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UFMylation Modulates OFIP Stability and Centrosomal Localization

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ABSTRACT

Background: OFIP, also known as KIAA0753, is a centrosomal and pericentriolar satellite protein implicated in ciliogenesis, centriolar duplication, and microtubule stability. In humans, genetic mutations affecting OFIP have been implicated in the pathogenesis of Oral-Facial-Digital (OFD) Syndrome and Joubert Syndrome. Ubiquitin-fold Modifier 1 (UFM1), the most recently identified ubiquitin-like protein, is covalently transferred to its substrates, in a process known as UFMylation. This modification has recently emerged as a key regulator of various biological processes by altering their stability, activity, or localization.

Methods: The interaction between UFL1 and OFIP, as well as the UFMylation of OFIP, were assessed through immunoprecipitation and immunoblotting analyses. The mRNA levels of *OFIP* were examined using reverse transcription quantitative PCR (RT-qPCR). Immunofluorescence microscopy was employed to examine the localization and distribution patterns of OFIP.

Results: Our findings demonstrate that UFL1 interacts with OFIP both in vivo and in vitro. We also found that OFIP undergoes UFMylation, and UFL1 promotes the OFIP UFMylation. Mechanistic studies demonstrate that OFIP UFMylation inhibits its protein stability and maintains its proper centrosomal localization. However, the efficacy of these regulatory mechanisms varies significantly between different cell types, being notably pronounced in HeLa cells but markedly reduced in RPE1 cells.

Conclusions: OFIP is identified as a novel substrate for UFMylation. UFL1-mediated OFIP UFMylation is essential for its stability and centrosomal localization in HeLa cells. However, these effects are not observed in RPE1 cells, highlighting cell type-specific heterogeneity in the role of OFIP UFMylation.

1 | Introduction

Centrosomes are multifunctional, membrane-less organelles that play a crucial role in various cellular processes, including centriolar duplication, microtubule nucleation, ciliogenesis, cell division, and polarity formation [1–5]. The proper function of these processes depends on the accurate regulation and activity of centrosomal proteins. Defects in these proteins have been implicated in severe diseases, such as pulmonary fibrosis, diabetic complications, cancer, Parkinson's disease, Huntington's disease, and retinopathy [6–9]. Post-translational modifications (PTMs) add significant complexity to the centrosomal proteome and are crucial for maintaining protein homeostasis within the centrosome [10, 11]. Among these PTMs, ubiquitin and ubiquitin-like (UBL) modifications are widespread PTMs that are involved in centrosomal biology. For example, the E3

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ligase Mindbomb1 (MIB1)-mediated polyubiquitination of PCM1 results in its proteasomal degradation, thereby impairing centrosomal integrity [12]. CYLD-mediated deubiquitination of the centrosomal protein CEP70 is required for cilia formation in multiple organs and has been linked to male infertility [13]. Additionally, SUMOylation, a type of UBL modification, regulates the centrosomal localization of Polo-like Kinase 1 (PLK1) during the cell cycle [14]. However, the understanding of other types of UBL modifications and their associated functions in centrosomal proteins remains elucidated.

UFMylation is a recently discovered UBL modification. In most eukaryotes, UFMylation involves the attachment of UFM1 to lysine residues on substrate proteins via a specialized enzymatic system, including a UFM1-specific activating enzyme (E1) UBA5, a UFM1-specific conjugating enzyme (E2) UFC1, and a specialized E3 ligase UFM1-ligase 1 (UFL1) [15–17]. This modification has recently emerged as a key regulator of various biological processes, including endoplasmic reticulum stress, DNA damage response, hematopoiesis, photoreceptor cilium integrity, neural development, tumorigenesis, and immunotherapy [17–21]. Defects in UFMylation have been linked to a range of human diseases, including early-onset encephalopathy, spinal dysplasia, hematopoietic abnormalities, heart disease, diabetes, intestinal exocrine dysfunction, schizophrenia, and cancer [15, 20–25]. Moreover, mutations in UFMylation-related genes cause embryonic lethality in mice [26], underscoring the critical biological role of UFMylation. However, the specific substrates and interactome of UFMylation remain largely unexplored.

The OFD1 and FOR20 interacting protein (OFIP), also known as MNR or KIAA0753, has been identified as a novel centrosomal protein [27]. OFIP localizes to the centrosomes and the ciliary basal body, where it plays critical roles in centriolar duplication, ciliogenesis, microtubule stability, and aggresome assembly [28–31]. Mutations in *OFIP* have been extensively linked to ciliopathies, including Jeune asphyxiating thoracic dystrophy, oral-facial-digital syndrome, skeletal dysplasias, and Joubert syndrome [32–36]. Recent studies have also associated OFIP with cancer, neuronal differentiation, and cerebellar development [37, 38]. Despite its pivotal roles in cellular activities and pathological processes, the PTMs of OFIP and their associated biological functions have yet to be investigated.

In this study, we identified OFIP as a novel substrate for UFMylation. Our findings demonstrate that UFL1 interacts with OFIP both in vivo and in vitro. Furthermore, UFL1 promotes the protein stability and centrosomal localization of OFIP. However, the regulation of OFIP by UFMylation is cell type-specific, highlighting the context-dependent regulatory mechanisms of UFMylation in cellular activities.

2 | Materials and Methods

2.1 | Cell Culture and Transfection

HEK293T cells, RPE1 cells and HeLa cells were obtained from the American Type Culture Collection (ATCC). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, #FSP500, ExCell Bio) at 37°C in a 5% CO_2 atmosphere. Plasmids were transfected into cells using polyethylenimine (PEI, #23966–1, Polysciences). siRNAs were transfected into cells using Lipofectamine RNAiMAX (#13778030, Invitrogen).

2.2 | Antibodies, Chemicals, siRNAs and Plasmids

Antibodies targeting γ-tubulin (#Ab11316) and UFL1 (#Ab226216) were purchased from Abcam. Antibodies targeting OFIP (#HPA023494), UFL1 (#HPA030559) and Flag (#F7425) were purchased from Sigma-Aldrich. Antibody targeting HA (#AB0025) was purchased from Abways. Antibody targeting UFM1 (#HY-P81832) was purchased from MedChemExpress. Antibody targeting β-actin (#66009-1-Ig) was purchased from ProteinTech. Horseradish peroxidase-conjugated secondary antibodies (#SE131, #SE134) were purchased from Solarbio. Fluor-conjugated secondary antibodies (#A10042, #A10037, #A-21206 and #A-21202) were purchased from Invitrogen. Anti-Flag nanobody magarose beads (#KTSM1335) and anti-HA nanobody magarose beads (#KTSM1338) were purchased from AlpaLifeBio. Mammalian plasmids expressing HA-OFIP, Myc-UFM1 and Flag-UFL1 were constructed by inserting the cDNAs into the pCMV-HA-C, pcDNA3.1-Myc and pCDNA3.1-Flag vectors, respectively. Human UFL1 siRNAs (#1: 5′-GGAACUUGUUAAUAGCGGA -3′; #2: 5′-GAGGAGUAAUUUUUACGGA −3′) were synthesized by RiboBio.

2.3 | Immunoprecipitation and Immunoblotting

Cells were lysed using a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.25 mM sodium pyrophosphate, 10% glycerol, 1% NP40, and complete EDTA-free protease inhibitor tablets (EASYpack, 04693132001, Roche) at 4°C. The lysates were then centrifuged at 12,000 rpm for 20 min at 4°C to obtain the supernatant. For immunoprecipitation, the supernatant was incubated with 5 μL of Nanobody Magarose Beads at 4°C for 4–6 h. After incubation, the beads were washed eight times, and the proteins captured by the beads were subjected to SDS-PAGE and immunoblotting analysis. For the in vitro binding assay, HA-OFIP and Flag-UFL1 proteins were expressed in HEK293T cells and purified with anti-HA nanobody magarose beads and anti-Flag nanobody magarose beads. Flag-UFL1 was incubated with HA-OFIP bound to beads at 4°C for 2 h with mild rotation. The bound proteins were boiled with loading buffer and subsequently detected by immunoblotting. During the immunoblotting procedure, the proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (66485, Pall Corporation). The membranes were blocked with Tris-buffered saline (TBS) containing 0.2% Tween-20 and 5% skim milk. The blocked membranes were incubated with primary antibodies diluted in the blocking solution for either 2 h at room temperature or overnight at 4°C. After four washes in TBS with 0.2% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature. The bound antibodies were detected using an enhanced chemiluminescence (ECL) detection reagent.

2.4 | Immunofluorescence Staining

Cultured cells were fixed and permeabilized with cold methanol at −20°C for 5 min. The samples were then blocked with 4% bovine serum albumin (BSA) for 1 h, followed by incubation with primary antibodies and Alexa Fluor-conjugated secondary antibodies. Imaging was performed using a Zeiss LSM710 confocal microscope, and fluorescence intensity was quantified with Zeiss ZEN Lite software.

2.5 | Quantification and Statistical Analysis

The data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA) and presented as mean ± SEM. Unpaired two-tailed Student's *t*-test was used to compare two groups. $p < 0.05$ was considered statistically significant.

3 | Results

3.1 | UFL1 Interacts With OFIP

To investigate the PTMs of OFIP, a series of immunoprecipitation assays were conducted. Cells were lysed using a mild co-immunoprecipitation buffer, and endogenous proteins were immunoprecipitated with anti-OFIP antibodies to identify OFIP-interacting proteins. Immunoblotting experiments demonstrated that UFL1 interacts with OFIP (Figure 1A). Additional immunoprecipitation experiments with anti-UFL1 antibody confirmed the interaction of UFL1 with OFIP in HeLa cells (Figure 1B). In addition, we co-expressed Flag-UFL1 and HA-OFIP in HEK293T cells, and cell lysates were immunoprecipitated using the corresponding nanobody agarose beads. The results demonstrated that exogenous Flag-UFL1 interacts with HA-OFIP (Figure 1C,D). Furthermore, to determine whether the two proteins have a direct interaction, we purified Flag-UFL1 and HA-OFIP from HEK293T cells. Immunoprecipitation revealed the interaction of OFIP and UFL1 in vitro (Figure 1E). These results indicate a strong interaction between OFIP and UFL1.

3.2 | UFL1 Mediates OFIP UFMylation

As the sole ligase mediating UFMylation, UFL1 mediates the UFMylation of its substrates and has been implicated in diverse cellular activities. To determine whether OFIP is a substrate of UFL1-mediated UFMylation, we overexpressed Flag-tagged UFL1 in HEK293T cells and observed a significant increase in the UFMylation level of OFIP (Figure 2A,B). Next, we performed immunoprecipitation analysis to examine the effect of co-expression of HA-tagged OFIP and Myc-tagged UFM1 with either the Flag vector or Flag-UFL1. Our results showed that co-expression of HA-OFIP and Myc-UFM1, along with Flag-UFL1, further elevated the UFMylation level of HA-OFIP (Figure 2C,D).

Given that UFL1 overexpression increased the UFMylation level of OFIP, we further investigated whether UFL1 knockdown would have the opposite effect. Indeed, immunoprecipitation analysis revealed that knocking down UFL1 using siRNAs significantly reduced the UFMylation level of HA-OFIP, both in the presence and absence of exogenous Myc-UFM1 (Figure 2E–H). Taken together, these results indicate that UFL1 promotes the UFMylation of OFIP.

3.3 | UFMylation Modulates OFIP Protein Stability

It is widely recognized that protein stability is largely regulated by ubiquitin and UBL modifications [39–41]. As one of the most recently identified UBLs, UFM1 has been extensively reported to influence protein stability [17, 20]. Given that UFL1 interacts with and UFMylates OFIP, we sought to investigate

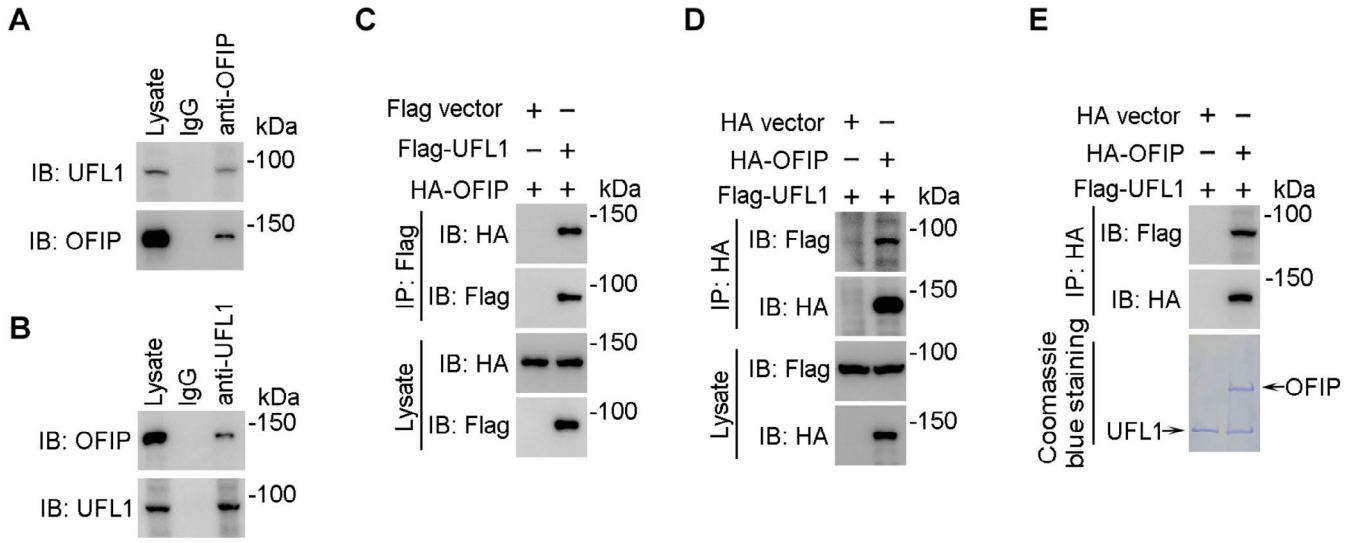

FIGURE 1 | UFL1 interacts with OFIP both in vivo and in vitro. (A and B) Immunoprecipitation and immunoblotting showing the interaction between endogenous UFL1 and OFIP in HeLa cells. (C and D) Immunoprecipitation and immunoblotting showing the interaction of Flag-UFL1 with HA-OFIP in HEK293T cells. (E) In vitro binding assay showing the interaction of purified HA-OFIP with purified Flag-UFL1.

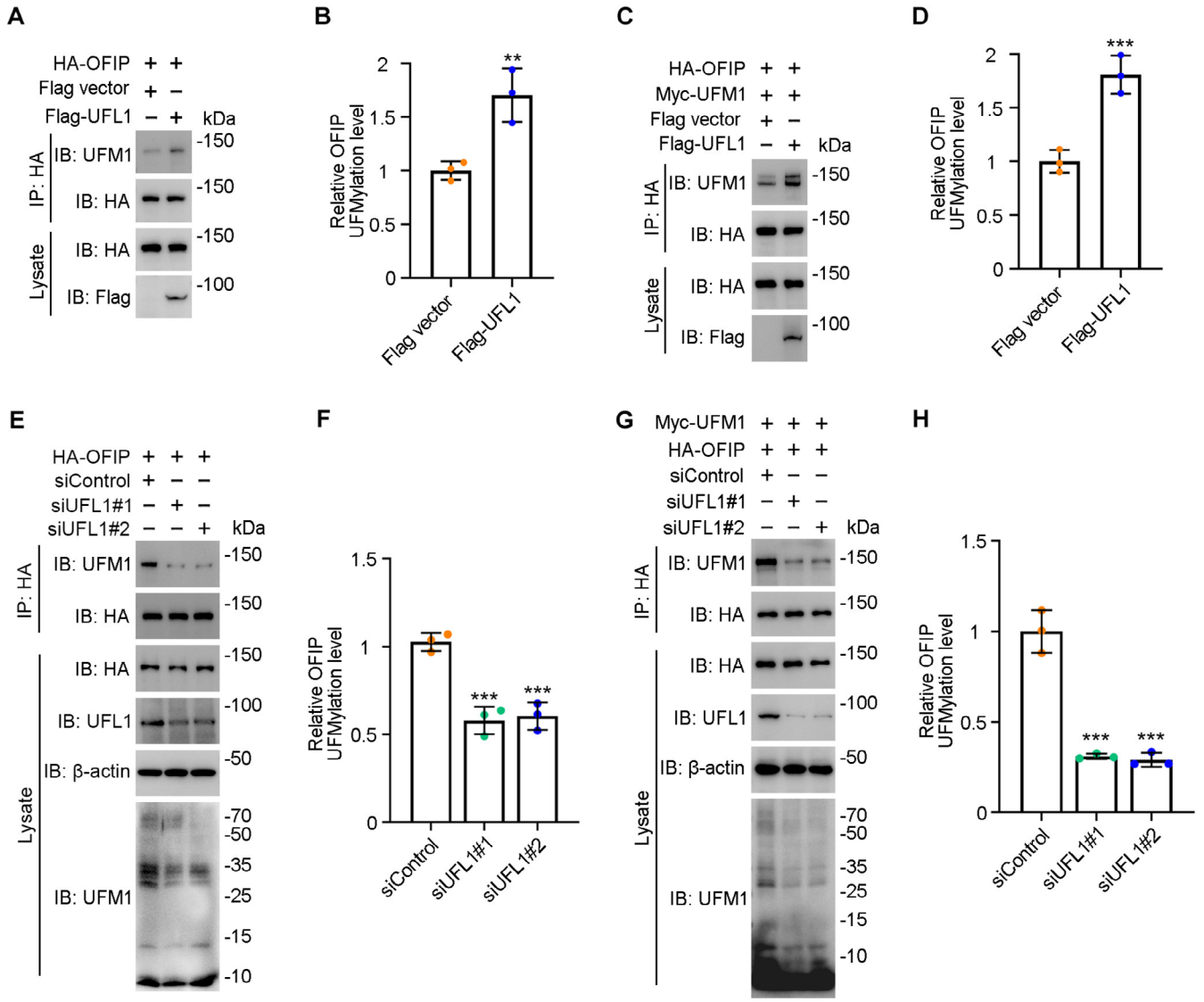

FIGURE 2 | UFL1 mediates OFIP UFMylation. (A) Immunoprecipitation showing HA-OFIP UFMylation in HEK293T cells transfected with Flag vector or Flag-UFL1. (B) Quantification of the relative HA-OFIP UFMylation level in cells treated as in (A). (C) Immunoprecipitation showing HA-OFIP UFMylation in HEK293T cells transfected with Myc-UFM1, together with Flag vector or Flag-UFL1. (D) Quantification of HA-OFIP UFMylation level in cells treated as in (C). (E) Immunoprecipitation showing HA-OFIP UFMylation in HEK293T cells transfected with control or UFL1 siRNAs. (F) Quantification of the relative HA-OFIP UFMylation level in cells treated as in (E). (G) Immunoprecipitation showing HA-OFIP UFMylation in HEK293T cells transfected with Myc-UFM1, together with control or UFL1 siRNAs. (H) Quantification of the relative HA-OFIP UFMylation level in cells treated as in (G). Data are presented as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

whether UFL1-mediated UFMylation affects OFIP protein stability. Immunoblotting assays revealed that knockdown of UFL1 using siRNAs significantly increased OFIP protein levels while decreasing its UFMylation level (Figure 3A,B). However, RT-qPCR analysis showed no noticeable changes in the relative *OFIP* mRNA levels upon UFL1 knockdown (Figure 3C). These findings suggest that UFL1-mediated UFMylation modulates OFIP protein stability.

Interestingly, when UFL1 siRNAs were transfected into retinal pigment epithelial-1 (RPE1) cells, no significant change in OFIP protein levels was observed while the UFMylation level of endogenous OFIP decreased remarkably (Figure 3D,E). Similarly, RT-qPCR analysis showed no notable alteration in *OFIP* mRNA levels following UFL1 knockdown (Figure 3F). These results suggest that UFL1 deficiency does not affect the protein stability of OFIP in RPE1 cells, indicating UFL1-mediated modulation of OFIP protein stability is cell type-specific.

3.4 | UFMylation Maintains OFIP Centrosomal Localization

Proper centrosomal localization of OFIP is crucial for maintaining the centrosomal integrity and homeostasis. We subsequently investigated whether UFMylation affects OFIP centrosomal localization. Immunofluorescence microscopy analysis revealed that UFL1 knockdown caused OFIP to be dispersed from the centrosomes in HeLa cells (Figure 4A–D). This aberrant localization may contribute to the excessive accumulation of OFIP following UFL1 knockdown. In contrast, in RPE1 cells,

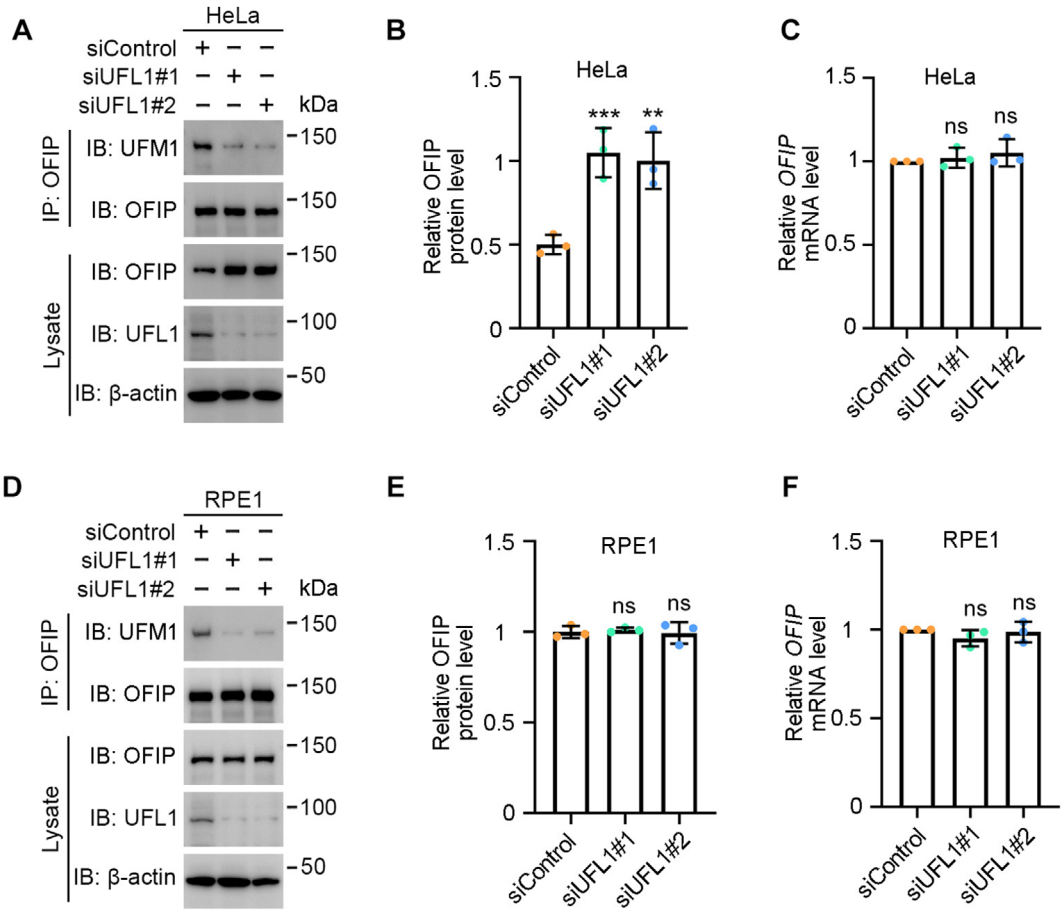

FIGURE 3 | UFL1-mediated UFMylation reduces OFIP protein levels. (A) Immunoprecipitation and immunoblotting showing the levels of OFIP and OFIP UFMylation in HeLa cells transfected with control or UFL1 siRNAs. (B) Quantification of the relative OFIP protein level in cells treated as in (A). (C) Quantitative RT-qPCR analysis of relative *OFIP* mRNA level in HeLa cells transfected with control or UFL1 siRNAs. (D) Immunoprecipitation and immunoblotting showing the levels of OFIP and OFIP UFMylation in RPE1 cells transfected with control or UFL1 siRNAs. (E) Quantification of the relative OFIP protein level in cells treated as in (F). (F) Quantitative RT-qPCR analysis of relative *OFIP* mRNA level in RPE1 cells transfected with control or UFL1 siRNAs. Data are presented as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$; ns not significant.

UFL1-mediated UFMylation did not affect the centrosomal localization of OFIP, which is consistent with our observations that UFMylation does not influence OFIP protein levels in these cells (Figure 4E–H). These results suggest that UFL1-mediated OFIP UFMylation maintains its proper centrosomal localization. However, this effect is also cell type-dependent, similar to its role in enhancing OFIP protein stability. This cell type-dependent regulation further supports the context-dependent regulatory mechanisms of UFMylation, which are similarly observed in its roles in immune responses and tumorigenesis [19, 20, 42, 43].

4 | Discussion

UFMylation was discovered in 2004, but its significance has only recently gained more attention. To date, a limited number of UFMylated proteins have been identified, highlighting the specificity of the UFMylation compared to ubiquitination [44]. Recent studies have shown that ribosomal and ER proteins are the primary cellular targets of UFMylation [45–47]. Additionally, in cells lacking UFL1 or UFBP1, the expression of several pro-apoptotic transcripts associated with chronic ER stress, such as C/EBP homologous protein (Chop), BCL2 associated X (Bax), p53 up-regulated modulator of apoptosis (Puma), and death receptor 5 (DR5), is significantly increased [26, 48]. While UFMylation predominantly affects ER and ribosome-related substrates, the UFM1 pathway may also regulate other noncanonical cellular processes.

Recent studies have highlighted the cytoskeleton-associated roles of UFMylation. During mitosis, UFM1 and Eg5 co-localize at the centrosome and spindle apparatus. Defects in UFMylation result in reduced spindle localization of Eg5, also known as kinesin-5 or KIF11, which is essential for proper spindle organization, mitotic progression, and cell proliferation [46]. Additionally, CDK5RAP3 (C53) interacts with the UFL1 at centrosomes, regulating microtubule nucleation. Knockout of UFL1 or C53 enhances centrosomal microtubule nucleation, accompanied by γ-tubulin accumulation and microtubule formation [49]. A recent study also demonstrated that the stability of KIF11 is regulated by the interplay between its UFMylation and ubiquitination, with KIF11 UFMylation being critical for maintaining photoreceptor cilium integrity and retinal homeostasis [17]. In Drosophila neuroblasts, the lack of UFMylation results in an increased mitotic index and an extended G2/M phase, indicating defects in mitotic progression. Knockdown

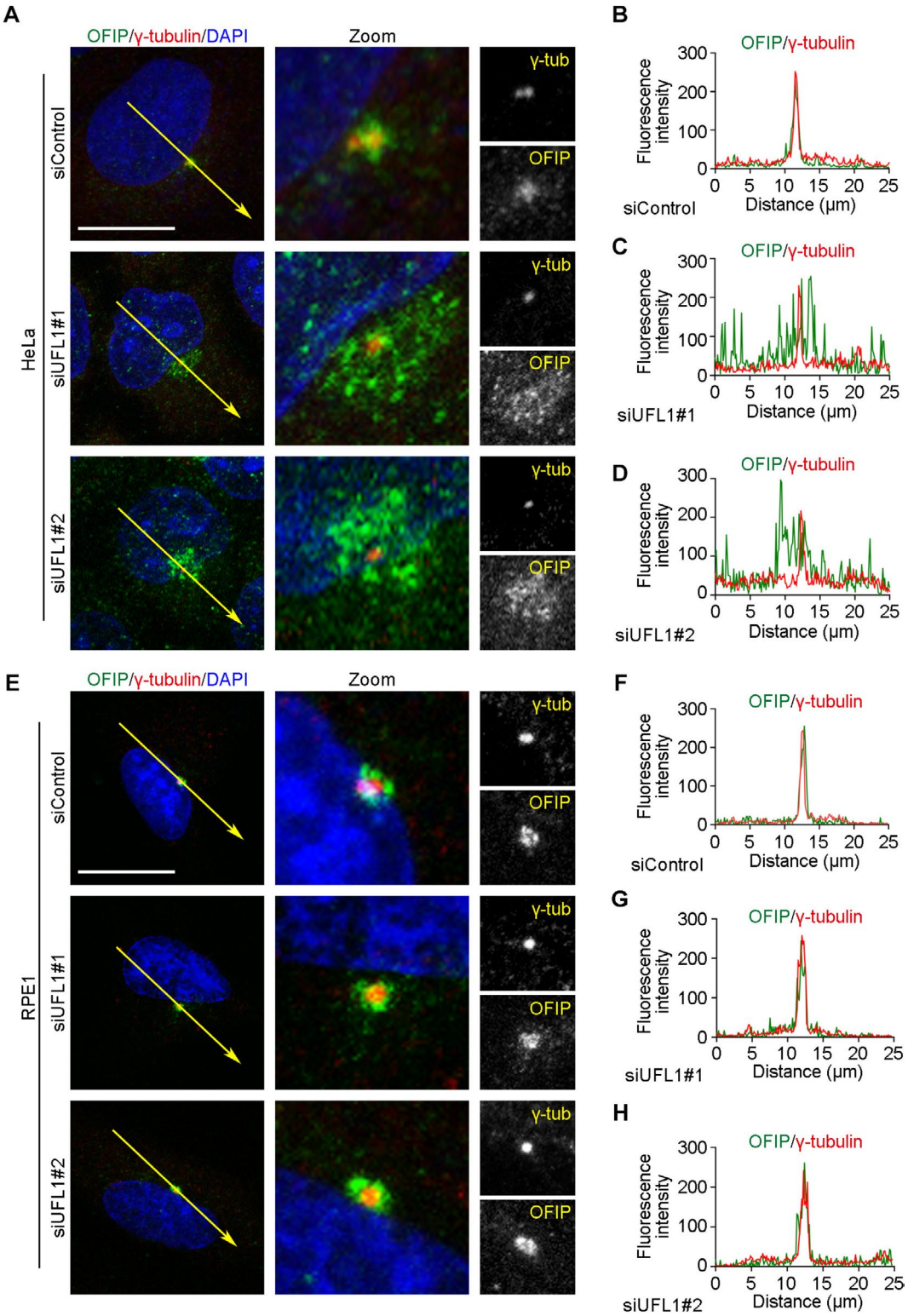

FIGURE 4 | UFL1 deficiency causes OFIP centrosomal mislocalization. (A) Immunofluorescence staining of OFIP and γ-tubulin in HeLa cells transfected with control or UFL1 siRNAs. Scale bar, 10 μm. (B–D) Quantification of fluorescence intensity for OFIP centrosomal localization in HeLa cells treated as in (A). The fluorescence intensity of OFIP and γ-tubulin across the cell was assessed along the yellow arrows. (E) Immunofluorescence staining of OFIP and γ-tubulin in RPE1 cells transfected with control or UFL1 siRNAs. Scale bar, 10 μm. (F–H) Quantification of fluorescence intensity for OFIP centrosomal localization in RPE1 cells treated as in (E). The fluorescence intensity of OFIP and γ-tubulin across the cell was assessed along the yellow arrows.

of UFMylation in fixed embryos leads to severe phenotypes, including detached centrosomes and defective microtubules [50]. In the present study, we identify the centrosome protein OFIP as a novel substrate of UFMylation. UFL1 deficiency results in defective centrosomal localization of OFIP. Given the significant role of OFIP in centrosomal and microtubule-associated functions,

further exploration is needed to uncover additional biological functions of UFMylation.

During the UFMylation cascade, UFL1 covalently attaches the C-terminal glycine of UFM1 to the lysine residue of the target protein, forming a peptide bond that links UFM1 to the substrate [15]. Generally, UFM1 modification and ubiquitination are in a competitive relationship. Loss of UFMylation often leads to an increase in ubiquitination, which typically results in the degradation of the substrate protein [17]. However, a recent study demonstrated that a decrease in PD-L1 UFMylation levels enhances its stability [20]. In our study, we show that reduced UFMylation of OFIP promotes its protein stability and leads to its mislocalization at the centrosome in HeLa cells. This aberrant localization and accumulation were not observed in RPE1 cells, suggesting that the impact of OFIP UFMylation on its localization and protein stability is cell type-dependent.

This cell type-dependent regulatory mechanism is also evident in the roles of UFMylation in immune responses and tumorigenesis. For example, PD-1 UFMylation stabilizes the protein by antagonizing its ubiquitination and subsequent degradation, leading to reduced activation of $CD8^+$ T cells and diminished anti-tumor immunity [19]. Conversely, UFMylation of PD-L1 in tumor cells promotes its degradation by synergizing its ubiquitination, thereby enhancing $CD8^+$ T cell-mediated antitumor immunity [20]. Additionally, UFMylation plays a multifaceted role in antiviral immune responses. It not only promotes interferon (IFN) induction in response to retinoic acid-inducible gene I (RIG-I) activation but also inhibits the proinflammatory activity of IFN-γ-activated macrophages. On the one hand, UFL1 and the UFMylation pathway are critical for the recruitment of 14–3-3ε to activated RIG-I, enhancing downstream signaling through mitochondrial antiviral signaling protein (MAVS) and increasing IFN production [42]. On the other hand, UFMylation acts as a negative regulator of IFN-γ and LPS responses in macrophages, preventing excessive inflammation and tissue damage [43]. Future research should focus on elucidating these regulatory mechanisms across different cell types and understanding the specific substrates and pathways involved in the complex interplay of UFMylation in these biological processes.

Author Contributions

Jie Ran, Dengwen Li and Mulin Yang designed the experiment and wrote the manuscript. Mulin Yang, Zihe Zhao, Jie Di, and Dan Dong performed experiments and analyzed data.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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