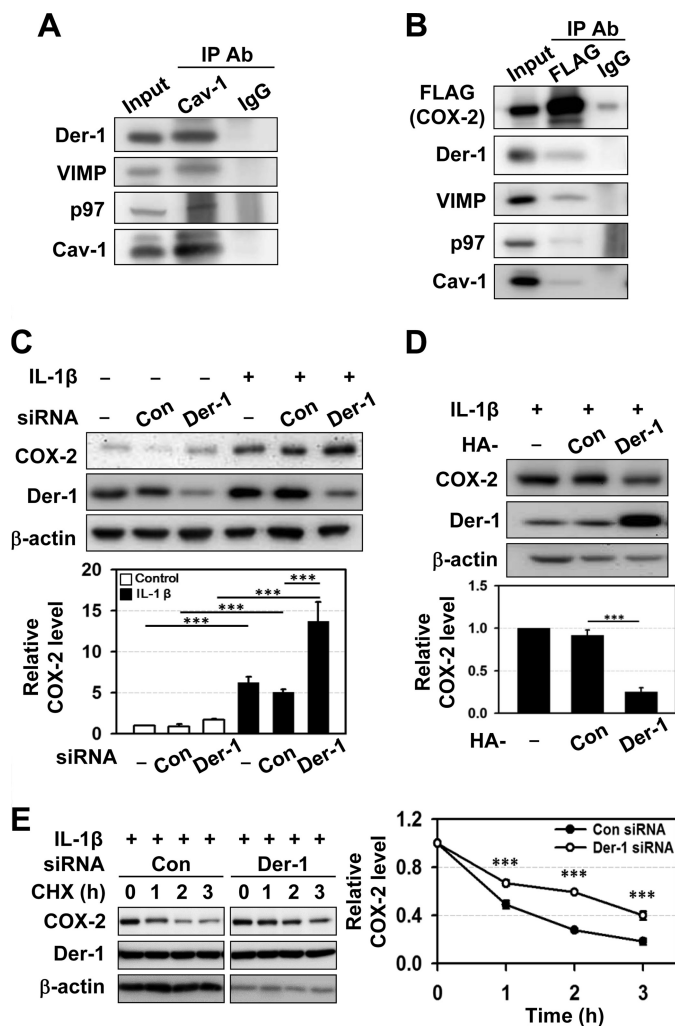
In this study we aimed to elucidate how Cav-1 interplays with the components of the ERAD complex to mediate COX-2 degradation. Our results suggest that Cav-1 interacts with Derlin-1 and COX-2 to facilitate COX-2 ubiquitination. Moreover, Cav-1 enhances interactions among Derlin-1, p97, and Ufd1 to assist in COX-2 extraction and degradation. Cav-1 may be a cofactor in the ERAD pathway promoting COX-2 degradation.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—Human foreskin fibroblast HS-68 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS (Hyclone). H1299 cells were maintained in RPMI 1640 medium containing 10% FBS.

**Antibodies and Reagents**—Cav-1, p97, and Ufd1 antibodies were from Santa Cruz Biotechnology. COX-2 antibody and recombinant COX-2 protein were from Cayman Chemical.  $\beta$ -Actin, Derlin-1, VIMP, FLAG, and HA-tagged antibodies were from Sigma. COX-1 antibody was from BD Biosciences. Interleukin-1 $\beta$  (IL-1 $\beta$ ), MG-132, and cycloheximide were from Calbiochem. Ufd1 and p97 siRNAs were from Santa Cruz Biotechnology. Cav-1 and Derlin-1 siRNA were from Dharmacon Technologies (Lafayette, CO). Control siRNA (GTACG CGGAA TACTTC GA) was from MDBio (Taiwan).

**Construction of Plasmids**—Plasmid vectors of pCMV14-3-FLAG and pXJN-HA and expression vectors encoding COX-1, COX-1-myc, COX-1<sup>+32aa</sup>-myc, COX-2, COX-2-FLAG, COX-2 <sup>$\Delta$ 32aa</sup>-myc, and Cav-1-HA were constructed as described (25). COX-2 N594A mutation was generated by PCR amplification with primers (GCTCA TTAA ACAGT CACCA TCGCT GCAAG TTCTT CC and GGAAG AACTT GCAGC GATGG TGACT GTTTT AATGA GC). Derlin-1 and Ufd1 cDNAs were obtained by PCR amplification from human cDNA and subcloned into pCMV14-3-FLAG and pXJN-HA to generate pUfd1-FLAG and pHA-Derlin-1, respectively. cDNA of mouse p97 (kindly provided by Dr. Y. Tomita, Osaka University Graduate School of Medicine) (33) was cloned into pXJN-HA to generate pHA-p97. p97 short hairpin RNA (shRNA; GACCC TGATT GCTCG AGCT) was annealed and cloned into pSuper



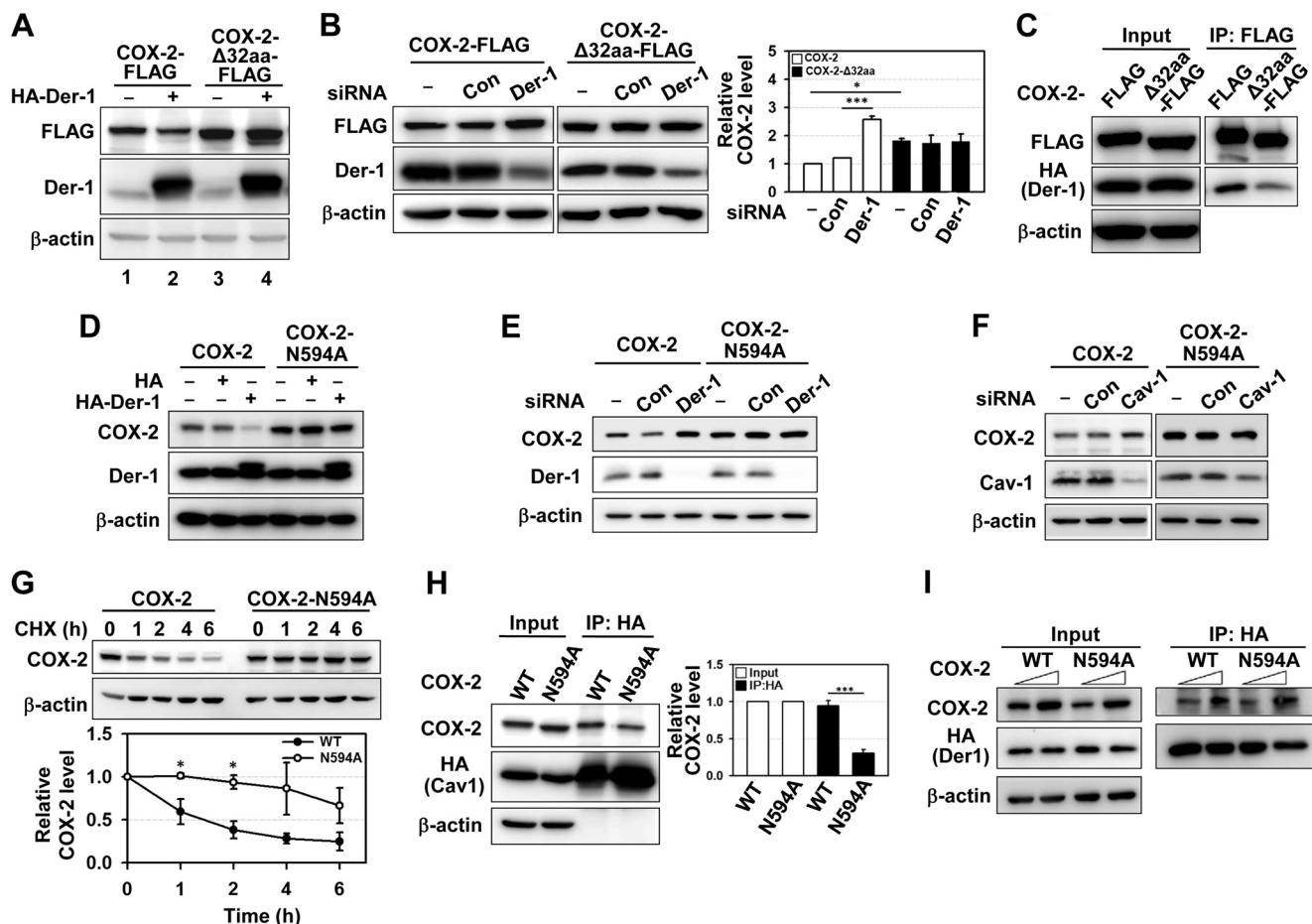
**FIGURE 1. Derlin-1 mediates COX-2 degradation.** *A* and *B*, Western blot analysis shows protein levels of interacting ERAD components Derlin-1 (*Der-1*), VIMP, and p97. *A*, cell lysates from H1299 cells were immunoprecipitated (*IP*) with anti-Cav-1 or IgG antibody (*Ab*). *B*, FLAG-tagged COX-2 was transiently expressed in H1299 cells for immunoprecipitation assay with anti-FLAG or IgG antibody. *C* and *D*, Western blot analysis and quantification were performed of protein levels in fibroblast HS-68 cells induced with IL-1 $\beta$  and transfected with siRNA of control (*Con*) or Derlin-1 (*C*) or transfected to express HA-tagged Derlin-1 (*D*) for 24 h. *E*, COX-2 level by the time of IL-1 $\beta$ -induced HS-68 cells transfected with control or Derlin-1 siRNA, then treated with cycloheximide (CHX) (20  $\mu$ g/ml) for 0–3 h is shown. Data are mean  $\pm$  S.E. (error bars; *n* = 3). \*\*\*, *p* < 0.001.

(OligoEngine, Seattle, WA). Luciferase shRNA (GTACG CGGAA TACTTC GA) in pSuper was a negative control.

Transfection involved the Lipofectamine 2000 method (Invitrogen). The amount of pCOX-2 for transfection was adjusted to ensure similar COX-2 levels in input samples for immunoprecipitation assay. To ensure an equal amount of protein expression, for co-transfection of a plasmid with siRNA the plasmid was transfected for 6 h, then cells were trypsinized and subcultured in smaller dishes overnight, followed by siRNA transfection for another 24 h.

**Sucrose Gradient Fractionation and Western Blot Analysis**—H1299 cells were lysed in radioimmunoprecipitation assay buffer, cell lysates underwent centrifugation by linear sucrose gradients (5–40% sucrose), and fractions were collected as described (34). Cell lysates or targeted proteins were resolved in

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**FIGURE 2. The C terminus of COX-2 is involved in Derlin-1- and Cav-1-mediated COX-2 degradation.** A–C, Western blot analysis of protein levels in H1299 cells transfected to express COX-2-FLAG or COX-2- $\Delta$ 32aa-FLAG with or without HA-Derlin-1 (A and C), siRNA of Derlin-1 (B), whole cell lysates (A and B), or cell lysates immunoprecipitated (IP) with anti-FLAG Ab (C). D–F, Western blot analysis of H1299 cells transfected to express COX-2 or COX-2-N594A with HA or HA-Derlin-1 (D), siRNA of Derlin-1 (E), and siRNA of Cav-1 (F). G, Western blot analysis of COX-2 protein level and quantification in H1299 cells transfected to express COX-2 or COX-2-N594A for 24 h then treated with cycloheximide (CHX) for 0–6 h. H and I, Western blot analysis of COX-2 levels interacting with Cav-1 or Derlin-1. H1299 cells were transfected to express HA-Cav-1 with COX-2 or COX-2-N594A (H) or HA-Derlin-1 with various amounts of COX-2 or COX-2-N594A (I). Cell lysates were immunoprecipitated with anti-HA antibody. Data are mean  $\pm$  S.E. (error bars;  $n = 3$ ). \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .

SDS-polyacrylamide gels and detected by Western blot analysis as described (35). GADPH or  $\beta$ -actin was an internal control. The protein levels were quantified by densitometry (Gel Pro v.3.1; Media Cybernetics).

**Immunofluorescence and Confocal Microscopy**—Cells were fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized with methanol for 15 min at room temperature, then blocked in PBS with 10% FBS for 30 min. Cells were double-stained by incubation with anti-COX-2 antibody along with anti-p97 or anti-Derlin-1 antibody for 1 h, washed three times, then incubated with FITC-conjugated donkey-anti-rabbit IgG and rhodamine-labeled donkey anti-mouse IgG for 1 h. After a wash, cells were examined by confocal microscopy.

**Immunoprecipitation Assay**—Cells were washed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM NaF, 2 mM NaVO<sub>4</sub>, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science)) and sonicated. Cell lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was incubated with antibodies for 2 h, then 50% A+G agarose beads were added for incubation for 1 h at 4 °C. The precipitates were washed three times with lysis buffer for Western blot analysis.

**Recombinant Proteins and in Vitro Protein Interaction**—Mouse p97 cDNA was subcloned into pRSET to generate pHis-p97. Cav-1 cDNA was cloned into pGEX-5X-1 to generate pGST-Cav-1. His-p97 and GST-Cav-1 were overexpressed in *Escherichia coli* BL21 and then purified by use of nickel-agarose (Invitrogen) and GST beads (Pharmacia), respectively. For *in vitro* protein interaction assay, GST and GST-Cav-1 were incubated with GST beads in immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM EDTA, 5 mM MgCl, and protease inhibitor mixture) for 1 h at 4 °C, then His-p97 or COX-2 was added for 2 h. After centrifugation at 1200 rpm for 2 min, bound proteins were washed with immunoprecipitation buffer three times and eluted for Western blot analysis.

**In Vivo Ubiquitination Assay**—HEK293 or H1299 cells were transfected to overexpress COX-2 and His-tagged ubiquitin plus Derlin-1 or p97 siRNA for 24 h and then treated with MG-132 (20  $\mu$ M) for another 8 h. The ubiquitinated proteins were bound by nickel-agarose beads for protein ubiquitination assay as described (25).

**Statistical Analysis**—One-way analysis of variance followed by a post hoc (Bonferroni) test was used to account for multiple

testing. Differences between two groups were tested by Student's *t* test. Statistical significance was set at  $p < 0.05$ .

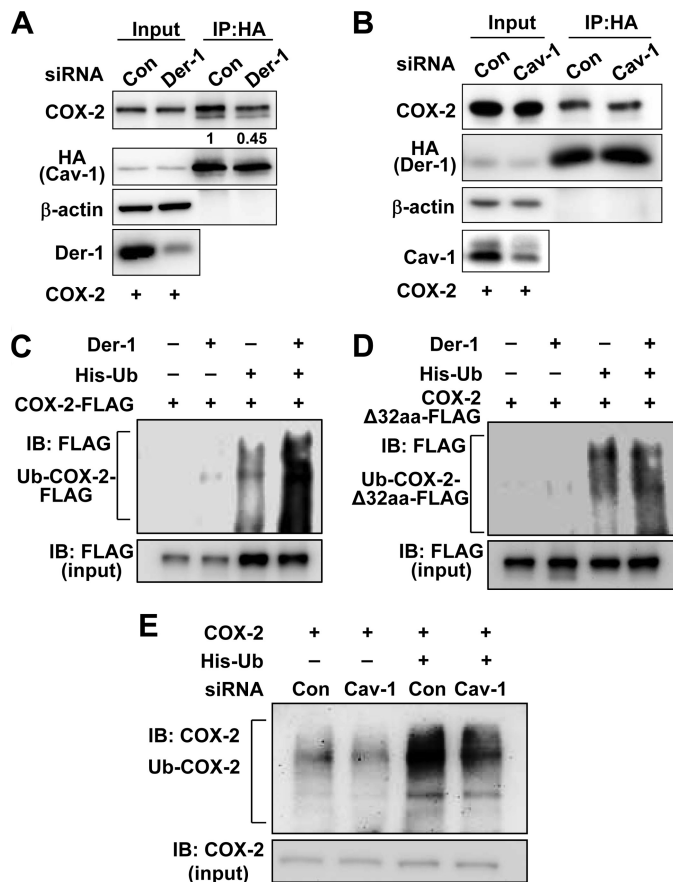
## RESULTS

**Derlin-1 Interacts with Cav-1 and Mediates COX-2 Degradation**—COX-2 interacts with the cellular membrane and resides in the lumen of the ER (36). ER-to-cytosol translocation of COX-2 is necessary to initiate its degradation. To investigate the possible interaction of COX-2 and Cav-1 with ERAD components, we used H1299 cells in immunoprecipitation assay. Cav-1 antibody co-precipitated Cav-1 with Derlin-1, VIMP, and p97 (Fig. 1A), and FLAG antibody co-precipitated COX-2-FLAG with Derlin-1, VIMP, p97, and Cav-1 in COX-2-FLAG-expressing H1299 cells (Fig. 1B). Therefore, Cav-1 and COX-2 may interact with Derlin-1, VIMP, and p97 of the ERAD complex.

Next, we examined whether Derlin-1 regulates COX-2 degradation. IL-1 $\beta$ -induced COX-2 was up-regulated with siRNA suppression of Derlin-1 in human fibroblast HS-68 cells (Fig. 1C) but was suppressed in HA-Derlin-1-expressing cells (Fig. 1D). Moreover, suppression of Derlin-1 prolonged the half-life of IL-1 $\beta$ -induced COX-2 from  $\sim 1$  to  $\sim 2.5$  h in HS-68 cells (Fig. 1E). Thus, Derlin-1 contributed to COX-2 degradation.

**C Terminus of COX-2 Is Involved in Derlin-1- and Cav-1-mediated Protein Degradation**—Compared with the C terminus of COX-1, that of COX-2 harbors a 19-amino acid (aa) unique sequence containing an *N*-glycosylation site at Asn-594 reported to be crucial for COX-2 degradation via ERAD (25, 32, 37). To determine whether this region is involved in Derlin-1-mediated COX-2 degradation, we co-expressed Derlin-1 with FLAG-tagged COX-2 (COX-2-FLAG) or FLAG-tagged COX-2 with C-terminal 32 aa deletion (COX-2 $^{\Delta 32aa}$ -FLAG), with Asn-594 and the adjacent 18 aa deleted, in H1299 cells. COX-2 $^{\Delta 32aa}$ -FLAG level was higher than the wild-type COX-2-FLAG level in control cells (Fig. 2A). Overexpression of HA-Derlin-1 decreased COX-2-FLAG level (Fig. 2A, lane 1 versus 2) but not COX-2 $^{\Delta 32aa}$ -FLAG level (Fig. 2A, lane 3 versus 4). siRNA suppression of Derlin-1 also up-regulated COX-2-FLAG but not COX-2 $^{\Delta 32aa}$ -FLAG in H1299 cells (Fig. 2B). These results suggest an important role of the COX-2 C terminus in Derlin-1-dependent COX-2 degradation. We further examined the role of this region in COX-2 and Derlin-1 interaction; less HA-Derlin-1 co-precipitated with COX-2 $^{\Delta 32aa}$ -FLAG than COX-2-FLAG (Fig. 2C). Therefore, the C-terminal 32 aa of COX-2 is responsible for the interaction of Derlin-1 with COX-2 and for Derlin-1-mediated COX-2 degradation.

To examine the role of Asn-594 in Derlin-1- and Cav-1-mediated degradation, we constructed a COX-2-N594A mutant. In agreement with previous results, the COX-2 level was higher with the N594A mutant than wild-type COX-2 (Fig. 2, D–F). Overexpression of HA-Derlin-1 down-regulated the wild-type COX-2 but not the COX-2-N594A level (Fig. 2D). siRNA suppression of Derlin-1 or Cav-1 also up-regulated COX-2 but had no effect on the COX-2-N594A level compared with controls (Fig. 2, E and F). Moreover, the N594A mutation markedly prolonged the COX-2 half-life from  $\sim 1.3$  h to longer than 6 h (Fig. 2G). Therefore, Asn-594 may play a key role in COX-2 degradation. In addition, the N594A mutation attenuated the interaction of COX-2 with HA-Cav-1, as evidenced by less COX-2-

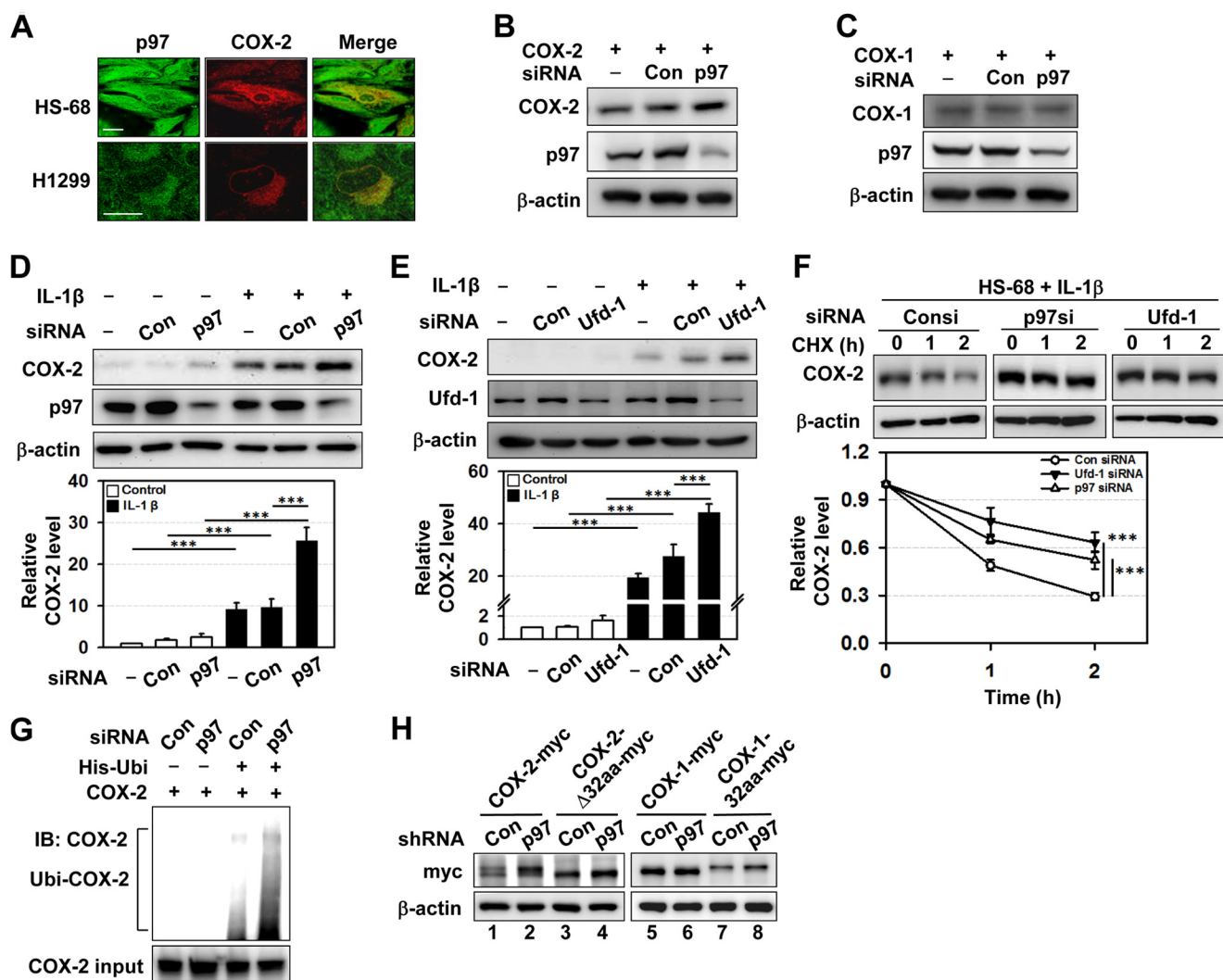


**FIGURE 3. Interplay of Derlin-1 and Cav-1 in COX-2 interaction and ubiquitination.** A and B, H1299 cells were transfected to overexpress COX-2 and HA-Cav-1 (A) or HA-Derlin-1 (B) along with siRNA of control (Con), Derlin-1 (A), or Cav-1 (B). Western blot analysis of cell lysates immunoprecipitated with anti-HA antibody is shown. Relative COX-2 levels were normalized to that of the control (A). C–E, H1299 cells were transfected to overexpress Derlin-1 (C and D), COX-2-FLAG (C), COX-2 $^{\Delta 32aa}$ -FLAG (D), or siRNA of Cav-1 plus COX-2 (E) and His-ubiquitin (Ub) for 24 h, then treated with MG-132 for 8 h. His-conjugated proteins were isolated for *in vivo* ubiquitination assay then Western blot analysis (IB) of COX-2 protein level by anti-FLAG or anti-COX-2 antibody.

N594A than wild-type COX-2 co-precipitated with HA-Cav-1 in H1299 cells (Fig. 2H). However, the N594A mutation did not reduce the interaction of Derlin-1 with COX-2-N594A (Fig. 2I). Therefore, the interaction of Cav-1 but not Derlin-1 with the C terminus of COX-2 depends on Asn-594. *N*-Glycosylation at Asn-594 may play a key role in the interaction of Cav-1 but not Derlin-1 with COX-2.

**Interplay of Derlin-1 and Cav-1 in COX-2 Retrotranslocation and Ubiquitination**—Contrary to the ER lumen localization of COX-2, Cav-1 is located in the outer membrane of the ER (14). Because Derlin-1 is a candidate for protein transmembrane translocation from the ER lumen to cytosol, we investigated the interplay of Derlin-1 and Cav-1 in interacting with COX-2 by immunoprecipitation assay. siRNA suppression of Derlin-1 reduced the interaction of Cav-1 and COX-2 (Fig. 3A); however, siRNA suppression of Cav-1 did not alter the interaction of Derlin-1 with COX-2 (Fig. 3B). Therefore, Derlin-1 enhances the interaction of Cav-1 and COX-2, but Cav-1 does not affect the interaction of Derlin-1 and COX-2. We further investigated the roles of Cav-1 and Derlin-1 in COX-2 ubiquitination. Overexpression of Derlin-1 increased the ubiquitination of

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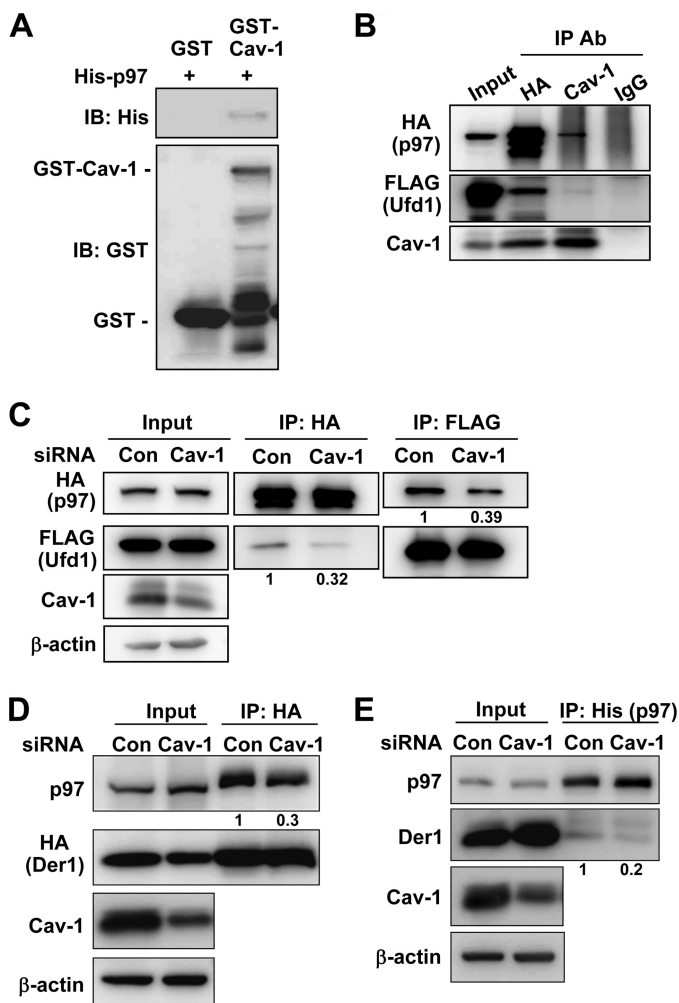
**FIGURE 4. p97 mediates COX-2 but not COX-1 degradation.** *A*, confocal microscopy of HS-68 cells treated with IL-1 $\beta$  (2 ng/ml) and H1299 cells expressing COX-2 and double-stained with FITC (against p97) and rhodamine red (against COX-2). *Merge* shows co-localization of both proteins. *Scale bars* denote 20  $\mu$ m. *B* and *C*, Western blot analysis of protein levels in H1299 cells transfected to express COX-2 (*B*) or COX-1 (*C*) and siRNA of p97. *D* and *E*, HS-68 cells induced by IL-1 $\beta$  and transfected with siRNA of p97 (*D*), or Ufd1 (*E*). *F*, Western blot analysis of COX-2 protein levels over time in IL-1 $\beta$ -induced HS-68 cells transfected with control (*Con*), p97 or Ufd1 siRNA and then treated with cycloheximide (*CHX*) for 0–2 h. *G*, HEK293 cells were transfected to overexpress COX-2 and His-tagged ubiquitin plus Derlin-1 or p97 siRNA. His-conjugated proteins were isolated for *in vivo* ubiquitination assay, then Western blot analysis (*IB*) of COX-2 protein level. *H*, Western blot analysis of myc protein level in H1299 cells transfected with control or p97 shRNA along with COX-2-myc, COX-2 $\Delta$ 32aa-myc, COX-1-myc, or COX-1 $\Delta$ 32aa-myc. Data are mean  $\pm$  S.E. (*error bars*; *n* = 3). **\*\*\***, *p* < 0.001.

COX-2-FLAG (Fig. 3C); however, overexpression of Derlin-1 did not alter the ubiquitination of COX-2 $\Delta$ 32aa-FLAG (Fig. 3D), which suggests that the C terminus of COX-2 is involved in Derlin-1-mediated retrotranslocation. Also, in line with our previous results (25), siRNA suppression of Cav-1 decreased the ubiquitination of COX-2 (Fig. 3E). These results suggest a sequential step of Derlin-1 and Cav-1 in COX-2 degradation, whereby Derlin-1 mediates the retrotranslocation of COX-2 from the ER to the cytosol to interact with Cav-1 for efficient ubiquitination.

**p97 Mediates COX-2 but Not COX-1 Degradation**—For ER protein degradation, the p97-Ufd1-Npl4 complex interacts with VIMP, Derlin-1, and ubiquitinated protein to facilitate retrotranslocation from the ER to cytosol for proteosomal degradation (6, 7, 38). We therefore investigated the role of p97 and Ufd1 in COX-2 degradation. Confocal microscopy revealed co-localization of COX-2 and p97 in HS-68 cells and H1299 cells in perinuclear and ER regions (Fig. 4A). Moreover, siRNA sup-

pression of p97 up-regulated the COX-2 level in H1299 cells (Fig. 4B). Conversely, siRNA suppression of p97 expression did not alter the COX-1 level (Fig. 4C). Similarly, the IL-1 $\beta$ -induced COX-2 level was markedly up-regulated in p97-attenuated HS-68 cells (Fig. 4D). siRNA suppression of Ufd1 up-regulated the IL-1 $\beta$ -induced COX-2 level (Fig. 4E). Also, siRNA suppression of p97 or Ufd1 prolonged the half-life of COX-2 from ~1 h to longer than 2 h in IL-1 $\beta$ -treated HS-68 cells (Fig. 4F). Therefore, the p97-Ufd1 complex mediated COX-2 degradation. We next examined the effect of p97 on the accumulation of ubiquitinated COX-2; siRNA suppression of p97 increased the ubiquitination of COX-2 (Fig. 4G), which suggests that p97 recognizes the ubiquitinated COX-2 and targets it for degradation.

We then examined the role of the C terminus of COX-2 in p97-mediated degradation. Transfection of p97 shRNA up-regulated both COX-2-myc and COX-2 $\Delta$ 32aa-myc levels but the



**FIGURE 5. Cav-1 interacts with p97 and enhances the interaction of p97 and Ufd1.** A, purified His-tagged p97 and GST-Cav-1 were incubated together for 2 h. GST-containing proteins were pulled down by GST beads. Western blot analysis (IB) of proteins eluted from GST beads with anti-His or anti-GST Ab was performed. B–E, H1299 cells were transfected to express HA-p97 and Ufd1-FLAG (B and C), HA-Derlin-1 (D), or His-p97 (E) along with control or Cav-1 siRNA. Western blot analysis of cell lysates immunoprecipitated (IP) with indicated Abs is shown. Relative protein levels, normalized with input control, are indicated under specific bands.

latter to a lesser extent (Fig. 4H, lane 1 versus 2 and lane 3 versus 4, respectively). In addition, we constructed a COX-1 insertion mutant with the 32 aa inserted into the C terminus of myc-tagged COX-1 (COX-1<sup>+32aa</sup>-myc). In H1299 cells, COX-1-myc levels were similar in cells transfected with control or p97 shRNA (Fig. 4H, lane 5 versus 6). The COX-1<sup>+32aa</sup>-myc level was lower than the COX-1-myc level with control shRNA (Fig. 4H, lane 5 versus 7) but was higher with p97 than control shRNA (Fig. 4H, lane 7 versus 8). Therefore, the p97-Ufd1 complex mediates COX-2 but not COX-1 degradation, and the C terminus of COX-2 plays an important role in p97-mediated degradation.

**Cav-1 Interacts with p97 and Enhances the Interaction of p97 with Ufd1, Derlin-1, and COX-2**—We investigated the role of Cav-1 in p97-mediated COX-2 degradation. Interaction of p97 with Cav-1 was supported by an *in vitro* pull-down assay showing His-tagged p97 interacting with GST-Cav-1 but not GST alone (Fig. 5A), which suggests a direct interaction of p97 with

Cav-1. Moreover, Ufd1 and Cav-1 co-precipitated with p97, and p97 and Ufd1 co-precipitated with Cav-1 in H1299 cells (Fig. 5B). Also, p97 co-precipitated less Ufd1 and Ufd1 co-precipitated less p97 in H1299 cells with Cav-1 siRNA knockdown than the control (Fig. 5C). Thus, Cav-1 interacts with the p97-Ufd1 complex and enhances the interaction.

We next examined whether Cav-1 promoted the interaction among p97, Derlin-1, and COX-2. Co-precipitation of p97 with HA-Derlin-1 and Derlin-1 with HA-p97 was lower in H1299 cells with Cav-1 siRNA knockdown than the control (Fig. 5, D and E), which suggests that Cav-1 enhances the interaction of p97 and Derlin-1. In addition, co-precipitation of COX-2 with HA-p97 and with HA-Cav-1 was lower in H1299 cells with Cav-1 and p97 siRNA knockdown, respectively, than the control (Fig. 6, A and B). Moreover, the GST pull-down assay revealed a direct interaction of GST-Cav-1 with COX-2, and the addition of His-p97 augmented this interaction (Fig. 6C). Thus, Cav-1 and p97 collaborated with each other to interact with and mediate COX-2 degradation.

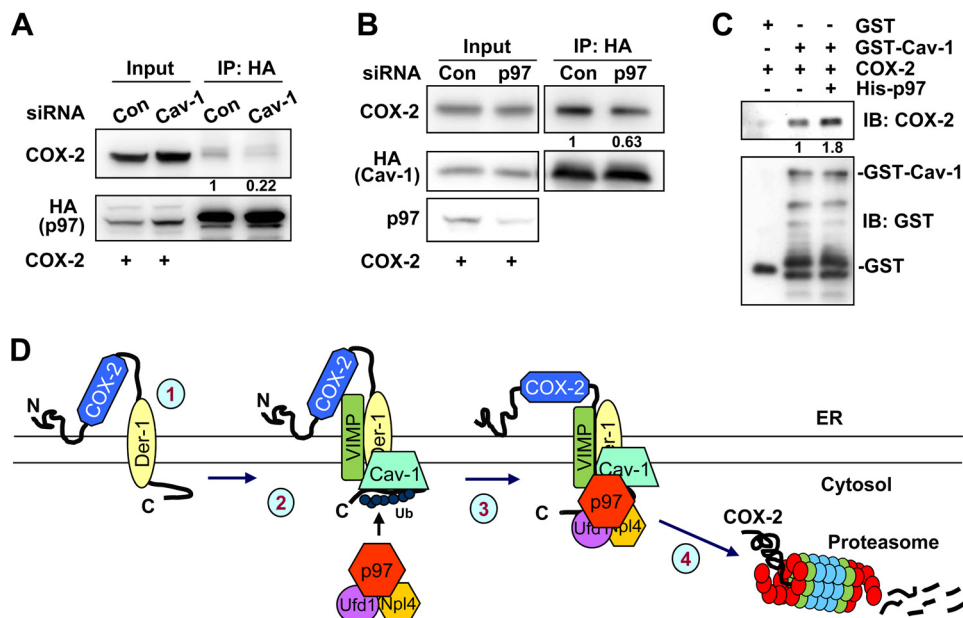
## DISCUSSION

In this study, we demonstrated a novel role of Cav-1 in interacting with Derlin-1, VIMP, p97, and Ufd1 to facilitate degradation of COX-2, a glycosylated protein. Our results suggest that COX-2 is targeted to Derlin-1 for initial retrotranslocation and, with the help of Cav-1, for ubiquitination. Although a number of studies show important functions of Cav-1 in caveolae near the plasma membrane, our results demonstrate a critical role of Cav-1 in the ER in interacting and enhancing the interaction among COX-2, Derlin-1, p97, and Ufd1 to facilitate COX-2 degradation.

COX-2 is a membrane-associated protein with a hydrophobic domain that interacts with the membrane and is located in the luminal leaflet of the ER membrane (36). Degradation of COX-2 may represent a specific category of ER proteins besides soluble and integral membrane proteins. Our results reveal a critical role of Derlin-1 in mediating COX-2 retrotranslocation and a sequential step of COX-2 degradation in that Derlin-1 mediates the initial translocation of COX-2 from the ER to the cytosol to interact with Cav-1. Cav-1 may interact with both Derlin-1 and COX-2 to help in Derlin-1-mediated retrotranslocation, thereby enhancing COX-2 ubiquitination.

N-Glycosylation of the Asn-594 residue in the C-terminal region of COX-2 has been reported to mediate COX-2 degradation (32, 37). Here, we further demonstrated that the C terminus of COX-2 plays a critical role in Derlin-1-, p97-, and Cav-1-mediated COX-2 degradation. Studies suggest that Derlin-1 interacts with and mediates nonglycosylated protein retrotranslocation (11), and knockdown of Derlin-1 stabilized the nonglycosylated mutant but not the wild type of human breast cancer-resistant protein (13). However, our results showed that knockdown of Derlin-1 increased the level of wild-type COX-2 but not that of the nonglycosylated N594A COX-2 mutant compared with the control (Fig. 2E). Notably, although deletion of the C terminus of COX-2 reduced its interaction with Derlin-1, the N594A mutation failed to suppress the interaction (Fig. 2I). Derlin-1 interacting with the C terminus of COX-2 did not depend on glycosylation. Conversely, Cav-1 interaction

## Caveolin-1 Assists Derlin-1/p97-mediated COX-2 Degradation



**FIGURE 6. Cav-1 collaborates with p97 to interact with COX-2.** A and B, H1299 cells were transfected to express COX-2 and HA-p97 (A) or COX-2 and HA-Cav-1 (B) along with siRNA of control, Cav-1 (A) or p97 (B). Western blot analysis of cell lysates immunoprecipitated (IP) with HA Ab is shown. Relative COX-2 levels, normalized with input control, are indicated under specific bands. C, purified His-tagged p97, COX-2, and GST-Cav-1 were incubated together for 2 h for GST pull-down assay. Western blot analysis (IB) of proteins eluted from GST beads with anti-COX-2 or anti-GST Abs was performed. Relative COX-2 levels are indicated. D, model of Derlin-1-, p97-, and Cav-1-mediated COX-2 degradation through ERAD. 1, Derlin-1 interacts with the C terminus of COX-2 to initiate retrotranslocation. 2, Cav-1 interacts with Derlin-1, VIMP, and the glycosylated C terminus of COX-2 to facilitate COX-2 ubiquitination. 3, Cav-1 interacts with and enhances the interaction of the p97-Ufd1-Npl4 complex with Derlin-1 and ubiquitinated COX-2. 4, the ERAD complex facilitates COX-2 retrotranslocation from the ER lumen to the cytosol for proteasomal degradation.

with the C terminus of COX-2 did depend on glycosylation because the COX-2 N594A mutation suppressed the interaction (Fig. 2H). These results support a novel model of Derlin-1-mediated glycosylated protein degradation in that after the initial translocation of COX-2 by Derlin-1, Cav-1 may interact with the N-glycosylated C terminus of COX-2 to assist with Derlin-1-mediated translocation and ubiquitination and then retrotranslocation and degradation.

Cav-1 has been suggested to interact with specific motifs containing aromatic residues such as  $\Phi X\Phi XXXX\Phi$  or  $\Phi XXXX\Phi XX\Phi$ , where  $\Phi$  is an aromatic residue and X is any amino acid (39). However, the C terminus of COX-2 contains no such motif. Cav-1 may interact with the specific secondary structure of the COX-2 C terminus containing the N-glycosylated residues (32). Of note, despite reduced interaction, the antibody of Cav-1 co-precipitated with COX-2 with the C-terminal 32 aa deleted (25) or with the N594A mutation (Fig. 2H), so Cav-1 may also interact with other regions of COX-2.

Although COX-1 and COX-2 are highly similar in protein sequence and structure (40), our results demonstrate that p97 mediates the degradation of COX-2 but not COX-1 (Fig. 4, B and C). Moreover, besides Derlin-1 and Cav-1, the C terminus of COX-2 plays an important role in p97-mediated COX-2 degradation, as evidenced by insertion of this region in the C terminus of COX-1 accelerating p97-mediated COX-1 degradation (Fig. 4H). Nevertheless, siRNA suppression of p97 augmented the COX-2 <sup>$\Delta$ 32aa</sup>-myc level compared with the control (Fig. 4H), but up-regulation or suppression of Derlin-1 did not alter COX-2 <sup>$\Delta$ 32aa</sup>-FLAG level (Fig. 2, A and B). Therefore, although the C terminus of COX-2 plays a key role in Derlin-1-mediated retrotranslocation,

p97 may also mediate the degradation of COX-2 <sup>$\Delta$ 32aa</sup> but with less efficiency than degradation of wild-type COX-2.

Interaction of p97 with Cav-1 has been reported in that p97 binds with monoubiquitinated Cav-1 in endosomes and mediates its transport to endolysosomes (41, 42). Our results support the direct interaction of Cav-1 with p97 and reveal that this interaction facilitates p97-mediated COX-2 protein degradation. Our results suggest a critical role of Cav-1 in p97-mediated COX-2 degradation in that Cav-1 enhances the interaction of p97 with Ufd1 (Fig. 5C) and Derlin-1 (Fig. 5, D and E) and Cav-1 and p97 collaborate with each other to interact with COX-2 (Fig. 6, A–C). These results reveal an important role of Cav-1: besides interacting with N-glycosylated COX-2, Cav-1 may be a cofactor to enhance and stabilize the interaction among COX-2, Derlin-1, and the p97-Ufd1 complex to facilitate ERAD-mediated COX-2 ubiquitination and degradation.

In conclusion, we have demonstrated a novel role of Cav-1 in mediating COX-2 degradation through ERAD (Fig. 6D). Derlin-1 interacts with the C terminus of COX-2 and mediates its initial translocation. Cav-1 interacts with Derlin-1 and N-glycosylated COX-2 to facilitate COX-2 ubiquitination and enhances the interaction of p97 with Derlin-1 and COX-2. Moreover, Cav-1 enhances the interaction of p97 with Ufd1 and collaborates with p97 to interact with COX-2, which may help with COX-2 retrotranslocation and degradation. Cav-1 may be a cofactor to enhance the interaction among ERAD components and COX-2 to facilitate COX-2 degradation.

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