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The E3 ligase RNF185 negatively regulates osteogenic differentiation by targeting Dvl2 for degradation

Ying Zhou [a,1], Hanqiao Shang [b,1], Chunli Zhang [a], Yan Liu [a], Yantao Zhao [a], Feng Shuang [a], Hongbin Zhong [a], Jiaguang Tang [a,*], Shuxun Hou [a,*]

[a] Institute of Orthopaedics, The First Affiliated Hospital of Chinese PLA General Hospital, No. 51 Fucheng Road, Beijing 100048, China
[b] State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

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ABSTRACT

Osteoblast plays a pivotal role in bone metabolism and bone remodeling by mediating bone formation and regulating the activity of osteoclast. Clarifying the regulators and regulation mechanisms of osteogenic differentiation of mesenchymal stem cells (MSCs) and pre-osteoblasts will provide tremendous promise for bone repair and bone regeneration. RNF185 was identified as a candidate of endogenous suppressors of osteogenic specification in human mesenchymal stem cells (hMSCs). Here we show that RNF185 down regulates osteogenic differentiation of mouse calvaria-derived MC3T3-E1 cells, confirmed by quantitative real-time-PCR (qRT-PCR) and alkaline phosphatase (ALP) activity. Further we confirm that RNF185 interacts with dishevelled2 (Dvl2), a key mediator of Wnt signaling pathway. Overexpression of RNF185 decreases the exogenous and endogenous level of Dvl2, promotes the ubiquitination and degradation of Dvl2 and inhibits Wnt signaling, which is evident from the down-regulation of β-catenin mediated transcriptional activity. And Dvl2 reverses the effect of RNF185 on osteogenic differentiation of MC3T3-E1 cells. Taken together, our results indicate that RNF185 negatively regulates osteogenesis through the degradation of Dvl2 and down-regulation of canonical Wnt signaling pathway and suggest a possible therapeutic target in osteoporosis.

1. Introduction

Osteoporosis and other bone-related disorders represent a major public health threat. One out of every two women and one in four men, aged 50 or older, is expected to develop an osteoporosis related fracture in their lifetime [1–3]. There is a huge demand for products enhancing bone regeneration. Understanding the mechanisms of osteoresorption and osteogenesis will provide tremendous promise for bone repair and bone regeneration. Recently, several high-throughput studies have been carried out in the search for novel osteogenic suppressors in human mesenchymal stem cells (hMSCs), novel promoters and inhibitors of osteogenic differentiation, and in the assessment of the osteogenic capacity of several compounds [4–7]. These work not only provide new candidates for clinical applications but also allow the discovery of unexpected signaling pathways and molecular mechanisms for a certain application.

During a high throughout siRNA library (targeting 5000 human genes) screen, RNF185 (also named as FLJ38628) was listed in the supplement as a candidate of endogenous suppressors of osteogenic specification in hMSCs, which when silenced could initiate differentiation of hMSCs into osteoblasts [4]. However, the authors did not make further confirmation.

To clarify the molecular mechanism through which RNF185 mediates osteoblast differentiation, we focused on the well-known signaling pathways regulating osteogenesis. It has been reported that Xenopus ortholog XRNF185, with 91% identical to human RNF185, interacts with Xdsh [8], the ortholog of human dishevelled/(Dvl), a central mediator of Wnt signaling pathway [9]. Wnt signaling has been demonstrated as a family of potent regulators of MSCs commitment to chondrogenic and osteogenic phenotypes [9–12]. Previous study has shown that osteoblast is one of the targets of Wnt/β-catenin signaling during bone formation and Wnt/β-catenin signaling is critical for osteoblasts to complete their differentiation cycle and synthesize bone [13]. The importance of Dvls in Wnt signaling and in embryogenesis has been greatly appreciated [14,15]. Three Dsh homologs (Dvl1, 2 and 3) have been

Abbreviations: RNF, RING finger; Dvl, dishevelled; MSCs, mesenchymal stem cells; ALP, alkaline phosphatase.

* Corresponding authors. Fax: +86 10 68989121.
E-mail addresses: tangjiaguang2013@163.com (J. Tang), instortho304@gmail.com (S. Hou).

[1] These authors contribute equally to the work.

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identified in humans and mice, and the proteins consist of about 750 amino acids and display high sequence homology. The phenotype of knockout mouse model suggests Dvl2 is essential for proper formation of ribs, vertebral bodies and sternum [16]. Due to the specific function of Dvl2 in skeleton development, we selected Dvl2 as the candidate to validate the interaction with RNF185 in mammalian cells and the possibility of regulating osteogenic differentiation.

Here we demonstrate that RNF185 negatively regulates osteogenesis in MC3T3-E1 cells through the degradation of Dvl2 and modulating canonical Wnt signaling pathway which may suggest possible therapeutic targets in osteoporosis.

2. Materials and methods

2.1. Reagents, antibodies and plasmids

Monoclonal anti-Flag antibody was purchased from Sigma (St. Louis, MO), anti-HA antibody from Roche (Basel, Switzerland), anti-Myc antibody from Clontech (Palo Alto, CA), anti-RNF185 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), anti-GFP antibody and anti-Dvl2 antibody from Cell Signaling Technology (Beverly, MA). Secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The DNA constructs of Wnt3a, Flag-Dvl2 and the Wnt/β-catenin responsive luciferase reporter gene TOPflash were generously provided by Prof. Zhijie Chang (School of Medicine, Tsinghua University, China).

Full length human RNF185 (GenBank accession No. NM_152267) was amplified from total RNA of human embryonic kidney HEK293T cells by reverse transcription (RT)–PCR, and the PCR products was subcloned in-frame into vectors pCMV-Myc.

The sequences for the siRNA oligos against mouse RNF185 are as following: siR-NC, sense 5′-UUCUCCGAACGUGUCACGUTT-3′, anti-sense 5′-ACGUGACACGUUCGGAGAATT-3′; siR-236: 5′-GGGAUUGUCUUGAGAAUCUTT-3′, anti-sense: 5′-AGAUUCUCAAGA CAAUCCCTT-3′; siR-721: 5′-GCCACAGCAUUUAACAUAATT-3′, anti-sense: 5′-UUAUG UUAAAUGCUGUGGCTT-3′; and the siRNA oligos against mouse Dvl2: siRNA-scrambled 5′-TTCTCCGAACGTGT-CACGT-3′; siRNA-Dvl2 5′-GCTTTCTTCGTACACCTAT-3′.

2.2. Cell culture and transfection

Human embryonic kidney HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin. Mouse calvarial pre-osteoblastic cells MC3T3-E1 were cultured in either control medium (modified α-MEM (Thermo Scientific, Carlsbad, CA) supplemented with 10% FBS or osteogenic inducing medium (OS, control medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid). All cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Transfections were performed with Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instructions.

2.3. RNA extraction, RT-PCR and quantitative real-time PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen). Quality and quantity of the total RNA was assessed by agarose gel electrophoresis and UV spectrophotometry. First-strand cDNA synthesis for each RNA sample was conducted using 2 μg DNase I-treated total RNA and random hexamer primer with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas).

Real-time PCR was performed using the SYBR® Premix Ex Taq™II (Takara) and the MyiQ Single-Color Real-Time PCR Detection System (BioRad) according to the manufacturer's instructions. The expression of GAPDH RNA was used as the reference primers are listed as follows. Data were expressed as the relative expression of the reference gene using the $2^{-(\Delta\Delta Ct)}$ method. All reactions were carried out in triplicate.

Runx2, sense 5′- CCTGAACTCTGCACCAAGTCCT-3′, anti-sense, 5′-TCATCTG GCTCAGATAGGAGGG-3′; ALP, sense 5′-CCAGAAAGA-CACCTTGACTGTGG-3′, anti-sense 5′-TCTTGTCCGTGTCGCTCAC-CAT-3′; Osx, sense 5′-GGCTTTTCTGCGGCAAGAGGTT-3′, anti-sense 5′-CGCTGATGTTTGCTCAAGTGGTC-3′; GAPDH, sense, 5′-CAT-CACTGCCACCCAGAAGACTG-3′, anti-sense, 5′-ATGCCAGTGA GCTTCCCGTTCAG-3′; Dvl2, sense, 5′-GCCCCTGCTGC CCACCTTCTC-3′, anti-sense, 5′-CTGCTCCGGCTC CCCTCACTGT-3′; RNF185, sense, 5′-TGATATGCCGCACCCACTCCTGT-3′, anti-sense, 5′-AATTGGGGG TTCCTT TGGTTTTGA-3′.

2.4. Immunoprecipitation and immunoblotting

For general cell lysis and co-immunoprecipitation of RNF185 and Dvl2, HEK293T cells were transfected with indicated expression vectors by Lipofectamine 2000. Cells were cultured for 2 days in DMEM medium, and lysed for 30 min on ice in NETN buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). The lysates were centrifuged at 15,000*g* for 15 min at 4 °C. Antibody was added to the supernatant and incubated for 2 h with rotation at 4 °C. Protein A/G-Agarose beads (Pierce) were added to the immunocomplex and the mixture was rotated for 1 h at 4 °C. The beads were washed four times each in NETN buffer for several seconds and centrifuged at 500*g* for 2 min at 4 °C. The precipitated samples were boiled and separated by 10–15% SDS–PAGE, then transferred to nitrocellulose membrane (Millipore). The membrane was probed with the indicated primary antibody over night at 4 °C after blocking with 10% skim milk in TBS-T (Tris-Buffered Saline supplemented with 0.05% Tween 20), and followed by incubation with an appropriate secondary HRP-conjugated antibody for 1 h at RT after washing with TBS-T. Bands were detected using enhanced chemiluminescence kit (Thermo Scientific, CA) according to the manufacturer's instructions.

2.5. In vivo ubiquitination assay

For *in vivo* ubiquitination assays, HEK293T cells were transfected with plasmids expressing Myc-RNF185, Flag-Dvl2 and HA-tagged ubiquitin in various combinations. 36 h later, cell extracts were prepared as detailed above and lysates were immunoprecipitated with the indicated antibodies and analyzed with immunoblotting.

2.6. ALP activity assay

MC3T3-E1 cells transfected with siRNAs were cultured for 5 days in either control medium or OS medium. On Day 5, cells received lysis buffer (deionized water containing 6.3% glycerol, 2% sodium dodecyl sulfate, 5% 1 M Tris (pH 6.8), and 0.5% protease inhibitor). PNPP (p-nitrophenol phosphate, Ameresco) was added to wells containing cell lysate or lysis buffer for 30 min at 37 °C, the reaction was then stopped with 2 N NaOH, and the sample absorbance at 405 nm was detected using a microplate reader (Bio-Rad). Sample absorbance values were normalized by total protein amount determined by Bradford method.

2.7. ALP staining

MC3T3-E1 cells transfected with siRNAs were cultured for 5 days in either control medium or OS medium. On Day 5, cells

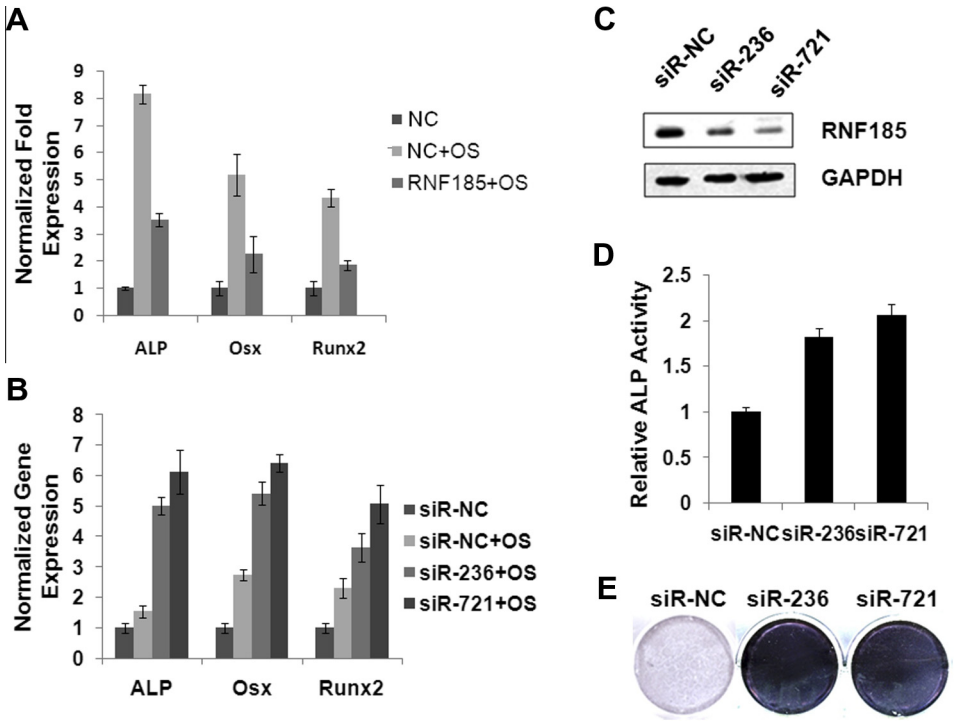

Fig. 1. RNF185 negatively regulates osteogenic differentiation of mouse pre-osteoblastic MC3T3-E1 cells. (A) Overexpressed RNF185 down-regulates expression of osteogenic markers (ALP, Osx and Runx2). MC3T3-E1 cells were transfected with pCMV-RNF185 and cultured in OS medium for 3 d, Then the total RNA were extracted and reverse transcribed. The mRNA levels of the indicated genes were analyzed using SYBR Green master mixture. Data were normalized to GAPDH and expressed as – fold changes relative to the mRNA levels of pCMV-Myc transfected-cells in OS ($\Delta\Delta$Ct method). (B) Knockdown of RNF185 induces expression of osteogenic markers (ALP, Osx and Runx2). MC3T3-E1 cells were transfected with siRNA against RNF185 (siR-236/siR-721) and cultured in OS medium for 3 d, Then the total RNA were extracted and reverse transcribed. The mRNA levels of the indicated genes were analyzed using SYBR Green master mixture. Data were normalized to GAPDH and expressed as – fold changes relative to the mRNA levels of siR-NC transfected-cells in OS. (C) siR-236/siR-721 could effectively inhibit endogenous RNF185 expression. MC3T3-E1 cells were transfected with siR-236/siR-721 and levels of RNF185 and GAPDH in lysates were assessed by SDS-PAGE and immunoblotting 48 h later. (D) Knowdown of RNF185 up-regulates ALP activity. MC3T3-E1 cells were transfected with siRNA and cultured in OS medium for 5 d, ALP activity were detected by pNPP. The total ALP luminescence was normalized to total protein amount. (E) Knowdown of RNF185 up-regulates ALP expression. MC3T3-E1 cells were transfected with siRNA and cultured in OS medium for 5 d, ALP activity were detected by NBT//BCIP staining.

were washed with PBS (Phosphate-Buffered Saline) and fixed for 30 min with 10% neutral buffered formalin at room temperature. Formalin was aspirated and cells were washed with deionized water. One-step nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyhosphate P-toluidine salt solution (BCIP) was added to each well for ALP staining (Pierce). Plates were incubated at 37 °C for 45 min. Staining solution was aspirated and cells were washed with deionized water. Plates were allowed to air-dry overnight and detected with scanner.

2.8. Luciferase reporter assay

HEK293T cells were transfected onto 24-well tissue cultured plates with a mixture of TOPflash reporter and pRL-TK (expressing Renilla luciferase) plasmids along with either RNF185 or Dvl2 constructs as indicated. At 24 h after transfection, the cells were harvested and subjected to dual luciferase assay according to the manufacturer's instructions. pRL-TK was used for co-transfection to normalize the data. All reactions were carried out in triplicate. Data are presented as mean ± SD.

3. Results

3.1. RNF185 negatively regulates osteogenic differentiation in MC3T3-E1 cells

In order to confirm the effects of RNF185 on osteogenic differentiation, we transiently transfected mouse pre-osteoblastic MC3T3-E1 cells with overexpressed RNF185 plasmid (Fig. 1A) or siRNA (Fig. 1B) and cultured in osteogenic inducing medium (OS). On day 4 after transfection qPCR analysis using the early osteogenic markers-specific primers (ALP, Osterix/Osx and Runx2) was carried out. As shown in Fig. 1A, cells transfected with RNF185 (RNF185) generated decreased ALP transcripts compared with the control cells transfected with empty vector (NC). In addition the expression of early osteogenic markers Osx and Runx2 were differentially down-regulated. Consistently, cells transfected with the siRNAs (siR-236 and siR-721) generated increased ALP, Osx and Runx2 transcripts compared with the control cells transfected with nonspecific siRNA (siR-NC) (Fig. 1B). Knockdown of the targeted gene was observed at 72 h after siRNA transfection (Fig. 1C). At the same time, the ALP activity was detected quantitatively (Fig. 1D) and qualitatively (Fig. 1E). Compared with the empty negative control, knockdown of RNF185 gave rise to a significant increase of ALP activity on day 5 after transfection up to two folds (Fig. 1D). Taken together, these results demonstrate that RNF185 could reduce osteogenic differentiation of MC3T3-E1.

3.2. RNF185 interacts with Dvl2 and decreases the protein level of Dvl2

To clarify the molecular mechanism through which RNF185 mediates osteogenic differentiation, we focused on the well-known signaling pathways regulating osteogenesis. It has been reported that XRNF185 interacts with Xdsh. gene [8]. The ortholog from Xenopus (NP_001088405.1) was compared with human RNF185 (NP_689480) by alignment of the amino acids sequences, and showed 91% identity, suggesting that RNF185 is evolutionarily

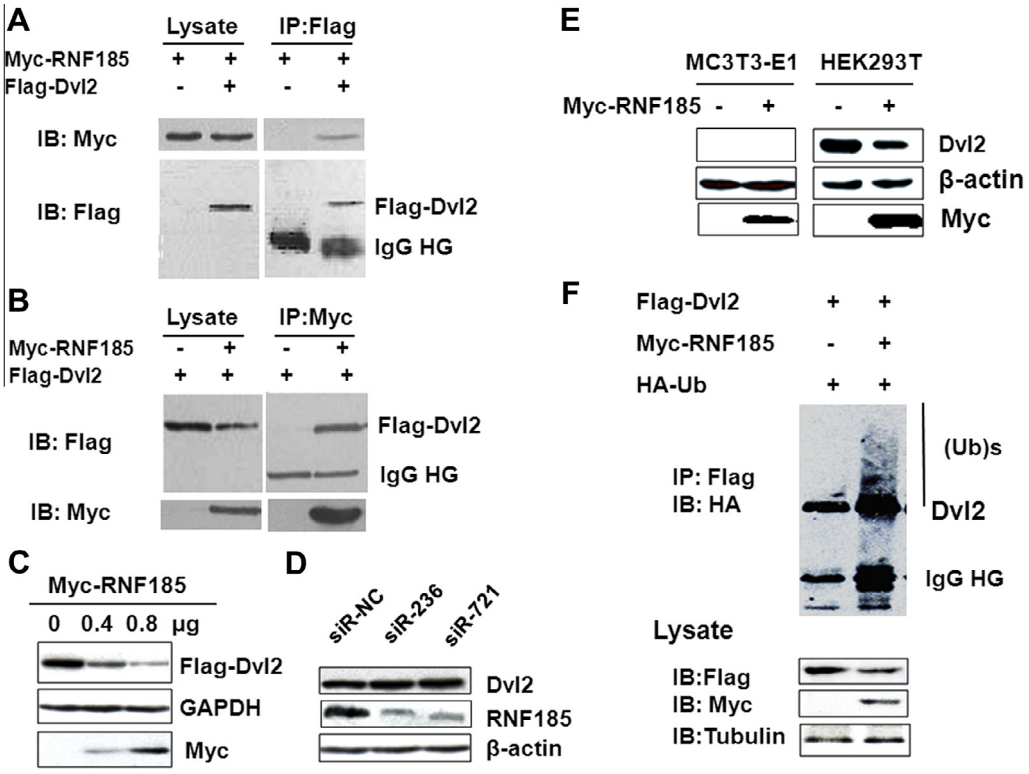

Fig. 2. RNF185 interacts with Dvl2 and promotes ubiquitination and degradation of Dvl2 *in vivo*. (A and B) RNF185 interacts with Dvl2 *in vivo*. HEK293T cells were transfected with expression plasmids for Myc-RNF185 together with Flag-Dvl2. Cell lysates were immunoprecipitated with monoclonal anti-Flag (A) or anti-Myc (B) antibody. 48 h later, the lysates and immunoprecipitates were detected by Western blot with anti-Myc and anti-Flag antibody. (C) Overexpressed RNF185 decreases the exogenous protein level of Dvl2 in a concentration-dependent manner. HEK293T cells were transfected with different concentrations of plasmid encoding Myc-RNF185 along with fixed amount of Flag-Dvl2. At 24 h post-transfection, the cells were lysed and subjected to immunoblotting analysis using anti-Flag, GAPDH and Myc antibodies. GAPDH was used as a loading control. (D) Knowdown of RNF185 increases the endogenous protein level of Dvl2. MC3T3-E1 cells were transfected with scramble siRNA (siR-NC) or siR-236/siR-721. 72 h later, the cells were collected and subjected to immunoblotting analysis using antibodies against Dvl2, RNF185 and β-actin. (E) Overexpressed RNF185 decreases the endogenous protein level of Dvl2. HEK293T cells or MC3T3-E1 cells were transfected with empty vector (control) or RNF185. 24 h later, the cells were collected and subjected to immunoblotting analysis using antibodies against Dvl2, Myc, and β-actin. (F) RNF185 promotes ubiquitination and degradation of Dvl2 *in vivo*. Myc-RNF185 was cotransfected into cells with plasmids encoding Flag-Dvl2, HA-Ub. Dvl2 were isolated by IP and analyzed by Western blot with HA antibody.

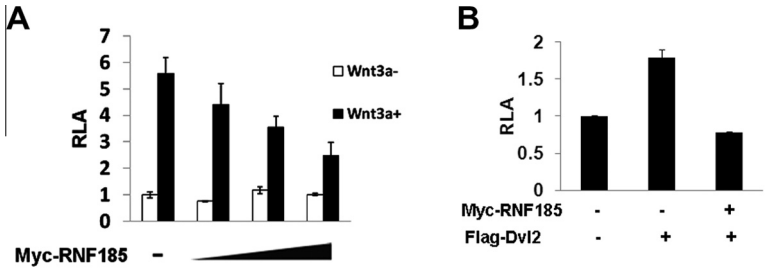

Fig. 3. RNF185 negatively regulates Wnt signaling. RNF185 inhibits β-catenin-dependent luciferase (TOPflash) activity in a concentration-dependent manner. HEK293T cells were plated onto 24-well tissue cultured plate, and on the following day the cells were transfected with increasing concentrations of plasmid encoding RNF185 along with a mixture of TOPflash and pRL-TK vectors with or without Wnt 3a (A) and Flag-Dvl2 (B). At 24 h post-transfection, the cells were collected and subjected to dual luciferase assay. The values are the means ± S.D. of three independent experiments each performed in triplicate.

conserved. Interaction between human RNF185 and Dvl2 was confirmed by co-immunoprecipitation assay in mammalian cells. HEK293T cells were transiently transfected with Myc-RNF185 and Flag-Dvl2, and the cell lysates were processed for co-immunoprecipitation using Flag antibody. Analysis of anti-Flag and anti-Myc immunoprecipitates derived from these cells revealed that Dvl2 immunocomplexes specifically contained RNF185 (Fig. 2A). On the other hand, Dvl2 could also be co-immunoprecipitated with RNF185. (Fig. 2B).

Due to the E3 activity of RNF185 [17,18], we further investigated the effect of RNF185 on Dvl2 protein level. HEK293T cells were transfected with different concentrations of plasmid encoding RNF185 along with fixed amount of Flag-Dvl2. As shown in Fig. 2C, ectopically expressed Dvl2 was decreased upon overexpression of RNF185 in a concentration-dependent manner. Furthermore the steady-state level of endogenous Dvl2 was increased in RNF185-depleted MC3T3-E1 cells (Fig. 2D), while it was decreased upon overexpression of RNF185 either in MC3T3-E1 or in HEK293T cells (Fig. 2E).

3.3. RNF185 promotes ubiquitination and degradation of Dvl2

To examine whether RNF185 leads to degradation of Dvl2, HEK293T cells were transiently transfected with Flag-Dvl2 and

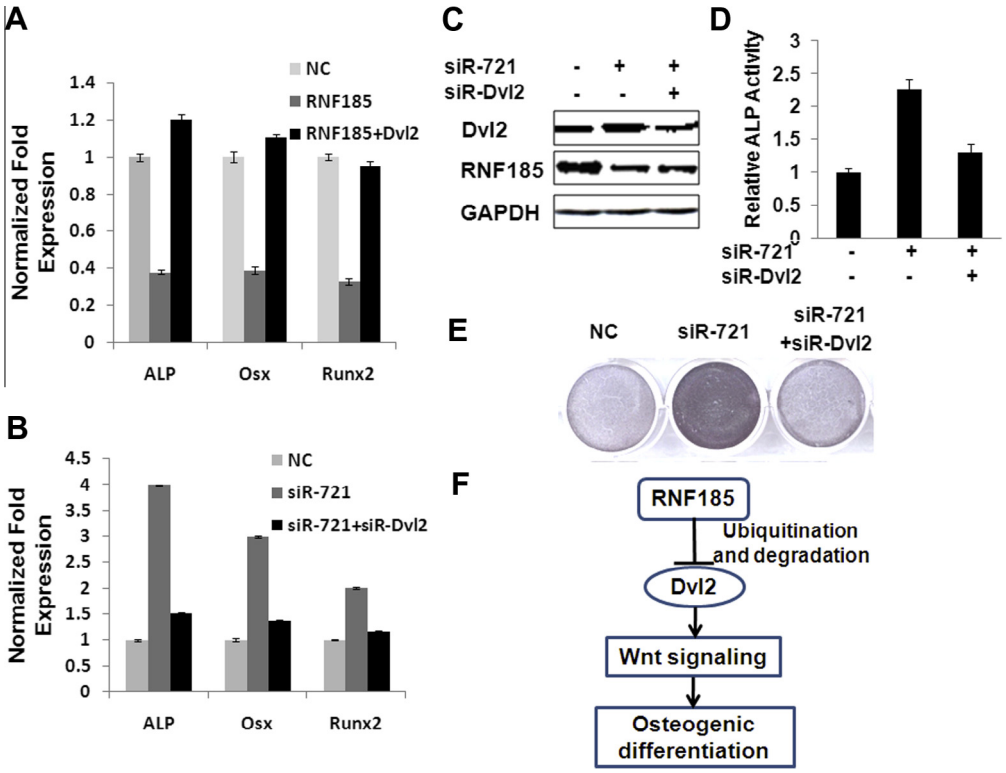

Fig. 4. RNF185 modulates osteogenic differentiation through regulation of Dvl2. (A) Overexpressed Dvl2 reverses the reduction of osteogenic markers (ALP, Osx and Runx2) in RNF185-overexpressing cells. MC3T3-E1 cells were transfected with RNF185 and Dvl2 and cultured in OS medium for 3 d, Then the total RNA were extracted and reverse transcribed. The mRNA levels of the indicated genes were analyzed using SYBR Green master mixture. Data were normalized to GAPDH and expressed as – fold changes relative to the mRNA levels of pCMV-Myc transfected-cells in OS. (B) Knockdown of Dvl2 reverses the induction of osteogenic markers (ALP, Osx and Runx2) expression in RNF185-depleted cells. MC3T3-E1 cells were transfected with siRNA against RNF185 (siR-721) and Dvl2 (siR-Dvl2) and cultured in OS medium for 3 d, Then the total RNA were extracted and reverse transcribed. The mRNA levels of the indicated genes were analyzed using SYBR Green master mixture. Data were normalized to GAPDH and expressed as –fold changes relative to the mRNA levels of siR-NC transfected-cells in OS. (C) siR-721 could effectively enhance endogenous Dvl2 expression. MC3T3-E1 cells were transfected with siR-721 and levels of Dvl2, RNF185 and GAPDH in lysates were assessed by SDS-PAGE and immunoblotting 48 h later. (D) Knockdown of Dvl2 decreases the induction of ALP activity in RNF185-depleted cells. MC3T3-E1 cells. MC3T3-E1 cells were transfected with siRNA and cultured in OS medium for 5 d, ALP activity were detected by pNPP. The total ALP luminescence was normalized to total protein amount. (E) Knockdown of Dvl2 decreases the induction of ALP, expression in RNF185-depleted cells. MC3T3-E1 cells were transfected with siRNA and cultured in OS medium for 5 d, ALP activity were detected by NBT//BCIP staining. (F) A proposed model for RNF185 mediated osteogenesis regulation. Our finding shows that RNF185 down-regulates Wnt pathway by promoting degradation of Dvl2, therefore, inhibits the osteogenic differentiation of pre-osteoblastic MC3T3-E1 cells.

HA-Ub together with/without pCMV-Myc-RNF185. Dvl2 conjugated in HA-Ub was purified by protein A/G agarose and detected by immunoblotting with HA monoclonal antibody. The result showed that a small proportion of Dvl2 was conjugated to ubiquitin *in vivo* and transfection of RNF185 increased Dvl2 ubiquitination (Fig. 2F). This result confirms that the Dvl2-RNF185 interaction leads to ubiquitination of Dvl2.

3.4. RNF185 negatively regulates Wnt signaling

Given that RNF185 can ubiquitinate Dvl2 to promote its degradation and that Dvl2 is required for canonical Wnt signaling, we asked whether RNF185 regulates the canonical Wnt pathway. We overexpressed RNF185 in HEK293T cells and evaluated its effects using the TOPflash reporter system. As shown in Fig. 3A and B, overexpression of RNF185 inhibited Wnt3a/Dvl2-induced TOPflash activity in a dose-dependent manner, indicating that RNF185 could down-regulate Wnt signaling transcriptional activity.

3.5. RNF185 modulates osteogenic differentiation through regulation of Dvl2

To determine whether RNF185 could modulate osteogenic differentiation through regulation of Dvl2, we examined the effect of Dvl2 on osteogenic differentiation in RNF185-overexpressing and depleted cells. It was shown that overexpression of Dvl2 partially increased the expression of ALP, Osx and Runx2 in RNF185-overexpressing cells (Fig. 4A), and reduction of Dvl2 level by siRNA decreased the expression of ALP, Osx and Runx2 (Fig. 4B), together with the ALP activity in RNF185-depleted cells (Fig. 4D and E). These results suggest that Dvl2 could reverse the effect of RNF185 on osteogenic differentiation of MC3T3-E1.

4. Discussion

Wnt signaling plays a critical role in a vast array of biological process, including cell proliferation, migration, polarity establishment and stem cell self-renewal [19,20]. Dvl is a key component of Wnt signaling and transmits Wnt signals from membrane receptors to downstream effectors. Its activity and stability is modulated dynamically by phosphorylation, ubiquitination and degradation. To date, several ubiquitin ligase (E3) have been identified, including HECT-type E3 NEDL1 [21], ITCH [22], KLHL12-Cullin-3 E3 complex [23]. pVHL, a component of an SCF-like E3 complex and RING-type ubiquitin ligase Malin [24]. A recent report demonstrates the degradation of Dvl2 through autophagy under metabolic stress and suggests that autophagy could potentially regulate Wnt signaling pathway [25]. These E3s target Dvl2 for degradation through both proteasome and autophagy under different physiological conditions. Our finding provides that RNF185 could be a new regulator

of Wnt signaling by promoting the degradation of Dvl2, although the underlying physiological conditions need to be investigated.

It has been reported that Xenopus XRNF185 promoted the degradation of ubiquitinated paxillin probably by directly bridging paxillin and the proteasome instead of promoting the ubiquitination of paxillin. The researchers also identified proteins commonly coprecipitated with both Xenopus and human RNF185 in HEK293 cells by mass spectrometry analysis. Among them there were several subunits of proteasome. Therefore, besides the activity of ubiquitin ligase for protein ubiquitination, RNF185 could also serve as a bridge between ubiquitinated proteins and the proteasome.

Recently, RNF185 was described as a mitochondria outer membrane (MOM) ubiquitin ligase involved in the regulation of selective autophagy through interaction with BNIP1(Bcl-2 Nineteen kilodalton Interacting Protein 1) [17]. Most recently, RNF185 was identified as a novel E3 ligase of Endoplasmic Reticulum-associated degradation (ERAD) that targets cystic fibrosis transmembrane conductance regulator (CFTR) for degradation in a RING and proteasome dependent manner [18]. Taken together, RNF 185 functions in both proteasome and autophagy-mediated degradation. Whether RNF185 may target Dvl2 to autophagy degradation needs to be further investigated. Identification of physiological conditions potentially regulating RNF185 localization and function will be of interest to further unravel functions of RNF185.

Osteogenic differentiation and bone formation play a pivotal role in bone regeneration. Cell-based assays can therefore be used to identify modulators of differentiation pathways (for example osteogenesis) in the physiological environment of the cell with all the intact regulatory networks and feedback control mechanisms present. Identification of the positive and negative regulators will provide potential therapeutic targets for osteoporosis.

In summary, our results identified Dvl2 as a new substrate of RNF185. RNF185 negatively regulates Wnt signaling through ubiquitination and degradation of Dvl2, therefore negatively modulates osteogenic differentiation (Fig. 4F). Our findings suggest a possible therapeutic target of osteoporosis.

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