This article has been withdrawn by Isha Kapoor, Kainat Khan, Gatha Thacker, Mohd. Parvez Khan, Nidhi Shukla, Jitendra Kumar Kanaujiya, Sabyasachi Sanyal, Naibedy Chattopadhyay, and Arun Kumar Trivedi. Yogesh Kumar could not be reached. The corresponding author identified some issues and brought them to the attention of the Journal. Subsequently, some issues were also noted in the article by the Journal. Based on the original data provided for Fig. 2C, the human Runx2 construct resolves at the size of β-actin. While they grossly differ in size (size of Runx2 is ~60 kDa while that of β-actin is 42 kDa), the withdrawing authors claim that the loading control was possibly probed with β-tubulin (~55 kDa), which separates at almost the same size as Runx2 and was erroneously labeled as β-actin. The Runx2 immunoblots from Figs. 2E and 2J were duplicated, and the FLAG Fbw7 immunoblot from Fig. 2J was inappropriately manipulated. Additionally, the actin immunoblots from Figs. 3B and 3C were duplicated, the graphs shown in Figs. 4A and 4B were duplicated, and a non-matching image due to overcropping was used in the Fbw7 panel for the Ovx + E2 group in Fig. 6A, which the withdrawing authors state were inadvertent errors. Given these errors, the withdrawing authors state that the responsible course of action should be to withdraw the article to maintain the high standards and rigor of scientific literature from the withdrawing authors’ group as well as the Journal. However, the withdrawing authors state that these errors do not change the underlying scientific findings of the article.
ligase for Runx2 based on following evidence: (a) phosphorylation of Runx2 negatively regulates its activity (13, 15, 25); (b) GSK3β negatively regulates osteogenesis by phosphorylating Runx2 and inhibiting its activity (25); (c) insulin signaling-mediated PI3K/AKT pathway promotes osteogenesis without phosphorylating Runx2 (26, 27); and (d) there are several putative CPD motifs within Runx2 protein, and some of these CPDs also harbor GSK3β consensus phosphorylation motifs known to be phospho-modified by GSK3β (25). Here, we studied whether Fbw7α serves as an E3 ubiquitin ligase for Runx2.

Materials and Methods

Cell Culture, Transfection, and Plasmids—HEK293T (human embryonic kidney cell line) and MC3T3-E1 (mouse preosteoblast cell line) were obtained from ATCC. HEK293T cells were cultured in DMEM high glucose (Sigma) with 10% FBS and 1% Antibacterial-Anti mycotic (Sigma). MC3T3-E1 cells were cultured in α-MEM (Sigma) supplemented with 10% FBS, 1% Antibacterial-Anti mycotic, and 1% MEM non-essential amino acid solution. Recombinant human core phospho-mutagenic protein-2 (rhBMP-2) was purchased from R&D Systems. DNAs were transfected in 293T with Lipofectamine 2000 while in MC3T3-E1 with Lipofectamine LTX (Invitrogen) as per the manufacturer’s protocol. For siRNA transfection, DharmaFECT transfection reagent was used per the manufacturer’s protocol (Dharmacon). ON-TARGETplus SMARTpool and human siGSK3β (L-003010-00-0005) and siFbw7 (sc37547) were purchased from Santa Cruz Biotechnology, respectively. Anti-Runx2 (Clone 1D8), anti-FLAG (Clone M2), and anti-Fbw7 (Clone 1D8), anti-FLAG (Clone M2) antibodies were purchased from Abcam. FLAG-Fbw7 (Clone 1D8) and anti-Runx2 (Clone 1D8) antibodies were used and incubated overnight at 4 °C. Slides were washed three times in PBS followed by incubation with fluorophore-tagged secondary antibodies for 1 h at room temperature. Alexa Fluor 488 donkey anti-rabbit IgG (A21206, Invitrogen) for Runx2 and Alexa Fluor chicken anti-mouse IgG (A2120, Invitrogen) for Fbw7 were used. Secondary antibodies were also prepared in 0.5% BSA in Milli-Q water. Primary antibody dilution was used according to the datasheet of the antibody. Runx2 (Abcam) and Fbw7 (Sigma) were used and incubated overnight at 4 °C. Slides were washed three times in PBS followed by incubation with fluorophore-tagged secondary antibodies for 1 h at room temperature. Alexa Fluor 488 donkey anti-rabbit IgG (A21206, Invitrogen) for Runx2 and Alexa Fluor chicken anti-mouse IgG (A2120, Invitrogen) for Fbw7 were used. Secondary antibodies were also prepared in 0.5% BSA in Milli-Q water. Slides were washed three times in PBS for 10 min each. 1 μg/ml DAPI (Sigma) was used as a nuclear stain. Slides were washed with PBS for 5 min and mounted with anti-fade chemical for subsequent confocal microscopy.

Mineralized Nodule Formation, Alkaline Phosphatase Assay, and Alizarin Staining—For mineralization studies, mouse calvarial osteoblasts were seeded in 6-well plates (20,000 cells/well) in osteoblast growth medium. After 72 h, siControl and siFbw7-transfected cells were then cultured in complete osteoblast growth medium and differentiation induction medium (DIM) containing α-MEM with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid supplemented with 10% FBS and cultured for 15 days with a change of medium every 2 days. At the end of the experiment, cells were fixed with 4% paraformaldehyde. Alizarin Red-S was used for staining of mineralized nodules followed by extraction of the stain using 10% cetylpyridinium chloride for colorimetric determination of the dye at 550 nm. Similarly, for mineralization studies in MC3T3-E1 cells, 60–70% confluent cells were plated and then transfected with
either siFbw7 or siControl and cultured in either osteoblast growth medium or DIM.

Animal Experiments and Procedures—Sprague-Dawley rats (4 months old; 220 ± 20 g) received ovariectomy (OVX) or were sham-operated. One week after surgery, rats were weight-randomized into three groups (n = 6) for 12 weeks: sham-operated (ovary intact) + vehicle, OVX + vehicle, and OVX + E2 (17β-estradiol, 5 μg/kg/day subcutaneously) (36). At the end of 12 weeks, all groups were killed, and lactating dams 10 days after parturition were also included (n = 6). Femurs were collected for protein isolation and sectioning. All animal care and experimental procedures performed were approved by Institutional Animal Ethical Committee guidelines. Sprague-Dawley rats (three per cage) were housed in temperature-controlled (22–24 °C) rooms with maintained fresh air supply, 100% air exhaust, and 60–70% relative humidity. Rooms had standard diffuse lighting (200–300 lux) with automatic maintenance of a diurnal 12-h light cycle. The animals were fed standard ad libitum (chow) diet and had free access to reverse osmosis water. The experimental procedures were approved and conducted in accordance with the Institutional Animal Ethics Committee of Council of Scientific and Industrial Research-Central Drug Research Institute (CSIR-CDRI). For ovariectomy and sham surgery, anesthesia was induced with xylazine (3–10 mg/kg) and ketamine (80–100 mg/kg) mixture (intraperitoneal injection). Euthanasia and disposal of carcass were in compliance with the Institutional Animal Ethics Committee guidelines.

Data Analysis and Statistics—Results are presented as mean ± S.E. All data were analyzed using GraphPad (San Diego, CA) software followed by Tukey’s multiple comparison test and considered statistically significant at p < 0.05. All data involving more than two groups.

Results

Fbw7 Negatively Regulates Steady State Levels of Runx2—Analysis of Runx2 protein sequence showed the presence of several potential CPD motifs that were recognized by Fbw7. Four such motifs starting at amino acid positions Ser14, Ser279, Thr305, and Thr387 matching with the CPDs in other known substrates of Fbw7 are present in Runx2 (Fig. 1a). These CPD sequences in Runx2 are conserved in all isoforms of human Runx2 as well as in mouse and rat, which prompted us to evaluate whether SCFFbw7 targets Runx2 for degradation. To this end, whole cell extracts (WCEs) of mouse preosteoblast cell line MC3T3-E1 transfected with three isoforms of Fbw7 (α, β, and γ individually) were immunoblotted with anti-Runx2 and anti-FLAG-M2 antibodies, and the results showed that all three Fbw7 isoforms substantially down-regulated endogenous Runx2 protein expression (Fig. 1b). Because Fbw7α is the dominant isoform of Fbw7, we next co-transfected Runx2 with increasing amounts of Fbw7α in HEK293T cells (hereafter 293T). Immunoblot with anti-Runx2 and anti-FLAG antibodies again showed a persistent down-regulation of Runx2 with increasing amounts of Fbw7α (Fig. 1c). This finding suggested that Fbw7α (hereby referred to as Fbw7) negatively regulated the steady state levels of endogenous as well as overexpressed Runx2.

We next determined whether Fbw7α also regulated steady state levels of Runx2 in osteoblasts. To address this, we transfected mouse preosteoblastic MC3T3-E1 cells with increasing amounts of Fbw7 in the presence or absence of proteasome inhibitors MG132 and lactacystin (LCN). Similar to 293T cells, increasing amounts of Fbw7 transfection in osteoblasts reduced endogenous Runx2 protein, whereas proteasome inhibitors abolished the inhibitory effect of Fbw7 on Runx2 protein (Fig. 1d). To further validate whether Runx2 suppression is Fbw7α-specific, we overexpressed Runx2 with Fbw7α and its non-functional deletion mutants Fbw7αΔF (which lacks the F box domain that binds directly to the SKP1 component of SCF ubiquitin ligase) and dnFbw7αWD (which contains only a stretch of WD40 repeats, the protein interaction domains in Fbw7 that bind to the substrate) in 293T cells. Contrary to wild type Fbw7α, the mutant constructs of Fbw7 instead enhanced Runx2 protein level, thus confirming that Runx2 suppression was indeed mediated by Fbw7α (Fig. 1e). Similar results were observed in MC3T3-E1 cells were transfection with wild type Fbw7 reduced endogenous Runx2 protein level, whereas the non-functional mutants stabilized it (Fig. 1f). To ascertain that Fbw7α regulates Runx2 protein stability, MC3T3-E1 cells were transfected to inhibit new protein synthesis with cycloheximide (CHX) and perfusion of endogenous Runx2 for protein isolation and western blot analysis (104–346 amino acids, two conserved CDPs) in the absence or presence of Fbw7 was assessed. Immunoblot followed by anti-FLAG showed rapid stabilization of endogenous Runx2 as well as Runx2ΔNΔC region transfected with Fbw7 (Fig. 1g), suggesting that Runx2 protein stabilization by Fbw7 was indeed due to loss of Runx2 proteasomal degradation. As an E3 ubiquitin ligase, Fbw7 is expected to target its substrate for ubiquitin-mediated proteasome degradation; we thus studied whether Fbw7 ubiquitinated Runx2. To address this, we performed in-cell ubiquitination of endogenous Runx2 by transiently overexpressing His-ubiquitin either alone or together with FLAG-Fbw7 or siFbw7 as indicated. Endogenous Runx2 co-immunoprecipitated with anti-Runx2 antibody and subsequently immunoblotted with anti-His, anti-Runx2, and anti-Fbw7 antibodies, respectively, by repeated stripping and probing the same membrane showed increased polyubiquitinated bands of Runx2 in Fbw7-overexpressed condition, whereas Fbw7 knockdown mitigated Runx2 polyubiquitination, suggesting that Fbw7 indeed promoted ubiquitin-mediated proteasome degradation of Runx2 (Fig. 1h). Taken together, these data demonstrated that Fbw7 mitigated steady state levels of Runx2 by ubiquitin-mediated proteasome degradation.

Fbw7 through Its WD Domain Physically Associates with Runx2 in Its Runx2ΔNΔC Region—Because Fbw7 is the substrate-binding moiety of the SCF-Fbw7 ubiquitin ligase complex, we therefore determined whether Fbw7 interacted with Runx2. To address this, we used wild type Runx2 (mouse isoform 5 and human isoform 2) and its deletion mutants schematically represented in Fig. 2a. Although Runx2ΔNΔC lacks N and C termini, it still contains two of the potential CDPs starting at Ser279 and Thr305 (Fig. 1a). Interestingly, Ser279 and Ser283 that are present in one of the CDPs starting at Ser359 have been shown.
to be phosphorylated by GSK3β, rendering Runx2 transcriptionally inactive (25). We therefore determined whether Fbw7 also targets this Runx2 mutant by co-transfecting it in 293T cells with Fbw7 and its mutants. Immunoblot with Runx2 and FLAG antibodies showed that wild type Fbw7 but not its mutants down-regulated both Runx2 and Runx2ΔNΔC, suggesting that CPDs present in Runx2ΔNΔC were important (Fig. 2b). However, the data did not rule out the possibility that the CPDs present in the N or C terminus of Runx2 were not involved in Fbw7-mediated targeting of Runx2. Co-transfec-
FIGURE 2. Fbw7 through its WD domain physically associates with Runx2 in its Runx2ΔNΔC region. a, Runx2 and its deletion mutants containing different regions with exact amino acids are schematically represented for both mouse Runx2 (mRunx2, isoform 5, 1–528 amino acids) and human Runx2 (hRunx2, isoform 2, 1–507 amino acids). b, Lysates of 293T cells transfected with 0.5 μg of Runx2 or Runx2ΔNΔC either alone or together with Fbw7α and its deletion mutants (Fbw7αΔΔF, Fbw7αWD) were immunoblotted (IB) with anti-FLAG-M2 and anti-Runx2 antibodies. c, Lysates of 293T cells transfected with 0.5 μg of human Runx2 or its deletion mutants (all contained two of the potential CPDs present in Runx2ΔNΔC) either alone or together with Fbw7α were immunoblotted with anti-FLAG-M2 and anti-Runx2 antibodies. d, Fbw7 co-immunoprecipitates using anti-FLAG-M2 antibody from lysates of MC3T3-E1 cells transfected with 0.5 μg of Fbw7 were immunoblotted with anti-FLAG-M2 and anti-Runx2 antibodies. Cells were treated with MG132 (10 μM) 6 h before lysate preparation. e, Fbw7 co-immunoprecipitates using anti-FLAG-M2 antibody from lysates of MC3T3-E1 cells transfected with 0.5 μg of Fbw7 were immunoblotted with anti-FLAG-M2 and anti-Runx2 antibodies. Cells were treated with MG132 (10 μM) 6 h before lysate preparation and lithium chloride (20 mM) 24 h before lysate preparation. f, Fbw7 co-immunoprecipitates from lysates of MC3T3-E1 cells transfected with 50 nM siGSK3β were immunoblotted with anti-Fbw7, anti-GSK3β, and anti-Runx2 antibodies. g, Fbw7 co-immunoprecipitates using anti-FLAG M2 antibody from lysates of 293T cells transfected with 1.0 μg of FLAG-Runx2N or FLAG-Runx2C either alone or together with FLAG-Fbw7 were immunoblotted with anti-FLAG-M2 antibody. h, i, Fbw7 co-immunoprecipitates using anti-Flag M2 antibody from lysates of HEK293T cells transfected with 1.0 μg of FLAG-Runx2N or FLAG-Runx2C either alone or together with FLAG-Fbw7 were immunoblotted with anti-FLAG-M2 antibody. j, Fbw7 co-immunoprecipitates using anti-FLAG M2 antibody from lysates of MC3T3-E1 cells transfected with 0.5 μg of FLAG-Fbw7αΔF or its deletion mutants (Fbw7αΔΔF or Fbw7αWD) were immunoblotted with anti-FLAG-M2 and anti-Runx2 antibodies. Data are representative of minimum three independent experiments.
tion of human Runx2 (human isoform 2) and its deletion mutants (these mutants also contained the two potential CPDs as present in Runx2ΔNΔC) either alone or together with Fbw7 in 293T cells showed similar results (Fig. 2c). Further, we assessed endogenous interaction between Runx2 and Fbw7 via co-immunoprecipitation of Fbw7 (anti-mouse) from WCEs of MG132-treated or untreated MC3T3-E1 cells as indicated. Immunoblot with anti-Runx2 antibody (anti-rabbit) showed a clear interaction with Fbw7 (Fig. 2d). Furthermore, we also confirmed this endogenous interaction between Runx2 and Fbw7 via co-immunoprecipitation of Fbw7 from WCEs of MC3T3-E1 cells transfected with FLAG-Fbw7 and treated with proteasome inhibitors MG132 and LiCl as indicated. Immunoblot with anti-Runx2 antibody again showed a clear interaction with Fbw7. An intense interaction was observed in conditions where cells were treated with MG132 or LiCl, which further affirmed that Fbw7 physically interacted with Runx2 under physiological conditions and regulated its protein stability in osteoblasts (Fig. 2e). To further validate the role of GSK3β in Fbw7-mediated Runx2 degradation, we transiently knocked down GSK3β (50 nM) in MC3T3-E1 cells and subsequently assessed interaction between Runx2 and Fbw7. Endogenous Fbw7 co-immunoprecipitated with anti-Fbw7 antibody followed by immunoblotting with anti-Runx2, anti-Fbw7, and anti-GSK3β antibodies, respectively, showed greater interaction of Runx2 with Fbw7 in siGSK3β-transfected cells compared with cells alone (Fig. 2f), suggesting that GSK3β rescued Runx2, leading to increased interaction between Runx2 and Fbw7. To further validate Runx2 interacting with Fbw7, we used immunoprecipitation with anti-Runx2 followed by immunoblotting with anti-Runx2, anti-Fbw7, and anti-GSK3β antibodies, respectively, showed greater interaction of Runx2 with Fbw7 when Runx2 was present in Fbw7 siRNA-treated cells (Fig. 2g). Furthermore, to rule out any physical interaction of Fbw7 with either Runx2N or Runx2C, we transfected HEK293T cells with FLAG-Runx2N or FLAG-Runx2C either alone or together with Fbw7. Fbw7 was co-immunoprecipitated with anti-Fbw7 antibody followed by immunoblotting with anti-FLAG antibody. Notably, no physical interaction was observed in either condition, suggesting that both the Runx2ΔNΔC region interacts with WD domain of Fbw7 in osteoblasts.

**GSK3β Is Required for Fbw7α-mediated Runx2 Degradation**—Several studies have previously reported that the majority of Fbw7 substrates such as KLF5 (19), c-Jun (37), c-Myc (16), cyclin E (38), Mcl (18), CCAAT-enhancer-binding protein α (C/EBPα) (39), and PGC-1α (20), having similar CPD motifs to those present in Runx2, are phosphorylated by GSK3β within their CPD motifs for recognition and subsequent degradation by Fbw7. Moreover, the general GSK3 substrate consensus sequence (STXXX(S/T)) also coincides with CPD motifs, where X is any residue (40). Furthermore, because GSK3β is known to inhibit osteoblast function by phosphorylating Runx2 (25), we next asked whether GSK3β is involved in Fbw7α-mediated Runx2 degradation. To address this, 293T cells were co-transfected with Runx2 and increasing amounts of GSK3β in the presence or absence of LiCl, the GSK3β inhibitor (41). Immunoblotting with Runx2 showed that similar to Fbw7, GSK3β dose-dependently down-regulated Runx2 protein level, whereas LiCl massively restored Runx2 protein even in the condition where GSK3β was expressed (Fig. 3a). Furthermore, constitutively active GSK3βA markedly down-regulated Runx2 (Fig. 3b), GSK3β also led to down-regulation of Runx2. Notably, the presence of increasing amounts of Fbw7α overexpression of GSK3β-mediated degradation of Runx2 (Fig. 3c), which further suggested an interdependence of Fbw7 and GSK3β-dependent, we next asked whether the Fbw7-mediated Runx2 degradation has any effect on Runx2 transactivation potential. To answer this, we performed a luciferase reporter assay using Runx2-responsive p6OSE2-Luc. We observed that co-transfection of Fbw7 with Runx2 in 293T cells strongly inhibited Runx2 transcriptional activity in a dose-dependent manner (Fig. 4a). In contrast, co-transfection of Fbw7 deletion mutants with Runx2 enhanced transactivation by Runx2. The same set of experiments when performed in osteoblastic cells (MC3T3-E1) also showed similar results (Fig. 4b). Furthermore, overexpression of Fbw7 inhibited Runx2-mediated transactivation, whereas knocking down Fbw7 led to a substantial increase in Runx2 transactivation potential as measured by luciferase activity (Fig. 4c). These data suggest that catalytically active Fbw7 negatively modulates Runx2 protein stability and thus inhibits its ability to transactivate the osteogenic target genes.
Fbw7 Negatively Regulates Runx2-dependent Osteogenesis

**Fbw7 Knockdown Restores Runx2 Expression and Enhances Osteoblast Differentiation**—Because Fbw7 negatively regulates Runx2 steady state levels by promoting its ubiquitin-mediated proteasomal degradation, we next assessed Runx2 expression and its functional consequences during osteoblast differentiation as well as upon Fbw7 knockdown in osteoblasts. Immunoblot analysis of Runx2 and Fbw7 from MC3T3-E1 cells cultured in DIM showed a sharp increase in Runx2 while showing a simultaneous decrease in Fbw7 protein expression between days 3 and 7, suggesting that down-regulation of Fbw7 is required for increased Runx2 expression (Fig. 5a). We next assessed Runx2 expression in MC3T3-E1 cells upon Fbw7 RNAi. Like in Fig. 5a, siRNA-mediated knockdown of Fbw7 led to a substantial increase in Runx2 protein expression (Fig. 5b) and significantly enhanced nodule formation by MC3T3-E1 cells even without DIM as compared with siControl (Fig. 5c, upper panel showing representative photographs). Reportedly, PI3K/Akt signaling is known to promote Runx2-dependent osteoblast differentiation by inhibiting GSK3β via its Ser9 phosphorylation (25). We hypothesized that inactivated GSK3β, in turn, fails to phosphorylate Runx2, thereby preventing its recognition and subsequent Fbw7-mediated proteasomal degradation. Moreover, increased Runx2 levels are known to up-regulate Akt protein levels, thereby regulating Runx2-dependent osteoblast differentiation (42, 43). We, therefore, determined the effect of Fbw7 depletion on GSK3β and Akt expression in MC3T3-E1 cells cultured in both growth medium and differentiation induction medium upon Fbw7 RNAi. Expectedly, Runx2 expression robustly increased in MC3T3-E1 after siFbw7 transfection under both growth-induced and differentiation-induced conditions (Fig. 5c, lower panel). We also observed enhanced Akt expression as well as increased GSK3β phosphorylation (inactive state of GSK3β) in siFbw7-transfected conditions (Fig. 5c, lower panel), suggesting that Fbw7 inhibition may restore Runx2 expression and its function. Increased Akt expression upon Fbw7 knockdown may apparently be attributed to stabilized Runx2, which is known to activate Akt (25, 27, 42, 43). Like MC3T3-E1, Fbw7 RNAi in mouse primary calvarial osteoblasts showed similar results (Fig. 5d). Fig. 5e shows Fbw7 silencing in primary cultures (Fig. 5e). These data thus suggest that Fbw7 RNAi promotes osteanabolic molecules (Runx2 and Akt) and antagonizes an anti-osteoblastic molecule (GSK3β), leading to osteogenesis.

Furthermore, BMP-2, an inducer of osteoblast differentiation, increased Runx2 with concomitant decrease in Fbw7 expression in MC3T3-E1 cells (Fig. 5f). In contrast, dexamethasone, an inducer of osteoblast apoptosis, resulted in decreased Runx2 and concomitantly increased Fbw7 levels in MC3T3-E1 cells.
Taken together, these data suggest that Fbw7 is a negative regulator of osteoblast differentiation and survival.

Reciprocal Relationship between Runx2 and Fbw7 in Vivo—Because our data so far indicated that Fbw7 was a negative regulator of osteoblast differentiation, we examined Fbw7 expression under the conditions of bone loss, viz. OVX and lactation (45). Bone sections from sham (ovary-intact) rats showed copious Runx2 but low levels of Fbw7 immunoreactivity (Fig. 6a). This pattern was reversed in the bone sections of lactating and ovariectomized rats, where Runx2 immunoreactivity was reduced, whereas Fbw7 was increased. E2 supplementation to ovariectomized rats resulted in reversal of the patterns of these two reciprocally expressed proteins and restored their expressions to that observed in the sham group (Fig. 6a).

Discussion

Although the regulation of Runx2 at the transcriptional level is well studied, its regulation at the protein levels is poorly understood. In the present study, we have identified the tumor suppressor Fbw7 as a novel regulator of Runx2. From several lines of experiments, it appears that the presence of multiple CPD motifs within Runx2 renders it a substrate for Fbw7. Furthermore, reduced protein stability of Runx2 and Runx2ΔNΔC in MC3T3-E1 cells co-transfected with Fbw7 and treated with cycloheximide indicated the involvement of Fbw7 ligase activ-
ity in down-regulation of Runx2 protein expression. As with other known substrates, Fbw7 through its substrate-interacting domain (WD repeats) interacted with Runx2 in its ΔNΔC region, which still contains two putative CPD domains (16, 19). Our data demonstrated that CPD starting at Ser279 might be crucial for this interaction. However, further investigation is required to confirm this. Nonetheless, as no interaction between Fbw7 and either of the Runx2 deletion mutants Runx2N or Runx2C was observed (Fig. 2, h and i), it is quite likely that the CPDs present in the ΔNΔC region of Runx2 are involved in its interaction and subsequent degradation by Fbw7.
As phosphorylation of CPD motifs within the substrates is required for their degradation by Fbw7 (16), we also demonstrate that GSK3β, a serine/threonine kinase, may phosphorylate Runx2 in its CPD motifs, leading to its degradation by Fbw7 based on two criteria. First, the majority of the known Fbw7 substrates having CPDs similar to Runx2 are phosphorylated by GSK3β. Second, GSK3β controls osteogenesis through regulating Runx2 activity (25). Our finding that GSK3β RNAi rescues degradation of Runx2 even in the presence of overexpressed Fbw7 does strengthen the notion that GSK3β apparently phosphorylated Runx2 to be recognized and degraded by Fbw7. This finding also gained support from previous studies showing that GSK3β inhibited DNA binding and transcriptional activity of Runx2 by phosphorylating Runx2 in GSK3β consensus sequence present at Ser369-Ser373-Ser377 (SPPWSYDQS) in mouse Runx2 isoform 5 (25). Interest-

**FIGURE 6.** Reciprocal relationship between Runx2 and Fbw7 in vivo. a, cross-sections (5 μm) were made from decalcified femur epiphysis of various groups as indicated, and immunofluorescence (40X confocal) was performed as indicated under “Materials and Methods” using anti-Fbw7 (mouse) and anti-Runx2 (rabbit) primary antibodies and anti-rabbit (Alexa Fluor 488) and anti-mouse (Alexa Fluor 594) secondary antibodies. Sham, ovary intact; Ovx, bilateral Ovx and maintained for 12 weeks; Ovx + E2, Ovx supplemented with 17β-estradiol (5 μg/kg/day subcutaneously). For the lactation group, rats 10 days after delivery were taken. b, protein extracts isolated from femurs (devoid of bone marrow) from various groups as in panel A were immunoblotted with anti-Runx2 and anti-Fbw7 antibodies. 293T cells transfected with or without Runx2 were used as positive and negative control, respectively, for Runx2 immunoblot, whereas MC3T3-E1 served as endogenous positive control. c, in 2-day-old BALB/c mice, MP (2 mg/kg/day subcutaneously) was injected for 4 and 8 days, and mice were subsequently sacrificed. Calvariae were lysed, and total protein was resolved on 12% SDS-PAGE followed by immunoblotting with anti-Fbw7 and anti-Runx2 antibodies. β-Actin was probed as a loading control. d, based on our study, schematic diagram depicting regulation of Runx2 protein stability by Fbw7 under physiological and bone loss conditions. Data are representative of minimum three independent experiments.
Fbw7 Negatively Regulates Runx2-dependent Osteogenesis

By analyzing the transcriptional regulation and protein stability of Runx2, we found that Fbw7 negatively regulates Runx2-dependent osteogenesis. Our studies suggest that Fbw7 plays a key role in maintaining bone mass by targeting Runx2 for ubiquitin-mediated degradation.

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