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The C-terminus of Ubl4A is critical for pro-death activity and association with the Arp2/3 complex

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Abstract

Ubl4A is a small ubiquitin-like protein involved in diverse cellular functions. We have shown that Ubl4A is critical for survival of the starvation-mediated cell death *in vivo*. The underlying mechanism for this is through interaction with the actin-related protein Arp2/3 complex and promotion of actin branching. Interestingly, "put-back" of Ubl4A to *Ubl4A*-deficient cells also results in cell death. Removal of the Ubl4A N-terminus significantly enhances its cytotoxicity, indicating that the pro-death activity of Ubl4A is mainly from its C-terminal region. *In vitro* protein pull-down assays show that the C-terminal region of Ubl4A can directly interact with the Arp2/3 complex. The single point mutation of an aspartic acid to alanine (D122A) in the Ubl4A C-terminus abolishes its ability to bind the Arp2/3 complex. This mutation also destabilizes Ubl4A proteins susceptible to protease degradation. Importantly, expression of wild-type Ubl4A can induce cell death in colon cancer cells, but such pro-death activity is diminished in the D122A mutant. These data suggest that Ubl4A C-terminus, especially D122, is critical for Ubl4A-Arp2/3 interaction and its pro-death function.

Keywords

Ubl4A; cell death; Arp2/3; protein interaction

1. Introduction

Ubl4A is a small protein encoded by a housekeeping gene [1]. Its yeast homolog, Get5, is a well-studied C-tail chaperone protein responsible for the translocation of nascent synthesized proteins from the ribosomes to the endoplasmic reticulum (ER) [2–4]. Ubl4A has 157 amino acids. The N-terminal half (amino acid 1 to 74) shares the homology with ubiquitin, but lacks ubiquitination activity. The C-terminal half of the protein (75 to 157) contains several conserved motifs and residues that are critical for interactions with its partner proteins, such as Get4 in yeast and BAG6 in human, and for recruiting target proteins for ER translocation [5,6].

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In mammals, in addition to the chaperone function, Ubl4A also involves in many other cellular functions, such as suppression of tumorigenesis and induction of DNA damage-mediated apoptosis [7,8]. Our previous work has shown that *Ubl4A*-deficient mice appeared to be normal with moderate low body weight, but the neonatal mortality was significantly higher than their wild-type littermates [9]. One of the defects of the *Ubl4A*-knockout mice was lack of Akt-mediated glycogen storage during the birth. Activation of Akt requires an immediate translocation from the cytosol to the plasma membrane upon stimulation [10]. This process requires formation of actin branches underneath of cell membrane. Ubl4A interacts with actin-related protein Arp2/3 and promotes actin branches, which serve as "bridges" bringing Akt to the plasma membrane for activation.

Actin-related protein 2/3 (Arp2/3) is a large protein complex containing seven subunits and its function in the formation of actin branches has been extensively studied [11,12]. Arp2/3 binds to the mother actin filaments and recruits daughter F-actin monomers to build up Y-shaped actin branches [13,14]. These actin branches are dynamic and essential for cell membrane ruffling, cell migration, and phagocytosis [15–17]. We have shown that knockout of *Ubl4A* significantly decreased the migration for both fibroblasts and macrophages [18]. The activity of Arp2/3 is regulated by several associated proteins. Two of the well-characterized activators are N-WASp (neural Wiskott-Aldrich syndrome proteins) and cortactin (a Src kinase substrate) [19,20]. Both proteins can simultaneously bind to the Arp2/3 complex in synergizing activation of Arp2/3 and actin assembly. Mutation of tryptophan 22 (W22) in cortactin abolishes both interaction and activation of the Arp2/3 complex by cortactin [19].

Interestingly, interactions of Ubl4A with other associated proteins are also mostly through the Ubl4A C-terminal region, such as binding BAG6 in human or dimerization with Get4 in yeast [5,6]. We wondered whether Ubl4A also used its C-terminal region for interaction with Arp2/3. In this report, we used genetic and biochemical approaches to identify the region in Ubl4A that is responsible for the direct interaction with Arp2/3. We also detected a residue in the Ubl4A C-terminal region that is critical not only for the interaction with Arp2/3 and stabilization of the protein, but also for Ubl4A pro-death activity.

2. Materials and methods

2.1 Materials

T4 DNA ligase, N-Ethylmaleimide (NEM) and Dithiothreitol (DTT) were obtained from Thermo Fisher Scientific. Glutathione-agarose and Dynabeads™, Leupeptin, Pepstatin and Aprotinin were purchased from Sigma-Aldrich. The Arp2/3 protein complex was obtained from Cytoskeleton. For immunoblotting, Pierce® ECL Western Blotting Substrate developing kit was obtained from Thermo Fisher Scientific. Rabbit antibody against Ubl4A was from Sigma-Aldrich. Mouse antibodies against Arp2 and green fluorescent protein (GFP) were from Santa Cruz Biotechnology. Horseradish peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Jackson ImmunoResearch.

2.2 Mutagenesis and cloning

All Ubl4A constructs used in this study were generated from mouse *Ubl4A*. QuikChange II site-directed mutagenesis kit (Agilent) was used for mutagenesis according to the manufacturer's protocol. For green fluorescence protein (GFP)-tagged protein expression, the constructs were cloned by insertion of PCR products into a pEGFP-N1 vector between the *EcoRI* and *BamHI* sites, in-frame fused with GFP at the C-terminus of Ubl4A. For expression of GST-fused recombinant proteins, the wild-type and mutant Ubl4A constructs were cloned into the pGEX-KG vector between the Bam*HI* and Xba*I* sites, with GST at the N-terminus of Ubl4A. All constructs were validated by DNA sequencing. Expression of the proteins was validated by immunoblotting using anti-Ubl4A or anti-GFP antibody accordingly.

2.3 Pull-down assay

Expression of GST recombinant proteins in BL21 (DE3) competent cells was induced with IPTG (1 mM) for 4 h according to the standard procedure. The cells were pelleted and resuspended in lysis buffer (50 mM HEPES, pH 7.2, 0.2% NP-40, 145 mM KCl, 5 mM $MgCl_2$, 20 mM EDTA, 1 mM EGTA, 10 mM DTT, 10 μg/ml Aprotinin, 10 mM Leupeptin, 10 mM NEM) with or without 10 μg/ml Pepstatin as indicated in the text. After lysis, the cell lysates were centrifuged to remove the cell debris. The clear supernatants were incubated with glutathione-agarose beads for 30 min on a rotator at room temperature. Then, the bead-protein complexes were washed four times with a high salt washing buffer (50 mM Tris-HCl, pH 8.3, 500 mM NaCl, 0.02% Tween 20). Purified Arp2/3 (250 ng for each reaction) complex was added into the Ubl4A-beads complex and mixed well. After incubation overnight on a nutator at 4 °C, the pulldown Arp2/3-Ubl4A-beads complexes were washed twice with a washing buffer (50 mM Tris, pH 8.3, 150 mM NaCl) and subjected to protein electrophoresis.

2.4 Cell culture and transfection

Mouse-embryonic fibroblast cells (MEFs) from *Ubl4A*-deficient mice (KO) and wild-type littermates (WT) were derived as previously reported [8]. Human colorectal cancer HCT116 cells were obtained from ATCC. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2 in a water-jacketed incubator. Cells were generally passed every two days for maintenance. For DNA transfection, cells were seeded into a 6-well plate and grown until a confluence of 60% prior to transfection. The cells were then washed with sterilized phosphate-buffered saline (PBS) and transfected with 3 μg DNA with Lipofectamine™ 3000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol.

2.5 Immunoblotting

The protein samples from the recombinant protein expressing bacteria, transfected cells, or pull-down assays were denatured by incubation of the samples at 95 °C for 3 min. An equal amount of protein of each sample was loaded onto a 12% or 15% SDS-PAGE gel depending on the sizes of the targeted proteins. The proteins on the gel were then transferred onto a 0.2 μm PVDF membrane. The membrane was blocked with 5% BSA in a washing buffer (0.2%

Tween 20 in PBS) before incubation with primary antibody against Arp2 (1:500 dilution), GFP (1:1000), Actin (1:3000), or Ubl4A (1:1000) overnight at 4 °C, followed by incubating for 1 h with appropriate HRP-conjugated secondary antibody at room temperature. The protein bands were visualized on X-ray films with Pierce® ECL Western Blotting Substrate developing kit (Thermo Fisher Scientific).

2.6 Caspase and cell death assays

For the caspase assay, MEFs cells were subjected to starvation by washing once with plain DMEM, followed by a 16 h incubation in plain DMEM. Cells were then treated with 2 μ NucView® 530 Caspase-3 substrate (Biotium) for 30 min in a 37 °C incubator. After incubation, both floating cells and attached cells were collected for imaging, and cells containing activated caspase-3 were counted for cell death. For transfection-induced cell death, cells were transfected with either GFP or GFP-tagged constructs as indicated in the text for 24 h or 48 h. Cells were then treated with 1 μM Ethidium homodimer-1 (EthD-1) (Invitrogen) for 15 min in a 37 °C incubator. After incubation, both floating cells and attached cells were collected for imaging or subjected to cell counting using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific). Ratio of EthD-1 positive cells within GFP-positive cells were calculated for cell death.

2.7 Statistics

All values represent the mean ± standard deviation (SD). One-way ANOVAs followed by Tukey test were performed using GraphPad Prism 7.03. *P* values < 0.05 were considered significant (*, < 0.05. **, < 0.01. ***, < 0.001).

3. Results

3.1 The Ubl4A protein has both pro- and anti- death functions and the cytotoxicity of Ubl4A is mainly from its C-terminus

We have previously shown that the newborns from *Ubl4A*-deficient mice were very sensitive to starvation stress during the birth, with a significantly higher neonatal mortality than that from the wild-type littermates [9]. Consistently, the *Ubl4A*−/− MEFs (mouse embryonic fibroblasts) were also more sensitive to starvation-induced cell death than the wild-type cells (Fig. 1A). Logically, put-back of wild-type Ubl4A would be expected to rescue the cell death phenotype. To our surprise, ectopic expression of Ubl4A in *Ubl4A*−/− MEFs resulted in a significant amount of cell death, similar to that seen under starvation treatment (Fig. 1B). To further study this, we generated several GFP-tagged Ubl4A constructs: full length (GFP-Ubl4A-F), N-terminus (GFP-Ubl4A-N), and C-terminus (GFP-Ubl4A-C) (Fig. 1C). Ubl4A-N encodes amino acid 1 to 74, which is a ubiquitin homologous region, and Ubl4A-C encodes amino acid 75 to 157, which is the region that contains conserved domains known for association with other proteins, such as BAG6 [6,8]. *Ubl4A*−/− MEFs were transfected with these constructs, and cell death was quantitated only from transfected GFP-positive cells. As shown in Figure. 1D, ectopic expression of full-length Ubl4A was indeed toxic to the cells. However, the toxicity from the C-terminal portion of the protein (Ubl4A-C) was significantly higher than either the full-length or N-terminus, although the amount of expressed N- and C- terminal proteins was similar in both (Fig. 1E). These results suggest

that removal of the N-terminus could enhance the cytotoxicity of Ubl4A, indicating that the cytotoxicity of Ubl4A is mainly from its C-terminus.

3.2 The C-terminal region of Ubl4A is responsible for interaction with the Arp2/3 complex

It is well-known that the C-terminus of the Ubl4A protein is essential for interaction with other proteins, such as BAG6, for its chaperone function [5,6]. We have previously shown that Ubl4A can directly interact with the Arp2/3 complex [9]. However, the interaction domain was unknown. As we noted here that ectopic expression of the C-terminal Ubl4A led to cell death, we wondered that whether the C-terminus of Ubl4A was also involved in binding with Arp2/3. To exam this, we generated the full-length, N-terminal, and C-terminal Ubl4A recombinant proteins, with a GST-tag at their N-terminal ends. The result from the GST-bead pull-down assay with the purified Arp2/3 complex showed that Arp2/3 could be pulled down by GST-Ubl4A-F, but not GST alone, as indicated by immunoblotting with anti-Arp2 antibody (Fig. 2A, top panel with anti-Arp2 antibody). GST-Ubl4A-C proteins were also able to pull down Arp2/3 equally well as that of GST-Ubl4A-F, while the GST-Ubl4A-N proteins were barely able to bind Arp2/3, even though the amount of expressed proteins was not less than the other two (Fig. 2A, bottom panel, Coomassie Blue-stained membrane). These results indicate that the C-terminus of Ubl4A can directly bind to Arp2/3.

Extensive structural studies have been well-documented and shown that the dimerization of Get5 (Ubl4A yeast homolog) with Get4 and the association of Ubl4A with BAG6 are both located in the Ubl4A C-terminus, involving a set of conserved hydrophobic residues [5,6]. There are three alpha helixes in this region, with both the α1 and α2 helixes containing several residues conserved across species (Fig. 2B). For the initial test, we generated two GST-tagged truncated proteins, Ubl4A-97 and Ubl4A-120. Ubl4A-97 was terminated in the beginning of the α1 helix; Ubl4A-120 construct contains intact α1 helix and the first half of α2 helix. The results from pull-down assays showed that neither of these two proteins was able to pull down Arp2/3 (Fig. 2C, top panel with anti-Arp2 antibody), although both proteins were expressed well relative to the full-length Ubl4A proteins (Fig. 2C, bottom panel with anti-Ubl4A antibody). Although Ubl4A-120 contains more than two-third of the Ubl4A peptide sequence and most of the conserved residues, it was not sufficient for Ubl4A to bind Arp2/3. These results suggest that the critical domain for Ubl4A association with Arp2/3 might be located at the very end of the C-terminus of Ubl4A.

3.3 A single mutation in the Ubl4A C-terminal putative acidic region abolishes its Arp2/3 binding ability

The Arp2/3-regulatory proteins, such as N-WASp and cortactin, typically interact with the Arp2/3 complex through a well-defined and conserved “acidic domain”, which is rich in acidic residues (95% in N-WASp, and 42% in cortactin). This region also contains a conserved “DDW” (two aspartic acids followed by tryptophan) motif, in which tryptophan has been shown to be critical for cortactin in binding and activating Arp2/3 [19]. For Ubl4A, there is a putative “acidic domain” region (about 33% of acidic residues) (Fig. 3A boxed). Also, Ubl4A has no “DDW” motif. Instead, Ubl4A has a “DYD” motif (tyrosine between two aspartic acids), in which the first “D” (D122) is well conserved with both N-WASp and cortactin (Fig. 3A, bolded amino acids). To test whether D122 is critical for Ubl4A

interaction with Arp2/3, we generated both single-mutation (D122A) and double-mutation (D122A/D124A) constructs. We had a difficulty in detecting both recombinant proteins by standard Coomassie Blue staining. However, immunoblot of GST-purified proteins showed that, in comparison with the full-length Ubl4A recombinant proteins, both mutant proteins were unstable with predominant degraded bands below the expected size (Fig. 3B, compare lane 4 with 5 and 6). The degradation process seemed to have already occurred inside the living cells given the fact that the immediately boiled cell lysates also showed that a significant amount of mutant proteins had degraded in comparison to the full-length Ubl4A proteins (Fig. 3B, compare lane 1 with 2 and 3). However, addition of the aspartic peptidase inhibitor, Pepstatin, (Fig. 3B, compare lane 5 and 6 with 7 and 8) seemed to significantly block the degradation.

As both mutant proteins were susceptible to degradation even in the presence of a standard cocktail of protease inhibitors (Leupeptin, Aprotinin, and NEM), we wondered whether such mutations could change the protein secondary structures, which resulted in instability. To test this, we analyzed both mutants using a secondary structure computational prediction software (Netsurfp, version 1.1) in comparison with the wild-type Ubl4A sequence. By this approach, we found that neither the single or double mutations appeared to alter the helicity for all three helixes in the C-terminus (Fig. 3C). We next performed the pull-down assay, and the result showed that both D122A and D122/D124A mutants were expressed at similar levels compared to the wild-type Ubl4A in the presence of pepstatin inhibitors (Fig. 3D, bottom panel with anti-Ubl4A antibody). However, neither of these mutants was able to pull down Arp2/3 (Fig. 3D, top panel with anti-Arp2 antibody). These results indicate that the D122 in Ubl4A at its C-terminus is critical for both association with Arp2/3 and stability of the proteins.

3.4 The Ubl4A Arp2/3-binding deficient mutant cannot induced cell death in cancer cells

The pro-survival role of Ubl4A is through its interaction with Arp2/3 [9]. It has been shown that Ubl4A has anti-tumorigenesis function [7]. As ectopic expression of Ubl4A also has a pro-apoptotic function, as we observed above (Fig. 1), we wondered whether the loss of Ubl4A's ability to associate with Arp2/3 also altered its pro-death function in cancer cells. To test this, we transfected colorectal cancer HCT116 cells with GFP-tagged wild-type, D122A mutant, as well as N- and C-terminal Ubl4A constructs as controls. As expected, the wild type full-length GFP-Ubl4A-F, but not GFP alone, caused significant cell death (Fig. 4A and B). Expression of GFP-Ubl4A-C proteins showed the most cytotoxicity, significantly greater than that seen with either the N-terminal or full-length proteins (Fig. 4B), which is consistent with the results we observed in *Ubl4A*−/− MEFs cells (Fig. 1). Importantly, unlike the wild-type control, the D122A mutant was unable to induce cell death in these cancer cells, although the amount of expressed proteins was similar for both proteins (Fig. 4C). These results indicate that the Ubl4A-Arp2/3 binding deficient mutation also abolishes its ability to induce cell death in colon cancer cells.

4. Discussion

Newborns from *Ubl4A* deficient mice are susceptible to starvation-stress during the birth and have a higher rate of mortality than that from their wild-type littermates [9]. We have demonstrated such Ubl4A function is through its interaction with the Arp2/3 complex, thereby promoting actin branching and activating survival factors, such as Akt kinase [9]. However, the region of Ubl4A that binds to Arp2/3 remained unknown. Here, we show that the Ubl4A C-terminal region has a pro-death activity and can directly interact with Arp2/3 (Fig. 1 and Fig. 2). Interestingly, a single point mutation (D122A) in the Ubl4A C-terminal abolished its binding ability for Arp2/3 (Fig. 3) and significantly diminished the ability of Ubl4A to induce cell death in colorectal cancer cells (Fig. 4).

Multiple regulatory proteins, such as N-WASp and cortactin, can bind to the Arp2/3 complex simultaneously to exert a synergistic effect [19,20]. Both proteins have a hallmark acidic domain, in which there is a conserved DDW (two aspartic acids followed by tryptophan) motif responsible for their binding with the Arp2/3 complex [19,20]. Cortactin interacts with only one of the subunits in the Arp2/3 complex, and mutation of W22 in the DDW motif within the acidic domain of cortactin abolishes both bind and activation of Arp2/3 [19]. On the other hands, N-WASp binds multiple subunits of Arp2/3, and single point mutation of the DDW motif does not affect its abilities for both binding and activating Arp2/3 [19]. Ubl4A does not have a typical DDW motif. Instead it has a DYD motif at the equivalent position with the first aspartic acid (D122) conserved. A single point mutation (D122A) in the DYD motif could abolish the ability of Ubl4A to interact with Arp2/3. This suggests that D122 is critical for the association between Ubl4A and Arp2/3. However, the subunit of Arp2/3 that Ubl4A associates with remains to be investigated. Whether Ubl4A interacts with Arp2/3 at multiple sites or shares its binding sites with either cortactin or N-WASp also remains to be explored.

The anti-tumor function of Ubl4A has been demonstrated in colorectal cancer *in vivo* [7]. The underlying mechanism is through regulating the phosphorylation status of STAT3, a transcriptional factor with a key role in tumorigenesis when constitutively phosphorylated [7]. Here, we showed that removal of the N-terminus of Ubl4A could “uncover” the cytotoxicity of Ubl4A, indicating that the C-terminus of Ubl4A contains a pro-death domain. Consistently, a couple of Ubl4A truncated mutations lack of the C-terminus were found in some pancreatic cancer patients. Whether such a pro-death function of Ubl4A is through regulating phosphorylation status of STAT3 remains to be explored. Interestingly, a single point mutation (D122A) significantly diminished the ability of Ubl4A to induce cell death in colorectal cancer cells. As the D122A mutant also lost its ability to bind Arp2/3, these results suggest that the association with Arp2/3 might be critical for Ubl4A to exert its pro-death activity in cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- The Ubl4A C-terminus has a pro-death function
- Ubl4A interacts with the Arp2/3 complex through its C-terminus
- A single mutation in Ubl4A C-terminus abolishes its association with Arp2/3
- The D122A mutation also diminishes Ubl4A cytotoxicity in cancer cells

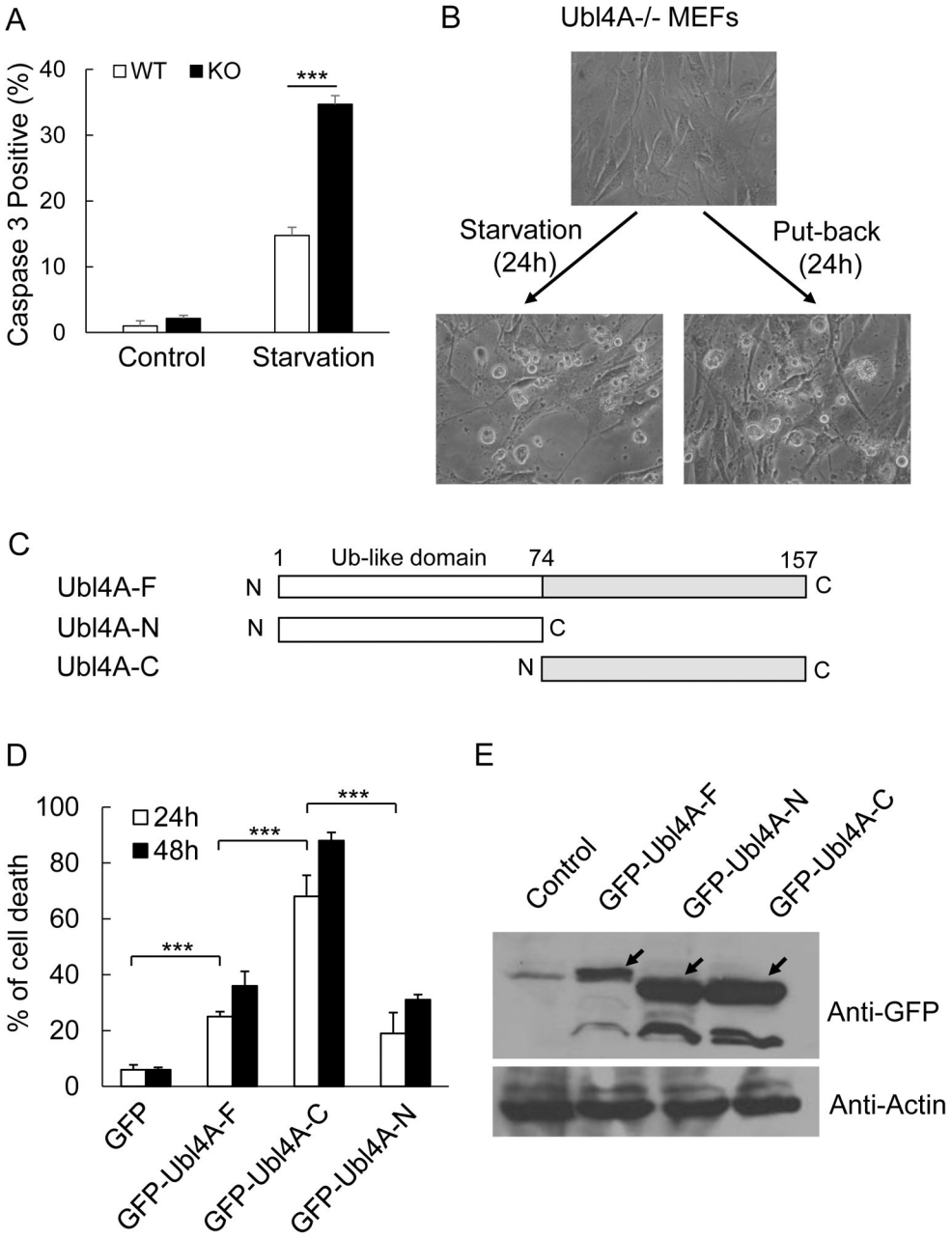

Fig. 1.

Ubl4A protein has both pro- and anti- death functions and the cytotoxicity of Ubl4A is mainly from its C-terminus. (**A**) Cell death assay of WT and *Ubl4A*−/− mouse embryonic fibroblasts (MEFs) with or without serum starvation for 16 h. Cell death was analyzed by measurement of active Caspase 3-positive cells. ***, $p < 0.001$. (**B**) Phase-contrast images of *Ubl4A*−/− MEFs at 0 h (before starvation) and after starvation for 24 h or transfection with wild-type *Ubl4A* for 24 h. Scale bar, 10 μm. (**C**) Cartoon representation of full-length or truncated Ubl4A proteins. (**D**) Cell death assay of *Ubl4A*−/− MEFs transfected with GFP-tagged constructs for 24 h or 48 h. Statistical analysis was performed based on the results of

24 h transfection. ***, $p < 0.001$. (**E**) Expression of GFP-tagged proteins used in (**D**) was measured by Immunoblotting using antibodies against GFP and actin.

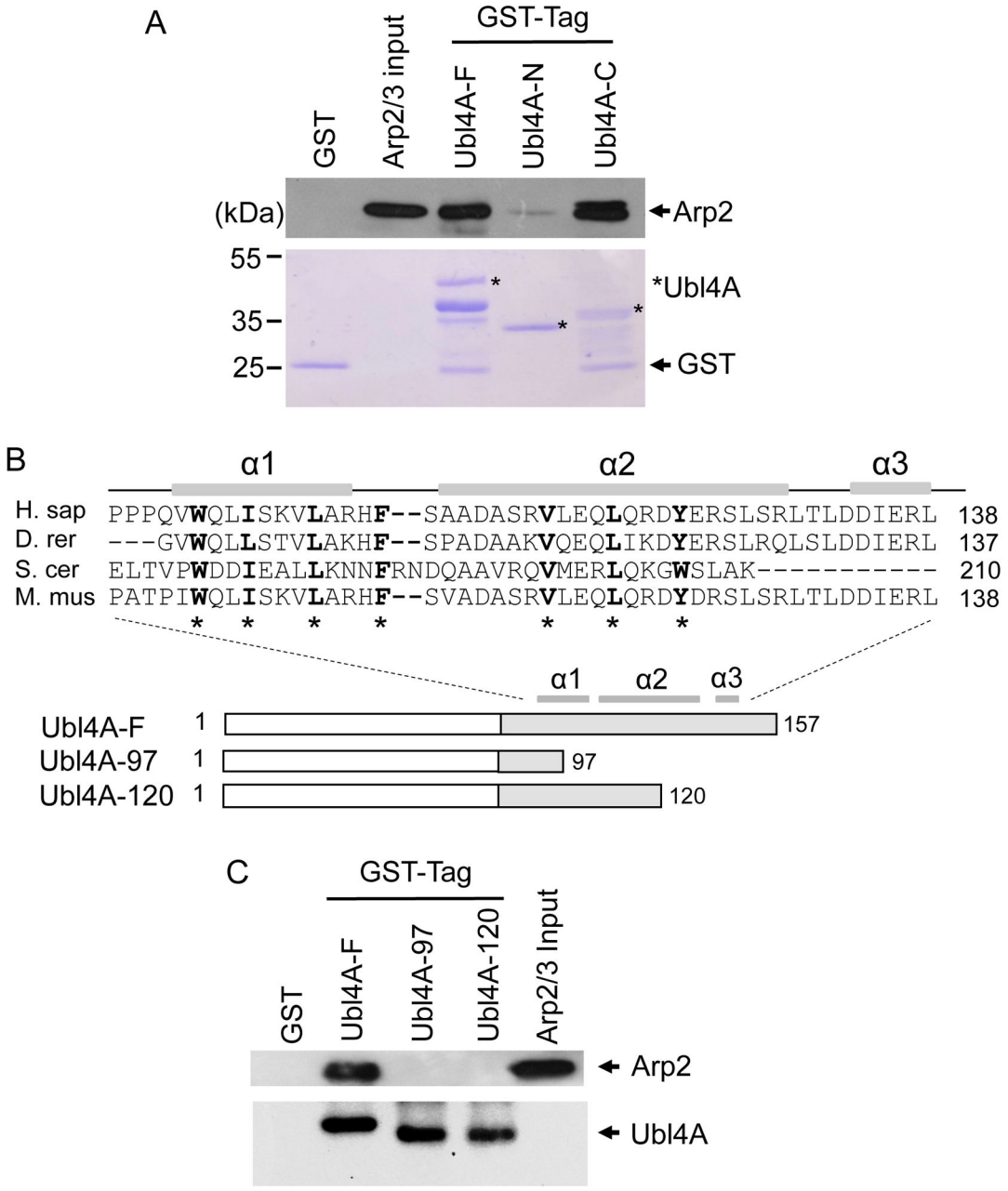

Fig. 2.

The C-terminal region of Ubl4A is responsible for interaction with Arp2/3. (**A**) GST-bead pull-down assay. The bound proteins were analyzed by immunoblotting using antibody against Arp2 (top panel). The immunoblot membrane was stained with Coomassie Blue. Asterisks indicate the predicted sizes of corresponding proteins. (**B**) Sequence alignment of the C-terminus of Ubl4A homologs (H.sap, *Homo sapiens*; D.rer, *Danio rerio*; S.cer, *Saccharomyces cerevisiae*; M.mus, *Mus musculus*). Conserved residues are indicated with asterisks and in bold. The locations of three helixes are shown on top of the sequence. (**C**) GST-bead pull-down assay. The pull-down products were analyzed with immunoblotting using antibodies against Arp2 and Ubl4A.

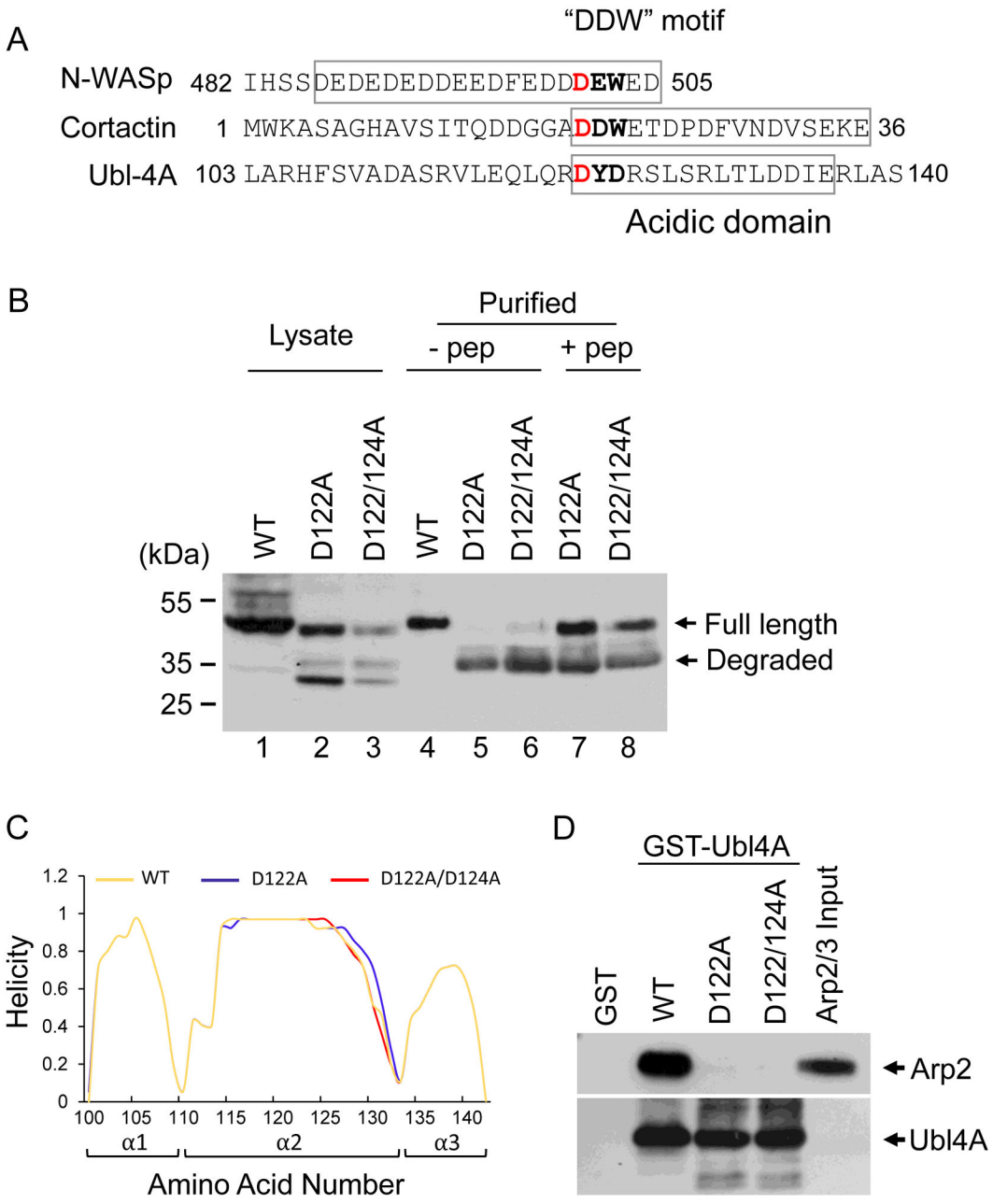

Fig. 3.

The Ubl4A D122A mutation abolishes its ability to bind Arp2/3. (**A**) Peptide sequence alignment of N-WASp, cortactin, and Ubl4A. The conserved motif is in bold. The acidic domain in both N-WASp and cortactin, as well as the Ubl4A putative acidic domain are boxed. (**B**) Immunoblotting using antibody against Ubl4A. (**C**) Helicity predication of the wild-type, D122A, and D122A/D124A Ubl4A C-termini using Netsurfp software. (**D**) Immunoblotting of the GST-bead pull-down products using antibodies against Arp2 and Ubl4A.

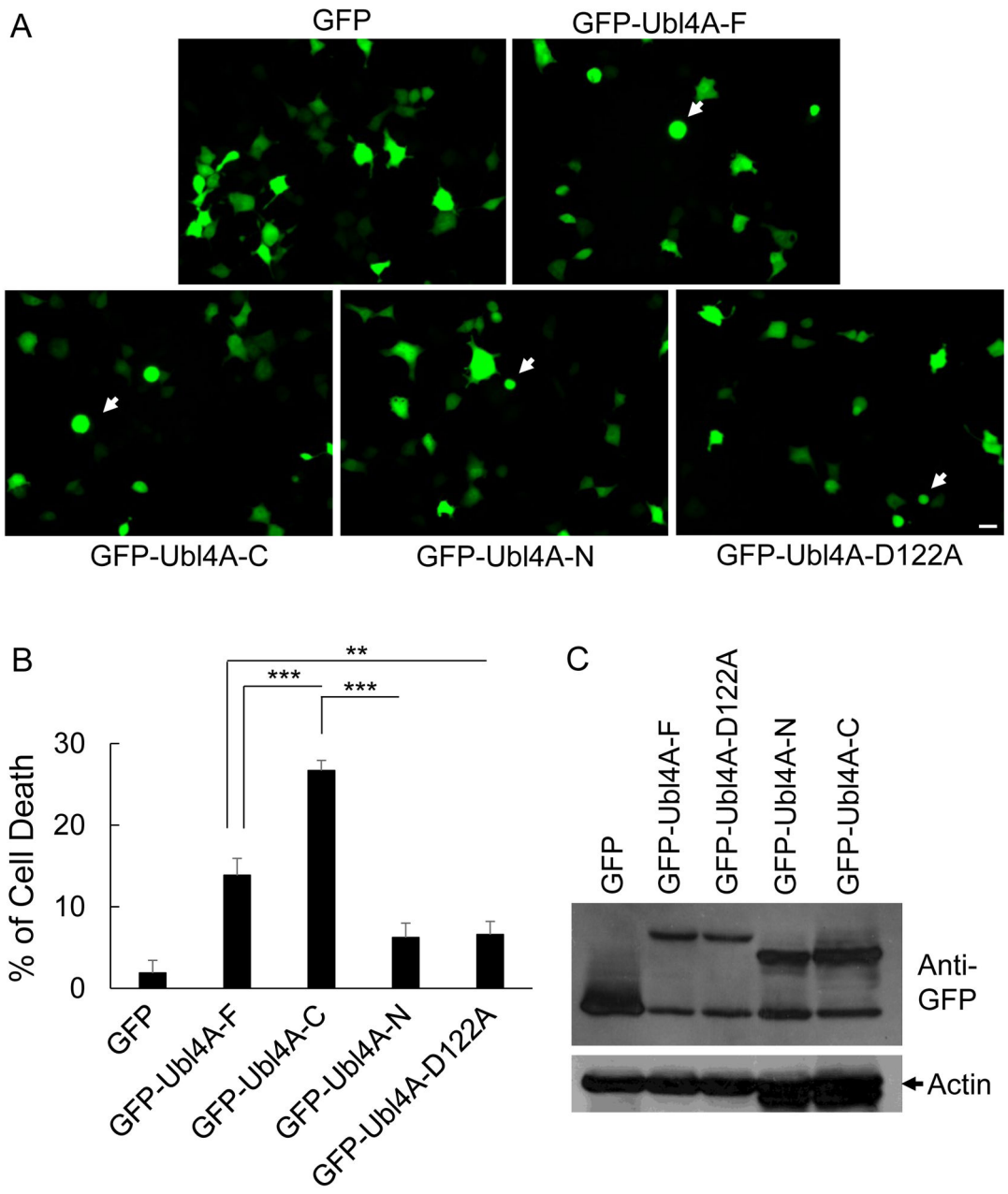

Fig. 4.

The Ubl4A D122A mutation abolishes its pro-death activity in cancer cells. (**A**) Fluorescent images of HCT116 cells transfected with indicated constructs for 24 h. Arrows indicate dead floating cells. Scale bar, 20 μm. (**B**) Cell death assay of (**A**). **, $p < 0.01$. ***, $p < 0.001$. (**C**) Immunoblotting of HCT116 cells transfected with indicated constructs for 24 h using antibodies against GFP and actin.