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Wwp2 targets SRG3, a scaffold protein of the SWI/SNF-like BAF complex, for ubiquitination and degradation

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ABSTRACT

SRG3 plays essential roles both in early mouse embryogenesis and in extra-embryonic vascular development. As one of the core components of the SWI/SNF-like BAF complex, SRG3 serves as the scaffold protein and its protein level controls the stability of the BAF complex, which controls diverse physiological processes through transcriptional regulation. However, little is known about how the protein level of SRG3 is regulated in mammalian cells. Previously, we identified a murine ubiquitin ligase (Wwp2) and demonstrated that it interacts with pluripotency-associated key transcription factor Oct4 and RNA polymerase II large subunit Rpb1, promoting their ubiquitination and degradation. Here, we report that Wwp2 acts as a ubiquitin ligase of SRG3. Our results show that Wwp2 and SRG3 form protein complexes and co-localize in the nucleus in mammalian cells. The interaction is mediated through the WW domain of Wwp2 and the PPPY motif of SRG3, respectively. Importantly, Wwp2 promotes ubiquitination and degradation of SRG3 through the ubiquitin-proteasome system. The expression of a catalytically inactive mutant of Wwp2 abolishes SRG3 ubiquitination. Collectively, our study opens up a new avenue to understand how the protein level of SRG3 is regulated in mammalian cells.

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1. Introduction

SRG3 (SWI3-related gene) is a mouse homolog of yeast SWI3, Drosophila MOIRA, and human BAF155. Previous study has shown that SRG3 plays essential roles in early mouse embryogenesis, brain development, T-cell development and tumor suppression [1–3]. It is also required in extra-embryonic vascular development [4]. As a core component of SWI/SNF-like BAF chromatin remodeling complex, SRG3 is required for the transcriptional regulation associated with various aspects of development, cellular proliferation, differentiation, and tumorigenesis [5–7]. SRG3 interacts directly with the major components of the BAF complex and protects them from proteasomal degradation [8]. To execute these functions, the protein level of SRG3 has to be tightly controlled. However, the factors responsible for SRG3 ubiquitination and degradation in mammalian cells remain unanswered up to date.

In mammalian cells, the majority of intracellular proteins are degraded through the ubiquitin proteasome [9], which regulates diverse biological processes ranging from protein degradation, protein–protein interactions, to cell-cycle progression, apoptosis, gene transcription and immune responses [10–14]. Among all these functions, marking proteins for degradation by the 26S proteasome is the one best studied. Ubiquitination is regulated by a cascade of enzymatic reactions resulting in the covalent addition of ubiquitin (Ub) to target proteins through the help of Ub-activating enzyme (E1), Ub-conjugating enzyme (E2) and Ub-protein ligase (E3). Through direct association with substrates, E3 ligase plays a critical role in determining enzymatic specificity in the ubiquitination reaction [15,16]. Two distinct E3 families have been identified: the RING (really interesting new gene) domain E3 family and the HECT (homologous to E6-AP carboxyl-terminus) domain E3 family [17]. In the HECT domain family, the Nedd4 subfamily has a common domain architecture: an N-terminal C2 domain, a C-terminal HECT domain and 2–4 tryptophan-based WW domains in the middle [18].

Previously, our laboratory identified Wwp2 as an E3 ligase of Oct4, a key transcription factor for the maintenance of pluripotency

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in mouse ES cells [19]. Our further study showed that human WWP2 also interacts with human OCT4 in human pluripotent stem cells [20]. In addition, we demonstrated that Wwp2 also serves as an Ub E3 ligase of the large subunit of RNA polymerase II, Rpb1 [21]. Since most E3 ligases have multiple substrates and additional substrates of Wwp2 have been reported afterwards [22–24], we attempted to find more substrates of Wwp2. We here report the identification of SRG3, the scaffold protein of the BAF complex, as a new substrate of Wwp2 through affinity chromatography using a glutathione S-transferase (GST) fusion protein of WW-HECT domains of mouse Wwp2 and nuclear extract from murine F9 embryonal carcinoma cells. Our results show that the WW domain of Wwp2 and the PPPY motif of SRG3 mediated the interaction between these two proteins. Wwp2 targeted SRG3 for ubiquitination and degradation both in vitro and in vivo. The enzymatic activity of Wwp2 was dependent on the active cysteine residue in the HECT domain. Our study uncovers a new regulator of SRG3 and indicates that Wwp2 may be involved in the regulation of functions of BAF complexes in mammalian cells.

2. Materials and methods

2.1. Plasmids

The cDNA sequence of mouse full-length SRG3 was amplified by RT-PCR using total RNA from CGR8 mouse embryonic stem (ES) cells (kindly provided by Austin Smith) and cloned into the pET-30a(+) vector (Novagen) for expression of His tagged fusion protein in bacteria, or into the pcDNA3 vector (Invitrogen) for expression of Flag tagged fusion protein in mammalian cells. The sequences of all constructs were verified by DNA sequencing. For construction of SRG3 truncation vectors, truncation fragments were amplified using pcDNA3-Flag-SRG3 as a template and the PCR products were cloned into the pGEX-4T-1 vector (Amersham Biosciences). To construct pGEX-4T-1-SRG3IIIAAAA plasmid with PPPY (Pro-Pro-Pro-Tyr) motif mutation to AAAA (Ala-Ala-Ala-Ala), amino acid residual mutants were generated by PCR-based site-directed mutagenesis. Plasmids used for expression of Wwp2, Wwp2CA mutation, or individual Wwp2 domains and His-ubiquitin (Ub) expression vector pMT107 have been described previously [19].

2.2. Reagents and antibodies

The E1, E2 and recombinant His-tagged Ub were from Calbiochem. MG132 and tetracyclin (Tc) were from Sigma. The antibodies used were as follows: SRG3 (Santa Cruz), a-Tubulin (Sigma), Flag epitope (Sigma), His epitope (Santa Cruz), EGFP epitope (Roche), and ubiquitin epitope (Cell Signaling). Antibodies against Wwp2 and GST were raised and affinity purified as described previously [19].

2.3. Cell culture and DNA transfection

F9 (mouse embryonal carcinoma cells) and CGR8 (mouse ES cells) were cultured as described previously [21]. HEK 293 cells were maintained under standard conditions and transfected with the calcium phosphate method or Lipofectamine 2000 (Invitrogen). For experiments testing whether Wwp2 or WWP2 (human homologue of murine Wwp2) promotes degradation of endogenous BAF155 (human homologue of murine SRG3), increasing amounts of pCB6-Flag-Wwp2 or pCMV-WWP2 (0, 0.08, 0.16, 0.32, 0.5 μg) were transfected by the calcium phosphate method in 6-well plates.

2.4. Expression and purification of fusion proteins

GST and His fusion proteins were expressed and purified according to the manufacturer's instructions from Amersham Biosciences and Novagen, respectively.

2.5. Nuclear extract preparation and affinity purification

The nuclear extract was prepared from F9 cells. GST or GST-Wwp2-WW-HECT proteins were bound to glutathione immobilized Sepharose beads in equal molar amounts. Affinity purification assays were performed as described previous [19].

2.6. In-gel digestion of proteins and capillary-high pressure liquid chromatography mass spectrometric analysis for protein identification

This experiment was carried out according to the procedures described previously [25].

2.7. GST pull-down and co-immunoprecipitation (Co-IP)

These experiments were performed as described previously [19]. For Co-IP experiment in HEK 293 cells, 4 μg of pcDNA3-Flag-SRG3 and 3 μg of pCMV-HA-Wwp2 were transfected in 10 cm dishes simultaneously or separately.

2.8. Immunofluorescence staining

Immunofluorescence staining was performed as described previously [19].

2.9. Western blot analysis

Cells were lysed in Co-IP buffer and the protein concentration was determined by the BCA kit (Pierce). For experiments involving transient transfection, the co-transfected pSV-β-galactosidase plasmid (kindly provided by Richard Baer) was used to normalize the transfection efficiency of each sample. Western blot analysis was conducted by enhanced chemiluminescence (Pierce).

2.10. Ubiquitination assay in vitro and in vivo

The ubiquitination assays in vivo and in vitro were performed as described previously [19]. For the ubiquitination assay in vivo, HEK 293 cells were transfected with 3 μg of pcDNA3-Flag-SRG3, 3 μg of pCMV-HA-Wwp2 or pCMV-HA-Wwp2CA, 4 μg of pMT 107 (vector for His-Ub expression, a kind gift from D. Bohmann), and 1 μg of pSV-β-galactosidase. For in vitro ubiquitination assays, His-SRG3 (1 μg), rabbit E1 (50 ng, Calbiochem), His-Ub-carrier enzyme H6 (0.4 μg, Calbiochem), His-Ub (2 μg, Calbiochem), and GST-Wwp2 or GST-Wwp2CA (0.5 μg) were incubated in the ubiquitination buffer.

3. Results and discussion

3.1. Identification and characterization of SRG3 as a novel Wwp2 interacting protein

As a typical Nedd4 like protein, Wwp2 has an N-terminal C2 domain, a C-terminal HECT domain, and four WW domains in the middle. To identify novel substrates of Wwp2, we performed affinity chromatography using bacterially expressed GST-WW-HECT (WW and HECT domains of Wwp2) fusion protein and nuclear extract from murine F9 embryonal carcinoma (EC) cells. As shown in Fig. 1A, one band of about 150 kDa was present only in the F9

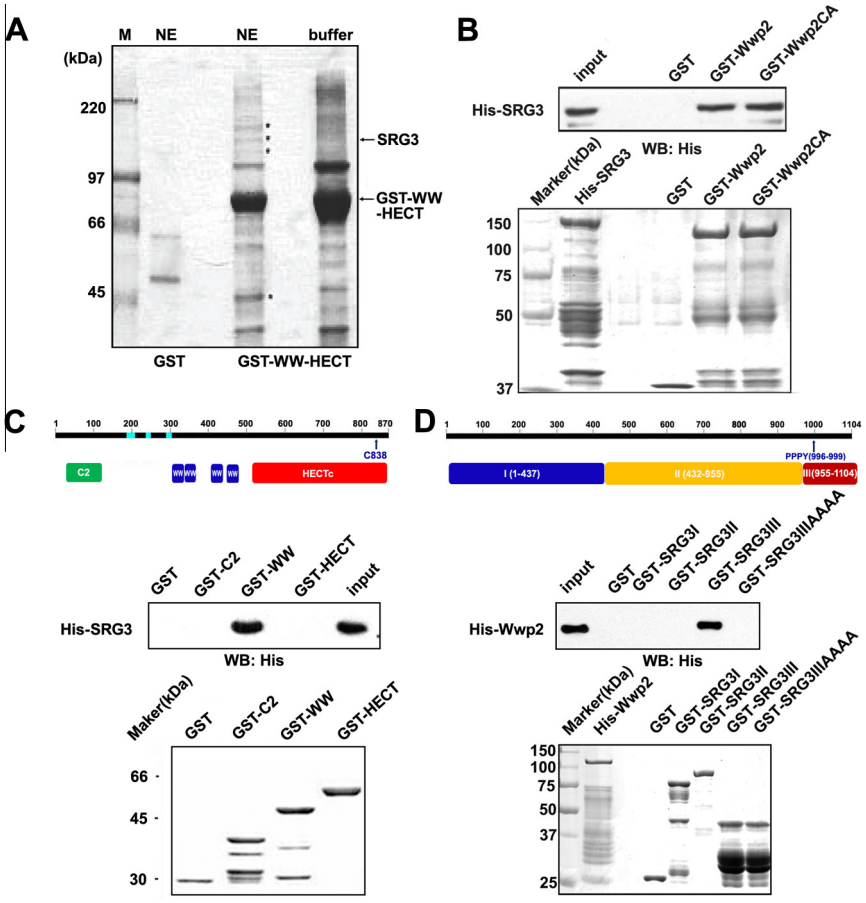

Fig. 1. Identification and characterization of the interaction between Wwp2 and SRG3 in vitro. (A) Purification of SRG3 from F9 nuclear extract (NE) using GST-WW-HECT fusion protein. Coomassie blue staining of SDS–PAGE gel containing the proteins from the indicated columns is shown. *association candidates of Wwp2 (B) GST pull-down assay to show that Wwp2 interacts with SRG3 directly in vitro (left panel). Coomassie blue staining of SDS–PAGE gel to show the purified proteins (right panel). (C) Schematic representation of Wwp2 domain structure (top panel). GST pull-down assay to show that the WW domain of Wwp2 mediates its association with SRG3 (middle panel). Coomassie blue staining of SDS–PAGE gel to show the purified proteins (bottom panel). (E) Schematic representation of SRG3 truncations (top panel). GST pull-down assay to show that PPPY motif of SRG3 mediates its association with Wwp2 (middle panel). Coomassie blue staining of SDS–PAGE gel to show the purified proteins (bottom panel).

nuclear extract-containing GST-WW-HECT column. Mass spectrometric analysis indicated that the band contained SRG3 protein.

To verify the association between SRG3 and Wwp2 detected by our affinity purification, GST pull-down assay was performed with bacterially expressed GST fusion protein of Wwp2 or Wwp2CA mutant (with a mutation of residue 838 cysteine to alanine to disrupt its ubiquitin ligase activity) and His fusion protein of SRG3. As shown in Fig. 1B, immobilized GST-Wwp2, but not GST alone, was able to pull down His-SRG3. In addition, mutation of cysteine to alanine at residue 838 of Wwp2 did not affect the association between SRG3 and Wwp2. This result validated the direct interaction between Wwp2 and SRG3 in vitro.

To determine which domain of Wwp2 interacted with SRG3, the three domains of Wwp2, namely C2 domain, WW domain (including four tandem ww domains), and HECT domain, were expressed as GST fusion proteins (Fig. 1C, bottom panel). In GST pull-down assays, the WW domain was found associated with SRG3, whereas C2 and HECT domains were not capable of binding to SRG3. Therefore, the interaction of Wwp2 with SRG3 was mediated through its WW domain.

Furthermore, to determine the region of SRG3 that was involved in its interaction with Wwp2, we fragmented full-length SRG3 into three parts, SRG3I (residue 1–437), SRG3II (residue 432–955), and SRG3III (residue 955–1104). Their GST fusion proteins were expressed bacterially (Fig. 1E, bottom panel). A previous study demonstrated that WW domains tend to bind to PPxY motifs, where P is proline, x is any amino acid and Y is tyrosine [26,27]. A PPPY motif (SRG3 residue 996–999) is located in SRG3III. We also expressed the GST tagged SRG3III where the PPPY motif was mutated into AAAA (A, Alanine). Then GST pull-down assays with His-Wwp2 were performed. As shown in Fig. 1E top panel, GST-SRG3III interacted with Wwp2 directly. As negative controls, neither GST alone nor GST-SRGI/II could pull down His-Wwp2. Specifically, the mutation of PPPY residues into AAAA residues in SRG3III abolished its binding ability to His-Wwp2 completely. Thus, the carboxyl-terminal proline- and glutamine-rich region of SRG3 mediated its association with Wwp2 and the PPPY motif was essential for the association.

3.2. Association of Wwp2 with SRG3 in mammalian cells

We next determined whether Wwp2 could interact with SRG3 in vivo. Co-IP experiments were performed using lysate of HEK 293 cells ectopically expressing Flag-tagged SRG3 and HA-tagged Wwp2 or vector alone. Both Flag-SRG3 and HA-Wwp2 proteins were immunoprecipitated by antibodies against Flag, indicating the association of Wwp2 with SRG3 in mammalian cells. As negative controls, HA-Wwp2 proteins were not detected in the Flag antibody-precipitated proteins in cells expressing HA-Wwp2 or Flag-SRG3 alone (Fig. 2A), providing evidence for the specific interaction of these two proteins in mammalian cells.

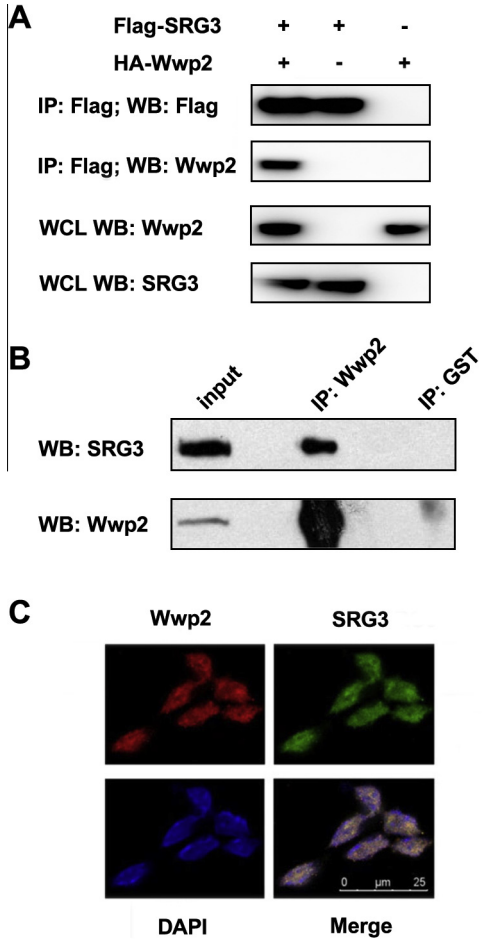

Fig. 2. Wwp2 binds to SRG3 specifically in vivo. (A) Co-IP assay in HEK 293 cells to demonstrate that ectopically expressed Wwp2 binds to SRG3 specifically in vivo. Whole cell lysates (WCL) were immunoprecipitated (IP) with anti-Flag antibody and analyzed by Western blotting with antibodies as indicated. (B) Association of endogenous Wwp2 with SRG3 in F9 cells. Nuclear extracts were subjected to IP with anti-Wwp2 antibody or control anti-GST antibody. (C) Co-localization of endogenous Wwp2 and SRG3 in CGR8 mouse ES cells. CGR8 cells were stained with anti-Wwp2 antibody (red), anti-SRG3 antibody (green) and DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Further, we examined whether endogenously expressed Wwp2 and SRG3 proteins form complexes (Fig. 2B). As both SRG3 and Wwp2 are known highly expressed in pluripotent stem cells, Co-IP experiments were carried out with nuclear extracts of F9 mouse EC cells. Anti-Wwp2 antibody, but not the control anti-GST antibody, was able to co-immunoprecipitate SRG3, indicating the existence of endogenous SRG3–Wwp2 complexes in embryonic pluripotent cells.

Last, to illustrate the associated cellular distribution of Wwp2 and SRG3, double immunofluorescence staining of endogenously expressed Wwp2 and SRG3 was performed. The co-localization of these two proteins was observed in CGR8 mouse ES cells (Fig. 2C), which further validated the physiological association between Wwp2 and SRG3 in pluripotent cells. Therefore, we conclude that SRG3 and Wwp2 do form complexes in a physiological context.

3.3. Wwp2 targets SRG3 for ubiquitination

Earlier studies from our group and others have demonstrated that Wwp2 is a ubiquitin E3 ligase. To investigate whether SRG3

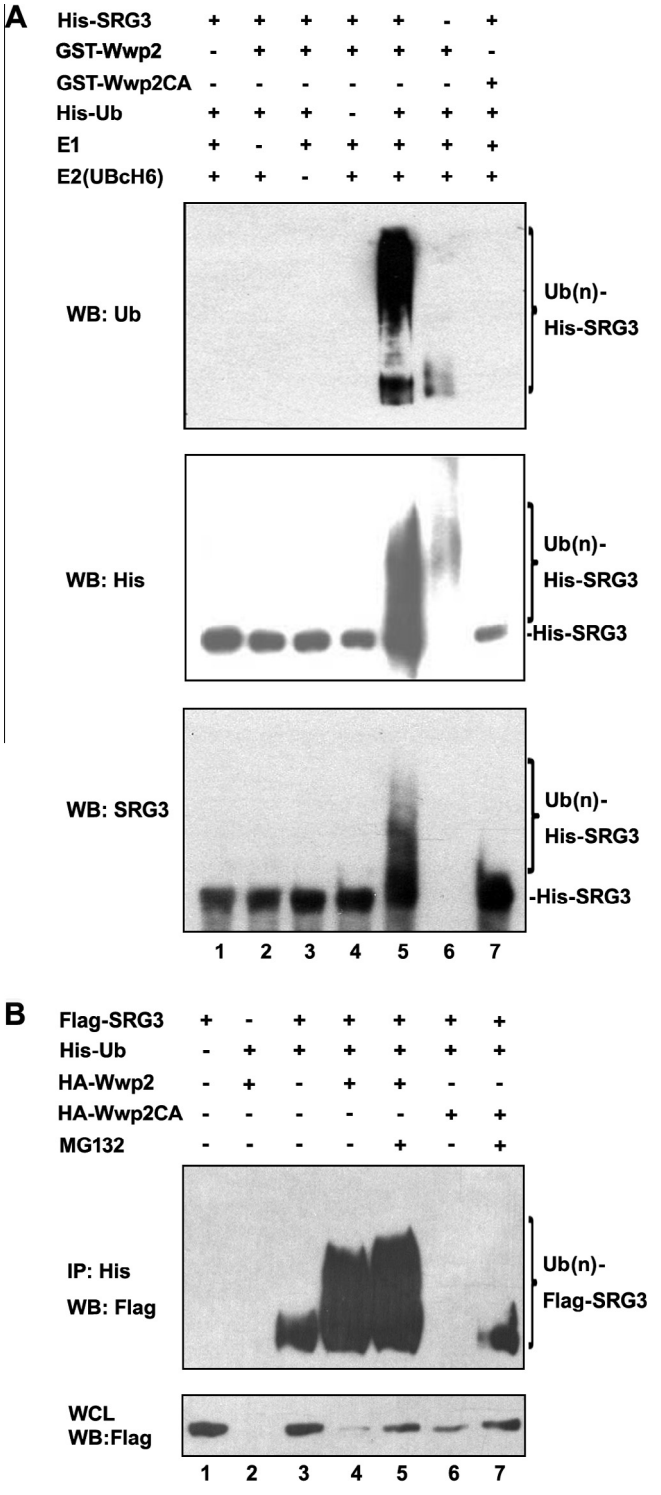

Fig. 3. Wwp2 targets SRG3 proteins for ubiquitination both in vitro and in vivo. (A) Wwp2 promotes SRG3 ubiquitination in vitro. Various bacterially expressed and purified proteins as indicated were incubated in the ubiquitination reaction buffer. Ubiquitinated SRG3 proteins were visualized by Western blotting with the antibody against ubiquitin (top panel), His (middle panel) and SRG3 (bottom panel). (B) Wwp2 enhances SRG3 ubiquitination in vivo. HEK 293 cells were transfected with expression vectors as indicated. Ubiquitinated polypeptides were isolated by nitrilotriacetic acid affinity beads and analyzed by Western blotting with the antibody against Flag (top panel). Protein levels of SRG3 in the cell lysates were measured by Western blotting with the antibody against Flag (bottom panel). Samples loaded in each lane were normalized by β-galactosidase activities in each individual specimen.

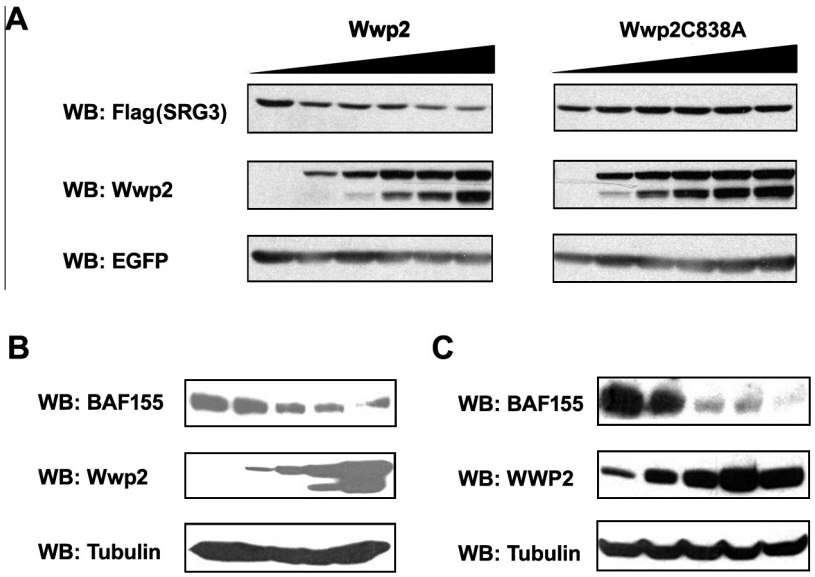

Fig. 4. Wwp2 regulates the protein level of SRG3 in mammalian cells. (A) Ectopic expression of Wwp2 reduces the protein level of SRG3 in a dosage-dependent manner. HEK 293 cells were transfected with a constant amount of Flag-SRG3 and EGFP together with increasing amounts of Wwp2 or Wwp2C838A mutant expression vectors. Cell lysates were analyzed by Western blotting with antibodies against Flag (detection of SRG3), Wwp2 and EGFP. (B) Ectopically expressed Wwp2 promotes degradation of endogenous BAF155 (human homologue of SRG3) in a dosage-dependent manner in HEK 293 cells. HEK 293 cells were transfected with increasing amounts of Wwp2 expression vector. 48 h later, cell lysates were measured by immunoblotting with antibodies against SRG3 (top panel) and Wwp2 (middle panel). (C) Ectopically expressed human WWP2 can also promote BAF155 degradation in a dosage-dependent manner in HEK 293 cells.

could be ubiquitinated by Wwp2, ubiquitination assay was conducted with a standard in vitro ubiquitination system, which contained purified proteins of GST-Wwp2, GST-Wwp2CA, His-SRG3, His-ubiquitin (His-Ub), E1, and E2 (UBcH6) as well as ATP. Higher molecular weight species indicative of the addition of ubiquitin moieties to His-SRG3 were observed in the presence of E1, E2, Ub, and wild-type Wwp2, when the reaction products were analyzed by Western blotting with an anti-Ub antibody (Fig. 3A, top panel, lane 5). These ubiquitinated products were His-SRG3 specific, since the ubiquitination signal was significantly different from that in the absence of SRG3 (top panel, lane 6). Our previous study had shown that the ubiquitination signal in the absence of substrates was a result of the self-ubiquitination of Wwp2 [28]. Of note, the ubiquitination of SRG3 was also dependent on the presence of Wwp2 and its intact HECT domain, since the ubiquitination signal disappeared when wild type Wwp2 was absent or replaced with a Wwp2CA mutant (top panel, lane 1 and lane 7), although the CA mutation of the HECT domain did not affect the association between Wwp2 and SRG3 (Fig. 1B). To further verify that the ubiquitinated proteins were indeed SRG3, similar ubiquitination assays were performed and the reaction products were examined by Western blotting with anti-His antibody (Fig. 3A, middle panel) or SRG3 antibody (Fig. 3A, bottom panel). Similarly, the higher molecular weight signals were detected in the presence of E1, E2, wild type Wwp2, Ub and His-SRG3 (middle and bottom panels, lane 5). The weak ubiquitination signal in the absence of SRG3 (middle panel, lane 6) was from Wwp2 self-ubiquitination, but not from SRG3, as the signals vanished when anti-SRG3 antibody was used to analyze the reaction product (bottom panel, lane 6). Again, Wwp2CA could not ubiquitinate SRG3 (middle and bottom panels, lane 7). These data clearly demonstrate that SRG3 can serve as a substrate for Wwp2-mediated ubiquitination in vitro and its ubiquitination requires normal activity of all three enzymes involved in the ubiquitination reaction.

We next detected whether Wwp2 could mediate SRG3 ubiquitination in vivo. HEK 293 cells were transiently transfected with expression vectors encoding His-Ub, Flag-SRG3, and Wwp2 or Wwp2CA. The cells were treated with the proteasome inhibitor MG132 for 16 h before harvest. Ubiquitinated proteins were isolated by nitrilotriacetic acid affinity beads from the cell lysate and analyzed by Western blotting with anti-Flag antibody (Fig. 3B, top panel). Co-expression of His-Ub with Flag-SRG3 caused ubiquitination of ectopically expressed SRG3 (lane 3). This could be caused by endogenous human WWP2 or other E3 activities present in the cells, as previously described [19]. As expected, the signal of ubiquitinated SRG3 increased when Wwp2 was co-expressed (lane 4). Meanwhile, the SRG3 protein level was significantly reduced in the presence of exogenous Wwp2 (lane 4, bottom panel), suggesting that the ubiquitination of SRG3 might promote its degradation. In the presence of proteasome inhibitor, MG132, the intensity of SRG3 ubiquitination was obviously enhanced (compare lane 5 with lane 4) and the SRG3 protein level was obviously recovered. These data reveal that Wwp2 targeted SRG3 for both ubiquitination and degradation through the 26S proteasome pathway in vivo. In contrast, the intensity of SRG3 ubiquitination was weaker in the presence of Wwp2CA than in its absence (compare lane 6 and lane3), suggesting a dominant negative effect of this mutated protein for SRG3 ubiquitination. There was no detectable ubiquitination signal when exogenous SRG3 was absent (Fig. 3B, top panel, lane 2), suggesting that the ubiquitination signals detected in the assay were specific to ectopically expressed SRG3 proteins. Taken together, Wwp2 can function as an E3 ligase of SRG3, regulating its ubiquitination and protein levels in vivo. Moreover, the cysteine residue 838 of Wwp2 plays an essential role in this process.

3.4. Wwp2 controls the protein level of SRG3

The E3 ligase and substrate relationship of Wwp2 and SRG3 shown above prompted us to investigate the role of Wwp2 in regulating SRG3 protein levels in mammalian cells. First, we further confirmed the regulation of Wwp2 on protein level of ectopically expressed SRG3. Consistent with aforementioned results, SRG3 protein levels decreased in a Wwp2 dose dependent manner, when Flag-SRG3 and EGFP together with increasing amounts of Wwp2

were co-expressed. As a negative control, protein levels of EGFP were not affected by Wwp2. Of note, Wwp2CA, an enzymatic activity dead mutation of Wwp2, lost its ability to control protein levels of SRG3, showing that the E3 ligase catalytic activity is required for Wwp2 to modulate protein levels of SRG3 (Fig. 4A).

To better probe the effect of Wwp2 on the steady state level of SRG3 proteins under a physiological context, we then detected the effect of Wwp2 on the protein level of endogenous SRG3. Increasing amounts of exogenous Wwp2 was over-expressed in HEK 293 cells and the endogenous protein levels of BAF155, SRG3 homologue in human cells, was examined by Western blotting. Our results indicated that protein levels of BAF155 decreased with increasing Wwp2 levels (Fig. 4B). Similar results were obtained when WWP2, the human homologue of Wwp2, was over-expressed (Fig. 4C). Hence, both mouse Wwp2 and human WWP2 promoted reduction in protein levels of endogenous BAF155 in HEK 293 cells.

Collectively, in the present study, we have identified and fully characterized Wwp2 as the first ubiquitin ligase that targets SRG3, a scaffold protein and one of the core component of BAF complex, for ubiquitination both in vitro and in vivo. Our study demonstrates that Wwp2 interacts specifically with SRG3, leading to its ubiquitination and degradation through 26S proteasomes, indicating that Wwp2 play an important role in regulating protein level of SRG3 in normal physiological conditions. Nevertheless, we do not rule out other E3 ligases play roles in regulation of SRG3 stability. There are several E3 ligases with a C2-WW-HECT domain structure similar to Wwp2 in mouse cells, such as Wwp1, Itch, Nedd4-1, Nedd4-2. Further investigation of the interaction between SRG3 and these ligases may address this possibility. The discovery of a new regulator for SRG3 provides new insights about how its stability is accurately modulated.

Acknowledgments

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