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WAVE3 Functions as a Negative Regulator of LDOC1

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WAVE3 belongs to the Wiskott-Aldrich syndrome protein family that mediates actin reorganization through activation of the Arp2/3 complex. However, the physiological function of WAVE3 is poorly understood. We found that LDOC1—encoded by a gene that is down-regulated in tumor cell lines—binds directly to the verprolin homology domain of WAVE3. Ectopically expressed LDOC1 is localized in the nucleus and induces apoptosis in the cells. This apoptosis is accompanied by an increase in the p53 protein level, but not in p53 transcription, suggesting that LDOC1 inhibits the degradation of p53. Further, the expression of WAVE3 induces the translocation of LDOC1 from the nucleus to the cytoplasm, resulting in the inhibition of LDOC1-induced apoptosis. Thus, it is possible that the LDOC1 function is negatively regulated by WAVE3.

Key words: apoptosis, Arp2/3, LDOC1, p53, WAVE3.

Abbreviations: WASP, Wiskott-Aldrich syndrome protein; WAVE, WASP family verprolin-homologous protein; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; MDCK, Madin-Derby canine kidney; dox, doxycycline; BrdU, 5-bromo-2′-deoxy-uridine; PBS, phosphate-buffered saline; GST, glutathione S-transferase.

WAVE3 was identified, along with WAVE1 and WAVE2 (WASP family Verprolin-homologous protein), as a member of the Wiskott-Aldrich syndrome protein (WASP) family of proteins (*1*, *2*). These proteins activate the Arp2/3 complex via their C-terminal verprolin-homology (V) domain, cofilin-homology (C) domain and acidic (A) region. The V domain binds to monomer actin, and the CA region binds to the Arp2/3 complex. The formation of the ternary complex comprising the actin monomer, Arp2/3 complex and VCA region is believed to be the basis of Arp2/3 complex-mediated actin polymerization (*3*, *4*). WAVE proteins are implicated in the formation of several types of ruffles downstream of small GTPase Rac (*5*). WAVE1 and WAVE2 were found to exist in a hetero complex that includes Sra1/PIR121, Nap1, Abi1/2 and HSPC300 (*6–8*). This complex is believed to be involved in the signal transduction from Rac to WAVE. WAVE2 is also known to bind activated Rac through IRSp53 (*9*). However, the regulatory mechanisms and functions of WAVE3 are poorly understood.

LDOC1 (a leucine zipper protein, down-regulated in cancer cells) has been identified as a novel gene that is down-regulated in a series of pancreatic and gastric cancer cell lines (*10*). The *LDOC1* gene, which was mapped on chromosome Xq27, encodes a 17-kDa protein that possesses a leucine zipper–like motif in the N-terminal region, a short proline-rich region containing an SH3-binding consensus sequence and an acidic region at the C-terminus. LDOC1 is reported to enhance phorbol 12-myristate 13-acetate (PMA)- or tumor necrosis factor-α (TNF-α)–mediated apoptosis through inhibition of NF-κB activation in pancreatic cancer cells (*11*).

We performed two-hybrid screening using the verprolin homology (VPH) region of WAVE3 as "bait" and identified LDOC1 as the binding partner of WAVE3.

MATERIALS AND METHODS

Two-Hybrid Screening—We used a Matchmaker Two-hybrid system (Clontech, CA). The DNA encoding the WAVE3 verprolin homology domain (amino acids 410–471) was amplified by PCR and subcloned into the pGBT-9 plasmid vector. This recombinant plasmid was transformed into the Y190 yeast, which was subsequently transformed with a library of human fetal brain cDNAs.

Cell Culture—COS7 cells and MDCK cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2.

Establishment of MDCK-tet-off-Myc-LDOC1 Cells—LDOC1 was constructed in pTRE2 with a Myc tag and transfected into MDCK-tet-off cells (Clontech) with Lipofectamine 2000 reagent. Stably transfected cells were selected in a medium containing 1 mg/ml doxycycline and 200 μg/ml hygromycin. LDOC1 expression was induced by removal of doxycycline by washing of the cells and then replacement of the medium with that without doxycycline. In all experiments, 0 h was set as when doxycycline was removed. The WAVE3-expressing plasmid was transfected into MDCK-tet-off-Myc-LDOC1 cells after 24 h of doxycycline removal as described previously (*12*). The cells were subsequently seeded onto coverslips and TUNEL stained at the indicated times after doxycycline removal.

BrdU Incorporation Assay—In this assay, a 5-bromo-2′-deoxy-uridine labeling and detection kit II (Roche, cat. number 1 299 964) was used. pCMV-Myc-LDOC1 or pcDL-SRα-LacZ was transfected into MDCK cells using Lipofectamine 2000 reagent. Thirty hours after

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transfection, 10 μM BrdU was added to the culture medium, and then the cells were incubated for another 30 min. For MDCK-tet-off-Myc-LDOC1 cells, 10 μM BrdU was added to the culture medium, and then the cells were incubated for 30 min at the indicated times of doxycycline removal. The cells were washed with phosphate-buffered saline (PBS) prior to methanol fixation. Subsequently, the cells were stained according to the manufacturer's instructions and then observed under a confocal laser-scanning microscope (Bio-Rad, model MRC 1024).

TUNEL Staining—TUNEL staining was performed with an in situ cell death detection kit (Roche, cat. number 2 156 792). At the indicated times after doxycycline removal, the cells were fixed with 3.7% formaldehyde in PBS and then stained according to the manufacturer's instructions. Cells were fixed with methanol, and then stained for simultaneous detection of TUNEL-positive and BrdU-positive cells.

Northern Blotting—RNA (18 μg) that had been extracted from cells using an RNeasy kit (Qiagen) was treated with formaldehyde and then separated on a 1% agarose gel under formaldehyde denaturing conditions. Subsequently, the RNA was transferred to GeneScreen Plus (NEN, MA) and then fixed on a membrane using a UV transilluminator. Full-length p53 and β-actin (as a control) were used as probes. The radioisotope-labeled probe was produced using a BcaBEST Labeling Kit (Takara, Japan). The membrane was placed in 10 ml of a hybridization solution containing 0.1 mg/ml salmon sperm DNA for 30 min at 65°C, and then in 5 ml of hybridization solution containing 0.1 mg/ml salmon sperm DNA and 2×10^6 cpm/ml probe overnight at 65°C. After washing, autoradiography was performed with a BAS2000 image analyzer (Fuji Film).

Analysis of DNA Fragmentation—2×10^6 MDCK-tet-off-Myc-LDOC1 cells were cultured 96 h with or without dox. Subsequently, the cells were harvested and incubated in a lysis buffer [20 mM Tris-HCl (pH 7.4), 20 mM EDTA and 0.5% Triton X-100] that contained proteinase K at 50°C for 5 h. The cellular DNA was extracted once with phenol, and then once with a mixture of phenol, chloroform and 3-methyl-1-butanol (25:24:1, v/v). After digestion with RNase at 37°C for 30 min, samples were subjected to electrophoresis on a 1% agarose gel and ethidium bromide staining.

GST-fusion Proteins and Pull-Down Assay—Glutathione S-transferase (GST)-fusion proteins of the full-length, N-terminal region, proline-rich region and C-terminal region for LDOC1 were constructed in pGEX plasmids. These GST-fusion proteins were first immobilized on glutathione-Sepharose beads and then mixed with protein samples such as cell lysates or purified proteins. After 2 h, the beads were washed five times with lysis buffer [40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100 and 5 mM EDTA] and then the SDS sample buffer was added. Samples were separated by SDS–polyacrylamide gel electrophoresis, followed by immunoblotting or Coomassie Brilliant Blue staining. Lysates of COS7 cells were obtained by lysing half-confluent cells in a dish (diameter, 150 mm) using 1 ml of lysis buffer.

Immunofluorescence Microscopy—Cells fixed in 3.7% formaldehyde in PBS for 15 min were permeabilized with 0.2% Triton X-100 in PBS for 5 min. After incubation with primary antibodies (anti-p53, anti-FLAG and/or anti-Myc antibodies), followed by anti-mouse and anti-rabbit antibodies conjugated to fluorescein and rhodamine-conjugated phalloidin, the cells were observed by fluorescence microscopy.

RESULTS

Identification of LDOC1 as a WAVE3-Binding Protein—In order to isolate the proteins binding to WAVE3, we performed two-hybrid screening using the VPH domain of WAVE3 as "bait." The screening of approximately 6×10^6 clones yielded eight positive clones encoding LDOC1.

LDOC1 was discovered in 1999 and was found to be ubiquitously expressed in normal tissues, but down-regulated in pancreatic and gastric cancer cells. LDOC1 has leucine-zipper motifs. The ectopically expressed LDOC1 is reported to be localized in the nucleus (*10*).

Inhibition of BrdU Incorporation by LDOC1 Expression—LDOC1 has been suggested to function as a tumor suppressor gene product because its expression was observed to be reduced in certain types of cancer cells. To examine the role of LDOC1, we transiently expressed LDOC1 in MDCK cells and then measured the BrdU incorporation rate in these cells. In the cells expressing LDOC1, the BrdU incorporation rate was significantly reduced (Fig. 1). This indicates that the DNA synthesis activity of LDOC1-expressing cells was reduced.

Induction of Apoptosis by LDOC1 Expression—To further investigate the effect of LDOC1 on cell proliferation, we established an MDCK-tet-off-Myc-LDOC1 cell line that stably expresses LDOC1 when doxycycline (dox) is removed from the culture medium. In these cells, LDOC1 expression on dox removal inhibited cell proliferation (Fig. 2A), and detachment of cells from the culture dish was observed. Thus, we examined whether LDOC1 induces cell apoptosis.

We next performed the BrdU incorporation assay as well as TUNEL staining to examine the proliferation and apoptosis of LDOC1-expressing cells. As shown in Fig. 2B, the number of BrdU-positive cells was decreased 60 h after dox removal. Subsequently, TUNEL-positive cells were found to be markedly increased 96 h after dox removal. These results suggest that the induction of LDOC1 expression caused the cells to undergo apoptosis, which is first observed as the inhibition of proliferation.

To confirm the apoptosis of LDOC1-expressing cells, we analyzed the genome of the cells that were believed to undergo apoptosis upon LDOC1 expression. A fragmented genome was observed in these cells, while a fragmented genome was absent in cells cultured with dox (Fig. 2C). Therefore, we concluded that the LDOC1-expressing cells undergo apoptosis.

LDOC1 and Apoptotic Signals—Several signal transduction pathways are known to be involved in apoptosis induction. One major pathway is known to be linked to the increase in p53 tumor suppressor expression. Therefore, we analyzed the expression of p53 upon LDOC1 expression. As shown in Fig. 3A, an increase in the amount of p53 was observed upon LDOC1 expression. The accumulation of p53 was observed in the nuclei of these cells (Fig. 3B). The accumulation of p53 in the nucleus correlates with the increase in TUNEL-positive cells. These results indicate that LDOC1 expression caused the accumulation of p53,

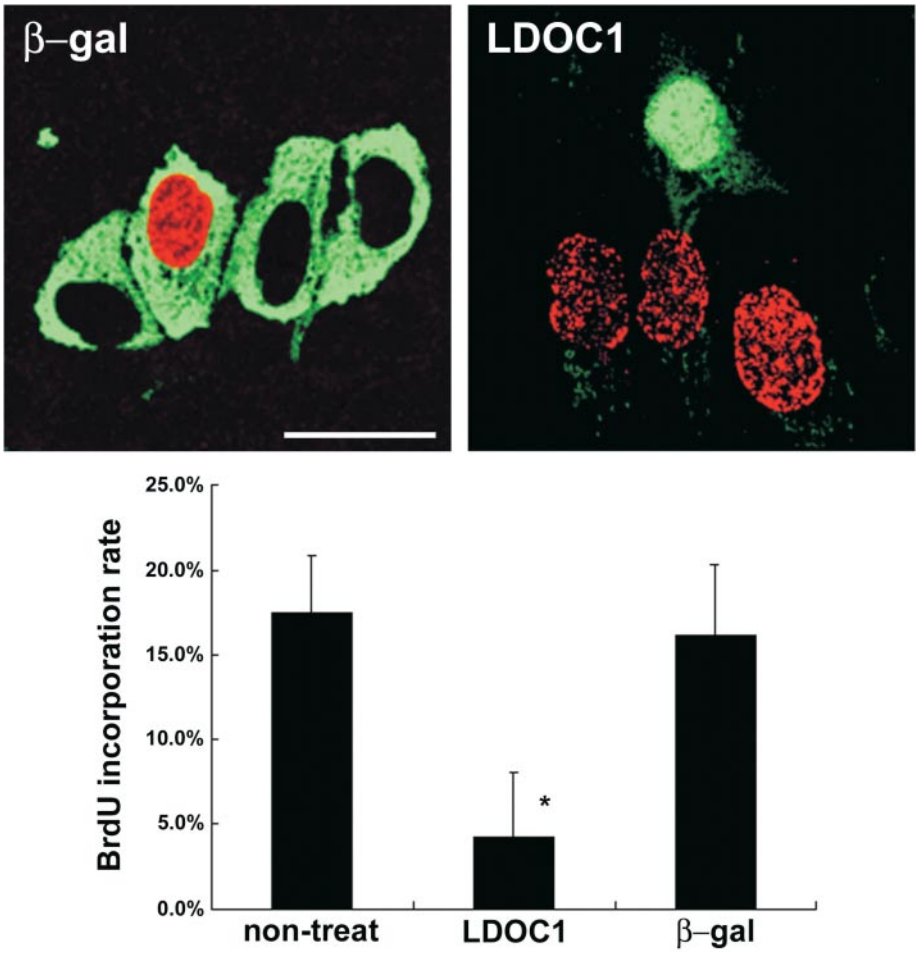

Fig. 1. **Inhibition of BrdU incorporation by LDOC1 expression.** The rate of incorporation of BrdU into LDOC1- or β-gal–expressing cells. MDCK cells were treated with BrdU and then stained with anti-BrdU (red), and anti-Myc or anti–β-gal antibodies (green). Bar, 20 μm. The bar-chart shows the rate of uptake of BrdU in MDCK cells. The BrdU incorporation rate in non-transfected cells was measured as a control. The results shown are averages of three independent experiments. Error bars represent standard deviations. * indicates statistical significance with student's *t*-test ($p < 0.05$).

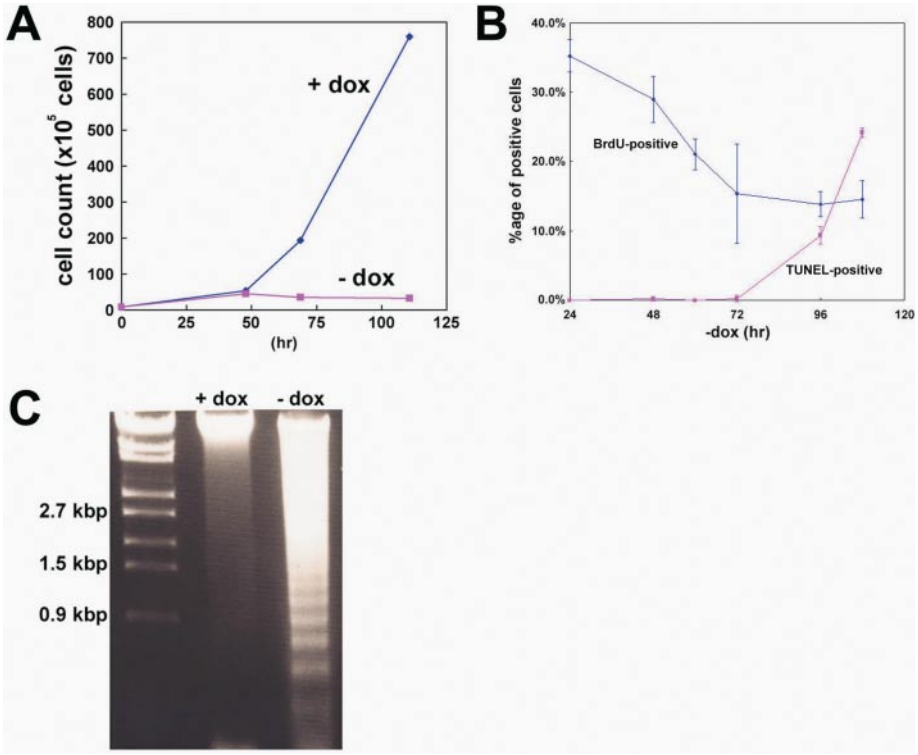

Fig. 2. **LDOC1-expressing MDCK cells undergo apoptosis.** (A) Proliferation of MDCK-tet-off-Myc-LDOC1 cells cultured with or without doxycycline (dox). The addition of dox suppressed LDOC1 expression, while removal of dox induced LDOC1 expression. (B) Apoptosis and DNA synthesis in cells exhibiting induced LDOC1 expression were simultaneously examined by TUNEL staining and BrdU incorporation assaying, respectively. BrdU and TUNEL double-positive cells were not observed. (C) Genomic DNA extracted from cells cultured with dox (+) (center) and ones cultured without dox (–) (expressing LDOC1) for 96 h was analyzed by agarose gel electrophoresis and ethidium bromide staining. The left lane shows the standards.

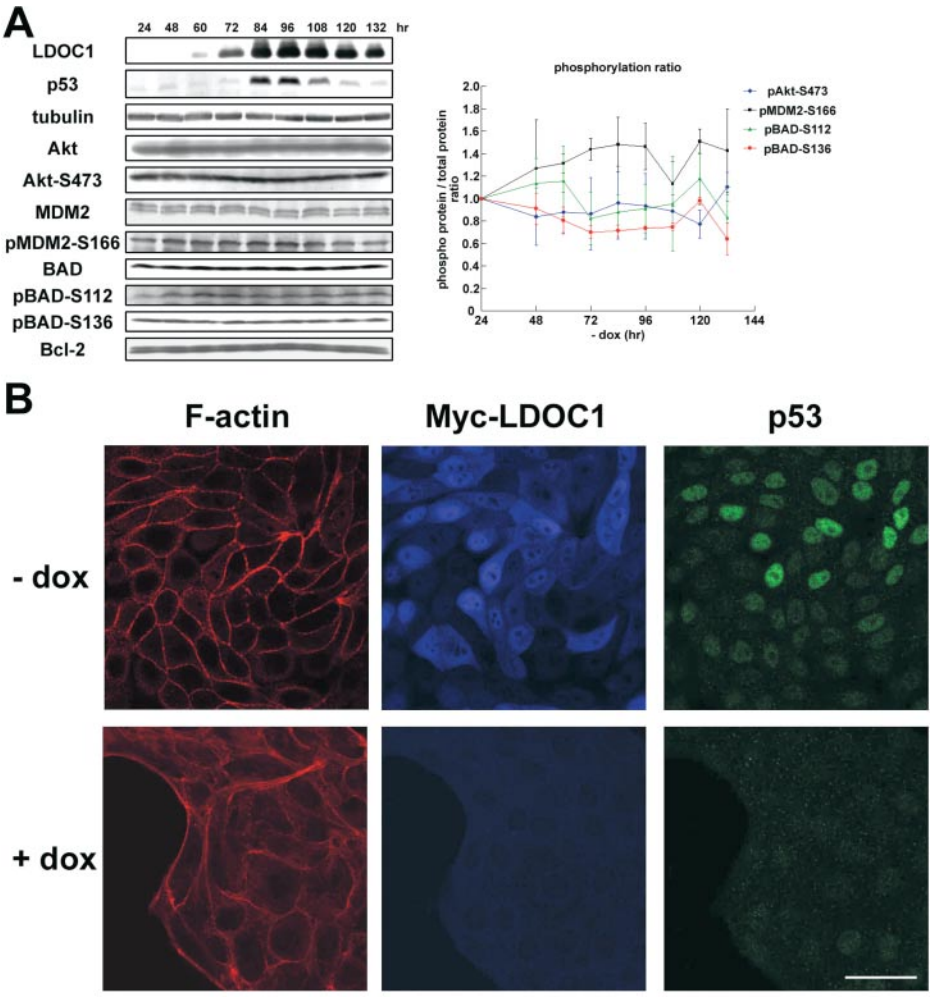

Fig. 3. **LDOC1 induces an increase in the p53 protein level.** (A) The expression of LDOC1 and proteins involved in apoptosis in MDCK-tet-off-Myc-LDOC1 cells. Cells were collected 24, 48, 60, 72, 84, 96, 108, 120 and 132 h after dox removal, and then immunoblotting with anti-Myc (LDOC1), p53, Akt, phospho-Ser473 Akt, MDM2, phospho-Ser166 MDM2, BAD, phospho-Ser112 BAD, phospho-Ser136 BAD or Bcl-2 was performed. Tubulin was also immunoblotted as a control. Densitometric analyses were carried out with ImageJ software. A line graph shows the phosphorylation ratios for Akt (Ser473), MDM2 (Ser166), and BAD (Ser112, Ser136). The phosphorylation ratio at 24 h after dox removal was set to 1.0, and the relative phosphorylation levels were plotted at the indicated times. (B) MDCK-tet-off-Myc-LDOC1 cells were cultured for 96 h with or without dox. Then, the cells were fixed and stained with phalloidin (for actin filaments, red), anti-Myc antibodies (blue), and anti-p53 antibodies (green). Bar, 40 μm. (C) Cell lysates and mRNAs were prepared from cells cultured with and without dox, and then Western and Northern blotting were performed to determine the amounts of p53 protein and mRNA, respectively.

thereby inducing apoptosis in the MDCK-tet-off-Myc-LDOC1 cells.

The amount of p53 protein was regulated at the mRNA transcription level and by protein degradation by proteases (*13*, *14*). Therefore, we next examined whether p53 mRNA was increased upon LDOC1 expression. Judging from the results of Northern blotting with RNAs from cells with and without LDOC1 expression, the amount of p53 mRNA did not change upon the expression of LDOC1 (Fig. 3C). Therefore, the increase in p53 caused by LDOC1 appears to be the result of suppression of the ubiquitin-proteasome system.

In order to examine the signals that are involved in LDOC1-induced apoptosis, we first focused on the signals that induce apoptosis of cells through p53. UV light or adriamycin treatment did not affect the LDOC1 expression in spite of p53 accumulation (data not shown). Oncogenic signals such as v-Ras, c-Myc and E2F-1 are also known to be involved in this process, but they did not affect the expression of LDOC1 (data not shown). The enhancement of p19/ARF was not induced by LDOC1 expression (data not shown).

We also examined the expression levels of other major mediators, such as Akt, MDM2, BAD and Bcl-2, involved in pro- or anti-survival signaling. Densitometric analyses revealed no significant changes in the amounts of these proteins upon LDOC1 expression. Moreover, we examined the phosphorylation levels of these proteins. Although not significantly, the phosphorylation levels of Akt-Ser473, BAD-Ser112 and BAD-Ser136 were slightly reduced, and that of MDM2-Ser166 was slightly increased (Fig. 3A).

Direct Interaction of LDOC1 with WAVE3—To confirm the binding of LDOC1 with WAVE3, we expressed GST-fused LDOC1 in bacteria. GST-LDOC1 immobilized on beads was mixed with a lysate of COS7 cells transfected with FLAG-WAVE1, -2, -3 or N-WASP. LDOC1 was likely to bind with these WASP-family proteins; however, it was likely to exhibit tight binding with WAVE3 (Fig. 4A). We also performed immunoprecipitation using a lysate of COS7 cells transfected with Myc-LDOC1 and either FLAG-WAVE3 or FLAG-WAVE3ΔV, the mutant with the VPH domain deleted. As shown in Fig. 4B, WAVE3ΔV

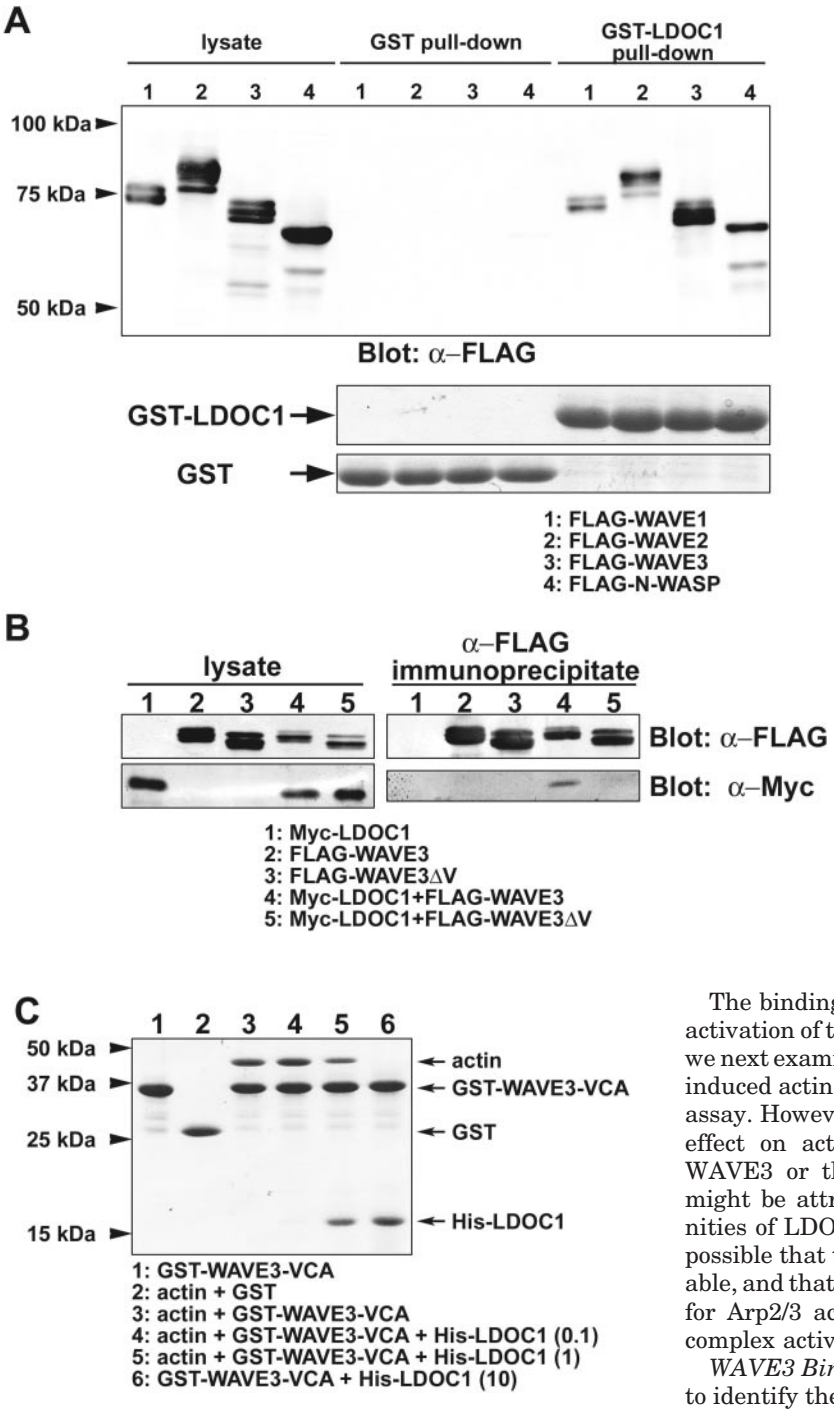

Fig. 4. **The direct interaction of LDOC1 with WAVE3.** (A) GST-fused LDOC1 immobilized on beads was mixed with a lysate of COS7 cells expressing FLAG-WAVE1, -2, -3 or -N-WASP. The precipitates were then analyzed by immunoblotting with anti-FLAG antibodies. The amount of immobilized GST or GST-LDOC1 was determined by Coomassie Brilliant Blue staining. (B) A lysate of COS7 cells expressing Myc-LDOC1 and either FLAG-WAVE3 or FLAG-WAVE3ΔV was immunoprecipitated with anti-FLAG antibodies. The precipitates were analyzed by Western blotting with anti-FLAG and anti-Myc antibodies. (C) GST-WAVE3 VCA immobilized on beads was subjected to a pull-down assay with purified LDOC1 and actin from rabbit muscle. The precipitates were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. The molar ratio of actin and LDOC1 was 1:0.1 in lane 4 and 1:1 in lane 5.

failed to bind with LDOC1, indicating that LDOC1 binds with WAVE3 through the VPH domain.

Since the VPH domain has been reported to bind with actin, we next examined whether the association of LDOC1 with the VPH domain has an effect on the actin binding ability of the VPH domain. The GST-WAVE3-VCA protein was immobilized on beads, and then the beads were mixed with either actin purified from rabbit muscle or His-tagged LDOC1. As shown in Fig. 4C, actin was competed out from the VPH domain by LDOC1, indicating that LDOC1 and actin bind to the VPH domain of WAVE3 in a mutually exclusive manner.

The binding of actin to the VPH domain is essential for activation of the Arp2/3 complex by the VCA domain. Thus, we next examined whether LDOC1 affects Arp2/3 complex–induced actin polymerization by means of the pyrene actin assay. However, the addition of LDOC1 had no significant effect on actin polymerization by the VCA domain of WAVE3 or the Arp2/3 complex (data not shown). This might be attributable to the difference between the affinities of LDOC1 and actin for the VCA domain. It is also possible that the binding of LDOC1 and actin is exchangeable, and that transient binding of actin to VCA is sufficient for Arp2/3 activation. The precise mechanism of Arp2/3 complex activation by the VCA domain is unknown.

WAVE3 Binding Region in LDOC1—We next attempted to identify the region of LDOC1 that binds to the WAVE3-VCA region. We divided LDOC1 into three fragments—the region containing the leucine-zipper motif (L), the proline-rich region (P), and the acidic region (A) (Fig. 5A). A GST-fusion protein of each region was constructed and immobilized on beads. The beads were mixed with a lysate of COS7 cells expressing FLAG-WAVE3, and the precipitates were analyzed by Western blotting. LDOC1 bound to WAVE3 through the acidic region of LDOC1, although its binding ability was reduced in comparison with that of the full-length LDOC1 (Fig. 5B).

When the full-length LDOC1 and fragments of each region were expressed in MDCK cells, the acidic region of LDOC1 was localized in the nucleus similar to the

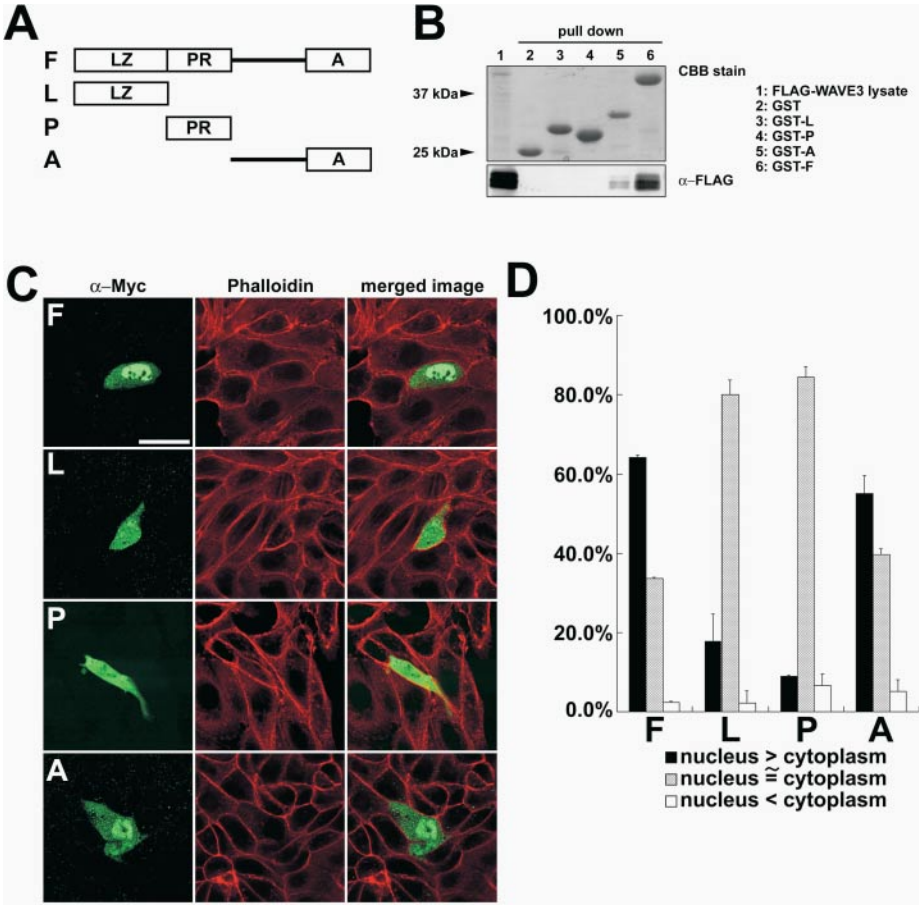

Fig. 5. **Direct association of WAVE3 with the C-terminal region of LDOC1.** (A) Schematic structures of LDOC1. The N-terminal region has a leucine zipper motif (L), the central region has a proline-rich sequence (PR), and the C-terminal region is a highly acidic region (A). LZ, PR and A indicate the leucine zipper motif, proline-rich region and acidic region, respectively. (B) GST-fusion proteins of LDOC1 deletion mutants were immobilized on beads and then mixed with a lysate of COS7 cells expressing FLAG-WAVE3. The precipitates were analyzed by immunoblotting using anti-FLAG antibodies. (C) Myc-tagged wild-type LDOC1 or LDOC1 deletion mutants were expressed in MDCK cells. The cells were stained with anti-Myc antibodies for LDOC1 (green) and phalloidin for actin filaments (red). Bar, 20 μm. (D) The percentage of cells with a greater amount of LDOC1 in the cytoplasm than in the nucleus, that with a greater amount of LDOC1 in the nucleus than in the cytoplasm, and that with LDOC1 equally distributed between the nucleus and the cytoplasm were determined for each experiment.

localization of the full-length LDOC1; however, the leucine-zipper motif or the proline-rich region of LDOC1 was localized in both the cytosol and nucleus (Fig. 5, C and D).

Effects of WAVE3 on LDOC1 Localization and LDOC1-Induced Apoptosis—We then examined the effect of WAVE3 on LDOC1 localization because LDOC1 is localized in the nucleus, whereas WAVE3 is expressed in the cytoplasm. When FLAG-tagged WAVE3 and Myc-tagged LDOC1 were transfected into MDCK cells, LDOC1 was localized in the cytoplasm on co-expression of WAVE3 (Fig. 6). Therefore, WAVE3 might determine the nuclear localization of LDOC1.

Finally, we investigated the effect of a change in the localization of LDOC1 caused by WAVE3 on LDOC1-induced apoptosis, because LDOC1 is localized in the nucleus and induces apoptosis. After dox removal, FLAG-WAVE3 was expressed in MDCK-tet-off-Myc-LDOC1 cells, and TUNEL staining was performed. While MOCK-transfected cells as well as non-transfected cells underwent apoptosis, the number of apoptotic cells among the WAVE3-expressing cells was reduced, as determined on TUNEL staining (Fig. 7). Therefore, it is possible that WAVE3 negatively regulates the function of LDOC1 by controlling its localization.

DISCUSSION

In certain types of cancer cells, the expression of LDOC1 is reduced, and thus it has been suggested that LDOC1 functions as a tumor suppressor gene product (*10*, *11*). In fact, LDOC1-expressing cells undergo apoptosis presumably via an increase in the p53 protein level (Figs 1, 2 and 3).

The mechanism underlying the increase in the p53 protein level caused by LDOC1 in MDCK cells is not yet understood. p53 exerts an anti-proliferative effect, involving growth arrest, genetic stability and apoptosis, through the induction of genes regulating cell cycle arrest and apoptosis. p53 is maintained an extremely low and often undetectable level due to its degradation, which is brought about by the ubiquitination of p53 by MDM2 and its subsequent degradation by proteasome (*13*, *14*). The level of p53 increases in response to various types of stress such as DNA damage induced by UV light or chemicals (*15*, *16*), oncogenic signals (*17*), hypoxia (*18*), and heat shock (*19*). In response to DNA damage, p53 is phosphorylated at Ser-15 by one of the subfamilies of the phosphoinositide-3-kinase–related kinase superfamily, namely, the ATM/ATR subfamily (*15*, *16*). p53 phosphorylated at Ser-15 exhibits reduced affinity for its inhibitor protein, MDM2; thus, p53 accumulates and is functionally activated. In response to particular oncogenic signals, p19/ARF can activate p53 by directly inhibiting MDM2, thereby, inducing cell-cycle arrest or apoptosis (*20*, *21*).

To clarify the upstream regulator of LDOC1 in p53 accumulation, LDOC1 expression was examined after stimuli for p53 accumulation, such as UV light, adriamycin treatment as well as oncogenic signals, such as v-Ras, c-Myc and E2F-1, were provided. However, no changes were observed

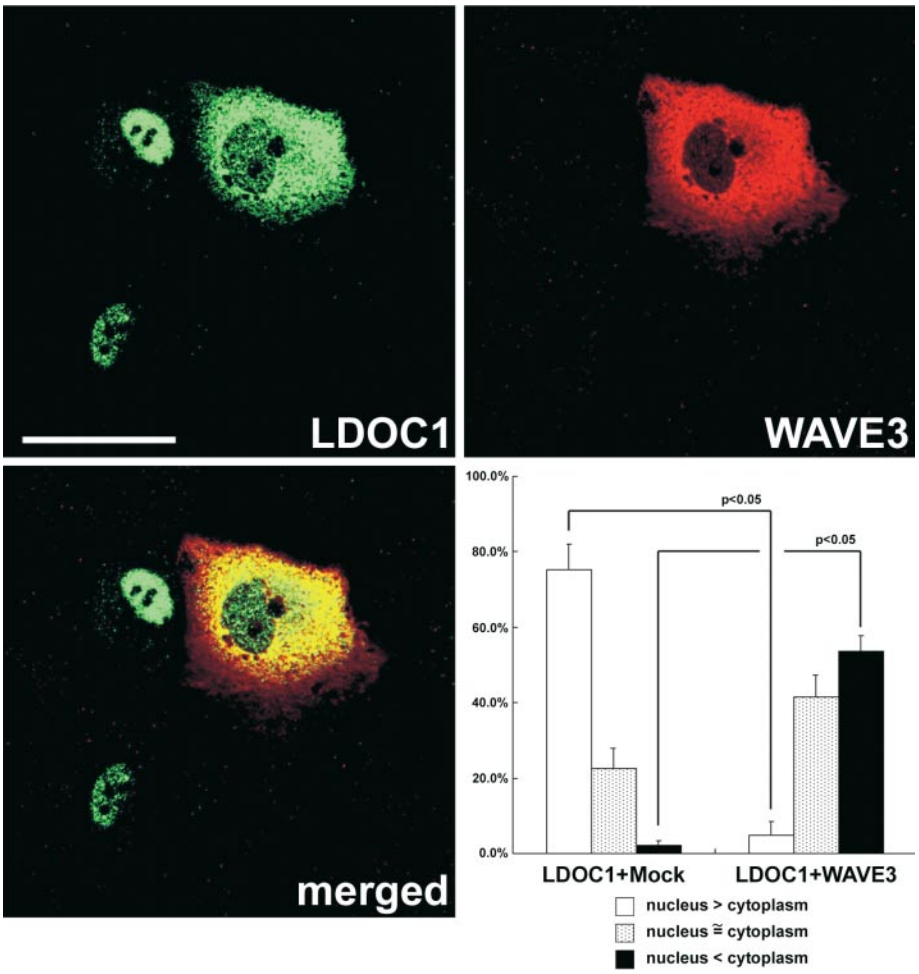

Fig. 6. **Ectopic expression of WAVE3 and LDOC1.** Myc-tagged LDOC1 and FLAG-tagged WAVE3 were expressed transiently in MDCK cells, and then the cells were stained with anti-Myc (green) and anti-FLAG (red). Bar, 20 μm. The percentage of cells with a greater amount of LDOC1 in the cytoplasm than in the nucleus, that with a greater amount of LDOC1 in the nucleus than in the cytoplasm and that with LDOC1 equally distributed between the nucleus and the cytoplasm were determined for each experiment. The significance of differences was determined by means of student's *t*-test.

in the expression of LDOC1 (data not shown). The enhancement of p19/ARF was not detected on expression of LDOC1 in the cells (data not shown). It is possible that LDOC1 directly influences MDM2; however, direct binding was not observed on immunoprecipitation (data not shown). The expression level of MDM2 did not change upon LDOC1 expression, while a slight increase was observed in that of phospho-MDM2. However, the degradation of p53 commenced 108 h after dox removal, that is, 48 h after LDOC1 expression. On the other hand, the increased phospho-MDM2 level was maintained during the experiment. At present, the reason for the prior accumulation of p53 is unknown. Thus, the association between LDOC1 and p53 accumulation through MDM2 remains to be determined. Moreover, when the MDCK cells were exposed to UV light, phosphorylated p53 (band shift) as well as an increase in p53 were observed. On the other hand, p53 was not phosphorylated in the MDCK cells expressing LDOC1 (data not shown). We also examined several proteins involved in apoptosis upon LDOC1 expression (Fig. 3A). No significant changes were observed in the Akt, BAD and Bcl-2 expression levels; however, slight reductions were observed in the phosphorylation levels of Akt and BAD. It is possible that LDOC1 expression directly affects the phosphorylation levels of these proteins through an unknown mechanism, leading to p53 accumulation. It is also possible that simply because of the effect on LDOC1-induced apoptosis through p53 accumulation, there are reductions in the phosphorylation levels of these proteins. However, it is unclear which pathway primarily acts to reduce these phosphorylated protein levels. A more detailed study of LDOC1-induced apoptosis is needed. In addition, an increase in p53 expression was not observed in NIH3T3 cells expressing LDOC1 (data not shown). These observations indicate that LDOC1 might function in a different manner, depending on the cell type.

Among the WASP family proteins, LDOC1 interacts with WAVE3 with the highest affinity (Fig. 4A), and the pattern of LDOC1 expression in tissues is similar to that of WAVE3 (*2*, *10*). Thus, LDOC1 might bind primarily with WAVE3 in vivo. We also found that the C-terminus acidic region of LDOC1 interacted with the WAVE3 VPH domain. Since the binding of LDOC1 with WAVE3 did not have much effect on in vitro actin polymerization (data not shown), WAVE3 may play a role as a carrier or scaffold for LDOC1. In fact, it has been reported that WAVE1 is translocated from the nucleus to the cell membrane in a stimulus-dependent manner. In this case, WAVE1-binding proteins, such as PKA and Abl, are recruited to the membrane upon WAVE1 translocation (*22*). Furthermore, it has been reported that endogenous WAVE1 and WAVE2 are localized in the nucleus as well as in the cytoplasm (*5*). Therefore, it is possible that shuttling of WAVE3 between the nucleus and the cytoplasm also

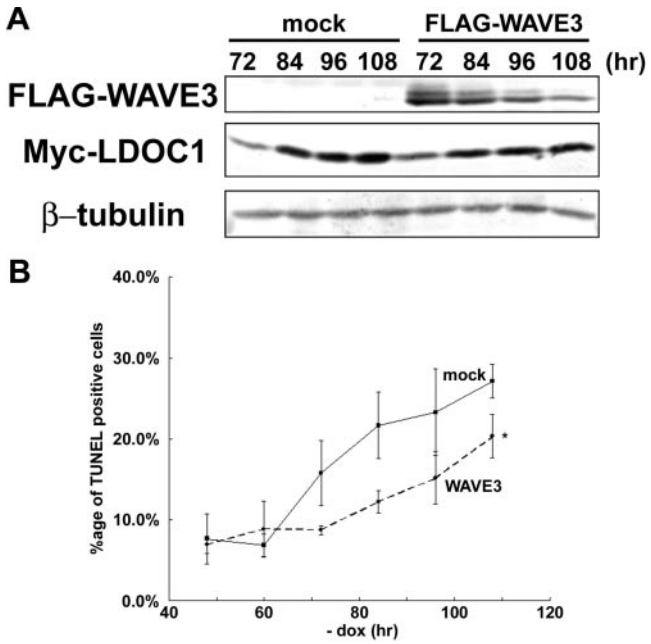

Fig. 7. **Inhibition of LDOC1-induced apoptosis in WAVE3-expressing cells**. (A) FLAG-tagged WAVE3 was expressed transiently in MDCK-tet-off-Myc-LDOC1 cells, 24 h after dox removal. The cells were fixed at the indicated times after dox removal, and then immunoblotting was performed. (B) Control and WAVE3-expressing MDCK-tet-off-Myc-LDOC1 cells were TUNEL stained. TUNEL-positive cells were counted at the indicated times. Error bars represent standard deviations. * indicates statistical significance with student's *t*-test ($p < 0.05$).

occurs. In this context, WAVE3 might transport LDOC1 out of the nucleus and inhibit its apoptotic function. However, we do not exclude the possibility that LDOC1 forms a complex with WAVE3 in the cytoplasm, because LDOC1 is localized not only in the nucleus but also in the cytoplasm. In this case, the complex of LDOC1 and WAVE3 probably is not translocated to the nucleus. As a result, the amount of nuclear LDOC1 is reduced, leading to prevention of cellular apoptosis. In any case, it is likely that the intracellular localization of LDOC1 is regulated by the localization of the exogenous WAVE3 (Fig. 6). WAVE3 might negatively regulate the function of LDOC1 in the nucleus through the translocation of LDOC1 from the nucleus to the cytoplasm.

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