Trp-Asp (WD) Repeat Domain 1 Is Essential for Mouse Peri-implantation Development and Regulates Cofilin Phosphorylation*

From the ‡State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, 100101 Beijing, the §Institute of Zoology, University of Chinese Academy of Sciences, Beijing 100049, the ¶State Key Laboratory of Pharmaceutical Biotechnology and MOE Key Laboratory of Model Animals for Disease Study, Model Animal Research Center, Nanjing University, Nanjing 210061, and the †Key Laboratory of Urban Agriculture (North) of Ministry of Agriculture, College of Biological Science and Engineering, Beijing University of Agriculture, Beijing 102206, China

Received for publication, September 21, 2016, and in revised form, December 16, 2016 Published, JBC Papers in Press, December 19, 2016, DOI 10.1074/jbc.M116.759886

Yi Xiao‡, Haixia Ma§, Ping Wan†, Dandan Qin‡, Xiaoxiao Wang‡§, Xiaoxin Zhang§, Yunlong Xiang§, Wenbo Liu§, Jiong Chen§, Zhaozhong Yi‡, and Lei Li‡§

Edited by Xiao-Fan Wang

Trp-Asp (WD) repeat domain 1 (WDR1) is a highly conserved actin-binding protein across all eukaryotes and is involved in numerous actin-based processes by accelerating Cofilin severing actin filament. However, the function and the mechanism of WDR1 in mammalian early development are still largely unclear. We now report that WDR1 is essential for mouse peri-implantation development and regulates Cofilin phosphorylation in mouse cells. The disruption of maternal WDR1 does not obviously affect ovulation and female fertility. However, depletion of zygotic WDR1 results in embryonic lethality at the peri-implantation stage. In WDR1 knock-out cells, we found that WDR1 regulates Cofilin phosphorylation. Interestingly, WDR1 is overdosed to regulate Cofilin phosphorylation in mouse cells. Furthermore, we showed that WDR1 interacts with Lim domain kinase 1 (LIMK1), a well known phosphorylation kinase of Cofilin. Altogether, our results provide new insights into the role and mechanism of WDR1 in physiological conditions.

The actin cytoskeleton is a highly dynamic structure that regulates cell motility, adhesion, division, and growth and has a fundamental function in embryonic development (1–3). In physiological conditions, actin filaments are continually assembled and disassembled in response to varied cellular activities (4, 5). The dynamics of F-actin is well orchestrated by a large number of actin-binding proteins (5, 6). Actin-depolymerizing factor Cofilin plays critical roles in the modulation of actin cytoskeleton dynamics (7). In vivo, Cofilin severs actin filament at low concentrations, whereas it fully decorates actin filaments and suppresses the severing activity at high concentrations (8, 9). In addition, Cofilin severing F-actin is not explained well for how the filaments of actin cytoskeleton can be rapidly disassembled in physiological conditions (4). Thus, the function of Cofilin in actin dynamics depends not only on its relative concentration to actin, but also on other regulatory proteins in vivo. Dysfunction of these regulating proteins results in aberrant actin cytoskeleton in various cellular and developmental processes (10–12).

Cofilin directly binds with actin to function as a regulator of the actin dynamics (13). However, the phosphorylation of Cofilin at Ser-3 blocks its binding site to actin (14, 15). Thus, the activity of Cofilin is controlled by phosphorylation and dephosphorylation processes, which are regulated by cascade reactions of several protein kinases and phosphatases. Lim domain kinases (LIMKs)4 and testis-specific protein kinases (TESKs) are responsible for the phosphorylation of Cofilin, whereas slingshot (SSH) family protein phosphatases and pyridoxal phosphate phosphatase (PDXP) catalyze the dephosphorylation reaction (16–20). These kinases and phosphatases are also regulated by their phosphorylation or localization in cytoplasm to maintain the level of Cofilin phosphorylation in response to extracellular stimuli (21–23).

The activity of Cofilin on actin disassembly is greatly accelerated by other actin-binding proteins (24, 25). WDR1 (WD40 domain repeat 1, also known as actin interaction protein 1), is conserved and was originally identified as an actin-binding protein by a yeast two-hybrid screen (26). WDR1 is involved in actin dynamics by promoting the actin disassembly activity of Cofilin. Mutations in WDR1 result in abnormal cytoskeletal behavior in many organisms (27–29). A Gene-trap vector inserted Wdr1 intron 2 disrupts its expression completely and results in embryonic lethality before E10.5 (embryonic day

4 The abbreviations used are: LIMK, Lim domain kinase; WDR1, Trp-Asp (WD) repeat domain 1; LIMK1, Lim domain kinase 1; TESKs, testis-specific protein kinases; SSH, slingshot; PDXP, pyridoxal phosphate; GV, germinal vesicle; MI, metaphase II; EB, embryoid body; IP, immunoprecipitation; oKd, oocyte-specific knock-out; MEF, mouse embryonic fibroblast; PI, pro-pidium iodide; iKO, inducible knock-out; LIF, leukemia inhibitory factor; EGF, enhanced green fluorescent protein; EdU, 5-ethynyldeoxyuridine; CTL, control.
WDR1 Functions in Cofilin Activity and Embryo Development

10.5) in mice, whereas a hypomorphic insertion mutation in this gene leads to autoinflammatory disease because of the impairment of cytoskeletal response in neutrophils and thrombopoiesis (27). WDR1-mediated actin disassembly is also required for spermatogenesis and myocardial growth in mice (30, 31). In humans, the aberrant expressions of WDR1 have been shown to be connected to several diseases, including gout, pancreatitis, and primary glioblastoma (32–34). Although WDR1 is critical for numerous physiological conditions, how it acts in early embryonic development remains largely unclear.

In our study, we demonstrated that zygotic, but not maternal, expression of WDR1 is important for mouse early embryonic development. We showed that WDR1 is essential for mouse peri-implantation development. Most interestingly, we found that WDR1 regulates the level of Cofilin phosphorylation through LIMK, a novel mechanism of WDR1 involved in Cofilin activity.

Results

Zygotic, Not Maternal, WDR1 Is Required for Mouse Development—To investigate the function of Wdr1 in oocyte maturation and pre-implantation embryogenesis, we first examined the expression of Wdr1 in mouse oocytes and pre-implantation embryos by quantitative RT-PCR and Western blotting analysis. Our results showed that Wdr1 mRNA was highly expressed in mouse germinal vesicle (GV) oocytes, decreased in metaphase II (MII) oocytes, 1-cell, 2-cell, and morula stage embryos, and then sharply increased in blastocyst stage embryos (Fig. 1A). By comparison, Western blotting analysis showed that WDR1 protein was comparatively highly expressed in GV oocytes, decreased in MII oocytes, increased in 1-cell and 2-cell embryos, and decreased in morula and blastocyst stage embryos again (Fig. 1B).

We generated Wdr1 oocyte-specific knock-out mice by mating Wdr1<sup>fl/fl</sup> mice with Zp3-cre transgenic mice to study the role of WDR1 in female fertility. The mRNA and protein of Wdr1 were dramatically decreased in the GV oocytes from Wdr1<sup>fl/fl</sup>; Zp3-cre (oocyte-specific knock-out, oKO) females as noted by quantitative RT-PCR and Western blotting analysis (Fig. 1, C and D). However, Wdr1<sup>fl/fl</sup>; Zp3-cre females delivered comparable offspring compared with the controls (Wdr1<sup>fl/fl</sup> and Wdr1<sup>fl/fl</sup>; Zp3-cre females) after mating with Wdr1<sup>fl/fl</sup> males (Fig. 1E). These data demonstrated that maternal WDR1 is not required for Wdr1<sup>fl/fl</sup>; Zp3-cre female fertility.

We also mated Wdr1<sup>fl/fl</sup>; Zp3-cre females with Wdr1<sup>−/−</sup> males. The litter size was decreased to about 50% (Fig. 1E), indicating that Wdr1 KO embryos was lost during embryonic development, consistent with a previous report in which severe loss function of WDR1 resulted in embryonic lethality before E10.5 (27). Thus, these results suggested that zygotic, but not maternal, expression of Wdr1 is required for mouse development.

WDR1 Is Essential for Mouse Peri-implantation Development—To examine the causes and time point of Wdr1 KO embryo lethality, Wdr1<sup>fl/fl</sup>; Zp3-cre and Wdr1<sup>fl/fl</sup> female mice mated with Wdr1<sup>−/−</sup> male mice and the deciduals were analyzed at E5.5. Our results showed that the number of deciduals per litter was 7–10 in both groups and were not obviously different from each other (Fig. 2A). However, after sectioning and H&E staining of these decidua, 53% of the deciduals from Wdr1<sup>fl/fl</sup>; Zp3-cre females had normal embryos (Fig. 2B (a)), and 47% had no embryos (Fig. 2B (b)), whereas the percentage of empty deciduals from Wdr1<sup>fl/fl</sup> females (control) was only 3% (Fig. 2B, right panel). These data indicated that the blastocysts lacking WDR1 were able to implant and induce the decidualization, but were absorbed at E5.5, suggesting that WDR1 is essential for mouse peri-implantation development.

To further investigate Wdr1 KO embryonic development, blastocysts were obtained from the uteruses of the Wdr1<sup>fl/fl</sup>; Zp3-cre females after mating with Wdr1<sup>−/−</sup> males at E3.5 and cultured in vitro. KO and control blastocysts could not be distinguished at E3.5 (Fig. 2C). All blastocysts grew, hatched, and adhered to the dish during the first 2 days of culturing. However, 55% (11 of 20) of embryos grew obviously smaller at the third day. No EdU incorporation was detected in the smaller embryos (Fig. 2D). We then examined the genotypes of these embryos by PCR with specific primers and found that the genotype of 81% (9 of 11) of the smaller embryos were Wdr1<sup>−/−</sup> (Fig. 2E). These data suggest loss of WDR1 has no obvious effect on embryo growth.
WDR1 Functions in Cofilin Activity and Embryo Development

Wdr1 KO embryos in peri-implantation development. A, after mating with Wdr1f/f males, oocyte specific knock-out females were injected with Chicago blue. Then, the uteruses were picked up at E5.5 and imaged by differential interference contrast. Scale bar, 5 mm. B, deciduous with embryos from A were frozen and sectioned (8 μm). The sections were stained by H&E and imaged. Two types such as (a) and (b) were observed. The percentage of the two types was counted from three pregnant females. Statistical results are represented as mean ± S.D. Scale bar, 50 μm. C, oocyte-specific Wdr1 KO females were mated with Wdr1f/f males. At E3.5, blastocysts were flushed from the uterus and imaged by differential interference contrast. Scale bar, 40 μm. D, blastocysts obtained from C were cultured in vitro for 3 days and treated with EdU for 2 h before being fixed. The outgrowth was stained for EdU (red) and Cofilin (P-Cofilin) by Western blotting analysis according to the protocol provided by the manufacturer. Nuclear DNA was stained by Hoechst 33342 (blue). Scale bar, 200 μm. E, Wdr1f/f (control, CTL) and Wdr1f/f (knock-out, KO) embryos were genotyped with specific primers.

on blastocyst development, but cause the arrested development after adherence in vitro.

Wdr1 Knock-out Decreases Cofilin Phosphorylation in Mouse ES Cells—To investigate WDR1 functions in early embryos, we established ES cell lines from Wdr1f/f (control, CTL) and Wdr1f/f (knock-out, KO) blastocysts from Wdr1f/f, Zp3-cre females after mating with Wdr1f/f males. The Wdr1 mRNA and protein depletion were examined by real-time RT-PCR and Western blotting analysis in these ES cell lines (Fig. 3, A and B). Wdr1 KO ES cells could be normally maintained in a 2i/leukemia inhibitory factor (LIF) medium and form typical clones (Fig. 3D). The expression of pluripotent markers such as NANOG, OCT4, and SOX2, and the cells proliferation were comparable in CTL and KO ES (Fig. 3, B and C).

To investigate their differentiation potentials, CTL and KO ES cells were labeled with EGFP and injected into normal blastocysts. These blastocysts were cultured for 3 days in an in vitro system of growing whole embryos. Our results showed that both CTL and KO ES cells incorporated into the cultured embryos detected by EGFP (Fig. 3D). The defect of Wdr1 disruption on differentiation was further examined by embryoid bodies (EB) formation system, a model of ESC differentiation into germ layer lineages. Compared with their expressions at day 0 (d0), germ layer makers (Sex17, endoderm; T, mesoderm; Pax6, ectoderm) were comparably up-regulated in both CTL and KO EB at day 3 (d3) (Fig. 3E). These results indicate that WDR1 depletion may not affect the ES cells potential of differentiation.

Previous reports showed that WDR1 partial dysfunction (decreased to 20%) affected actin structure and depolarized Cofilin localization in migrating cells (27). Then, we examined F-actin and Cofilin in Wdr1 KO ES cells by immunofluorescence. F-actin (red) was concentrated in the membrane in both knock-out ES cells and controls (Fig. 3F). The detailed actin structure could not be observed clearly in these cells. Surprisingly, different from WDR1 dysfunction in migrating cells (27), Cofilin was more concentrated to the membrane and co-located with actin filaments in Wdr1 KO ES cells, whereas it was more diffused in control (Fig. 3F). We also investigated phosphorylated Cofilin (P-Cofilin) by Western blotting analysis. Our results showed that the P-Cofilin level was much lower in Wdr1 KO ES cells than that in control (Fig. 3G). Then, we separated G-actin and F-actin from KO and control ES cells (35). Distinct from a previous report in Wdr1 knockdown cells (36), F-actin level was not obviously increased in Wdr1 KO ES cells as compared with the control (Fig. 3H). Altogether, Wdr1 KO has no obvious impact on ES pluripotency and growth, but results in dramatically decreased P-Cofilin in these cells.

WDR1 Regulates the Phosphorylation of Cofilin and Controls F-actin in Mouse Embryonic Fibroblast (MEF) Cells—Interestingly, WDR1 expression is much higher in MEF cells than that in ES cells (Fig. 4A). To further investigate the effects of WDR1 on actin cytoskeleton, knock-out MEF cell lines were established by a tamoxifen induction system. Wdr1f/f; Cre-ER™ fetuses were used to establish inducible knock-out (iKO) MEF
lines, using Wdr1+/−; Cre-ERTM MEF cells as the control (CTL). After 72 h treatment with tamoxifen, Wdr1 mRNA and protein were decreased to 0.3 ± 0.018% and 4.5 ± 0.2%, respectively, compared with control (CTL) (Fig. 4, B and C). Actin plaques were observed in Wdr1 iKO MEF cells, whereas the clear linear F-actin structure was shown in the control (Fig. 4D). However, we did not detect a significant increase in the overall F-actin level in Wdr1 iKO MEF cells (Fig. 4E). P-Cofilin was observed to be dramatically decreased in Wdr1 iKO MEF cells (Fig. 4F). We also examined the cell cycle by flow cytometry. Our results showed that control MEF cells had complete G1, S, and G2 cell stages, whereas Wdr1 iKO MEF cells lost S stage and expanded G2 stage (Fig. 4G). These data suggested that the WDR1 loss affected MEF cell proliferation. Altogether, our results indicate that WDR1 regulates the formation of special actin plaques, the proliferation, and Cofilin phosphorylation in MEF cells.

**WDR1 Regulates Phosphorylation of Cofilin in a Dose-independent Manner**—The dramatically decreased P-Cofilin in Wdr1 KO cells was inconsistent with a previous report for Wdr1 knockdown cells (36), suggesting WDR1 might be overdosed in cells to regulate Cofilin phosphorylation. To test this hypothesis, we used the tamoxifen induction system to gradually reduce WDR1 content and examined the level of P-Cofilin during this process. WDR1, P-Cofilin, and Cofilin were detected in Wdr1f+/−/H11002; Cre-ERTM and control MEF cells after 0, 24, 48, and 72 h tamoxifen treatments. In Wdr1f+/−/H11002; Cre-ERTM MEF cells, WDR1 protein gradually reduced at the time course manner and almost failed to be detected at 72 h (Fig. 5A). Although the protein levels of WDR1 were obviously decreased at 24 and 48 h, P-Cofilin was comparable with the control at these time points. The decreased P-Cofilin was only clearly observed at 72 h after tamoxifen treatment (Fig. 5A). Consis-

**FIGURE 3. Characterization of Wdr1 KO ES cells.** A, total RNA samples from Wdr1+/− (control, CTL) and Wdr1−/− (knock-out, KO) ES were subjected to real-time RT-PCR to examine Wdr1 transcription. Gapdh mRNA was used as a control. B, WDR1 and pluripotential markers NANOG, OCT4, and SOX2 were analyzed by Western blotting in CTL and KO ES cell lines, with GAPDH as a loading control. C, two KO and two CTL ES cell lines were cultured in a 12-well plate and the cells were counted every 24 h. Three wells for each cell line were examined at each time point. D, CTL and KO ES cells were labeled with EGFP and injected into normal blastocysts. After culturing 3 days, the embryos were examined by microscopy. Scale bar, 20 μm. E, Pax6, T, and Sox17 transcription were examined by real-time RT-PCR in CTL and KO ES cells. Gapdh mRNA was used as a control. F, both CTL and Wdr1 KO ES cells were fixed and incubated with a primary antibody to Cofilin. Then, these cells were incubated with FITC-tagged second antibody (Cofilin, green), Alexa Fluor® 546 Phalloidin (F-actin, red), and Hoechst 33342 (DNA, blue), and imaged. Scale bar, 20 μm. G, P-Cofilin and total Cofilin were detected by immunoblotting in CTL and Wdr1 KO ES cells. GAPDH was used as a loading control. H, G-actin and F-actin were separated in control and Wdr1 KO ES cell lines and probed with mouse anti-actin antibody.
WDR1 Functions in Cofilin Activity and Embryo Development

FIGURE 4. Characterization of Wdr1 mutant MEF cells. A, WDR1 was analyzed by Western blotting in ES and MEF cells, with GAPDH as a loading control. B, Wdr1 mRNA was detected by real-time RT-PCR in Wdr1+/−; Cre-ERT2 (control, CTL) and Wdr1−/−; Cre-ERT2 (KO) MEF cells after being treated with tamoxifen for 72 h. C, immunoblot analyzed CTL and iKO MEF cells using WDR1 and GAPDH (loading control) antibodies. D, CTL and iKO MEF cells were stained by Alexa Fluor® 546 Phalloidin (F-actin, red) and Hoechst 33342 (DNA, blue), and imaged. Scale bar, 80 μm. E, G-actin and F-actin in CTL and iKO MEF cells were separated and probed by Western blotting with actin antibody. F, P-Cofilin, total-Cofilin, and WDR1 were detected in CTL and iKO MEF cells by Western blotting, using GAPDH as a loading control. G, after being treated with tamoxifen for 72 h, CTL and iKO MEF cells were digested, stained with PI, and examined by flow cytometry.

...tently, the actin plaques were obviously observed at 72 h, but could not be found at 24 h and only a small number of actin plaques were observed at 48 h (Fig. 5B). These results suggest that WDR1 regulating Cofilin phosphorylation is overdosed in cells.

To further examine the regulation of WDR1 on Cofilin phosphorylation, WDR1 was overexpressed in both ES and MEF cells. Cofilin phosphorylation was not obviously different in both WDR1 overexpressed and control cells (Fig. 5, C and D). When WDR1 was re-expressed in knock-out ES cells, P-Cofilin was obviously increased and comparable with the control (Fig. 5E).

WDR1 Regulates Cofilin Phosphorylation via LIMK1—To investigate how WDR1 regulates Cofilin phosphorylation, WDR1 with a FLAG tag was overexpressed in MEF cells and an immunoprecipitation (IP) experiment was performed with FLAG antibody. Our results showed that WDR1 precipitated LIMK1, but not SSH1, PDXP, or TESK1 (Fig. 6A). We further docked the binding domains of LIMK1 in 293T cells (Fig. 6B). Our results showed that LIMK1−FL and LIMK1−415, but not LIMK1−766, pulled down WDR1 (Fig. 6B). We also examined the levels of P-LIMK1 and total LIMK1 in control and Wdr1 KO MEF cells. Our results showed that both P-LIMK1 and total LIMK1 levels were almost similar in control and KO MEF cells (Fig. 6C).

Previous reports showed that LIMK1 activity was inhibited by binding with BMP-RII (bone morphogenetic proteins receptor II) or microtubules (21, 37, 38). Because the LIMK1 antibody did not work well for immunostaining, LIMK1 with the Myc tag was overexpressed in control and Wdr1 KO MEF cells to examine whether WDR1 regulates LIMK1 by changing its localization. Our results showed that LIMK1 was diffused in both control and KO MEF cells, but was more concentrated to microtubules in Wdr1 KO MEF cells. We then treated the MEF cells to lightly damage the microtubule structure with a small dosage of nocodazol (1.25 μg/ml), a microtubules depolymerization reagent, and examined P-Cofilin by Western blotting analysis in these cells. Our results showed that P-Cofilin was up-regulated in Wdr1 KO MEF cells, but was virtually unchanged in the controls (Fig. 6F). Our IP experiment showed that WDR1 interacts with the PDZ domain of LIMK1, which is a microtubule binding domain (17). Thus, we concluded that WDR1 might regulate LIMK1 function by inhibiting its binding to microtubules.

Lacking of WDR1 Decreases Cofilin Phosphorylation and Disturbs Actin Structure in Both Mouse Blastocyst Outgrowth and Oocytes—To examine effects of WDR1 loss on F-actin and Cofilin phosphorylation during embryonic development, the blastocysts cultured in vitro for 3 days were stained by phalloidin. Actin filaments were orderly and homogeneous in the control (CTL), whereas random actin plaques were observed in Wdr1−/− (knock-out, KO) blastocyst outgrowth (Fig. 7A). The P-Cofilin level in KO outgrowth was much lower than that in control (Fig. 7B). F-actin and P-Cofilin were also examined in mouse GV oocytes by staining and Western blotting analysis. Our results showed that actin was concentrated to some small plaques in the cytoplasm of Wdr1−/− (oKO) GV oocytes (Fig. 7C, arrowheads), and uniformly distributed in Wdr1+/+ (CTL) (Fig. 7C). P-Cofilin was also dramatically decreased in Wdr1−/− GV oocytes compared with the control (Fig. 7D).

Discussion

WDR1 and its involvement in actin cytoskeleton play important roles in multiple processes during mouse development (27, 30, 31). However, the role of WDR1 in mouse early embryogen-
esis is still largely unknown. In the present study, we showed
that depletion of Wdr1 in oocytes by Zp3-cre has no impact on
ovulation and pre-implantation embryogenesis, and the
oocyte-specific KO females deliver offspring comparable with
the controls when they are mated with wild type males. Fur-
thermore, Wdr1 KO ES cells can be established and have no
obvious defects in pluripotency and proliferation. Mouse
embryos depleted with zygotic Wdr1 are lost at a very early
stage of development at E5.5, although these embryos can
implant and induce decidualization. Consistently, Wdr1
KO blastocysts can hatch and adhere, but have impaired outgrowth
and cannot be labeled with EdU, accompanying the severe
defects of actin cytoskeleton. Wdr1 KO MEF cells have growth
defects such as losing S phase and abnormal actin plaques, sim-
ilar to the phenotypes observed in Wdr1−/−; Cre-ERTM and Wdr1f−/−; Cre-ERTM MEF cells (30, 31). For successful develop-
ment, mouse trophoblast cells dramatically differentiate and
proliferate, accompanying tremendous changes in cytoskeleton
after implantation (39, 40). Although the exact reason that
WDR1 loss of function results in the embryonic arrest at E5.5 is
unclear, the role of WDR1 in actin cytoskeleton might be crit-
ical in the differentiation and growth of trophoblast cells. Thus,
the function of WDR1 regulating actin disassembly might be a
cell-type specific manner for cell proliferation and survival.

Taken together, our results demonstrate that WDR1 plays
essential roles in mouse peri-implantation development.

Cofilin is important for spatial and temporal regulation of
F-actin dynamics in cells and its activity is tightly controlled by
several mechanisms (7, 41, 42). However, which mechanism is
the most important process for Cofilin severing activity is still
unclear. Cofilin binding and severing F-actin activity is de-
pendent upon its phosphorylation, which is tightly regulated by
the protein kinases and phosphatases in vivo, such as LIMK
and SSH (15, 42). In response to the stimuli, the kinases
phosphorylate, whereas the phosphatases dephosphorylate
Cofilin to control its interaction with actin for optimal sev-
ering activity to regulate the dynamics of actin cytoskeleton
(14, 44). In addition, some small molecules or actin-binding
protein ligands can competitively displace Cofilin to
decrease the binding density of this protein on F-actin into a
range that favors severing activity (43, 44). These results sug-
gest that the regulation of Cofilin binding to F-actin is controlled
by multiple mechanisms and might be the primary process for
Cofilin severing activity in vivo.

Most studies have shown that WDR1 significantly acceler-
ated Cofilin dissembling of actin cytoskeleton. However, how
WDR1 regulates Cofilin severing activity remains obscure. In
the current study, we established Wdr1 KO ES, MEF cells,
oocytes, and early embryos to investigate the regulation of WDR1 on Cofilin. Unexpectedly, we found that loss of WDR1 down-regulates P-Cofilin in all examined cells. Consistent with this, a very recent report showed that P-Cofilin is decreased in the cells of some patients with \textit{Wdr1} mutation (45). Interestingly, WDR1 seems to be present in an excessive amount in the cells, which well explains the discrepancies of Cofilin phosphorylation levels observed in knockdown and KO cells (36). We also found that WDR1 interacts with the LIMK1 PDZ region, which was reported to bind to microtubule (17). In addition, LIMK1 is concentrated to the microtubules in \textit{Wdr1} KO MEF cells and the disassembly of microtubules can partly rescue the phosphorylated Cofilin in these cells. Previous reports show that the PDZ domain is a cytoskeleton binding domain and the PDZ domain can localize LIMK1 to the microtubules (21, 37, 38). Thus, these results suggest that WDR1 might inhibit the binding of LIMK1 to microtubules and release LIMK1 to the cytoplasm to catalyze Cofilin phosphorylation, therefore regulating the binding of F-actin to Cofilin.

Previous results show that WDR1 can bind to Cofilin-actin and induces F-actin structural changes to accelerate Cofilin severing F-actin activity (9). In addition, another report shows that WDR1 might simply compete with Cofilin and dissociate redundant Cofilin from F-actin to accelerate severing activity (44). Furthermore, this regulation of Cofilin severing activity is dependent on the pH (46). In our study, we found WDR1 regulated Cofilin phosphorylation through LIMK1 to control the binding of Cofilin with actin filaments. Thus, WDR1 regulates Cofilin severing F-actin activity at multiple layers, suggesting the interrelationship of WDR1 and Cofilin is very complex \textit{in vivo}.

It is worth mentioning that WDR1 depletion decreases the level of Cofilin phosphorylation and disrupts the actin structure in mouse oocytes, but does not affect oocyte maturation and fertilization. Similar observations are also reported in some other cytoskeleton relative proteins in mouse oocytes (47). These results indicate mouse oocytes and very early embryos might have tolerance to some level of actin deficiency. Taking together, our results demonstrate that WDR1 is essential for...
mouse peri-implantation development and regulates Cofilin phosphorylation through LIMK1 to optimize its F-actin severing activity in vivo.

**Experimental Procedures**

**Mice Maintenance**—In compliance with the guidelines of the Animal Care and Use Committee of the Institute of Zoology at the Chinese Academy of Sciences, the Wdr1f/f; Zp3-cre and Wdr1f/f; Cre-ERTM mouse lines were maintained on a mixed background (129/C57BL/6). A pair of Flox sequences was inserted in both ends of the fourth exon of Wdr1 to establish Wdr1f/f mice as previously described (31). Zp3-cre mice (48) were mated with Wdr1f/f mice to obtain the oocyte-specific Wdr1 knock-out females. To establish Wdr1 inducible knock-out MEF cell lines, Wdr1f/f mice were mated with Cre-ERTM transgenic mice (49) to obtain Wdr1f/f; Cre-ERTM offspring. E13.5 fetuses from the mating of Wdr1f/f; Cre-ERTM females with Wdr1f/f males were used to derive the MEF cells. All mice were maintained in SPF animal houses with a 12-h light/12-h dark cycle. Mouse tail tip or cells were lysed by an alkaline lysate buffer at 95 °C for 1 h. Then genotyping PCR was conducted with specific primers (Table 1).

**Collection and Culture of Mouse Eggs and Embryos**—GV oocytes were collected using M199 medium from ovaries. After female mice were stimulated by injecting PMSG and hCG (Ningbo Second Hormone Factory), metaphase II (MII) oocytes were collected in M2 medium from oviducts after hCG 13 h; 1-cell, 2-cell embryos, and morulas were flushed from oviducts at E0.5, E1.5, and E2.5 days; and blastocysts were collected from uteruses at E3.5. Blastocysts were cultured in 1066 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 µg/ml of penicillin/streptomycin at 37 °C in 5% CO₂ (50, 51). The culture dishes for blastocysts were pre-treated with 0.2% gelatin. For the E5.5 embryos section, the deciduas were frozen at −80 °C.

**Cell Culturing**—Wdr1 inducible knock-out MEF cell lines were derived from Wdr1f/f; Cre-ERTM fetuses at E13.5 and Wdr1f/f; Cre-ERTM fetuses as the control. Tamoxifen was added to the medium to induce the Wdr1 knock-out. The 293T cell line was cultured in DMEM with 10% FBS.

Wdr1 knock-out ES cell lines were established from Wdr1f/f blastocysts as in a previous report (52), Wdr1f/f ES cells were used as control. Briefly, blastocysts were cultured in 0.2% gelatin-pretreated dishes in N2B27 medium supplemented with PD0325901 (1 µM, catalogue number P-9688, LC Laboratories), CHIR99021 (3 µM, catalogue number C-6556, LC Laboratories), and LIF (1000 units/ml, catalogue number...
ESG1107, Millipore) (2i/LIF medium) for 5 days. Then, the inner cell mass clones were digested by trypsin substitution and planted onto MEF cell feeders in 2i/LIF medium or DMEM containing 15% fetal bovine serum, 2 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 μg/ml of penicillin/streptomycin, 1 mM β-mercaptoethanol, and 10 ng/ml of LIF (ES serum medium). For cell number counting, ES cells cultured on gelatin were digested to single cells and resuspended in DMEM with 15% FBS, 2 mM sodium pyruvate, and 0.1 mM nonessential amino acids, and then cultured with regular medium. For protein expression, MEF or ES cells were incubated with lentivirus for 12 h and then cultured by seeding 600 cells in dishes for 3 days. ES cells in the chimeras were detected by injection pipette. The injected blastocysts were cultured in gelatin-pretreated hanging droplet of ES serum medium without LIF. ES cells were labeled with EGFP, and injected into one blastocyst cavity by an injection pipette. Blastocysts were collected from the uterus of pregnant recipient mice on day 9 of pregnancy. The injected blastocysts were cultured in gelatin-pretreated M2 medium and kept on ice. Blastocysts were collected from the uterus of pregnant recipient mice on day 9 of pregnancy. After collection, blastocysts were transferred to M2 medium without LIF to recover from the cold shock. The recovered blastocysts were cultured in M2 medium for 3 days and then transferred to M2 medium with 15% FBS. ES cells cultured on gelatin were digested to single cells and counted in a blood counting chamber. All cell lines were cultured at 37 °C in 5% CO2.

ES Cell Differentiation—ES cells were labeled with EGFP, digested to single cells, resuspended in DMEM with 15% FBS, and kept on ice. Blastocysts were collected from the uterus of E3.5 superovulated ICR females. 10–15 ES cells were picked up and injected into one blastocyst cavity by an injection pipette. The injected blastocysts were cultured in gelatin-pretreated dishes for 3 days. ES cells in the chimeras were detected by fluorescence confocal microscopy. For EB formation, ES cells were digested to single cells and cultured by seeding 600 cells in one 30-μl hanging droplet of ES serum medium without LIF.

Real-time RT-PCR—Total RNA was extracted from cells with the RNAzol® RT RNA Isolation Reagent (Molecular Research Center) and isolated from oocytes and pre-implantation embryos with the Dynabeads mRNA Direct Micro Kit (Ambion). The RNA was then reversely transcribed with the PrimerScript™ RT-PCR kit (Takara). The primer sequences were listed in Table 1. Real-time PCR was carried out in the All-In-One RT Master Mix G490 system (Applied Biological Materials). GAPDH was used as a reference gene for normalization of the mRNA levels. Data were processed using the 2−ΔΔCt method.

Plasmid Construction and Lentivirus Transfection—293FT cells were transfected with the envelope plasmid pMD2G, packaging plasmid psPAX2, and core expression plasmid pHIV-EGFP (Addgene) by PEI. After culturing for 48 and 72 h, the lentivirus supernatant was collected and concentrated through ultracentrifugation at 17,000 rpm for 2 h. For transfection, MEF or ES cells were incubated with lentivirus for 12 h and then cultured with regular medium. For protein expression in 293FT cells, genes were linked to HA-pCMV or Myc-pCMV vector. Wdr1 was added at the FLAG sequence in the forward primer, and linked to pHIV-EGFP through Xbal and SmaI sites. Full-length Limk1, Limk1-415 (deleted two Lim domains), and Limk1-766 (deleted two Lim domains and PDZ domain) were added by the respective forward primer and a common reverse primer, and then linked to pcMV-HA vector through xhoI and NotI sites. The primer sequences for vector construction are listed in Table 1.

| Gene                      | Forward | Reverse | Sequence (5′-3′) |
|---------------------------|---------|---------|-----------------|
| Genotyping                |         |         |                 |
| Wdr1-Flox                 |         |         |                 |
| Wdr1-deletion             |         |         |                 |
| Zp3-cre                   |         |         |                 |
| Global cre                |         |         |                 |
| qRT-PCR                   |         |         |                 |
| Wdr1                      |         |         |                 |
| Pax6                      |         |         |                 |
| T                         |         |         |                 |
| Sox17                     |         |         |                 |
| Gapdh                     |         |         |                 |
| Plasmid construction      |         |         |                 |
| Flag-Wdr                  |         |         |                 |
| Wdr1                      |         |         |                 |
| Limk1-FL                  |         |         |                 |
| Limk1-415                 |         |         |                 |
| Limk1-766                 |         |         |                 |

TABLE 1
Primer sequences for vector construction are listed in Table 1.
gated secondary antibodies. The signals were developed by SuperSignal West Dura Extended Duration Substrate (Pierce).

Primary antibodies included: rabbit anti-WDR1 (1:1,000, catalogue number 13676-1-AP, Proteintech), rabbit anti-phospho-Cofilin (Ser-3, 1:1,000, catalogue number 3313, Cell Signaling Technology), rabbit anti-Cofilin (1:1,000, catalogue number 5175, Cell Signaling Technology), rabbit anti-LIMK1 (1:1,000, catalogue number 3842, Cell Signaling Technology), rabbit anti-phospho-LIMK1 (1:1,000, catalogue number 3841, Cell Signaling Technology), rabbit anti-PDXP (1:1,000, catalogue number 4686, Cell Signaling Technology), rabbit anti-TESK1 (1:1,000, catalogue number 4655, Cell Signaling Technology), rabbit anti-SSH1 (1:1,000, catalogue number 13578, Cell Signaling Technology), rabbit anti-NANOG (1:1,000, catalogue number ab80892, Abcam), mouse anti-SOX2 (1:1,000, catalogue number 3579, Cell Signaling Technology), goat anti-OCT4 (1:1,000, catalogue number sc-8629, Santa Cruz Biotechnology), mouse monoclonal anti-GAPDH (1:5,000, catalogue number 30201ES20, Yeason), mouse monoclonal anti-pan-actin (1:2,000, catalogue number ab75373, Abcam), mouse anti-Myc (1:2,000 catalogue number M20002, Abmart), mouse anti-FLAG (1:1,000 for Western 1:500 for IP, catalogue number M20018, Abmart), and mouse anti-HA (1:500 for IP, catalogue number M2003, Abmart). The second antibodies included: goat anti-rabbit, goat anti-mouse, and rabbit anti-goat, which were conjugated with HRP (1:4,500, Abmart).

G-actin and F-actin Separation—G-actin and F-actin were separated by a two-step extract and ultracentrifugation protocol. The samples from the separated G-actin and F-actin fractions were proportionally loaded and analyzed by Western blotting using an antibody to pan-actin. G-actin was extracted by a cold lysis buffer 1 (10 mM K$_2$HPO$_4$, 100 mM NaF, 50 mM KCl, 2 mM MgCl$_2$, 1 mM EGTA, 0.2 mM DTT, 0.5% Triton X-100, 1 mM sucrose, pH 7.0) and centrifuged at 15,000 × g for 30 min. Soluble actin (G-actin) was measured in the supernatant. F-actin in the pellet was resuspended in lysis buffer plus an equal volume of buffer 2 (1.5 mM guanidine hydrochloride, 1 mM sodium acetate, 1 mM CaCl$_2$, 1 mM ATP, and 20 mM Tris-HCl, pH 7.5) and incubated on ice for 1 h to convert F-actin into soluble G-actin, with gentle mixing every 15 min. The samples were centrifuged at 15,000 × g for 30 min, and F-actin was measured in this supernatant (35).

EdU and PI Staining—Embryos were incubated with 500 µM EdU for 2 h and then fixed with 4% paraformaldehyde. Detection of incorporated EdU was performed using the Cell-Light EdU Apollo 567 In Vitro Imaging Kit (RiboBio) according to the manufacturer’s protocol.

For cell cycle detecting, MEF cells were digested to single cells and fixed by 70% ethanol at −20 °C overnight. Cells were permeabilized in PBS containing 0.5% Triton X-100 at room temperature for 30 min and then stained by propidium iodide (PI) in PBS at 4 °C for 30 min. PI signals were detected by flow cytometry and the results were analyzed by Flowjo software (Tree Star).

Immunohistochemistry—Cells or blastocysts were fixed by 4% paraformaldehyde in PBS at room temperature for 10 min and permeabilized in PBS containing 0.5% Triton X-100. Cells and embryos were blocked by 5% normal donkey serum at room temperature for 1 h and then incubated in primary antibodies at 4 °C overnight. After being washed with PBS three times to remove unconjugated primary antibodies, second antibodies, Hoechst 33342 (Invitrogen) and Alexa Fluor 546-labeled phaloidin (Invitrogen), were added and incubated at room temperature for 1 h. For Myc-Limk1 staining, MEF cells were fixed by 0.1% glutaraldehyde, 0.1 mM sodium cacodylate, pH 7.3. For GF oocye F-actin staining, oocytes were fixed with 130 mM KCl, 25 mM HEPES, pH 7, 3 mM MgCl$_2$, 4% paraformaldehyde, 0.15% glutaraldehyde, and 0.06% Triton X-100 at room temperature for 2 h and permeabilization was extended to 30 min.

Primary antibodies included: rabbit anti-Cofilin (1:100), rabbit anti-Myc (1:100, catalogue number 2278, Cell Signaling Technology), and mouse anti-α-tubulin (1:200, catalogue number F2168, Sigma). The secondary antibodies included: donkey anti-mouse conjugated with Alexa Fluor 549 (1:200), donkey anti-rabbit conjugated with Alexa Fluor 488 (1:500), and donkey anti-rabbit conjugated with Alexa Fluor 633 (1:500) (Invitrogen, Life Technologies). Immunofluorescent images were obtained on an Axioplan Zeiss microscope (LSM780, Carl Zeiss, Thornwood, NY) and the intensity of the immunofluorescent signal was analyzed with ZEN lite 2011 (Carl Zeiss).

Statistical Analyses—Quantitative analyses were carried out with GraphPad Prism software with at least 3 independent biological samples and expressed as the mean ± S.D. p values of comparisons between 2 groups were calculated using Student’s t test. p <0.05 was considered as significant.

Author Contributions—Y. X. designed and performed major experiments, analyzed the data, and wrote the manuscript. H. M. conducted Limk1 overexpression experiments. W. P. established the Wdr1$	ext{−/−}$ mouse line. D. Q. harvested GV oocytes and stained F-actin structure. X. W., Z. X., Y-L. X., and W. L. established MEF cell lines, injected ES cells into blastocysts, and performed H&E staining of embryo sections. L. L., Y. Z., and C. J. initiated and organized this project, analyzed the data, and wrote the manuscript. All authors commented on the manuscript.

References
1. Scheijer, E. D., and Wieschaus, E. (1993) Functional elements of the cytoskeleton in the early Drosophila embryo. Annu. Rev. Cell Biol. 9, 67–99
2. Heller, E., and Fuchs, E. (2015) Tissue patterning and cellular mechanics. J. Cell Biol. 211, 219–231
3. Yae, K., Keng, V. W., Koike, M., Yusa, K., Kouno, M., Uno, Y., Kondoh, G., Gotow, T., Uchiyama, Y., Horie, K., and Takeda, J. (2006) Sleeping beauty transposon-based phenotypic analysis of mice: lack of Arpc3 results in defective trophoblast outgrowth. Mol. Cell. Biol. 26, 6185–6196
4. Briere, W. (2013) Mechanisms of actin disassembly. Mol. Biol. Cell 24, 2299–2302
5. Bugyi, B., and Carlier, M. F. (2010) Control of actin filament treadmilling in cell motility. Annu. Rev. Biophys. 39, 449–470
6. Pollard, T. D. (2016) Actin and actin-binding proteins. Cold Spring Harbor Perspect. Biol. 8, pii: a018226
7. Bernstein, B. W., and Bamberg, J. R. (2010) ADF/cofilin: a functional node in cell biology. Trends Cell Biol. 20, 187–195
8. Andrianantoandro, E., and Pollard, T. D. (2006) Mechanism of actin filament turnover by severing and nucleation at different concentrations of ADF/cofilin. Mol. Cell 24, 13–23
9. Gressin, L., Guillotin, A., Guérin, C., Blanchon, L., and Michelot, A. (2015) Architecture dependence of actin filament network disassembly. Curr. Biol. 25, 1437–1447
10. Ohashi, K. (2015) Roles of cofilin in development and its mechanisms of regulation. Dev. Growth Differ. 57, 275–290
11. Bamberg, J. R., and Wiggan, O. P. (2002) ADF/cofilin and actin dynamics in disease. Trends Cell Biol. 12, 598–605
12. Bamberg, J. R., Bernstein, B. W., Davis, R. C., Flynn, K. C., Goldsbury, C., Jorgensen, J. R., Maloney, M. T., Marsden, I. T., Minamide, L. S., Nakayama, K., Nakayama, K., and Wiggan, O. (2003) ADF/Coilin rod in neurodegenerative diseases. Curr. Alzheimer Res. 7, 241–250
13. Nishida, E., Maekawa, S., and Sakai, H. (1994) Cofilin, a protein in porcine brain that binds to actin filaments and inhibits their interactions with myosin and tropomyosin. Biochemistry 23, 5307–5313
14. Kaji, N., Muramoto, A., and Mizuno, K. (2008) LIM kinase-mediated cofilin phosphorylation during mitosis is required for precise spindle positioning. J. Biol. Chem. 283, 4983–4992
15. Mizuno, K. (2013) Signaling mechanisms and functional roles of cofilin phosphorylation and dephosphorylation. Cell Signal. 25, 457–469
16. Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393, 805–809
17. Edwards, D. C., and Gill, G. N. (1999) Structural features of LIM kinase that control effects on the actin cytoskeleton. J. Biol. Chem. 274, 11152–11156
18. Edwards, D. C., and Gill, G. N. (1999) Structural features of LIM kinase that control effects on the actin cytoskeleton. J. Biol. Chem. 274, 11152–11156
19. Niwa, R., Sagata-Ohashi, K., Takeichi, M., Mizuno, K., and Uemura, T. (2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell 108, 233–246
20. Toshima, J., Toshima, J. Y., Amano, T., Yang, N., Narumiya, S., and Mizuno, K. (2001) Cofilin phosphorylation by protein kinase testicular protein kinase 1 and its role in integrin-mediated actin reorganization and focal adhesion formation. Mol. Biol. Cell 12, 1131–1145
21. Golahi, A., Birkenfeld, J., and Bokchod, G. M. (2005) Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. Nat. Cell Biol. 7, 21–29
22. Foletta, V. C., Lim, M. A., Soosairajah, J., Kelly, A. P., Stanley, E. G., Shann, M., He, W., Das, S., Massague, J., Bernard, O., and Soosairajah, J. (2003) Direct signaling by the BMP type II receptor via the cytoskeletal regulator LIMK1. J. Cell Biol. 162, 1089–1098
23. Döppler, H., Bastea, L. I., Eiseler, T., and Storz, P. (2013) Neuregulin mediates F-actin-driven cell migration through inhibition of protein kinase D1 via Rac1 protein. J. Biol. Chem. 288, 455–465
24. Oleinik, N. V., Helke, K. L., Kistner-Griffin, E., Krupenko, N. I., and Kruke, K., Wu, J., Wu, X., Huang, X., and Chen, J. (2015) AIP1-mediated actin polymerization and neutrophil dysfunction in WDR1 deficiency. Blood 125, 2570–2576
25. Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393, 805–809
26. Rodal, A. A., Tetreault, J. W., Lappalainen, P., Drubin, D. G., and Amberg, M. B. (2005) Direct signaling by the BMP type II receptor via the cytoskeletal regulator LIMK1. J. Cell Biol. 162, 1089–1098
27. Lee-Hoeflich, S. T., Causing, C. G., Podliowa, M., Zhao, X., Wran, J. L., and Attisano, L. (2004) Activation of LIMK1 by binding to the BMP receptor, BMPRII, regulates BMP-dependent dendritogenesis. EMBO J. 23, 4792–4801
28. Gorovoy, M., Niu, J., Bernard, O., Proifirovic, J., Minshall, R., Neamu, R., and Voyno-Yasenetskaya, T. (2005) LIM kinase 1 coordinates microtubule stability and actin polymerization in human endothelial cells. J. Biol. Chem. 280, 26533–26542
29. Parast, M. M., Aeder, S., and Sutherland, A. E. (2001) Trophoblast giant-cell differentiation involves changes in cytoskeleton and cell motility. Dev. Biol. 230, 43–60
30. Wang, H., and Dey, S. K. (2006) Roadmap to embryo implantation: clues from mouse models. Nat. Rev. Genet. 7, 185–199