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Ubiquitin-Conjugating Enzyme UBE2D2 Is Responsible for FBXW2 (F-Box and WD Repeat Domain Containing 2)-Mediated Human GCM1 (Glial Cell Missing Homolog 1) Ubiquitination and Degradation[1]

Meng-Hsiu Chiang,[3,4] Liang-Fu Chen,[3,4] and Hungwen Chen[2,4,5]

Graduate Institute of Biochemical Sciences,[4] National Taiwan University, Taipei 106, Taiwan
Institute of Biological Chemistry,[5] Academia Sinica, Taipei 115, Taiwan

ABSTRACT

Glial cell missing homolog 1 (GCM1) is an important transcription factor regulating placental cell fusion. Recently, we have demonstrated that GCM1 is a labile protein and that the F-box protein FBXW2 (F-box and WD repeat domain containing 2) mediates GCM1 ubiquitination for proteasomal degradation. Multiple factors are involved in the ubiquitin-proteasome degradation system. Therefore, in order to better understand the mechanism regulating GCM1 stability, we further isolated and characterized the E2 ubiquitin-conjugating enzyme responsible for FBXW2-mediated ubiquitination of GCM1 in this study. We prepared and screened a variety of E2 proteins in an in vitro ubiquitination assay system for GCM1 and found that UBE2D2 is required for the SCF^{FBXW2} E3 ligase in regulation of GCM1 ubiquitination. We also demonstrated that the enzyme activity of UBE2D2 is required for GCMa ubiquitination and for association with the SCF^{FBXW2} complex. Moreover, knocking down UBE2D2 expression by RNA interference not only suppressed FBXW2-mediated GCM1 ubiquitination, but also prolonged the half-life of GCM1 in vivo. Our results suggest that UBE2D2 is a functional E2 protein which, together with FBXW2, regulates GCM1 stability in the placenta.

GCM1, pregnancy, placenta, syncytiotrophoblast, trophoblast, ubiquitination

INTRODUCTION

Proteolysis of intracellular proteins can be conducted in the specialist organelle, the lysosome, or by a nonlysosomal machinery, namely, the ubiquitin-proteasome system. For lysosomal degradation, cellular proteins are segregated into a membrane-bound compartment, which then fuses with a lysosome, or an organelle can be engulfed by a lysosome for degradation. The ubiquitin-proteasome system comprises a distinct and rapid means of specific protein degradation, which may allow the cell to fine tune the level of critical cellular proteins in order to meet its requirements. Protein ubiquitination involves a three-step reaction to achieve a covalent conjugation of the 76-amino acid polypeptide ubiquitin to a lysine in a target protein. To begin the process, a ubiquitin molecule is activated by the E1 ubiquitin-activating enzyme to form a high-energy thioester intermediate with E1. The activated ubiquitin molecule is then transferred to an E2 ubiquitin-conjugating enzyme to form a high-energy thioester intermediate with E2. Finally, association of this charged E2 with an E3 ubiquitin-protein ligase facilitates the conjugation of the ubiquitin molecule to the target protein, which is specifically recognized by one of many different E3 enzymes [1, 2]. When the target protein is polyubiquitinated, it is recognized by the 26S proteasome and subject to proteolysis. It is generally believed that a single E1 gene, *UBA1*, (also known as *UBE1* in humans), works with some tens of E2 genes and hundreds of E3 genes, setting up a hierarchical system of protein ubiquitination. Interestingly, Jin et al. [3] have recently identified a second human E1 gene, termed *UBA6*, with distinct preferences for E2 charging, suggesting that humans have two ubiquitin activation pathways.

GCM1 is a member of the labile transcription factor family GCM, originally found in *Drosophila,* and is essential for placental development [4, 5]. Indeed, GCM1 (also known as GCMa) regulates the expression of a fusogenic protein called syncytin, which is very likely to mediate placental cell-cell fusion in the formation of the syncytiotrophoblast layer on the surface of human placental villi [6, 7]. Another study by Chang et al. [8] has demonstrated that GCM1 regulates the expression of placental growth factor, a key factor in placental development. Our recent studies investigating the regulation of GCM1 activity in terms of protein turnover have shown that the protein level of GCM1 is regulated by the ubiquitin-proteasome system [9]. Moreover, we have identified the E3 ligase responsible for GCM1 degradation: it is the F-box protein, FBXW2, of the SCF^{FBXW2} E3 ligase, which recognizes and promotes GCM1 ubiquitination [9]. Protein ubiquitination involves a cascade of ubiquitin transfer reactions carried out by E1, E2, and E3 enzymes. GCM1 is the first known substrate for SCF^{FBXW2}, and the identity of the collaborator E2(s) of SCF^{FBXW2} has remained elusive. In this study we isolated and characterized an E2 involved in FBXW2-mediated GCM1 ubiquitination in order to further understand the molecular mechanism underlying the regulation of the cellular level of GCM1 protein. We identified the ubiquitin-conjugating enzyme E2D2 (UBE2D2, also known as UbcH5B or UBC4) as the E2 collaborating with the SCF^{FBXW2} E3 complex in GCM1 ubiquitination. We demonstrated that the enzyme activity of UBE2D2 is required for GCMa ubiquitination and for association with the SCF^{FBXW2} complex. Moreover, knocking down the expression of UBE2D2 stabilized the GCM1 protein level in vivo. Our results provide a better understanding of the mechanistic steps involved in the protein ubiquitination and degradation that regulate GCM1 activity in the placenta.

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[2]Correspondence: Hungwen Chen, Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 115, Taiwan. FAX: 886 2 27889759; e-mail: hwchen@gate.sinica.edu.tw
[3]These authors contributed equally to this work.

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MATERIALS AND METHODS

Plasmid Constructs and Preparation of Recombinant E2 Proteins

The cDNA fragments of human UBE2s A, B, D1, D2, E1, E3, C, M, N, K, and T were cloned into pGEX6P1 (GE Healthcare Bio-Sciences, Piscataway, NJ) for recombinant protein preparation in *Escherichia coli*. Recombinant UBE2s D1, D3, H, L3, L6, and CDC34 proteins were purchased from Boston Biochem (Cambridge, MA) or Sigma (St. Louis, MO). The pHA-GCM1-Flag expression plasmid encodes full-length GCM1 plus an N-terminal triple HA tag and a C-terminal FLAG tag, whereas the pGCM1-Flag expression plasmid encodes full-length GCM1 with a C-terminal triple FLAG tag. The pCUL1-Flag, pHA-SKP1, pFBXW2-Myc, pHA-Ub, and pHis-Ub expression plasmids have been described previously [9]. The pT7-UBE2D2 expression plasmid encodes full-length UBE2D2 with an N-terminal T7 tag. The pT7-UBE2D2C85A expression plasmid was similar to pT7-UBE2D2, except that the active site Cys85 residue was changed into alanine by two-step PCR. The aforementioned mammalian expression constructs were all under control of the cytomegalovirus promoter.

Cell Culture, Transfection, and Baculovirus-Insect Cell Expression

The 293T cells were obtained from American Type Culture Collection (Manassas, VA) and maintained at 37°C in minimal essential medium alpha medium, 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin. Establishment and culture of stable BeWo cells expressing HA-tagged GCM1 have been described previously [9, 10]. For transient expression, 293T cells were transfected with expression plasmid(s) using the calcium phosphate coprecipitation method. Sf9 cells were maintained at 28°C in Sf-900 II SFM (Invitrogen, Carlsbad, CA). For expression of HA-CUL1, SKP1-HA, HA-RBX1, and FBXW2-Flag, Sf9 cells were coinfected with the corresponding recombinant baculovirus strains obtained from Orbigen (San Diego, CA). Two days after infection, cells were harvested for extract preparation in a lysis buffer (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2 mM 2-mercaptoethanol, 0.1% Nonidet P-40, 10% glycerol, 2 mM PMSF, and a protease inhibitor cocktail [Sigma]).

In Vitro Ubiquitination Assay

HA-GCM1-FLAG substrate proteins were purified from 293T cells transfected with pHA-GCM1-FLAG by the FLAG M2 monoclonal antibody (mAb) affinity column (Sigma). For an in vitro ubiquitination reaction with HeLa S-100 fraction (Boston Biochem), immunopurified HA-GCM1-FLAG was incubated with 15 μg HeLa S-100 fraction and 5 μg $(His)_6$-Ub in a reaction buffer (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM KCl, 1 mM Na_3VO_4, 5 mM NaF, 1 mM dithiothreitol, 1 mM ATP, 20 mM phosphocreatine, 3 μg creatine kinase, 0.5 μg ubiquitin aldehyde, 10 μM MG132, and a protease inhibitor cocktail [Sigma]) at 30°C for 2 h. The reaction mixture was then analyzed by immunoblotting using a mouse anti-HA mAb (Sigma). For E2 screening, in vitro ubiquitination reactions were performed using 50 ng E1 (Boston Biochem), 500 ng E2, and 45 μg SCF^{FBXW2}-containing extracts under the aforementioned conditions.

Immunoprecipitation and Immunoblotting

To study the association between UBE2D2 and the SCF^{FBXW2} complex, 293T cells were transfected with 1 μg pFBXW2-Myc, 0.5 μg pCUL1-Flag, pHA-SKP1, and pHA-RBX1 plus different combinations of 0.5 μg pHis-Ub, 2 μg pT7-UBE2D2, and pT7-UBE2D2C85A. Two days after transfection, cells were harvested in a lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.5 mM NaF, and a protease inhibitor cocktail [Sigma]) for immunoprecipitation with a mouse anti-Myc mAb (Santa Cruz Biotechnology, Santa Cruz, CA). The immune complexes were further analyzed by immunoblotting with anti-Myc mAb or a mouse anti-T7 mAb (Novagen, Madison, WI). Band intensities were determined by densitometric analysis using an Eastman Kodak DC290 zoom digital camera and Kodak 1D image analysis software. The Student *t*-test was used to determine statistical significance for differences between means. A P value of less than 0.05 was considered significant.

RNA Interference and In Vivo Ubiquitination Assay

Construction of the pshRNA 910 and 1112 plasmids was described previously [9]. For generation of small interfering RNA (siRNA) for *UBE2D2*, the gene-specific oligonucleotides 12 (5′-AAGTACTCTTGTCCATCTGTT-3′) and 34 (5′-TCCAGATGATCCTTTAGTGCC-3′) were synthesized and cloned into the pSuppressorRetro plasmid (Imgenex, San Diego, CA) to generate pshRNA 12 and 34 plasmids. The efficacy of both constructs was tested by transfecting the constructs into 293T cells for 48 h, followed by analysis of *UBE2D2* mRNA by RT-PCR (RT-PCR). The primer sequences for *UBE2D2* were 5′-AGAATCCACAAGGAATTGAATGAT-3′ (forward) and 5′-TGGACAAGAGTACTTTTGAAATAG-3′ (reverse). As a complementary approach for evaluation of the efficacy of pshRNA constructs, 293T cells were transfected with pT7-UBE2D2 together with pshRNA 12 or 34 for 48 h, followed by immunoblotting using anti-T7 mAb. For generation of retroviruses harboring different pshRNA constructs, 293T cells were transfected with the indicated pshRNA plasmid and the packaging plasmid pCA10 according to the instructions of the manufacturer. Target cells were then transduced with packaged retrovirus stock for 72 h before immunoblotting analysis.

For in vivo ubiquitination assay of GCM1, 293T cells were transfected with pGCM1-Flag, pHA-Ub, and the indicated pshRNA plasmid and treated with the proteasome blocker MG132. Analysis of ubiquitinated GCM1 by immunoprecipitation using anti-FLAG mAb and by immunoblotting using anti-HA mAb, as well as pulse-chase experiments to analyze GCM1 stability were performed as described previously [9].

RESULTS

In Vitro Ubiquitination of GCM1

To identify the E2 for FBXW2-mediated ubiquitination of GCM1, we first established an in vitro ubiquitination assay system for GCM1. To this end, immunopurified GCM1 was prepared from 293T cells transfected with pHA-GCM1-Flag and tested in an in vitro ubiquitination assay using HeLa S-100 extracts, which have been known to contain the enzymes for ubiquitination. As shown in Figure 1A, a significant level of polyubiquitinated GCM1 was detected in the presence of HeLa S-100 extracts, suggesting that the immunopurified GCM1 proteins were an appropriate substrate for in vitro ubiquitination assay. We next prepared the recombinant SCF^{FBXW2} ubiquitin E3 ligase complex (comprising SKP1A, RBX1, cullin1 [CUL1], and FBXW2) by a baculovirus-insect cell expression system in order to reconstitute in vitro GCM1 ubiquitination. Successful expression of human CUL1, SKP1, RBX1, and FBXW2 proteins was detected in insect cells coinfected with the recombinant baculoviral strains (Fig. 1B). Attempts to purify the whole SCF^{FBXW2} complex from the infected insect cells by affinity purification of its FBXW2 component were without success. We speculated that the complex may be labile because the level of the intact SCF^{FBXW2} protein complex was extremely low after purification (data not shown). For this reason, we used SCF^{FBXW2}-containing cell extracts for the E3 ligase in the in vitro GCM1 ubiquitination reactions in the rest part (Figs. 1C, 2A, and 2B) of this study.

Identification of the E2 for GCM1 Ubiquitination

According to the HUGO Gene Nomenclature Committee (http://www.genenames.org), 50 E2 genes, including 12 pseudogenes, have been documented in the human genome [11]. We screened a panel of 16 E2 proteins by in vitro ubiquitination assay using recombinant E1 (UBA1), E2, and $(His)_6$-Ub proteins, supplemented with the SCF^{FBXW2}-containing extracts. As shown in Figure 1C, a significant level of polyubiquitinated GCM1 proteins was only detected in the presence of recombinant UBE2D2. The functional specificity of UBE2D2 to SCF^{FBXW2}-mediated GCM1 ubiquitination was further confirmed by the fact that omission of UBA1 or incubation with mock-infection extracts failed to significantly promote GCM1 ubiquitination (Fig. 2A). Moreover, a mutant UBE2D2 (C85A) harboring a cysteine-to-alanine mutation in the active site Cys85 of UBE2D2 also failed to promote GCM1

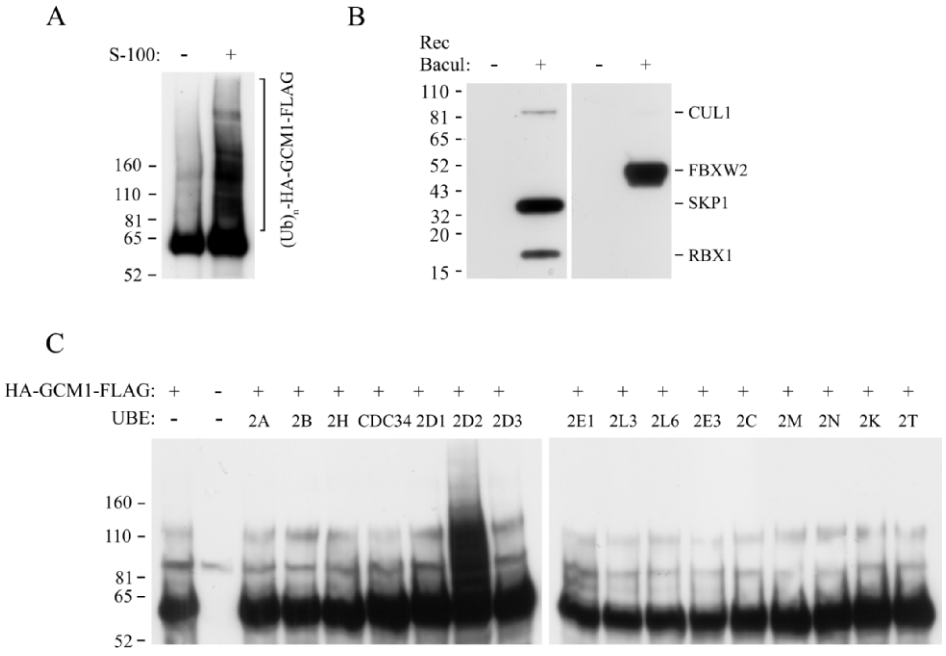

FIG. 1 In vitro ubiquitination of GCM1. **A**) HeLa S-100 (S-100) fraction promotes polyubiquitination of GCM1 in vitro. Immunopurified HA-GCM1-FLAG proteins were incubated with or without HeLa S-100 fraction for in vitro ubiquitination reactions as described in *Materials and Methods*. $(Ub)_n$-HA-GCM1-FLAG, polyubiquitinated HA-GCM1-FLAG. **B**) Expression of the SCF^{FBXW2} complex in Sf9 insect cells. Sf9 cells were coinfected with recombinant baculovirus (Rec Bacul) strains for HA-CUL1 (CUL1), SKP1-HA (SKP1), HA-RBX1 (RBX1), and FBXW2-FLAG (RBX1) for 48 h, when cell extracts were prepared and immunoblotted using anti-HA mAb (left) and anti-FLAG mAb (right), respectively. **C**) Functional screening of the E2 enzyme essential for GCM1 ubiquitination. A panel of 16 E2 enzymes was screened for in vitro ubiquitination of the immunopurified HA-GCM1-FLAG proteins as described in *Materials and Methods*. Numbers to the left of the blots are in kDa.

ubiquitination compared with the wild type (Fig. 2B). We further tested whether UBE2D2 can associate with the SCF^{FBXW2} complex in vivo; 239T cells were cotransfected with pCUL1-Flag, pHA-SKP1, pHA-RBX1, and pFBXW2-Myc plus different combinations of pHis-Ub, pT7-UBE2D2, and pT7-UBE2D2C85A, followed by immunoprecipitation and immunoblotting. When the components of SCF^{FBXW2} were expressed, UBE2D2 was coimmunoprecipitated with FBXW2. Interestingly, this association was further enhanced when ubiquitin was overexpressed in cells. On the other hand, the UBE2D2C85A mutant failed to associate with FBXW2, regardless of whether ubiquitin was overexpressed or not (Fig. 2C). Taken together, these results suggest that UBE2D2 is a bona fide E2, which works together with UBA1 and SCF^{FBXW2} to mediate GCM1 ubiquitination.

Regulation of GCM1 Degradation by UBE2D2

We next investigated the role of UBE2D2 in GCMa degradation by RNA interference. Two functional short hairpin RNA expression plasmids, pshRNAs 12 and 34, capable of suppressing *UBE2D2* gene expression and reducing UBE2D2 protein level were generated (Fig. 3A). We performed in vivo ubiquitination analysis of GCM1 by transfecting 293T cells

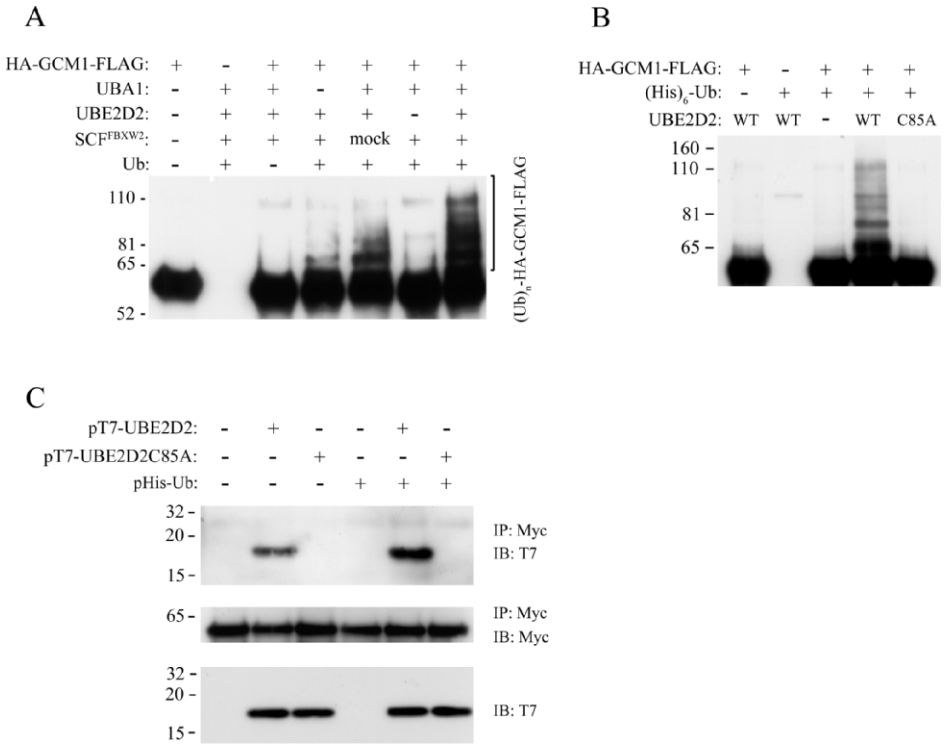

FIG. 2. UBE2D2 is the functional E2 for SCF^{FBXW2}-mediated GCM1 ubiquitination. **A**, **B**) Characterization of UBE2D2 in GCM1 ubiquitination. Omission of E1 (UBA1) or the SCF^{FBXW2}-containing extracts or replacement of the wild-type (WT) UBE2D2 with the active-site mutant UBE2D2C85A (C85A) in ubiquitination reactions abolished GCM1 ubiquitination. SCF^{FBXW2}, extracts prepared from Sf9 cells coinfected with the recombinant baculovirus strains described in the legend of Figure 1B; Ub, ubiquitin; mock, extracts of noninfected Sf9 cells. **C**) Association between UBE2D2 and the SCF^{FBXW2} complex. The 293T cells were transfected with 1 μg pFBXW2-Myc, 0.5 μg pCUL1-Flag, pHA-SKP1, and pHA-RBX1 plus different combinations of 0.5 μg pHis-Ub, 2 μg each of pT7-UBE2D2 and pT7-UBE2D2C85A for 48 h, followed by sequential immunoprecipitation (IP) with anti-Myc mAb and immunoblotting (IB) with anti-T7 or anti-Myc mAb. The protein levels of UBE2D2 and UBE2D2C85A in whole-cell lysate were detected by immunoblotting with anti-T7 mAb. Numbers to the left of the blots are in kDa.

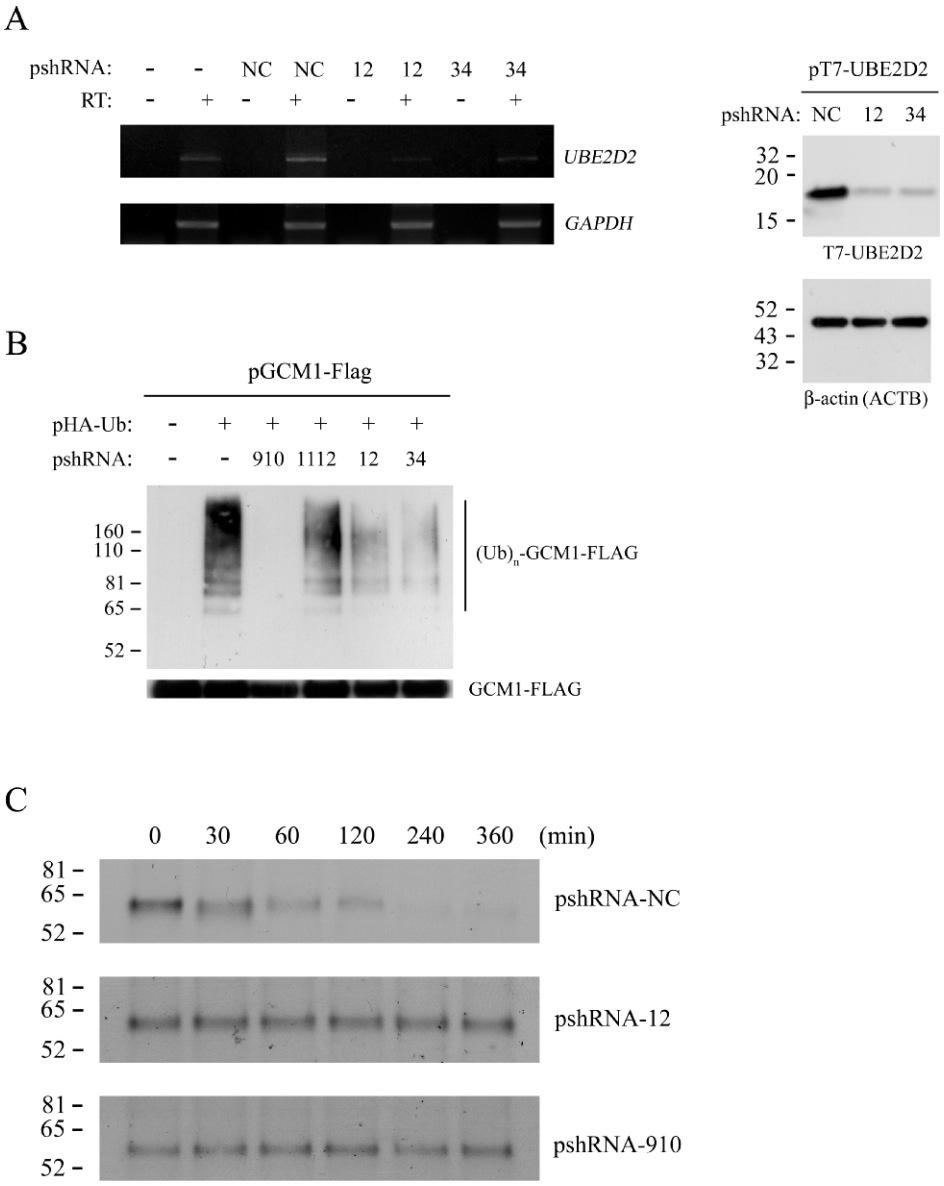

FIG. 3. Silencing of *UBE2D2* gene expression increases GCM1 stability. **A**) *UBE2D2* gene silencing by RNAi. The 293T cells were transfected with 2 μg of the indicated pshRNA plasmid for 48 h. The level of *UBE2D2* mRNA was analyzed by RT-PCR (left). In a separate experiment (right), the 293T cells were transfected with 2 μg pT7-UBE2D2 plus 4 μg of the indicated pshRNA plasmid for 48 h. The level of T7-UBE2D2 was detected by immunoblotting with anti-T7 mAb. The pshRNA-12 and pshRNA-34 constructs are functional for suppressing *UBE2D2* expression, whereas pshRNA-NC with a sequence not matching any human genome sequence is a negative control. **B**) Knockdown of *UBE2D2* decreases the level of polyubiquitinated GCM1. The 293T cells were transfected with 3 μg pGCM1-Flag alone or together with 3 μg pHA-Ub plus 4 μg of the indicated pshRNA. At 24 h after transfection, cells were treated with 40 μM MG132 for another 10 h before immunoprecipitation and immunoblotting as described in *Materials and Methods*. The pshRNA-910 and pshRNA-1112 constructs, which carry different sequences from *FBXW2*, are functional and nonfunctional for suppressing *FBXW2* expression, respectively. **C**) Knockdown of *UBE2D2* prolongs the half-life of GCM1. The 293T cells were transfected with 3 μg pHA-GCM1 plus 3 μg pshRNA NC, 12, or 910. At 36 h after transfection, cells were analyzed by pulse-chase analyses as described in *Materials and Methods*. Numbers to the left of the blots are in kDa.

with different combinations of pGCM1-Flag, pHA-Ub, and pshRNAs 910, 1112, 12, and 34. Our previous studies have shown that pshRNA-910, but not pshRNA-1112, effectively knocked down *FBXW2* expression; therefore, these two constructs served as positive and negative controls, respectively [9]. As shown in Figure 3B, knocking down *UBE2D2* gene expression by pshRNA 12 or 34 significantly decreased the level of polyubiquitinated GCM1. Pulse-chase experiments were performed to compare the half-life of GCM1 in the presence or absence of pshRNA NC, 12, or 910. The construct pshRNA-NC, which carries a sequence not matching any human genome sequence, was used as a control. The half-life of GCM1 in cells transfected with pshRNA-NC was about 60 min, which was consistent with previous studies [9]. In contrast, the half-life of GCM1 in cells transfected with either pshRNA 12 or 910 was significantly prolonged (Fig. 3C). These results suggest that UBE2D2 is an essential E2 component for SCF^{FBXW2}-mediated GCM1 ubiquitination and degradation.

We further studied the roles of UBE2D2 and FBXW2 in GCM1 stability in placental cells. A previously established BeWo stable line expressing HA-tagged GCM1 was transduced with retroviruses harboring pshRNAs NC, 12, and 910, respectively. The steady-state protein levels of HA-GCM1 in the three groups of cells were compared by immunoblotting analysis. As shown in Figure 4, compared with cells expressing the negative control shRNA, the level of HA-GCM1 was significantly elevated in cells expressing shRNA against UBE2D2 or FBXW2. These results provide further supporting evidence that UBE2D2 and FBXW2 are the functional E2 and E3 components in regulation of GCM1 stability in placental cells.

DISCUSSION

GCM1 is a zinc-containing transcription factor expressed primarily in placenta [12–15]. We have recently identified the F-box protein, FBXW2, as the important substrate recognition component of the SCF^{FBXW2} E3 ligase for GCM1 ubiquitination. We have also shown that GCM1 is a cellular phosphoprotein and that FBXW2 interacts with the phosphorylated form of GCM1 [9]. To better understand the degradation mechanism of GCM1, in the present study we first set up an in vitro assay system for GCM1 ubiquitination and then screened

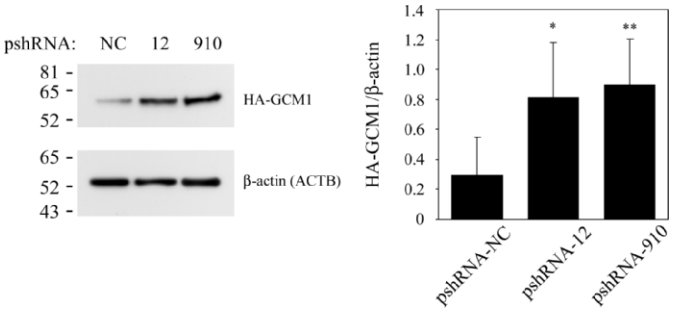

FIG. 4. Suppression of UBE2D2 and FBXW2 expression stabilizes GCM1 proteins in placental cells. Stable BeWo cells expressing HA-GCM1 were transduced with the indicated retroviruses harboring pshRNA NC, 12, or 910 for 72 h. Left: The protein level of HA-GCM1 was analyzed by immunoblotting with anti-HA mAb. Numbers to the left of the blot are in kDa. A representative of three independent experiments is presented. Right: In addition, the HA-GCM1 and β-actin protein levels were quantified by densitometry as described in *Materials and Methods*. The HA-GCM1:β-actin band intensity ratio is presented. Values are mean and SD obtained from three independent transduction experiments. Asterisks denote significant differences ($*P < 0.05$; $**P < 0.01$) between the cells transduced with retroviruses harboring pshRNA-NC and the cells transduced with retroviruses harboring pshRNA 12 or 910.

for the E2(s) involved in SCF^{FBXW2}-mediated GCM1 ubiquitination. We demonstrated that immunopurified GCM1 is a pertinent substrate for this in vitro ubiquitination assay using the HeLa S-100 fraction, which is enriched with E1, E2s, E3s, and proteasomes. We then further refined the assay system by replacing the HeLa S-100 fraction with recombinant E1 and the SCF^{FBXW2}-containing insect cell extracts in order to identify the important E2 enzyme in the three-step ubiquitination reaction of GCM1. A panel of 16 candidate E2 enzymes was screened.

Accordingly, we identified UBE2D2 as an essential E2 for GCM1 ubiquitination. Two lines of evidence support this conclusion. First, omission of UBE2D2 or its replacement with the active-site mutant UBE2D2C85A in the reconstituted ubiquitination reaction abolished GCM1 ubiquitination. Second, gene silencing of *UBE2D2* by RNAi suppressed GCM1 ubiquitination and prolonged the half-life of GCM1 in placental cells. Studies in transgenic mice have shown that GCM1 is required for labyrinthine layer formation and fusion of trophoblasts to syncytiotrophoblasts [4, 5]. Regulation of GCM1 activity is important to maintain human placental functions, because GCM1 target genes such as syncytin and placental growth factor mediate trophoblast fusion and proliferation, respectively. Indeed, the activity of human GCM1 can be positively or negatively regulated by different posttranslational modifications. Acetylation of Lys367, Lys406, and Lys409 stabilizes GCM1, and sumoylation of Lys156 inhibits GCM1 DNA-binding activity, leading to increased and decreased cellular GCM1 activity, respectively [10, 16]. In addition, GCM1 is susceptible to ubiquitination and degradation, which accounts for the short half-life of GCM1 in stability analysis (Fig. 3C). It is feasible to speculate that GCM1 ubiquitination and degradation are important for maintaining appropriate GCM1 activity in placenta, because abnormal GCM1 activity may result in improper trophoblast fusion and proliferation and, hence, impose adverse effects on placental functions. Identification of UBE2D2 as the collaborator E2 of SCF^{FBXW2} in the present study helps to elucidate the E1-E2-E3 reaction cascade underpinning GCM1 ubiquitination.

The E2 ubiquitin-conjugating enzymes are structurally related and share a conserved ~150-residue core domain harboring the catalytic cysteine residue required for ubiquitin-E2 thioester formation [17–19]. The E2 core domain consists of an N-terminal α helix, followed by a four-stranded antiparallel β sheet, a short 3_{10} helix, and three α helices. The α1 helix of E2 is involved in binding to E1 and E3. Recent structural studies of the E1s for ubiquitinlike polypeptides, NEDD8 and SUMO, have shown that a ubiquitin-like domain in the C-terminus of E1 is responsible for interaction with E2 [20, 21]. Binding of E2 to the RING-type E3 depends on a RING finger domain in the E3, which comprises eight cysteines and histidines that coordinate two zinc ions [22, 23]. Although different E2s share structural similarities in their E2 core domains, as do different E3s in their RING domains, structural analyses have revealed that different amino acid residues and, hence, different kinds of forces are involved in the interaction between the RING domain and the E2 enzyme in different E2-E3 pairings [19]. This may account for specific pairings between E2s and E3s in ubiquitination reactions. SCF^{FBXW2} belongs to the Cullin-RING ligase family and is composed of the RING protein RBX1, cullin1 (CUL1), SKP1, and the F-box protein FBXW2. CUL1 functions as a scaffold protein, with its N- and C-termini associating with the adaptor proteins SKP1 and RBX1, respectively. RBX1 recruits the ubiquitin-loaded UBE2D2 via its RING domain. The N-terminus of SKP1 binds CUL1 and the C-terminus binds FBXW2 via the F-box motif of FBXW2. FBXW2 recruits the phosphorylated form of GCM1 via its WD40 domain to facilitate the transfer of ubiquitin from UBE2D2 to GCM1.

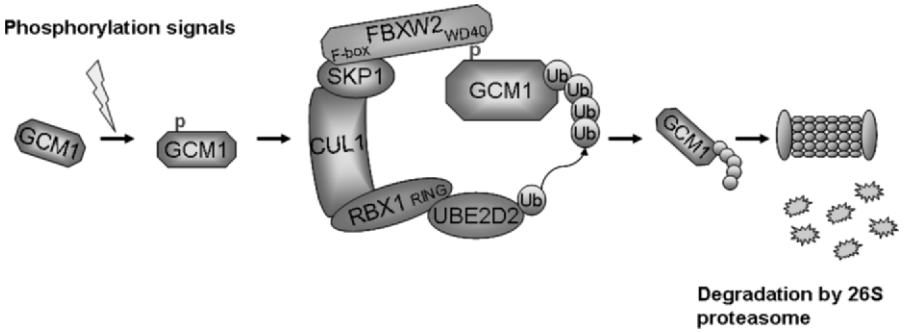

FIG. 5. Model of GCM1 ubiquitination by UBE2D2 and the multisubunit SCF^{FBXW2} complex. Environmental cues are speculated to modify the phosphorylation status of GCM1. Phosphorylated GCM1 proteins are recognized by the SCF^{FBXW2} complex, which is composed of the RING protein RBX1, cullin1 (CUL1), SKP1, and the F-box protein FBXW2. RBX1 recruits the ubiquitin-loaded UBE2D2 via its RING domain. FBXW2 recruits phosphorylated GCM1 via its WD40 domain to facilitate the transfer of ubiquitin from UBE2D2 to GCM1. After several cycles of ubiquitination, GCM1 becomes polyubiquitinated and is recognized and degraded by the 26S proteasome.

After several cycles of ubiquitination, GCM1 becomes polyubiquitinated and is recognized and degraded by the 26S proteasome (Fig. 5). Interestingly, in the present study we also found that the enzyme activity of UBE2D2 is not only required for GCM1 ubiquitination (Fig. 2B), but also for association with the SCF^{FBXW2} complex (Fig. 2C). This may explain why UBE2D2C83A failed to impose any dominant negative effect on GCM1 stability, that is, prolonging the half-life of GCM1, when coexpressed with GCM1 (data not shown).

Involvement of different E2s in the ubiquitination of a substrate protein has been reported. Gonen et al. [24] have shown that any one of UBE2D2, UBE2D3, or CDC34 is able to support IκBα ubiquitination. Although UBE2D1 shares a high sequence homology with UBE2Ds 2 and 3, it is not an efficient ubiquitinator of IκBα. Yeast Ubc4 and Ubc1 are two key E2s for the anaphase-promoting complex (APC) in regulation of cell cycle progression. Interestingly, the two E2s collaborate with each other to facilitate ubiquitination of APC targets such that Ubc4 is involved in the rapid monoubiquitination of multiple lysines on APC targets, whereas Ubc1 promotes polyubiquitination on preattached ubiquitins [25]. In the present study, we demonstrated that CDC34, UBE2D1, and UBE2D3 are unable to support GCM1 ubiquitination in vitro. The reasons for the lack of functional redundancy between UBE2Ds 1, 2, and 3 for GCM1 ubiquitination are currently unknown. In addition, because UBE2D2 shares sequence similarity with yeast UBC4, it is possible that collaborator E2s of UBE2D2 may exist to promote GCM1 polyubiquitination, as with yeast Ubc4 and its APC targets. It is known that an F-box protein may recognize different substrate proteins. For example, FBXW1 (also known as βTrcp) recognizes β-catenin and NFKBIA (also known as IκBα), and SKP2 (also known as FBXL1) recognizes p21, p27, p57, and p130 [26]. It is feasible that FBXW2 may have additional substrates and, hence, may regulate other biological functions by controlling the stabilities of the substrate proteins. To our knowledge, GCM1 is the only substrate of FBXW2 known so far. Therefore, this discovery of a functional interaction between UBE2D2 and FBXW2 in GCM1 ubiquitination increases our understanding of the molecular mechanism underlying the stability regulation of new FBXW2 substrate proteins.

Although the enzymatic function of E2 is largely known, E2 may play additional biological roles for different physiological functions. For example, UBE2C (also known as UBCH10) is highly expressed in a variety of human primary tumors, and it is able to promote cell proliferation and malignant transformation [27]. Because UBE2C is functionally associated with the APC E3 ligase in regulating cyclin ubiquitination and degradation, abnormal UBE2C activity may perturb cell cycle progression and promote cell growth. Plaques containing amyloid-β peptide (Aβ) are a pathological feature in Alzheimer disease patients. Interestingly, the expression of an E2 enzyme, UBE2K (also known as E2-25K/HIP2), is upregulated in primary cortical neurons exposed to $Aβ_{1–42}$ [28]. It is believed that functional interaction between UBE2K and the frameshift ubiquitin mutant, UBB+1, which is commonly found in Alzheimer disease patients, inhibits proteasome activity, resulting in neurotoxicity. Genetic ablation of mouse *UBE2B* (also known as *HHR6B*) caused male infertility by impairment of spermatogenesis [29]. The yeast homolog of UBE2B, RAD6, mediates ubiquitination of histones 2A and 2B and may modulate chromatin structure essential for DNA repair and replication [30]. Therefore, the defect in spermatogenesis in the *UBE2B* knockout mice may be due to abnormal histone ubiquitination, which is required for proper postmeiotic chromatin remodeling. Currently, it is unknown whether UBE2D2 expression is regulated during placental development and whether abnormal UBE2D2 expression is associated with any pregnancy disorder, given that UBE2D2 is a key E2 enzyme in regulation of GCM1 stability. Nevertheless, this study furthers our understanding of the molecular mechanism underlying the regulation of GCM1 activity by the ubiquitin-proteasome system and may also provide a basis for future investigation of additional functional roles of UBE2D2 and FBXW2 in placenta.

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