RACK1 (receptor for activated C-kinase 1) interacts with FBW2 (F-box and WD-repeat domain-containing 2) to up-regulate GCM1 (glial cell missing 1) stability and placental cell migration and invasion

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GCM1 (glial cell missing 1) is a short-lived transcription factor essential for placental development. The F-box protein, FBW2 (F-box and WD-repeat domain-containing 2), which contains five WD (tryptophan–aspartate) repeat domains, recognizes GCM1 and mediates its ubiquitination via the SCF\(^{FBW2}\) E3 ligase complex. Although the interaction between GCM1 and FBW2 is facilitated by GCM1 phosphorylation, it is possible that this interaction might be regulated by additional cellular factors. In the present study, we perform tandem-affinity purification coupled with MS analysis identifying RACK1 (receptor for activated C-kinase 1) as an FBW2-interacting protein. RACK1 is a multifaceted scaffold protein containing seven WD repeats. We demonstrate that the WD repeats in both RACK1 and FBW2 are required for the interaction of RACK1 and FBW2. Furthermore, RACK1 competes with GCM1 for FBW2 and thereby prevents GCM1 ubiquitination, which is also supported by the observation that GCM1 is destabilized in RACK1-knockdown BeWo placental cells. Importantly, RACK1 knockdown leads to decreased expression of the GCM1 target gene HTRA4 (high-temperature requirement protein A4), which encodes a serine protease crucial for cell migration and invasion. As a result, migration and invasion activities are down-regulated in RACK1-knockdown BeWo cells. The present study reveals a novel function for RACK1 to regulate GCM1 activity and placental cell migration and invasion.

Key words: cell migration, cell invasion, F-box and WD-repeat domain-containing 2 (FBW2), glial cell missing 1 (GCM1), placenta, receptor for activated C-kinase 1 (RACK1).

INTRODUCTION

GCM1 (glial cell missing 1) is a transcription factor essential for placental development. Gcm1-knockout mice are embryonic lethal due to defective placental development at the labyrinthine layer, where gas/nutrient exchange takes place [1,2]. The known target genes of human GCM1 include PGF (placental growth factor), syncytin-1 and -2, and HtrA4 (high-temperature requirement protein A4), which participate in placental vasculogenesis and trophoblastic fusion and invasion [3–6]. Our previous study identified FBW2 (F-box and WD-repeat domain-containing 2) as a GCM1-binding protein that mediates GCM1 ubiquitination [7]. The human FBW2 polypeptide contains an N-terminal F-box motif and a C-terminal domain of five WD (tryptophan–aspartate) repeat domains. A WD repeat is a motif typically consisting of 44–60 amino acids with a signature tryptophan and aspartic acid dipeptide at its C-terminus. The WD repeats in FBW2 recognize GCM1 in a way that is facilitated by glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation of Ser\(^{322}\) in GCM1 [8]. Consequently, GCM1 is ubiquitinated by the SCF\(^{FBW2}\) E3 ligase complex and degraded by the 26S proteasome.

RACK1 (receptor for activated C-kinase 1) is an anchoring protein for the active conformation of protein kinase C βII and consists of seven WD repeats [9]. In addition, RACK1 is a scaffold protein that modulates a wide range of cellular processes through interacting with many factors, including PDE4D5 (phosphodiesterase 4D5), G-protein G\(_{\beta}\) subunit, Src, dynamin-1 and ion channels [10–13]. We wished to search cellular factors that interact with FBW2 to regulate GCM1 activity in terms of GCM1 recognition and ubiquitination. To this end, we performed TAP (tandem-affinity purification) coupled with MS and identified RACK1 as an FBW2-interacting protein that stabilizes GCM1. Specifically, we have demonstrated that RACK1 binds to the WD-repeat domain of FBW2 to interfere with the recognition and ubiquitination of GCM1 by FBW2. In addition, RACK1 knockdown decreases the stability of GCM1 and the expression of the GCM1 target genes PGF and HTRA4 in BeWo placental cells. Because HtrA4 is a serine protease crucial for placental cell migration and invasion, we show further that RACK1-knockdown BeWo cells exert decreased migration and invasion activities. Our results reveal a novel mechanism underlying the regulation of GCM1 stability and placental cell migration and invasion by RACK1.

EXPERIMENTAL

Plasmid constructs

The pFBW2-Myc and pHA-GCM1 expression plasmids and the pG(t)E1Bluc reporter plasmid have been described previously [7,14]. The pRACK1-FLAG and pHA-RACK1 expression plasmids were constructed in a pEF expression vector (Invitrogen) to encode RACK1 with a C-terminal triple HA (haemagglutinin) tag respectively.

Abbreviations used: FBW, F-box and WD-repeat domain-containing; GCM1, glial cell missing 1; HA, haemagglutinin; HEK, human embryonic kidney; HtrA4, high-temperature requirement protein A4; MBP, maltose-binding protein; PGF, placental growth factor; RACK1, receptor for activated C-kinase 1; TAP, tandem-affinity purification.

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Cell culture, transfection, reporter gene assay and RNAi

Cell culture of HEK (human embryonic kidney)-293T, JAR, BeWo and BeWo31 cells has been described previously [14]. For transient expression, cells were transfected with the indicated reporter and expression plasmids using Lipofectamine™ 2000 reagent (Invitrogen). Luciferase assays were performed as described previously [15]. For RNAi, pLKO.1-Puro shRNA expression plasmids harbouring a scramble sequence (5′-CTTAAAGGTTAAGTCCCTCG-3′, Addgene plasmid 1864) and two separate sequences for RACK1, RK92 (5′-TACCCCTGGGTTGTGGCAAATA-3′) and RK72 (5′-GATGTTGGTTATCTCCTCAGAT-3′), were acquired from the National RNAi Core Facility of Taiwan.

Tandem-affinity purification and mass spectrometry

To isolate cellular proteins associated with GCM1, we established JAR cells stably expressing FBW2 with a C-terminal TAP tag containing two tandem streptavidin-binding peptides plus a FLAG peptide [16]. Cells were harvested for purification of FBW2 protein complex, followed by trypsin digestion and MS analysis (LTQ-Orbitrap, Thermo Scientific).

Interaction between FBW2 and RACK1

To study the interaction of FBW2 and RACK1 in vivo, HEK-293T cells were transfected with pHA-RACK1 and pFBW2-Myc for 48 h. Cells were then harvested in lysis buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10 % glycerol, 0.5 % Nonidet P40, 1 mM DTT, 5 mM NaF, 1mM Na3VO4, and a protease inhibitor cocktail (Sigma–Aldrich) for consecutive immunoprecipitation and immunoblotting with anti-HA and anti-Myc antibodies (Sigma–Aldrich). The interaction of endogenous RACK1 and FBW2 was studied by immunoprecipitation of HEK-293T cells transfected with pFBW2-FLAG using anti-RACK1 antibody (Santa Cruz Biotechnology) and immunoblotting using anti-FLAG antibody (Sigma–Aldrich). In a separate experiment, BeWo and JAR cells and term placental extracts were subjected to consecutive immunoprecipitation and immunoblotting with anti-FBW2 and anti-RACK1 antibodies. The anti-FBW2 antibody has been described previously [7]. Placental samples were obtained after defective Caesarean section in healthy women with informed written consent according to a protocol approved by the Cathay General Hospital institutional review board. In vitro interaction of RACK1 and FBW2 was studied by incubation of GST or GST–FBW2 pre-bound glutathione-conjugated agarose beads with MBP (maltose-binding protein) or MBP–RACK1 at 4 °C for 1 h before washing three times with lysis buffer. The proteins pulled down were analysed by immunoblotting with anti-MBP and anti-GST antibodies (New England Biolabs and Santa Cruz Biotechnology respectively). The recombinant HA–GCM1–FLAG protein was purified from HEK-293T cells transfected with an expression construct encoding GCM1 with an N-terminal triple HA tag and a C-terminal FLAG tag.

Mapping the RACK1-interacting domain in FBW2

To map the FBW2 domain that interacts with RACK1, HEK-293T cells were transfected with pHA-RACK1 and full-length pFBW2-Myc or the indicated deletion mutant pFBW2-Myc plasmid. At 48 h post-transfection, cells were harvested in lysis buffer for consecutive immunoprecipitation and immunoblotting with anti-HA and anti-Myc antibodies.

In vivo ubiquitination analysis

To study the effect of RACK1 on FBW2-mediated ubiquitination of GCM1, HEK-293T cells were co-transfected with pGCM1-FLAG, pHA-Ub, and the expression plasmid for scramble, RK92 or RK72 shRNA for 36 h and then treated with 40 μM MG132 for another 10 h at 37 °C. Cells were harvested for immunoprecipitation with anti-FLAG antibody and immunoblotting with anti-HA antibody.

Quantitative real-time PCR

BeWo cells stably expressing RK92 shRNA were harvested for RNA isolation using RNeasy reagents (Qiagen) and then transcribed into cDNA using SuperScript III reagents (Invitrogen) with an oligo-(dT)20 primer. Quantification of the transcript levels of GCM1 target genes was performed in a LightCycler 480 real-time PCR instrument (Roche Applied Science) using a commercial SYBR Green reaction reagent (Qiagen) and specific primer sets. The sequences of primer sets were 5′-ACTGTCGAGATGAGGAG-3′ and 5′-TTGCTGGCCCTCAAGGC-3′ for RACK1, 5′-GAGATTGAGAATGAGAGAA-3′ and 5′-CTGGATGCCATCAACCCG-3′ for FBW2, 5′-GACGAGCGCGATTC-3′ and 5′-TCAGAGGTGGAAATGGTCACCT-3′ for PGF, 5′-GTCAGACCCACACACGCG-3′ and 5′-GGAGATCCATGTCCTGCCCC-3′ for HtrA4, and 5′-AATTCATCATGGAAGGTTGCACGC-3′ and 5′-GAGCATCATCTTGACGCGAGGC-3′ for β-actin.

Immunohistochemistry

Detection of GCM1, FBW2 and RACK1 proteins in placenta was performed by immunohistochemistry as described previously [6]. In brief, formalin-fixed paraffin-embedded tissue sections of human term placenta were deparaffinized and subjected to immunostaining by incubation with anti-GCM1, anti-FBW2 and anti-RACK1 antibodies respectively. The sections were then incubated sequentially with biotinylated secondary antibody and HRP (horseradish peroxidase)-conjugated streptavidin. Antigenic detection was performed using chromogenic substrate DAB (3,3′-diaminobenzidine tetrahydrochloride) and the sections were counterstained further by haematoxylin. The anti-FBW2 antibody was prepared from rabbits immunized with His6-tagged FBW2 recombinant protein.

Cell migration and invasion analysis

To study the effect of RACK1 knockdown on the migration and invasion activity of placentonal cells, BeWo cells stably expressing scramble or RK92 shRNA were plated into fibronectin-coated chambers and Matrigel™ invasion chambers (BD Biosciences) respectively. After 16 h, migrated or invaded cells in the lower surface of the filters were fixed with paraformaldehyde and visualized by Crystal Violet stain and counted. Five microscopic fields per sample were randomly selected for quantification in each of three independent experiments. Images were prepared for presentation using Adobe Photoshop version 7.0.

Statistical analysis

Statistical analysis of the data was performed using Student’s t test. Statistical significance was classified as *P < 0.05 or

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RACK1 is a potential FBW2-interacting protein. We characterized the interaction between FBW2 and RACK1 by co-immunoprecipitation analysis. The pFBW2-Myc and pHA-RACK1 plasmids were transfected into HEK-293T cells, followed by immunoprecipitation with anti-FBW2 antibody and immunoblotting with anti-Myc antibody. As shown in Figure 1(A), specific interaction between FBW2-Myc and HA-RACK1 was detected. This observation was also supported by a reciprocal experiment using anti-Myc antibody for immunoprecipitation and anti-HA antibody for immunoblotting (Figure 1B). We further tested whether FBW2 interacts with endogenous RACK1 by performing co-immunoprecipitation analysis in HEK-293T cells transfected with pFBW2-Myc. Specific interaction between endogenous RACK1 and FBW2–FLAG was detected when anti-RACK1 antibody (but not an irrelevant anti-GFP antibody) was used in the immunoprecipitation step (Figure 1B, left-hand panel). Using immunofluorescence microscopy, we demonstrated that RACK1 is co-localized with FBW2–GFP in the cytoplasm of HEK-293T cells (Figure 1B, right-hand panel). Interaction between endogenous FBW2 and RACK1 in placenta was confirmed further by co-immunoprecipitation analysis in BeWo and JAR placental cells and term placental extracts using anti-FBW2 and anti-RACK1 antibodies (Figure 1C). We next studied whether FBW2 interacts directly with RACK1 in vitro by incubation of GST–FBW2 with MBP or MBP–RACK1. Indeed, MBP–RACK1 was pulled down by GST–FBW2, but not GST (Figure 1D).

To map the interaction domain in FBW2 for RACK1, co-immunoprecipitation analysis was performed in HEK-293T cells transfected with pHA-RACK1 and pFBW2-Myc or its derivatives encoding different regions of the FBW2 polypeptide (Figure 2A). As shown in Figure 2(B), HA–RACK1 interacted with the full-length FBW2 and the region of amino acids 140–351 in the FBW2 polypeptide, which harbours five WD repeats. These results suggest that RACK1 interacts with the WD-repeat domain of FBW2.

RACK1 impedes the binding and ubiquitination of GCM1 by FBW2. Because the WD-repeat domain of FBW2 is required for binding its substrate(s), we examined the effect of RACK1 on the interaction between FBW2 and its currently known substrate, GCM1. To this end, HEK-293T cells were transfected with pFBW2-Myc, pHA-GCM1 and increasing amounts of pRACK1-FLAG, followed by immunoprecipitation with anti-Myc antibody and immunoblotting with anti-Myc, anti-FLAG and anti-HA antibodies respectively. As shown in Figure 3(A), interaction between HA–GCM1 and FBW2–Myc was detected in the absence of RACK1–FLAG. Interestingly, this interaction gradually decreased in the presence of increasing amounts of RACK1–FLAG (Figure 3A), suggesting that RACK1 competes with GCM1 for interaction with FBW2. This hypothesis was tested with a GST pull-down assay using recombinant GST–FBW2, HA–GCM1–FLAG and MBP–RACK1 proteins. As shown in Figure 3(B), the amount of HA–GCM1–FLAG pulled down by GST–FBW2 gradually decreased in the presence of increasing amounts of MBP–RACK1.

We then tested the effect of RACK1 on FBW2-mediated ubiquitination of GCM1. In vivo ubiquitination assays were...
Figure 2  Mapping of the interaction domain of FBW2 for RACK1

(A) Schematic representation of FBW2 domain structure. (B) The WD-repeat domain of FBW2 is recognized by RACK1. HEK-293T cells were transfected with pHA-RACK1 and pFBW2-Myc encoding full-length or truncated FBW2, followed by immunoprecipitation (IP) with anti-HA antibody and immunoblotting (IB) with anti-Myc antibody. Molecular masses are indicated in kDa.

performed in HEK-293T cells transfected with pGCM1-FLAG, pHA-Ub and an shRNA expression plasmid harbouring scramble or RACK1 sequence. As shown in Figure 3(C), ubiquitination of GCM1–FLAG was detected in cells expressing scramble shRNA (SC), which was enhanced further in cells expressing a functional (RK92), but not a non-functional (RK72), RACK1 shRNA. Correspondingly, transfection of pGCM1-FLAG with increasing amounts of pHA-RACK1 into HEK-293T cells resulted in increasing levels of GCM1–FLAG (Figure 3D). The observed positive effect of RACK1 on GCM1 protein levels was unlikely to be due to differential transcription as the levels of GCM1–FLAG transcript were not affected by RACK1 according to quantitative real-time PCR analysis (results not shown). We also examined whether RACK1 affects GCM1-mediated transcriptional activation on p(GBS)₄E1BLuc, which is a GCM1 reporter plasmid. Indeed, the stimulation of luciferase reporter gene expression by GCM1 was enhanced by increasing amounts of RACK1 (Figure 3E). Taken together, these results suggest that RACK1 up-regulates GCM1 activity through interaction with FBW2 to prevent GCM1 ubiquitination.

RACK1 stabilizes GCM1 in placental cells

We studied further the effect of RACK1 on GCM1 stability in placental cells. The placental BeWo31 cells, which stably express HA-tagged GCM1, were transduced with lentivirus harbouring the scramble or RK92 shRNA expression cassette to generate control or RACK1-knockdown cells. In comparison with the control BeWo31 cells, the protein levels of both HA–GCM1 and endogenous GCM1 decreased in RACK1-knockdown BeWo31 cells (Figure 4A, left-hand panel). Importantly, both HA–GCM1 and GCM1 transcript levels were comparable in the control and RACK1-knockdown cells (Figure 4A, right-hand panel), suggesting that RACK1 may affect the stability of HA–GCM1 and GCM1. To test this hypothesis, we compared the half-life of HA–GCM1 in control and RACK1-knockdown BeWo31 cells by cycloheximide chase experiments. As shown in Figure 4(B), the half-life of HA–GCM1 was shortened from 3.5 h for the control cells to 2.9 h for the RACK1-knockdown cells. Therefore RACK1 may enhance GCM1 stability in placental cells.

Expression of GCM1, FBW2 and RACK1 in placenta

We investigated GCM1, FBW2 and RACK1 expression in placenta by immunohistochemistry. Tissue sections of human term placenta were immunostained using anti-GCM1, anti-FBW2 and anti-RACK1 antibodies respectively. In line with our recent study [6], GCM1 protein was primarily detected in the nuclei of villous and extravillous trophoblasts (Figure 5A). Expression of FBW2 and RACK1 proteins was also observed in both villous and extravillous trophoblasts (Figures 5C and 5E). In extravillous trophoblasts, FBW2 was primarily detected in the nuclei and cytoplasm, whereas RACK1 was only detected in the cytoplasm (Figures 5D and 5F). Therefore it is likely that interaction of RACK1 and FBW2 may regulate GCM1 activity in the invasive extravillous trophoblasts of human placenta.

Regulation of placental cell migration and invasion by RACK1

Because RACK1 regulates GCM1 stability, we examined the effect of RACK1 knockdown on GCM1 target gene expression in BeWo placental cells. The transcript levels of the GCM1 target genes *PGF* and *HTRA4* were measured by quantitative real-time PCR analysis in BeWo cells stably expressing scramble or RK92
Figure 3 Regulation of FBW2 substrate binding by RACK1

(A) RACK1 blocks binding of GCM1 to FBW2. HEK-293T cells were transfected with pFBW2-Myc, pHA-GCM1 and increasing amounts of pRACK1-FLAG for immunoprecipitation (IP) and immunoblotting (IB) with the antibodies indicated. WCL, whole-cell lysate. (B) In vitro competition between RACK1 and GCM1 for FBW2. Increasing amounts of MBP–RACK1 were incubated with agarose beads pre-loaded with GST–FBW2 in the presence of HA–GCM1–FLAG for pull-down analysis, followed by immunoblotting with anti-HA antibody. Note that increasing amounts of MBP–RACK1 gradually decrease the binding of HA–GCM1–FLAG to GST–FBW2. Molecular masses are indicated in kDa. (C) RACK1 impairs FBW2-mediated GCM1 ubiquitination. HEK-293T cells were transfected with pHA-Ub, pGCM1-FLAG and the expression plasmid encoding scramble (SC), RK92 or RK72 shRNA for 36 h. Cells were then treated with 40 μM MG132 for another 10 h, followed by immunoprecipitation and immunoblotting with the antibodies indicated. (D) GCM1 protein levels are increased by RACK1 overexpression. HEK-293T cells were transfected with pGCM1-FLAG and increasing amounts of pHA-RACK1. Cells were harvested for immunoblotting with the antibodies indicated. (E) RACK1 up-regulates GCM1 transcriptional activity. HEK-293T cells were transfected with p(GBS)4E1BLuc, pGCM1-FLAG and increasing amounts of pHA-RACK1. Cells were harvested for luciferase reporter assay. Results are means ± S.D. for three independent experiments. *P < 0.05; **P < 0.01; ns, not significant.

Our recent study indicated that HtrA4 is a serine protease expressed in extravillous trophoblasts and is crucial for mediating BeWo cell invasion [6]. Because RACK1 knockdown decreases HtrA4 expression, we tested further whether RACK1 affects the migration and invasion activities of BeWo cells. To this end, control and RACK1-knockdown BeWo cells were plated into fibronectin- and Matrigel™-coated transwells for migration and invasion assays respectively. As shown in Figure 6(C), RACK1 knockdown decreased both the migration and invasion activities of BeWo cells. These results suggest that RACK1 may regulate placental cell migration and invasion through control of GCM1-mediated HTRA4 gene expression.

DISCUSSION

Trophoblast cells migrate and invade the uterus after implantation to facilitate the establishment of fetal–placental circulation, which is essential for gas and nutrient exchange between fetus and mother. Recently, we have shown that GCM1 regulates placental...
Figure 4 Regulation of GCM1 stability by RACK1

(A) Decrease of GCM1 protein level by RACK1 knockdown. BeWo31 cells stably expressing scramble (SC) or RK92 shRNA were harvested for immunoblotting (IB) with anti-GCM1, anti-HA, anti-RACK1 and anti-β-actin antibodies respectively (left). In a separate experiment, cells were harvested for quantitative real-time PCR of RACK1, GCM1 and HA–GCM1 transcript levels. Molecular masses are indicated in kDa. Results are means ± S.D. for three independent experiments. **P < 0.01; ns, not significant. (B) GCM1 half-life is shortened by RACK1 knockdown. BeWo31 cells stably expressing scramble (SC) or RK92 shRNA were treated with 75 μM cycloheximide (CHX) and chased for the periods of time indicated. Cells were harvested for immunoblotting with anti-HA and anti-β-actin antibodies respectively. Densitometry analysis of two independent experiments was performed using ImageJ software (NIH) to quantify the relative HA–GCM1 protein level normalized by β-actin.

Figure 5 Expression of GCM1, FBW2 and RACK1 in human placenta

Tissue sections of human term placenta were immunostained with anti-GCM1 (A and B), anti-FBW2 (C and D) and anti-RACK1 (E and F) antibodies and counterstained further with haematoxylin. Note that (B), (D) and (F) are higher magnification images of the extravillous trophoblasts in (A), (C) and (E).
cell invasion through activation of HtrA4 expression [6]. Along this line, it is crucial to properly regulate GCM1 activity to ensure successful trophoblastic invasion. Indeed, GCM1 activity can be negatively regulated by FBW2-mediated ubiquitination.

In the present study, we have identified RACK1 as a novel regulator for GCM1 ubiquitination and placental cell migration and invasion. Specifically, we identified RACK1 as an FBW2-interacting protein that protects GCM1 from ubiquitination by FBW2. This conclusion is based on the following observations. First, RACK1 binds to the WD-repeat domain of FBW2, which is involved in substrate binding of GCM1. Secondly, RACK1 competes with GCM1 for interaction with FBW2. Correspondingly, we demonstrate that RACK1 knockdown enhances GCM1 ubiquitination by FBW2. Thirdly, knocking down RACK1 expression by RNAi decreases the endogenous GCM1 protein level in the placental BeWo cells, whereas co-expression of RACK1 and GCM1 in the non-placental HEK-293T cells increases the exogenous GCM1 protein level. Furthermore, the protein level of the GCM1 target gene HTRA4 is decreased when RACK1 is knocked down in the placental BeWo cells. Consequently, the migration and invasion activities of BeWo cells are attenuated by RACK1 knockdown. Together with our immunohistochemistry study that RACK1 and FBW2 are expressed in extravillous trophoblasts, it is feasible to speculate that the interplay between RACK1 and FBW2 may modulate GCM1-mediated HtrA4 expression in extravillous trophoblasts. As a scaffolding protein, RACK1 interacts with cellular proteins via its WD-repeat domain. The Gβ subunit of heterotrimeric G protein contains seven WD repeats and adopts a seven-bladed β-propeller structure with each blade containing a four-stranded antiparallel β-sheet [17]. Because RACK1 shares significant sequence similarity with the Gβ subunit, RACK1 is predicted to adopt a similar seven-bladed β-propeller structure. Interestingly, RACK1 was identified as a binding protein of the Gβγ subunit of heterotrimeric G-protein in a yeast two-hybrid screen and interaction between RACK1 and Gβγ requires the WD-repeat domains in both proteins [11,18]. Further characterization indicated that dynamin-1, another RACK1-interacting protein, is able to compete with Gβγ for RACK1 [11]. By analogy, we have shown in the present study that GCM1 competes with RACK1 for
binding to FBW2, which probably impairs the interaction between the WD-repeat domains of RACK1 and FBW2.

The F-box protein FBW7, which contains eight WD repeats, mediates c-Jun ubiquitination and degradation. Zhang et al. [19] have shown recently that RACK1 interacts with both c-Jun and FBW7 to facilitate ubiquitination of N-terminal non-phosphorylated c-Jun by FBW7. Apparently, the interaction between FBW7 and RACK1 through their WD-repeat domains does not impair the recruitment of c-Jun by FBW7. On the contrary, RACK1 promotes the interaction between c-Jun and FBW7 and the resulting RACK1–c-Jun–FBW7 heterotrimer enhances c-Jun ubiquitination.

Interestingly, we have demonstrated in the present study that the interaction between RACK1 and FBW2 impedes the binding and ubiquitination of GCM1 by FBW2. Unlike c-Jun, GCM1 does not bind to RACK1, therefore the interaction between RACK1 and FBW2 probably blocks the binding of GCM1 to the WD-repeat domain of FBW2. This hypothesis is supported by the in vivo and in vitro competition assays (Figures 3A and 3B). Therefore RACK1 may positively or negatively modulate the biological activity of F-box protein depending on how RACK1 interacts with the F-box protein and its substrate. The mechanisms underlying these intriguing observations may await structural studies of the RACK1–FBW2 complex and the RACK1–c-Jun–FBW7 complex. Overall, our study revealed a novel function for RACK1 in the regulation of FBW2-mediated GCM1 ubiquitination and placental cell (e.g. extravillous trophoblast) migration and invasion mediated by the GCM1 target gene HTRA4 (Figure 6D).

**AUTHOR CONTRIBUTION**

Chang-Chun Wang performed the experiments and analysed the results. Hsiao-Fan Lo constructed the plasmids and performed the experiments. Shu-Yu Lin analysed the results and contributed to the discussion. Hungwen Chen designed the experiments, analysed the results, and prepared the paper.

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SUPPLEMENTARY ONLINE DATA
RACK1 (receptor for activated C-kinase 1) interacts with FBW2 (F-box and WD-repeat domain-containing 2) to up-regulate GCM1 (glial cell missing 1) stability and placental cell migration and invasion

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Figure S1 Identification of RACK1 as a candidate FBW2-associated protein

Cultured JAR cells stably expressing FBW2 with a C-terminal TAP tag were subjected to immunopurification and MS analysis. The MS/MS spectra on the [M + 3H]3++ (m/z 597.0049) ion for the peptide IIVDELKQEVISTSSK and the [M + 2H]2++ (m/z 530.3019) ion for the peptide VWQVTIGTR from RACK1 are provided respectively. The b and y ion series represent fragment ions containing the N- and C-termini of the peptide respectively. The protein sequence of RACK1 is listed with the MS/MS-detected peptides underlined.