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# AIP1/WDR1 supports mitotic cell rounding☆

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**Abstract**

The actin cytoskeleton plays a fundamental role in configuring cell shapes and movements. Actin interacting protein 1 (AIP1)/tryptophan-aspartate-repeat protein 1 (WDR1) induces actin severing and disassembly cooperating with ADF/cofilin. We found that mitotic cell flattening but not rounding was manifested by suppression of AIP1/WDR1 in cells. This mitotic cell flattening was not due to any changes in phosphorylation and distribution of cofilin in cells. We carried out a direct observation of actin filament severing/disassembly assay and found that phosphorylated cofilin still somewhat severs/disassembles actin filaments and that AIP1/WDR1 effaces this in vitro. We suggest that the phosphorylation of ADF/cofilin will be insufficient to completely inhibit actin turnover during mitosis, and that AIP1/WDR1 could abort the severing/disassembly activity somewhat still carried out due to phosphorylated ADF/cofilin. This mechanism could be required to induce cell morphologic changes, especially mitotic cell rounding.


*Keywords:* Actin turnover; Mitotic cell rounding; Cofilin; AIP1/WDR1

Cells extend and shrink for movement, ruffle and smooth for endocytosis/exocytosis, and round up and flatten down for proliferation cycles. These morphologic changes require cytoskeletal remodeling in which actin plays a central role. The actin remodeling system is controlled by its binding proteins [1], much the same as microtubule modification is controlled by its binding partners [2]. Profilin binds to the actin monomer to catalyze the exchange of nucleotides and enhances filament turnover [1,3]. Gelsolin severs and caps actin filaments, and nucleates actin filament assembly in a $Ca^{2+}$-dependent manner [1,4]. Among the actin-binding proteins, ADF/cofilin play one of the most fundamental roles; they augment actin turnover via treadmilling, depolymerization, and severing [1].

Mitotic cells round up from the bottom of culture dishes in vitro. In histological tissue sections, we can observe round mitotic cells partially detached from basement membrane where interphase cells are arranged in polygonal, spindle or rod-like shapes in situ. The mechanism and purpose of this mitotic cell rounding have not been fully understood. Actin but not myosin is imperative in retraction of cortex during cell rounding

☆ *Abbreviations:* AIP1, actin-interacting protein 1; WDR1, tryptophan-aspartate repeat protein 1; ADF, actin depolymerizing factor; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAPI: 4′,6-diamidino-2-phenylindole; PCR, polymerase chain reaction; siWDR1, siRNA for WDR1.

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[5]. Actin polymerization and filament cross-linking by gelsolin and filamin control actin-filled lipid vesicles [6]. Recently, a multi-directional cell signaling element Rho was shown to execute important roles in this cryptic phenomenon achieved by actin regulation [7]. Some researchers theorize that, to maintain cell rigidity at the cortex, actin cross-linking takes place with cross-linkers such as α-actininin, filamin, and spectrin [7]. During this step, actin turnover should be turned strictly off to assure tight cross-linking of the filament.

We found and first cloned a human homologue of a yeast actin-binding protein AIP1 [8] from a lymphokine-activated killer (LAK) cell subtraction library, which was enriched with cytotoxic T-cell activation-related genes [9,10], and we deposited the data to the GenBank as NORI-1 protein in 1998 (GenBank Accession No. AB010427) [11]. Subsequently, a study on this protein appeared in which it was given the name of WDR1, meaning a WD-repeat containing protein number 1 [12]. The functions of AIP1/WDR1 have been analyzed, and it was identified as an important cofactor of ADF/cofilin; AIP1/WDR1 binds to both actin and ADF/cofilin, and facilitates actin turnover [13,14]. In this study, we further addressed the functional roles of AIP1/WDR1 using a gene-silencing technology with siRNA, aiming for functional elucidation of this protein in LAK/T-cells.

## Materials and methods

*Plasmids*. DNA fragments corresponding to open reading frames of human AIP1/WDR1 and cofilin were amplified using cDNA prepared from HEK293 human kidney cells by polymerase chain reaction (PCR). Primers used were: WDR1: 5′-CGCGGATCCATGCCG TACGAGATCAAGAA-3′ and 5′-CCGGAATTCAGTAGGTGATT GTCCACTCCT-3′; cofilin: 5′-CGCGGATCCATGGCCTCCGGT GTGGCTGT-3′ and 5′-CCGGAATTCTCACAAAGGCTTGCCC TCC-3′. Amplified fragments were digested by proper restriction enzymes and ligated to pGEX-6P-2 (Amersham Biosciences, Piscataway, NJ) vectors. Cofilin serine-3 was changed to aspartic acid (cofilin S3D) using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic primers were: cofilin S3D: 5′-CCGGAT CCATGGCCGACGGTGTGGCTGTC-3′ and 5′-GACAGCCACAC CGTCGGCCATGGATCCGG-3′. DNA sequences were verified using a 310 Genetic Analyzer (Applied Biosystems, Urayasu, Japan).

*RT-PCR*. mRNA was prepared by ISOGEN (NIPPON GENE, Tokyo, Japan). cDNA was synthesized by RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) and used as a template for mRNA expression analysis [10] for AIP1/WDR1, cyclin B1, and α-tubulin. Primers used were: AIP1/WDR1: 5′-GAAGGA GCACCTGTTGAAGTATGAG-3′ and 5′-CCGTTTTTATGCACC GTCAGAC-3′; cyclinB1: 5′-ATGTGGATGCAGAAGATGGAGC-3′ and 5′-AGATGCTCTCCGAAGGAAGTGC-3′; and α-tubulin: 5′-GGTTCCCAAAGATGTCAA-3′ and 5′-CCGCTCGAGTTAGT ATTCCTCTCCTTCTTCC-3′.

*Proteins*. AIP1/WDR1, cofilin, and mutants were expressed as glutathione *S*-transferase (GST) fusion proteins [9,10]. Expressed proteins were clipped off the GST part by PreScission protease (Amersham Biosciences) followed by dialysis to phosphate-buffered saline (PBS). Protein concentrations were estimated using a DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA). Rabbit skeletal muscle actin was purchased from Sigma (St. Louis, MO), Alexa Fluor 488-actin was from Molecular Probes (Eugene, OR), and biotinylated actin was from Cytoskeleton (Denver, CO). Phosphorylated cofilin was prepared using recombinant cofilin and LIMK1 (Upstate, Lake Placid, NY) as described elsewhere [15]. Phosphorylated cofilin was purified using a Phosphoprotein Purification kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. The purity of phosphorylated cofilin was confirmed by 2D electrophoresis.

*Antibodies*. Antisera against AIP1/WDR1 were prepared by immunization to rabbits with recombinant AIP1/WDR1 in Freund's adjuvant and were purified by affinity chromatography using recombinant AIP1/WDR1 coupled with CNBr-activated Sepharose 4B (Amersham Biosciences). Anti-cofilin rabbit polyclonal and anti-α-tubulin mouse monoclonal antibodies were purchased from Sigma, anti-phosphorylated serine-3 cofilin rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-biotin mouse monoclonal antibody was from Molecular Probes, and anti-cyclin B1 was from BD Biosciences (San Jose, CA).

*Cells*. U-2 OS human osteosarcoma, HeLa human cervical cancer, and MDA-MB231 human breast cancer cells were cultivated with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml ampicillin (10% DMEM). For synchronization of HeLa cells, the cell-cycle was blocked in the early S phase by the thymidine double block method. HeLa cells were incubated with 2.5 mM thymidine in 10% DMEM for 22 h at 37 °C in 5% $CO_2$, followed by two washings with PBS, and a further incubation for 10 h in 10% DMEM at 37 °C in 5% $CO_2$. Cells were then incubated with 1 mM hydroxyurea for 15 h at 37 °C, 5% $CO_2$ followed by two washings with PBS, and released from the block by an incubation with fresh medium. U-2 OS cells were transfected with plasmids using TransFast reagent (Promega, Madison, WI) according to manufacturer's instructions. For microinjection study, anti-AIP1/WDR1 antibody (5 mg/ml) or recombinant WDR1 protein (1 μM) in PBS was injected to U-2 OS cells or AIP1/WDR1 knocked-down cells cultivated on a glass coverslip using a micromanipulator (Narishige, Tokyo, Japan). For Y-27632 treatment, cells were incubated with 10 μM Y-27632 (CALBIOCHEM, San Diego, CA) for 2 h and subjected to analyses. For immunofluorescent histochemistry, cells were fixed with 3.7% paraformaldehyde in PBS for 10 min followed by incubation with 0.1% Triton X-100 in PBS for 5 min and then washed with PBS. After blocking with 1% BSA, 0.02% $NaN_3$ in PBS for 30 min, cells were incubated with primary antibodies for 1 h at room temperature followed by a reaction with secondary anti-mouse or anti-rabbit IgG antibody labeled with Alexa Fluor 488. F-actin was stained by Alexa Fluor 568-labeled phalloidin for 20 min. Nucleus DNA was stained by 1 μg/ml, 4′,6-diamidino-2 phenylindole (DAPI) (Nakarai Tesque, Tokyo, Japan). Images were captured using a fluorescence microscope with a DC70 CCD camera (Olympus, Tokyo, Japan) or an Eclipse confocal laser scanning microscope (Nikon, Tokyo, Japan). Western blotting was carried out using a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and an ECL detection system (Amersham Biosciences) [9,10]. Samples were subjected to SDS–PAGE and transferred to membrane using a semi-dry blotter (BioCraft, Tokyo, Japan) for 60 min at 1 mA/$cm^2$. After blocking by 5% skim milk, 0.1% Tween 20 in PBS for 1 h at room temperature, membrane was incubated with primary antibodies as indicated, followed by a reaction with secondary antibodies labeled with horseradish peroxidase. The membrane was finally developed using ECL solution and exposed to a Hyper Film (Amersham).

*Knock-down cells*. For short RNA interference to AIP1/WDR1 gene in cells, psiRNA hH1neo vector (InvivoGen, San Diego, CA) was ligated with an annealed oligonucleotide made of 5′-TCCCAACGG CGGCAAGTCCTACATTTCAAGAGAATGTAGGACTTGCCGC CGTTT-3′ and 5′-CAAAAAACGGCGGCAAGTCCTACATTCT CTTGAAATGTAGGACTTGCCGCCGTT-3′ according to manufacturer's instructions. Elaborated psiRNA vector was transfected to

cells using TransFast reagent and cultivated for 5 days with occasional passage using 10% DMEM supplemented with G418, 500 μg/ml (Nakarai Tesque).

*Direct observation of actin filament severing/disassembly assay*. Microscopic direct observation of polymerized actin filament severing and disassembly assay was carried out with modifications [16]. First, 1.4 μM unlabeled actin, 0.4 μM Alexa Fluor 488-labeled actin, and 0.2 μM biotin-labeled actin were mixed and polymerized in ISAP buffer (50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, and 20 mM Hepes–KOH, pH 7.2) for 2 h at room temperature. A cover glass was mounted onto a 0.1% nitrocellulose-coated slide glass with two pieces of double sticky tape to make a perfusion chamber. Anti-biotin monoclonal antibody was diluted to 30 μg/ml with ISAP buffer, perfused into the chamber, and incubated for 5 min. After being washed twice with 0.5 mg/ml BSA in ISAP buffer, polymerized actin was diluted fivefold (0.4 μM final concentration) with anti-bleaching buffer (ISAP buffer containing 5 mg/ml BSA, 0.036 mg/ml catalase, 0.2 mg/ml glucose oxidase, 6 mg/ml glucose, and 100 μM dithiothreitol) and perfused into the chamber. After incubation for 5 min at room temperature, the chamber was washed twice with anti-bleaching buffer and images of actin filaments were taken using a fluorescent microscopy system. Polymerized actin filaments were then treated by samples composed of cofilin, mutants or phosphorylated cofilin (2 μM) with or without AIP1/WDR1 (2 nM) dissolved in anti-bleaching buffer in a perfusion chamber for 5 min. The chamber was washed with anti-bleaching buffer and images were observed.

## Results and discussion

### *Knock down of AIP1/WDR1 in cells*

We carried out a gene knock-down study by transfecting a psiRNA-hH1neo vector that produces siRNA in cells [17]. Suppression of intrinsic AIP1/WDR1 at both mRNA and protein levels was manifested during day 5–7 (Fig. 1A). In immunofluorescent histochemical analysis, AIP1/WDR1 protein expression disappeared in about 50–60% of transfected cells on day 5 (Fig. 1B). We prepared vectors with two different inserts for this study, and both were comparatively active, with similar results observed (data not shown).

### *Induction of mitotic cell flattening by suppression of AIP1/WDR1 in cells*

During interphase, there were no evident changes in cell shapes or F-actin figures of AIP1/WDR1 knocked-down cells. No aggregation of F-actin filament or thickening of actin stress fibers was observed. Commonly, mitotic cells are rounded up and chromosomes are aligned to the equator with a flower-like arrangement. However, AIP1/WDR1 knocked-down cells demonstrated a flattened cell shape as compared with the control cells (Fig. 2A). Here, mitotic cell shapes were compared by height and long axis diameter. As shown, a 20% decrease in height and a 30% increase in long axis were evident, both of which were statistically significantly different. To further confirm this mitotic cell change, we microinjected anti-AIP1/WDR1 antibodies directly into cells. While control rabbit IgG-injected U-2 OS cells showed the normal rounded-up shape during mitosis, anti-AIP1/WDR1 rabbit IgG injected cells showed a flattened shape as AIP1/WDR1 knocked-down cells (Fig. 2B). We next microinjected recombinant AIP1/WDR1 into AIP1/WDR1 knocked-down cells and observed their shapes. Cells rounded up by injecting recombinant AIP1/WDR1 protein to cells treated with psiWDR1 vector (Fig. 2C). As shown, the proportion of rounding cells was significantly elevated by the injection. We tried knock-down study using other cells such as HeLa human cervical cancer cells and HEK293 human kidney cells, and the same result was observed (data not shown).

Flattened cell shapes by RhoA suppressive treatment quite resemble those observed in this study [7]. By RhoA suppressive treatment using Y-27632 or C3, LIM kinases remain deactivated, phosphorylation of cofilin is suppressed, and cofilin should remain active in cells, which could be one of the mechanisms in cell flattening [7]. We examined and confirmed moderate suppression of cofilin phosphorylation in cells with Y-27632 treat-

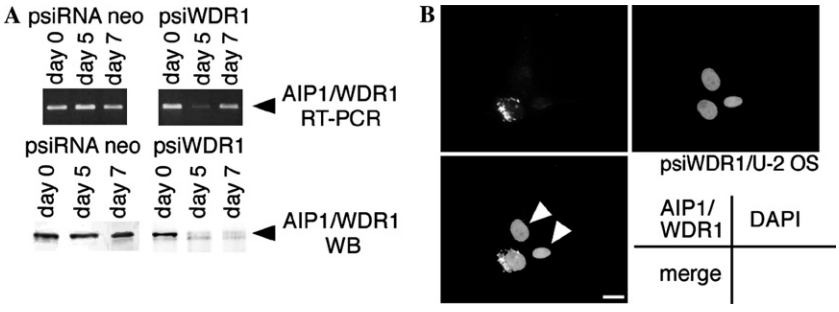


Fig. 1. Knock down of AIP1/WDR1 mRNA expression in cells was carried out using psiRNA hH1neo vector containing a suppressive sequence (psiWDR1) to U-2 OS cells. (A) Suppression of AIP1/WDR1 in both mRNA and protein levels was manifested between day 5 and 7. (B) Immunofluorescent histochemical analysis showed that about 50–60% of cells were thus depleted of protein expression. Arrowhead indicates the AIP1/WDR1-depleted cells. White bar indicates 20 μm.

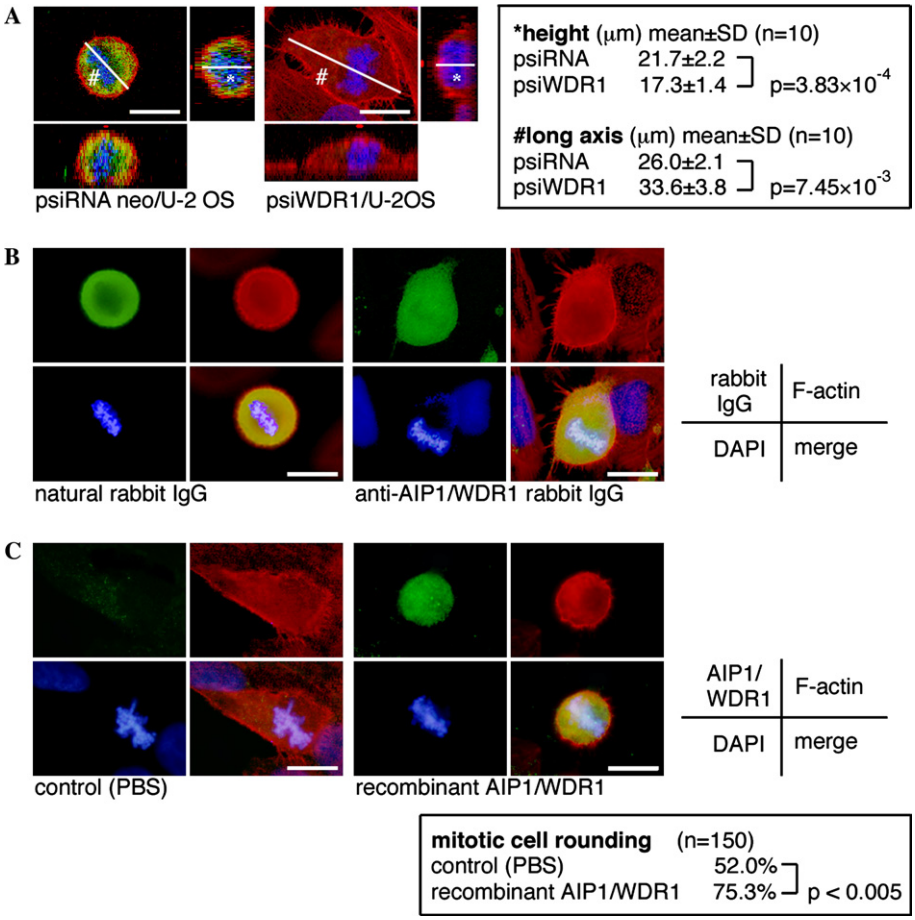


Fig. 2. Mitotic cell flattening was induced by suppression of AIP1/WDR1 in cells. (A) Representative figure of flattened mitotic cells by AIP1/WDR1 knock-down and estimation of dimensions using a confocal microscope. Control cells showed rounding, while AIP1/WDR1 knocked-down cells flattening. A statistically significant difference was evident between these cells in height and long-axis diameter, as shown in the box. Experiments were repeated and the same result was observed every time. (B) Mitotic cell flattening was also evident by microinjection of anti-AIP1/WDR1 antibody into cells. Under microscopy, antibodies were injected into cells directly and cultured at 37 °C, 5% $CO_2$ overnight. Cells were fixed and stained as indicated. (C) psiWDR1 vector was transfected to U-2 OS cells and recombinant AIP1/WDR1 was injected to these cells. The proportion of rounding cells significantly increased by AIP1/WDR1 injection. The typical figures were represented. White bar indicates 20 μm.

ment (data not shown). While, in this study, we suppressed AIP1/WDR1, which has been thought to be an ADF/cofilin supporting cofactor; suppression of AIP1/WDR1 should bring forth a suppression of ADF/cofilin's actin disassembling function if this were a cofactor possessing an exclusive actin destabilizing nature. We first anticipated that mitotic cells would round up and be more rigid than controls in response to AIP1/WDR1 suppression. However, the opposite occurred: when AIP1/WDR1 was suppressed, the mitotic cell rounding failed and flattening followed.

### *Intrinsic gene and protein expression of AIP1/WDR1 during cell-cycle*

We next analyzed intrinsic AIP1/WDR1 expression in cultured cells to address if there were any cell-cycle-specific changes that could affect mitotic figures. No evident change in expression of AIP1/WDR1 at either mRNA or protein levels was seen during any part of the cell-cycle (Fig. 3A). Cyclin B1 expression was elevated at both mRNA and protein levels during the G2-M phase, as seen during 6–12 h after the release from thymidine double block in this study. Also not seen was any band shift in Western blotting during the cell-cycle, which suggests that post-transcriptional modifications such as phosphorylation would not be the case for AIP1/WDR1. Also not seen was any specific change in intracellular localization of AIP1/WDR1 (Fig. 3C), which is localized in cytosol throughout cell-cycle. Thus, expression and localization of AIP1/WDR1 were simply constant during cell-cycle.

### *Mitotic cell flattening induced by AIP1/WDR1 knock-down was irrelevant to cofilin phosphorylation*

To rule out the possibility that cofilin phosphorylation could be blocked in AIP1/WDR1-suppressed cells, we carried out analyses using anti-human phosphorylated cofilin-specific antibody. Western blotting showed

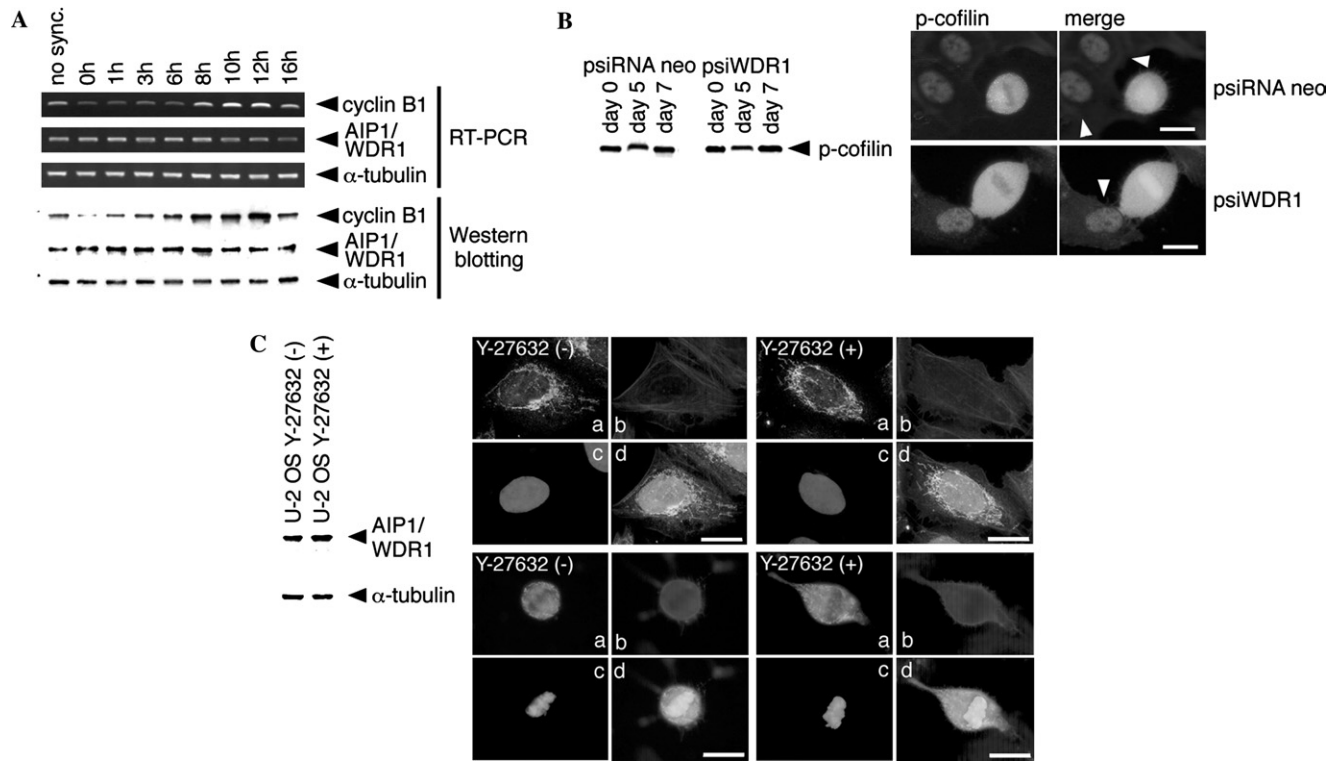


Fig. 3. Intrinsic gene and protein expression of AIP1/WDR1 was regular during the cell-cycle, mitotic cell flattening induced by AIP1/WDR1 knocked-down was irrelevant to cofilin phosphorylation, and flattening induced by RhoA inhibitor was irrelevant to AIP1/WDR1 expression. (A) Analyses of mRNA and protein expressions of AIP1/WDR1 during the cell-cycle by RT-PCR and Western blotting, respectively. (Upper panel) RT-PCR analysis of cyclin B1, AIP1/WDR1, and α-tubulin. Cyclin B1 expression was up-regulated during the G2-M phase, while no change in AIP1/WDR1 expression was seen. (Lower panel) Western blotting analysis. Expression patterns of these proteins were the same as those of RT-PCR. (B) Phosphorylated cofilin expression in AIP1/WDR1 knocked-down cells using phosphorylation-specific antibody. (Left panel) Western blotting analysis of whole cell lysate manifested no suppression of phosphorylated cofilin expression in AIP1/WDR1 knocked-down cells. (Right panel) Immunofluorescent histochemical analysis showed that intrinsic expression of phosphorylated cofilin in AIP1/WDR1-knocked-down cells during mitosis was also comparable with that of the control cells. Please note that the staining patterns of interphase cells were comparable in both (arrowheads). (C) AIP1/WDR1 expression in RhoA suppressed cells by Y-27632. In U-2 OS cells treated with Y-27632 for 2 h, expression of AIP1/WDR1 was analyzed by Western blotting and immunofluorescent histochemistry. No changes in AIP1/WDR1 expression were observed by these analyses as compared with control cells. (Left panel) Western blotting. (Right panel) Immunofluorescent histochemistry (a, AIP1/WDR1; b, α-tubulin; c, DAPI; and d, merge). White bar indicates 20 μm.

that phosphorylated cofilin was comparably expressed in AIP1/WDR1 knocked-down cells compared with control cells (Fig. 3B). To address mitotic cells specifically, we achieved an immunofluorescent histochemistry and confirmed an upregulation of phosphorylated cofilin during mitosis (Fig. 3B). Please note that interphase cells were also stained comparatively in AIP1/WDR1 knocked-down cells and control cells. Also not observed were any specific changes in distribution of phosphorylated (Fig. 3B) or total cofilin (data not shown) in cells. These data indicated that cofilin phosphorylation and distribution advance with the cell-cycle in AIP1/WDR1 knocked-down cells as control cells in vitro.

### *Mitotic cell flattening induced by RhoA inhibitor was irrelevant to AIP1/WDR1 expression*

Next we addressed the possibility that AIP1/WDR1 expression might be decreased by RhoA inhibitor, which could cause the cell flattening. We examined the expression of AIP1/WDR1 in RhoA-inhibited cells by Y-27632 for 2 h, but we found that AIP1/WDR1 protein expression was comparable to that of control cells in Western blotting (Fig. 3C). This result indicates that post-translational modification of AIP1/WDR1 protein such as protein degradation is not the case. Mitotic cell-specific analysis by immunofluorescent histochemistry also showed no evident difference in the expression level and distribution pattern of AIP1/WDR1 at either the interphase or mitosis (Fig. 3C).

### *AIP1/WDR1 inhibits actin filament severing/disassembly activity of phosphorylated but not dephosphorylated cofilin in vitro*

Thus, mitotic cell flattening induced by AIP1/WDR1 knock-down was suggested not to be related with the intrinsic change of this protein expression or not to be

due to the extent and distribution of cofilin phosphorylation. We here hypothesized that AIP1/WDR1 may exert an actin-stabilizing effect once ADF/cofilin is phosphorylated. We performed a direct microscopic observation of an actin severing and disassembly assay in vitro [16], and our hypothesis was displayed by this method. Recombinant AIP1/WDR1 did not change polymerized actin filament by itself (Fig. 4A). Recombinant cofilin disassembled actin filament evidently, on which recombinant AIP1/WDR1 showed a destabilization-augmenting effect. Please note the concentrations used in this experiment: the molar concentration of WDR1 (2 nM) is 103 times less than that of cofilin (2 μM). This result was the same as reported previously [16]. Dose-dependent analysis of AIP1/WDR1 on the depolymerization-augmenting effect was carried out (data not shown). Ten times lower concentration of AIP1/WDR1 (0.2 nM) did not evidently augment it or only marginally enhanced it. The augmenting effect of AIP1/WDR1 forms a plateau after more than 2 nM (data not shown). Surprisingly, cofilin S3D showed the severing/disassembly activity more significantly than we expected in this assay, which AIP1/WDR1 clearly effaced (Fig. 4A). The same results were observed repeatedly. When using phosphorylated recombinant cofilin instead of cofilin S3D, the same result was observed (Fig. 4B). Thus, AIP1/WDR1 was suggested to antagonize the severing/disassembly activity somewhat carried out by the phosphorylated cofilin to actin polymer. Next, we mixed wild-type and S3D cofilin, and performed a severing/disassembly assay with the addition of AIP1/WDR1. Inhibition of actin severing/disassembly activity disappeared according to the molar ratio of wild-type to S3D cofilin (Fig. 4B). Thus, this inhibitory activity of AIP1/WDR1 appeared to depend on the quantity of cofilin S3D to actin. We also carried out this experiment using phosphorylated cofilin and observed the same result (data not shown). These results could illustrate the cause of mitotic cell flattening in AIP1/WDR1-depleted cells; phosphorylated cofilin, which should be exactly inactive in actin turnover during mitosis, still possesses some actin severing/disassembly activity. AIP1/WDR1 will totally efface this. We carried out experiments using recombinant ADF instead of cofilin and obtained basically the same results (data not shown).

A previous hypothesis was that phosphorylated ADF/cofilin would be detached from actin soon after phosphorylation and would not then associate with actin filament [18,19]. But, some phosphorylated cofilin molecules may exist on actin filament that could still induce a moderate actin-severing activity. Even though it is not as strong as dephosphorylated cofilin, some affinity of phosphorylated cofilin to actin has been detected in previous data in vitro [15,20,21], and once there is an association, phosphorylated cofilin will depolymerize actin as dephosphorylated cofilin [21]. In mitotic cell rounding, actin filament should not be destabilized to advance actin filament cross-linking tightly and to get cell rigidity at the cortex. Mitotic flattening shapes in

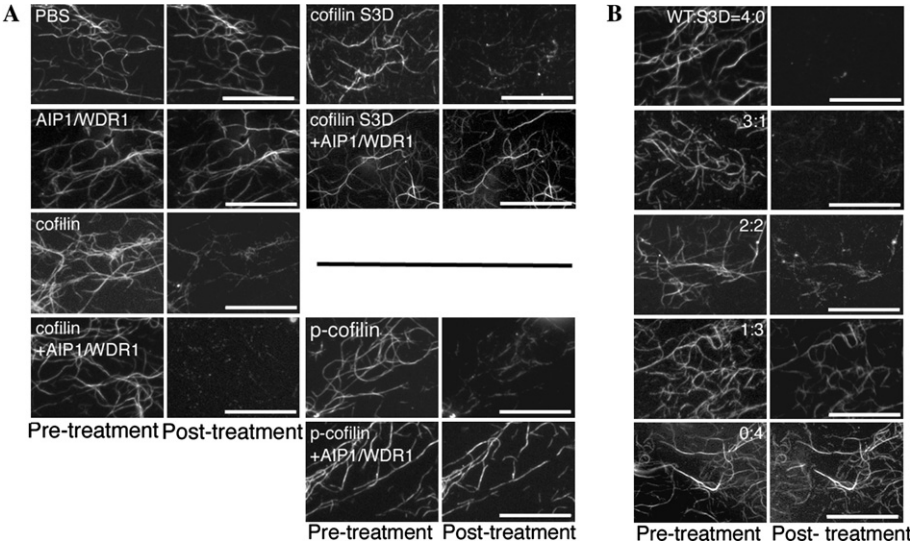


Fig. 4. AIP1/WDR1 inhibits actin severing/disassembly activity of phosphorylated but not that of dephosphorylated cofilin in vitro. (A) Microscopic direct observation of actin filament severing/disassembly assay. Recombinant AIP1/WDR1 (2 nM) did not change the figure of the actin filament by itself. Wild-type cofilin (2 μM) disassembled actin filament and AIP1/WDR1 augmented this activity. Phosphorylation-mimic cofilin mutant S3D (2 μM) disassembled filament distinctly in this assay, and AIP1/WDR1 effaced this activity (right upper panel). Phosphorylated cofilin also depolymerized actin filament as cofilin S3D and AIP1/WDR1 effaced it (right lower panel). (B) Microscopic direct observation of actin filament at various cofilin wild-type:S3D ratios. Recombinant cofilin wild-type and S3D were mixed at various ratios (final cofilin concentration 2 μM) with AIP1/WDR1 (2 nM) and applied to the assay. The actin stabilization effect of AIP1/WDR1 appeared to depend on cofilin S3D quantity. Without AIP1/WDR1, all of these cofilin mixtures sever/disassemble actin filament comparably, similar to what is shown in (A). White bar indicates 20 μm.

AIP1/WDR1 knocked-down cells and anti-AIP1/WDR1 antibody-microinjected cells indicate that cofilin phosphorylation by itself may not be enough to stop actin depolymerization that is essential for tight actin filament cross-linking.

Cell flattening by suppression of AIP1/WDR1 was also seen in suspension culture cells even in interphase. When AIP1/WDR1 was knocked down in T-cell lines, the cell shape changed to flat and untidy (data not shown). Without AIP1/WDR1, phosphorylated cofilin at the cell cortex may still possess some actin-destabilizing activity, which consequently could change the shape of the floating cell from round to flat. In fact, F-actin, AIP1/WDR1, and phosphorylated ADF/cofilin all exist in the cortical region of floating cells (data not shown) similar to what occurs in adherent cells during mitosis. On the other hand, once dephosphorylated, cofilin is soon transported into the nucleus in T-cells [22], which will be important to keep dephosphorylated ADF/cofilin away from the cortex, maintain cell rigidity, and preserve the round shape of floating cells. If dephosphorylated but not phosphorylated ADF/cofilin meets with AIP1/WDR1 at the cell cortex, actin could be destabilized and cell rigidity would not be maintained.

It was reported that 14-3-3 stabilizes actin dynamics via scaffolding phosphorylated cofilin [23]. The actin turnover stabilizing effect of AIP1/WDR1 will not be the same as that of 14-3-3. First, no depolymerization inhibitory activity of 14-3-3 protein was seen in microscopic actin depolymerization assay at the same concentration as AIP1/WDR1 used in this study (data not shown). Next, no actin aggregate or stress fiber thickening in AIP1/WDR1 transfected cells was evident (data not shown). If AIP1/WDR1 simply binds and stabilizes phosphorylated cofilin as 14-3-3 in vivo, actin aggregates or stress fiber thickening will be observed [23] by AIP1/WDR1 over-expression in cells. Affinity of 14-3-3 protein to phosphorylated cofilin is high enough to co-sediment by an immunoprecipitation analysis [23], while that of AIP1/WDR1 is not high enough to be observable (data not shown). It is conceivable that AIP1/WDR1 will bind to both actin and cofilin at the same time, at the same place, but not to either one singly and not at different places singly; divalent binding of AIP1/WDR1 may generate significant avidity when compared to the single affinity to each protein individually, like generating avidity by antibody. Thus, AIP1/WDR1 will not bind to free cofilin and G-actin in cytosol easily, but it may form trimeric complex on actin filament dynamically.

### *Abnormal chromosomal segregation and cytokinetic failure in AIP1/WDR1 knocked-down cells*

We finally addressed biological significance of mitotic cell rounding from aspects of chromosomal segregation and cytokinesis (Fig. 5). We counted abnormal segregation such as asymmetric cell division into three or more parts, and we found the rate was elevated by six times in AIP1/WDR1 knocked-down cells. This evidence was consistent with that of a previous report, where *Dictyostelium* showed segregation failure in AIP1 gene mutant and irregular progression of mitosis was observed in Aip1-null cells [24]. We assume that mitotic cell rounding and resultant balanced chromosomal alignment are essential for an exact chromosomal segregation. The flower-like arrangement of chromosomes during mitosis would be disoriented in flattened mitotic cells. Next, the frequency of multi-nucleated cells due to cytokinetic failure was three times that of controls in AIP1/WDR1 knocked-down cells (Fig. 5). This was also consistent with a previous report where cytokinesis was prolonged in DAip1 null cells and they became multi-nucleated [25]. AIP1/WDR1 will be mandatory for the formation of an actin contractile ring during cytokinesis [26], which is supported by a dynamic actin turnover [27]. In this case, however, AIP1/WDR1 will be working as an actin depolymerization augmentator exclusively, because cofilin is vigorously dephosphorylated by sling shot phosphatases at the actin contraction ring, and the dephosphorylated cofilin there activates actin turnover [28,29]. Thus, AIP1/WDR1 will be essential for exact chromosomal segregation and cytokinesis, where stopping of actin turnover is necessary in the former but not the latter. Elucidation of the precise mechanisms of both these phenomena has not yet been achieved.

AIP1/WDR1 had been thought to be an exclusive augmentator of actin turnover cooperating with ADF/

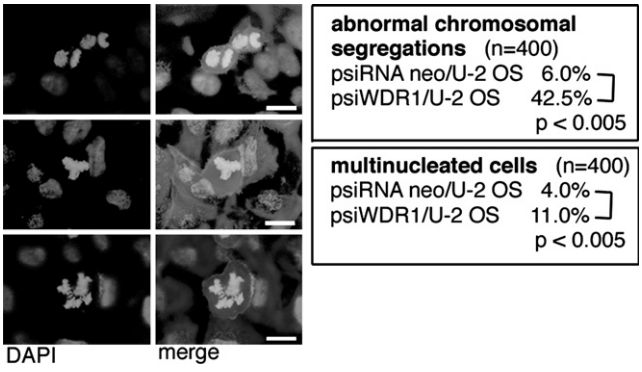


Fig. 5. AIP1/WDR1 knocked-down cells showed abnormal chromosomal segregation and cytokinetic failure. Immunofluorescent histochemistry: representative figure of abnormal chromosomal segregation by AIP1/WDR1 knock-down. (Right upper panel) Abnormal chromosomal segregation was obvious in AIP1/WDR1 knocked-down cells, and its rate was severely elevated in AIP1/WDR1 knocked-down cells. (Right lower panel) Due to cytokinetic failure, number of multi-nucleated cells increased by twice the normal number in AIP1/WDR1 knocked-down cells. White bar indicates 20 μm.

cofilin [1,30,31]. However, this study suggests that the action of AIP1/WDR1 could be more sophisticated than that.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.11.156.

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