ADF/cofilin is not essential but critically important for actin activities during phagocytosis in *Tetrahymena thermophila*

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Running title: *Tetrahymena* ADF/cofilin

Abstract

ADF/cofilin is a highly conserved actin-modulating protein. Reorganization of the actin cytoskeleton in vivo through severing and depolymerizing F-actin by this protein is essential for various cellular events such as endocytosis, phagocytosis, cytokinesis and cell migration. We show that in the ciliate *Tetrahymena thermophila*, the ADF/cofilin-homologue, Adf73p, associates with actin on nascent food vacuoles. Overexpression of Adf73p disrupted the proper localization of actin and inhibited the formation of food vacuoles. In vitro*,* recombinant Adf73p promoted the depolymerization of filaments made of *T. thermophila* actin, Act1p. Knockout cells lacking *ADF73* gene are viable but grow extremely slowly and have a severely decreased rate of food vacuole formation. Knockout cells have abnormal aggregates of actin in the cytoplasm. Surprisingly, unlike in animals and yeasts, in *Tetrahymena*, ADF/cofilin is not required for cytokinesis. Thus, the *Tetrahymena* model shows promise for future studies on the role of ADF/cofilin *in vivo*.

(146 words)

Introduction

Dynamic reorganization of the actin cytoskeleton is associated with membrane deformations such as phagocytosis, endocytosis, ameboid movement, and cytokinesis in eukaryotes. ADF (actin-depolymerizing factor)/cofilin (AC) is the principal actin-modulating protein involved in these events, and its biochemical activities have been well studied in animal, yeasts, and higher plants (1). By binding to F-actin, canonical AC induces a twist in the arrangements of subunits (2), which produces distortion at the boundary between twisted and non-twisted polymer regions (3, 4). Consequently, F-actin is severed, and dissociation of monomeric actin (G-actin) is accelerated from the pointed end of filaments (5). On the other hand, at higher concentration, AC increases the rate of actin nucleation and induces abnormal actin structures, called bars or rods, probably because AC binding bridges longitudinal contacts between actin subunits (3, 6, 7). Thus, it has been considered that AC exerts distinct effects on F-actin depending on the AC:actin stoichiometry. In addition, AC binds preferentially to ADP-G-actin and inhibits nucleotide exchange to form an assembly competent ATP-actin (8). This activity may be involved in maintaining the G-actin pool cooperatively with other G-actin-binding proteins such as thymosin β4 and

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profilin.

Protists also show dynamic reorganization of the actin cytoskeleton accompanying unique cell behaviors but neither their fundamental molecular mechanisms nor regulatory systems have been uncovered. One exceptional is the malaria parasite, a lineage of apicomplexa, which expresses AC-like protein ADF1 with unusual biochemical properties (9). ADF1 does not bind or sever F-actin but strongly stimulates the nucleotide exchange on G-actin to promote rapid actin polymerization (10). These unique properties of ADF1 could co-evolve with malaria-specific actin molecule that is extremely unstable as a polymer as compared with conventional actin of other eukaryotes (11). Their functional relationship probably enables the malaria parasite to rapidly turn over F-actin in gliding motility during host invasion (9).

Ciliates form the phylogenic group alveorates, together with apicomplexa and dinoflagellates, are highly diverged from the animal and yeast lineage of opistkonts (12). *Tetrahymena thermophila* is well studied model ciliates with a sequenced genome (13). This unicellular organism vigorously internalizes extracellular solutions and particles as nutrients by forming food vacuoles (FVs) at the funnel-shaped oral apparatus located in the anterior region of the cell cortex (14). Electron microscopic observations revealed

that FVs are formed along the deep fiber, a fibrous microtubule-rich structure that protrudes from the bottom of the oral apparatus (14). After absorption of nutrients, undigested materials are egested at the organelle called the cytoproct in the posterior region of the cell (14). One of the *Tetrahymena* actin Act1p (15) and actin-bundling proteins including fimbrin (16) and eEF-1A (15) all localize near the deep fiber and nascent FVs. Moreover, strong accumulation of actin is seen around the cytoproct (17). Consistently, actin-binding drugs block the formation of FVs in the oral apparatus (18) and membrane retrieval after FV ejection in the cytoproct (17). Thus, the actin cytoskeleton is possible to play an important role in the deformation of membranes associated with the formation and ejection of FVs. Meanwhile, profilin, which promotes the nucleotide exchange on G-actin and hence is generally involved in accelerating actin turnover, is not visibly associated with the FV formation (19). Overall, the exact function of actin dynamics accompanying the FV cycle remains poorly understood. In addition, the *Tetrahymena* actin is highly divergent from conventional actin in the preservation of the amino acid sequence and shows unique biochemical characteristics (20).

After completion of the macronuclear genome project on *T. thermophila* (13), it

has been revealed that this organism has only one gene encoding an AC homolog, *ADF73*. Previously, we purified the gene product, Adf73p (21). Biochemical studies using rabbit muscle actin showed that Adf73p has F-actin-severing activity, but unexpectedly, accelerates the nucleotide exchange on G-actin in a manner similar to malaria parasite ADF1. Here we investigated the cellular function of Adf73p by determining its localization and the consequences of overexpression and deletion. We show that Adf73p is required for phagocytosis, likely via its role in the reorganization of the actin cytoskeleton during FV formation by severing and depolymerizing F-actin in *Tetrahymena thermophila*. Surprisingly, unlike animal cells, *Tetrahymena* cells do not require AC for cytokinesis.

Materials and Methods

Cell culture

Wild-type *Tetrahymena thermophila* was cultured in NEFF (0.25% proteose peptone, 0.25% yeast extract, 0.55% D-(+)-glucose, 33 μM $FeCl_2$) or SPP (1% proteose peptone, 0.2% glucose, 0.1% yeast extract, 0.003% EDTA-ferric sodium salt) (22) medium at 30°C. To study the effects of *ADF73* deletion, wild-type and *adf73*KO strains were cultured in MEPP (2% proteose peptone, 0.06% sodium citrate·$2H_2O$, 0.027% $FeCl_3$·$6H_2O$, 0.0003% $CuSO_4$·$5H_2O$, and 0.0001% folinic acid) (23) medium, which promotes the growth of cells lacking the ability to form FVs.

Mitotic index was defined as a percentage of the number of cells undergoing micro- and macro-nuclear division against the total number of cells. Nuclear DNA was microscopically observed by staining cells with 1 μM 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) after fixation with 1% formaldehyde (Wako).

Antiserum

To produce anti-Adf73p antibodies, recombinant GST-Adf73p was purified and Adf73p was separated from GST using thrombin (21). Adf73p was dialyzed in the

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phosphate-buffered saline (PBS) overnight at 4°C, mixed with adjuvant and emulsified

with Freund's complete adjuvant (Wako Pure Chemical Industries, Ltd.) for the first

injection and Freund's incomplete adjuvant (Wako Pure Chemical Industries, Ltd.) for

the following injections by sonication (UD-201; Tomy). The antigen was

hypodermically injected into the back of a rabbit. One month after the first injection,

additional injections were performed once every 2 weeks. After third additional

injection, the antisera were collected and diluted 10 times with PBS and incubated with

a PVDF membrane bound with recombinant Adf73p overnight at 4°C. The membrane

was washed 3 times with 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.05% Tween 20 and

soaked in 50 mM glycine-HCl (pH 2.5). Eluted affinity-purified antibodies were

neutralized with 0.5 M Tris (pH 8.8). A guinea pig antiserum against *T. thermophila*

actin has previously been described (15). An anti-GFP antibody was purchased from

Roche Diagnostics. The mouse anti-chicken α-tubulin antibody was purchased from

Calbiochem.

Immunoblotting

T. thermophila cells cultured in NEFF medium at 30°C were collected by centrifugation

for 3 min at 750 × g and washed twice with NKC solution (34 mM NaCl, 1 mM KCl, 1

mM CaCl$_2$). The cells were suspended in NKC containing 1 mM ATP, 0.5 mM DTT, 1

mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml TLCK and smashed with

sonicater (UD-201; Tomy). After adding one-third volume of the loading buffer (0.25

mM Tris (pH 6.8), 4% SDS, 40% glycerol, and 8% β-mercaptoethanol) followed by

incubation at 95°C for 5 min, the cell lysate was subjected to SDS-PAGE and

transferred onto a PVDF membrane. The membrane was blocked with Tris-buffered

saline (TBS; 20 mM Tris-HCl, 0.9% NaCl, pH 7.5) containing 0.5% Tween-20 (TTBS)

with 1% skim milk and incubated with 0.1% affinity purified anti-Adf73p antiserum for

2 h. After washing with TTBS with 1% skim milk, the membrane was incubated with

1% goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Biosource

International) for 1 h. Immunoblots were developed using the BCIP/NBT phosphatase

substrate kit (Kirkegaard & Perry Laboratories, Inc.).

Fluorescence microscopy

T. thermophila cells were fixed with cold methanol (−20°C) for at least 30 min. After

washing cells with PBS 3 times, cells were permeabilized with PBS containing 0.1%

Triton X-100 for 1 min and then washed with PBS 3 times. Cells were incubated with

PBS containing 1% skim milk for 30 min, and then with 1% anti-actin antiserum and/or

25% affinity purified anti-Adf73p antiserum in PBS containing 1% skim milk for more

than 6 h at room temperature. After washing cells with PBS containing 1% skim milk 3

times, cells were incubated with 1% Rhodamine-labeled goat anti-guinea pig IgG

(Kirkegaard & Perry Laboratories, Inc.) and/or 1% FITC-conjugated goat anti-rabbit

IgG (Jackson Immune Research Laboratories, Inc.) in PBS containing 1% skim milk for

more than 6 h at room temperature. After washing with PBS 3 times, cells were

observed by a confocal laser scanning microscope, LSM510 (Carl Zeiss, Inc.).

Immunofluorescence staining with an anti-α-tubulin was performed by the method of

Wloga et al. (24).

Gene overexpression

A plasmid vector for overexpressing *ADF73*, pMTT1-GFP-*ADF73*, was obtained by

inserting *ADF73* cDNA into the *Bam*HI-*Hin*dIII site of pMTT1-GFP (25). CU522 cells

were transformed with pMTT1-GFP-*ADF73* as described (26). Overexpression of

ADF73 was induced by the addition of 2.5 μg/ml $CdCl_2$ to the medium for 3 h.

Gene knockout

To obtain a targeting plasmid for knockout of *ADF73*, 1.5 kb DNA fragments of 5' and 3' untranslated regions of the *ADF73* gene were amplified by PCR and cloned into the *Apa*I-*Sma*I site and *Pst*I-*Sac*I sites of p*Neo4* (27), respectively. Log-phase growing B2086 and CU428 cells in SPP were washed, starved in 10 mM Tris-HCl (pH 7.4) for 16–24 hours at 30°C, and mixed for conjugation. p*Neo4-ADF73* was introduced into micronuclei of the conjugating cells by biolistic transformation (28). Germ-line transformants were selected by the addition of 10 μg/ml paromomycin, 15 μg/ml 6-methylpurine and 1 μg/ml CdCl$_2$. Disrupted loci in the macronuclei were eliminated by phenotypic assortment in the absence of paromomycin (29). Heterozygous heterokaryons were mated with a "star" strain (A*III) to make homozygous heterokaryon strains with a double disrupted loci in the micronucleus and wild-type genes in the macronucleus by uniparental pronuclear transfer. Two heterokaryons were mated to obtain homozygous homokaryon strains (*adf73*KO).

Isolation and fractionation of cell bodies and cilia

Cell bodies and cilia were isolated by the method of Ueno et al. (30).

Co-sedimentation assay

Purification of recombinant Adf73p and the co-sedimentation assay were as described

by Shiozaki et al. (21).

Swimming behavior of cells

To investigate swimming behavior, 20 µL cell culture was placed in a basic chamber

assembled from a slide glass with two slits of thin vinyl tape arranged in parallel as a

spacer and covered with cover glass. In this chamber, cells can swim around freely.

Swimming behavior was recorded at 15 Hz for 30 sec using CCD camera (DFK41AU02,

Argo) under the inverted microscopy (Olympus IX70) equipped with ×10 objective lens.

Swimming velocity was determined using Manual tracking-plugin tool on Image J

software. To record the ciliary beating of swimming cells at 1 kHz for 1 s, ×100

objective lens and high-speed CCD camera (Focuscope SV-200i, Photron limited) were

used. All observations were performed at room temperature.

Results

Adf73p is a component of F-actin structures associated with FVs

In *T. thermophila*, dynamic reorganization of the actin cytoskeleton accompanies the course of phagocytosis (Fig. S1). AC is known as the major actin-modulating protein (1). The macronuclear genome contains only a single gene putatively encoding an AC homologue (13, 21). To investigate the cellular localization of its gene product Adf73p, we prepared an antiserum against the recombinant protein. After affinity purification, immunoblotting against the total lysate of *T. thermophila* cells showed that the serum most strongly recognized a band of 15 kDa, corresponding to the estimated molecular weight of Adf73p (Fig. 1A). This signal is increased by Adf73p overexpression and diminished by *ADF73* gene knockout (Figs. 2A, 4C). In immunofluorescence microscopy with an affinity-purified anti-Adf73p serum, a strong signal colocalized with F-actin protruding from the oral apparatus, actin dots around the nascent FV, and the actin clump in the posterior region of the cell, which would be consistent with localization to an older FV ejecting its contents from the cytoproct (Fig. 1B). These signals were gone by *ADF73* gene knockout (Fig. 4D). Furthermore, genetically tagged GFP-Adf73p showed a similar pattern of fluorescence (Fig. 1C). Taken together, we

concluded that Adf73p localized to actin structures associated with FVs around the oral apparatus and a cytoproct. Consistently, brief incubation with the actin inhibitor Latrunculin B (Lat-B), which sequestered actin monomer from polymerization, greatly reduced the signal of both Adf73p and actin (Fig. 1B). In addition, F-actin associated with Adf73p in those structures of *T. thermophila* cells appeared to possess a high turnover rates of polymerization and depolymerization because of its disappearance by a short period of incubation with Lat-B (Fig. 1B).

Adf73p affects actin in vivo and in vitro

To evaluate the activity of Adf73p against the actin cytoskeleton in vivo, we constructed a strain that over expresses Adf73p under the Cd^{2+}-inducible *MTT1* promoter. The levels of Adf73p were markedly elevated after the addition of Cd^{2+} (Fig. 2A). Overproduction of Adf73p diminished actin associated with FVs and frequently induced thick actin bars, probably because of the excessive amount of Adf73p associated with F-actin (Fig. 2B). Importantly, the formation of FVs was strongly suppressed by overproduction of Adf73p (Fig. 2C); thus, proper expression levels of Adf73p are important for organization of actin structures and phagocytosis in *T.*

thermophila.

We have previously studied the biochemical activities of Adf73p against actin derived from rabbit skeletal muscle; however, *Tetrahymena* actin isotypes are quite evolutionarily divergent from skeletal actin and possess unique biochemical features (20). *T. thermophila* has 4 actin genes in its genome (13), and Act1p is the most abundant actin expressed in vegetative growing cells (KN and ON, manuscript in preparation). Therefore, we investigated the biochemical activity of Adf73p on the recombinant *T. thermophila* Act1p purified from the expression system using the slime mold *Dictyostelium discoideum* (supplemental material and Fig. S2). Polymerized Act1p was precipitated by ultracentrifugation (Fig. 3). After incubation with Adf73p, the amount of Act1p in the supernatant was significantly increased. Thus, Adf73p directly depolymerize F-actin made of Act1p.

Adf73p is required for vigorous cell growth

To investigate the cellular function of Adf73p, we made an *adf73* gene knockout strain by replacing the coding region with the *neo* marker in the micronucleus (see Fig. 4A). Homozygous knockout cells were obtained as progeny of mating heterokaryons.

Initially, we failed to obtain viable progeny of knockout heterokaryons in SPP, the standard medium for *T. thermophila*. However, putative gene knockout homozygotes were isolated in MEPP, a medium that allows the growth of cells that lack the ability to phagocytose (33). Gene replacement was confirmed by PCR using gene-specific primers (Fig. 4B). Moreover, the gene product was not detected in the *adf73* gene knockout strain (*adf73*KO) by immunoblotting (Fig. 4C). It is generally thought that AC is essential for cell viability in animals and yeast probably because its activity is required for cytokinesis (31-35). In contrast, *Tetrahymena adf73*KO populations were able to grow albeit extremely slowly as compared to wild-type cells (Fig. 4E). This decrease in the multiplication rate is not caused by the prolonged cytokinesis process since the mitotic index, which was generally elevated by cytokinesis defects, was almost the same in *adf73*KO cells (15.8%) and control cells (16.8%)(n >200). Moreover, a giant cell containing multiple nuclei, representing failed cytokinesis, was never seen in *adf73*KO strains (data not shown). Consistently, we have not detected any signal of Adf73p near the cleavage furrow in dividing cells (Fig. S3; our unpublished data). Therefore, the function of Adf73p is dispensable for cytokinesis in *Tetrahymena*. On the other hand, prolonged incubation of *adf73*KO cells entered the stationary phase at a

much lower cell densities than wild-type cells (see Fig. 4E). This difference suggests

that *adf73*KO cells are unable to take up enough of nutrients for cell proliferation as

compared with wild-type cells.

We then examined the ability of *adf73*KO cells to form FVs. Within 30 min after

incubation with India ink, the numbers of filled FVs was greatly increased in control

cells but not in *adf73*KO cells (Fig. 5A). Moreover, small black ball-shaped aggregates

of India ink, that represents FV materials ejected from cytoprocts after the phagocytic

process, were abundant in the cultures of wild-type cells but not in *adf73*KO cultures

after prolonged incubation with India ink (Fig. 5B). The result shown in Fig. 5C also

suggested that phagocytic activity was markedly reduced by gene knockout of *ADF73*.

Thus, Adf73p is required for efficient formation of FVs.

What is the primary defect in phagocytosis induced by loss of Adf73p function?

By examining the cellular localization of actin, we noticed large aggregates of actin in

the posterior region of *adf73*KO cells, which were not seen in wild-type cells in which

actin is mostly localized around a nascent FV near the oral apparatus located in the

anterior part of a cell (Fig. 6). It seems that the remodeling of the actin cytoskeleton

during FV formation (see Fig. S1) is severely affected in the absence of *ADF73*.

As mentioned previously, gene knockout of *T. thermophila* actin *ACT1* abrogated

phagocytosis ability, potentially because of the dysfunction of ciliary motility that

affects the undulation of the oral membranelles that produces a current of extracellular

materials toward the bottom of the oral cavity (36). Actin is a component of the inner

dynein arm of cilia in *T. thermophila* (37). Interestingly, a homologue of AC is found in

flagella in *Leishmania* where it is required for axoneme assembly (38). However, we

did not detect any Adf73p signal in isolated cilia by immunoblotting whereas actin was

detected in both the cell and cilia (Fig. S4). Also, the *adf73*KO cells are motile (*see*

supplemental movies), in contrast to *act1*KO cells that are paralysed (36). Although the

average swimming velocity was somewhat lower in *adf73*KO cells than wild-type cells,

the difference was not statistically significant (Table 1). Interestingly, *adf73*KO cells

turn more frequently than wild-type cells.

Discussion

Adf73p regulates the reorganization of the actin cytoskeleton required for formation of FVs

Here we show that Adf73p colocalizes with actin dots associated with the nascent FVs and a fibrous structure extending from the oral apparatus in *T. thermophila*. *Tetrahymena* internalizes extracellular liquid with particles into a FVs every 1-2 min. The actin cytoskeleton is implicated in the formation of FVs since actin inhibitors potently block FV formation (18). Moreover, this study shows that the formation of FVs is greatly reduced in cells that assemble abnormal actin structures due to overexpression of GFP-Adf73p (Fig. 2). Taking into account that Adf73p has depolymerizing activity against filaments made of Act1p (Fig. 3), a major isoform of *T. thermophila* actin, it is conceivable that Adf73p-mediated turnover of the actin cytoskeleton is associated with the formation of FVs. Importantly we found that the formation of FVs was significantly inhibited in cells lacking Adf73p (Fig. 5A) and that the organization of the actin cytoskeleton was abnormal in those cells (Fig. 6). Accordingly, the *adf73*KO cells failed to proliferate in the standard SPP medium but grew in the MEPP medium that supported mutants lacking ability to phagocytose (23). Thus, the phagocytosis-mediated uptake of

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nutrients is severely affected in cells lacking Adf73p while the endocytosis-mediated

uptake of nutrients is probably functional. Indeed, it has been demonstrated that

endocytosis at the coated pits near basal bodies is not actin-dependent in *T. thermophila*

(39); however, in the natural environment, actin-based phagocytosis from the oral

apparatus is unambiguously indispensable for preying on microorganisms such as

bacteria for *T. thermophila*.

In other eukaryotic cells such as macrophages and slime molds, the actin

cytoskeleton promotes invagination or protrusion of the plasma membrane during

phagocytosis (40). In contrast, it has not been exactly understood how the actin

structures, such as actin dots and fibers, induce the formation of FVs, in part because

the source membrane for the nascent FV seems to be distinct from the plasma

membrane (41). Uncovering how the actin cytoskeleton functions in phagocytosis in *T.*

thermophila may shed light of the general question of how the actin cytoskeleton

regulates the plasma membrane dynamics.

In addition, we found that Adf73p colocalized with actin to FV near a cytoproct.

Sugita et al. (17) previously showed that the cytoproct-associated "actin clump" is a

transient structure engaged in the membrane recycling from old FV; therefore,

Adf73p-mediated reorganization of the actin cytoskeleton may be involved in this

process.

Adf73p is not essential for cytokinesis in *T. thermophila*

In the cytokinesis of animal and yeast cells, the cleavage furrow is induced by the

contraction of a ring-made of actin within the division plane (42-45). Many

actin-modulating proteins, including AC, are involved in the assembly and dynamics of

the contractile ring. *Tetrahymena* divides by binary fission and assembles an actin-rich

contractile ring (46). Several actin-modulating proteins, including fimbrin (16), eEF1A

(47), and profilin (48), localize to the cleavage furrow. On the other hand, we failed to

detect Adf73p in the cleavage furrow (Fig. S3 and our unpublished data). Supporting

this observation, *adf73*KO cells multiply without a major increase in the mitotic index

(Fig. 4E); therefore, Adf73p is dispensable for cytokinesis in *T. thermophila*. Moreover,

cells lacking Act1p can develop an advanced cleavage furrow (36) but fail to perform

scission, the terminal stage of cytokinesis, by which the cell bridge linking the two

daughter cells is broken by rotokinesis, a cilia-dependent rotation of daughter cells (49).

This is probably because of the essential function of Act1p in ciliary movement in the

inner arm dynein complex (36, 37). It is therefore likely that isotypes of actin distinct

from Act1p are involved in the contractile ring and that their functions do not require

Adf73p. Alternatively, it is possible that another type of actin-severing or

depolymerizing protein dominantly functions in *Tetrahymena* cytokinesis. Very recently,

it was reported that cyclase-associated protein CAP, known as a synergic factor for AC

function, can sever F-actin without AC, although the activity is weak (50). We have

found a gene encoding a CAP-homologous protein in the *T. thermophila* genome (our

unpublished data), although the function of this gene remains to be uncovered. In

addition, a myosin II heavy chain, an essential component of the contractile ring in

animal and yeast cells, is not present in *T. thermophila* (51, 52). Therefore, the

formation of the contractile ring in *Tetrahymena* is likely to involve divergent forms of

actin and actin regulators. Interestingly, it has been demonstrated that *Giardia*

intestinalis, the intestinal parasite lacking most of the genes encoding canonical

actin-modulating proteins including AC and myosin in the genome, requires actin

function for cytokinesis (53). Further studies on protists such as *Giardia* and

Tetrahymena will provide important evolutionary insights into the molecular

mechanism of cytokinesis. In addition, the divergence of the cytokinetic apparatus

creates an experimental opportunity in *Tetrahymena*. Namely, regulators of important

actin-dependent functions, such as AC, can be studied in vivo as their function is not

required for survival.

Acknowledgements

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Figure Legends

Fig. 1. Adf73p colocalized with F-actin in *T. thermophila*

(A) Western blots with anti-Adf73p antiserum. Cell lysate of *T. thermophila* was subjected to SDS-PAGF and transferred to PVDF membrane. The membrane was slit and subjected to immunoblots with pre-immune serum (slit 1), anti-Adf73p antiserum before (slit 2) and after (slit 3) affinity purification. Arrow indicates a band consistent with the predicted size of Adf73p. Arrowheads indicate extra bands not eliminated after the process of affinity purification. (B) Immunofluorescence microscopy for cellular localization of Adf73p. Cells were processed for immunofluorescence staining after black ink was loaded on their FVs for 30 min. Right column shows bright field microscopic images of cells merged with immunofluorescence images (red, actin; green, Adf73p). Blue dotted line shows outline of the cell. In top row, Adf73p localizes to actin dots located on nascent FVs (small arrows) near the oral apparatus (OA) and an actin clump near a cytoproct (CP) (large arrow). Mid-size arrow indicates an actin cable protruding from OA. This cable-like structure seems to be transiently formed during phagocytosis since it is found only in limited populations of cells forming FV. Adf73p associates with this structure as well. Cells shown in the bottom two rows of panels were treated with DMSO (solvent only) or actin-polymerization inhibitor Lat-B (final conc. 10 μM) for 10 min, respectively. Note that localization of Adf73p was diminished in cells incubated with Lat-B. (C) Cells transiently expressing GFP-Adf73p under exogenous promoter of *MTT1* were stained with an anti-actin antiserum and anti-GFP antibody. Right column shows merged image (red, actin; green, GFP-Adf73p). GFP-Adf73p showed similar localization with Adf73p observed in (B). Bars, 10 μm.

Fig. 2. Overexpression of *ADF73* disorganizes actin cytoskeleton *in vivo*

(A) Addition of Cd^{2+} to cell culture induces Adf73p-overexpression in *MTT1-ADF73* strain. Immunoblotting of cell lysate from CU522 (a parental cell) as the control and *MTT1-ADF73* cells using anti-Adf73p antiserum and anti-α-tubulin antibody. The expression of Adf73p in *MTT1-ADF73* cells was specifically increased by adding 2.5 μg/ml $CdCl_2$ to medium. (B) Immunofluorescence images of CU522 (control) and *MTT1-ADF73* cells (Adf73p-OE) stained with anti-actin antiserum (left and green in merged images) and affinity-purified anti-Adf73p antiserum (middle and red in merged images). Several actin bars were produced by overexpression of Adf73p while control cells showed normal localization of actin and Adf73p, as mentioned in Fig. 1. Arrow and arrowhead indicate a nascent FV and an actin cable, respectively. (C) FV formation was significantly suppressed in *ADF73*-overexpressing cells. Cells were incubated with 0.1% India ink for 1 h. Bars, 10 μm.

Fig. 3. Adf73p depolymerizes filaments of *T. thermophila* actin Act1p

Act1p (2 μM) was polymerized and mixed with various amounts of Adf73p for 2 h at 25°C and then centrifuged at $100,000 \times g$ for 30 min. The supernatant (S) and pellet (P) were subjected to SDS-PAGE. Note that the amount of Act1p in supernatant increased in the presence of Adf73p.

Fig. 4. *ADF73*-gene knockout severely affects cell growth

(A) Scheme of disruption of *ADF73* gene using DNA homologous recombination. Open

reading frame of *ADF73* was completely replaced with *NEO4* marker gene. (B) Confirmation of gene replacement by PCR. Genomic DNAs prepared from a parental strain (CU428) and two independent clones of *ADF73* knockout cells (KO) were amplified using two sets of PCR primers (primer 1 and 2 or primer 1 and 3). Annealing sites of the primers are indicated in A. (C) Expression of Adf73p was abolished in the knockout strain. Immunoblotting was performed against cell lysate using affinity-purified anti-Adf73p serum. Membrane area corresponding to the molecular weight of Adf73p was trimmed and shown. Expression level of actin was unaffected by *ADF73*-gene knockout. (D) Immunofluorescence microscopy was performed against *adf73*KO cells using affinity-purified anti-Adf73p serum. Right image is rather overexposed for showing a non-specific fluorescence signal. Bar, 10 μm. (E) Cell growth of CU428 strain as the wild-type (WT) and three independent clones of *adf73*KO strains. Each strain was cultured in MEPP medium at 30°C. Note that both the cell growth rate and maximum number of cells in the steady state were significantly reduced in all of the *adf73*KO strains.

Fig. 5. Phagocytosis activity is severely affected in *adf73*KO cells

(A) FV-forming ability is significantly reduced by gene knockout of *ADF73*. Wild-type and *adf73*KO cells were incubated with 0.1% India ink in MEPP medium at 30°C. At 0.5, 2, 4.5 and 7.5 h after the India ink was loaded, cells were fixed and the average number of FVs per cell was evaluated (>10 cells). (B) Microscopic images of cells after 2 h of incubation with India ink were shown. Arrows indicate aggregates of India ink that have been ejected from a cytoproct through a process of phagocytosis. (C) Colloid

of India ink was significantly reduced by long incubation with wild-type *Tetrahymena* cells but not *adf73*KO cells. Microtubes containing cell culture with India ink as in A were left to stand and observed. Note that almost all India ink settled into wild-type cells but not in *adf73*KO after prolonged incubation for 24 h, since the colloid was mostly precipitated with cells as the FV contents and India ink aggregated as a result of phagocytosis.

Fig. 6. Abnormally large accumulation of actin was seen in *adf73*KO cells

Immunofluorescence images of a parental strain CU428 and *adf73*KO stained with anti-actin antiserum and anti-α-tubulin antibody for MTs. Arrowheads indicate normal localization of actin dots surrounding a nascent FV located close to the oral apparatus (yellow arrows). White arrows indicate abnormal large accumulation of actin in cytoplasm, which is frequently observed in *adf73*KO cells but never in wild-type cells. Bar, 10 μm.

actin   Adf73p   merged

OA
CP

control

OA
CP

+ Lat-B

actin   GFP-Adf73p   merged

OA

OA

1 2 3

100
75

50

37

kDa

Adf73p

Fig.1

Fig.2

CBB

Adf73p

α-tubulin
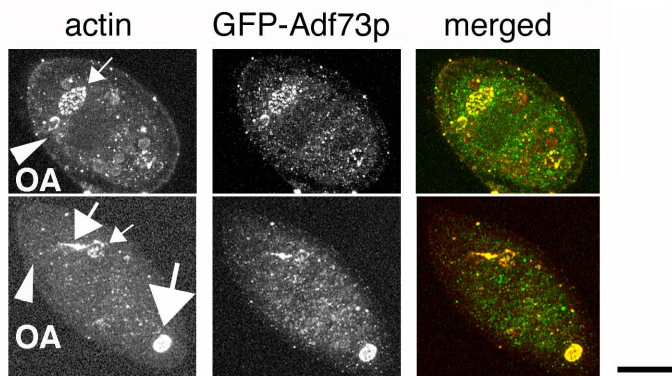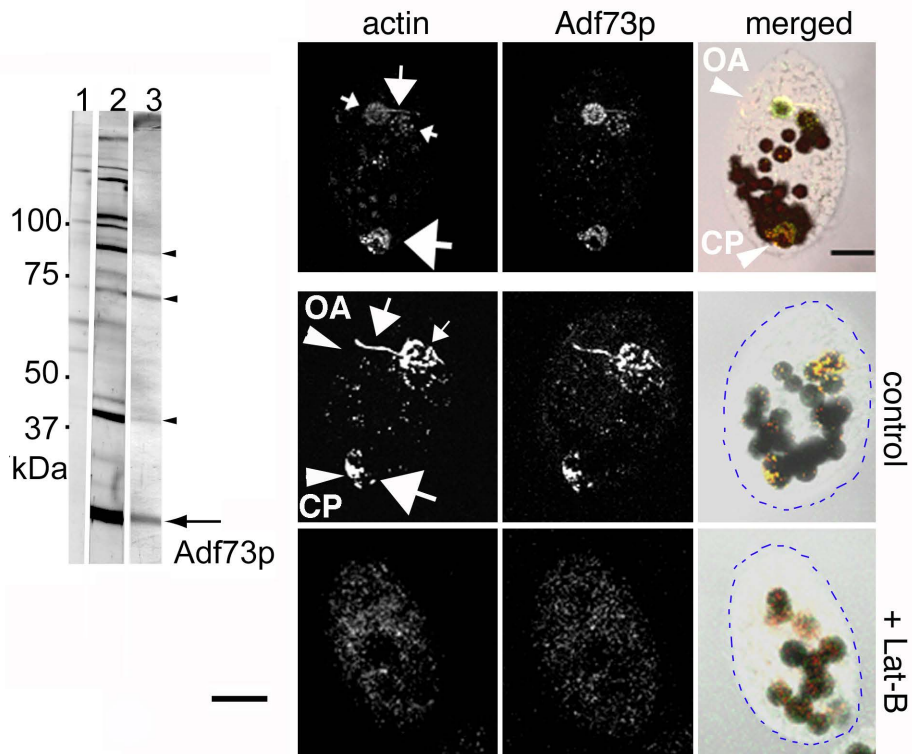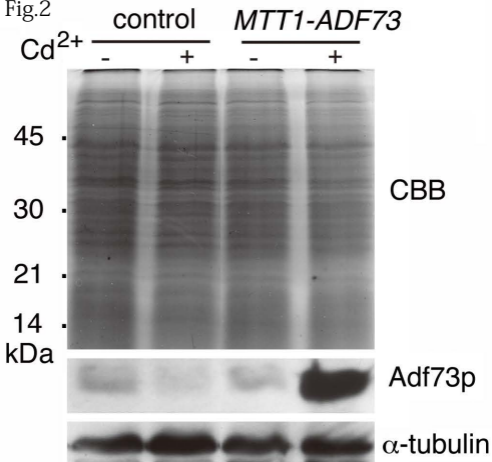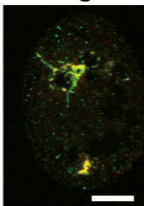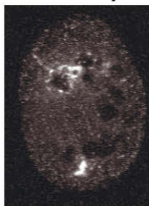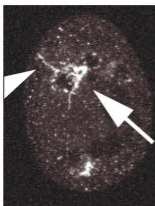
actin | Adf73p | merged
control
Adf73p-OE
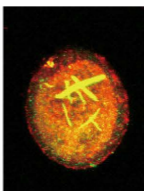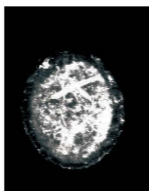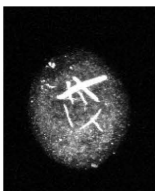
control | Adf73p-OE

Fig.3

								actin (2 μM)
+		+		+		-		
0		1		2		2		Adf73p (μM)
S	P	S	P	S	P	S	P	

◄ Act1p

◄ Adf73p

A *ADF73* gene

1 kb

5' UTR | ORF | 3' UTR

1 →   ← 3

Apa I   *Sac* I

5' UTR | *NEO4* | *MTT1* | 3' UTR

← 2   ← 3

B

| | CU428 | KO | CU428 | KO |

(kbp)
19
7.7
6.2
3.5
2.7
1.9

primer 1+2   1+3

C

CU428   KO

(kDa)
80
58
46
25

17

CBB

Adf73p

actin

D

DIC

anti-Adf73p

*adf73*KO cells

E

● WT (CU428)
△ adf73KO#1
□ adf73KO#2
◇ adf73KO#3

20

15

*10⁶ cells/ml

10

5

0   10   20   30   40   50   60
time (h)

Fig.4

Fig.5
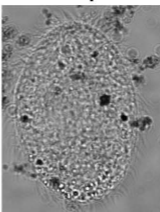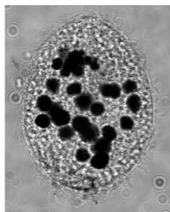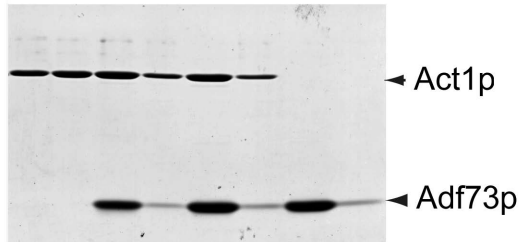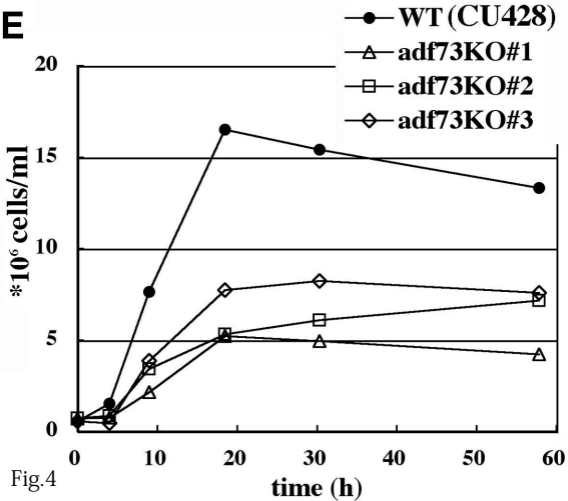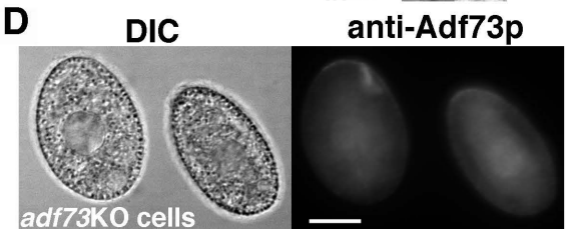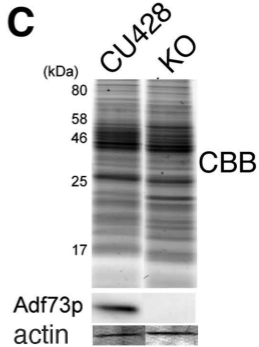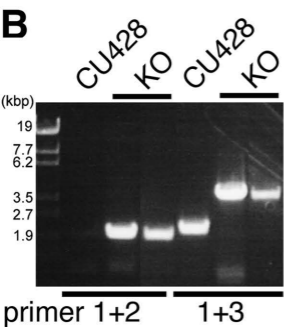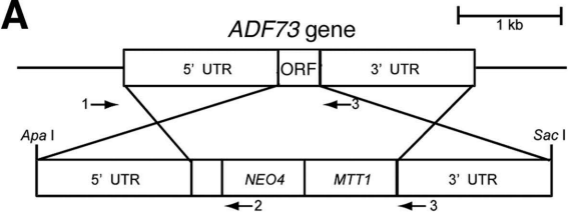
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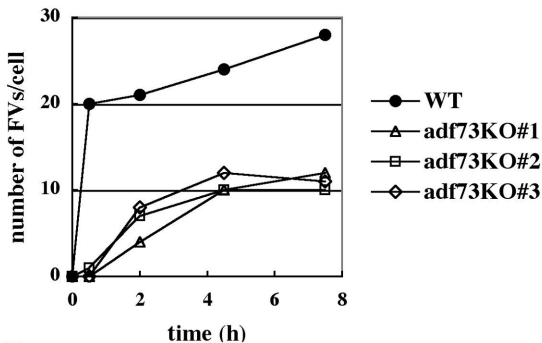
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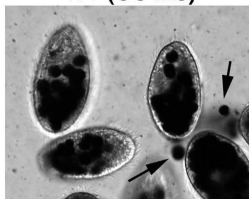
WT (CU428)   *adf73*KO

particles of India ink

OA

FVs

CP

ejected materials

C

WT   KO   WT   KO
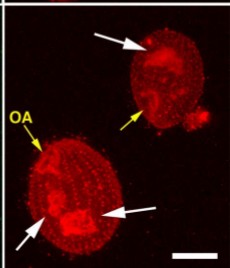
time 0.5 h   24 h

Fig.6
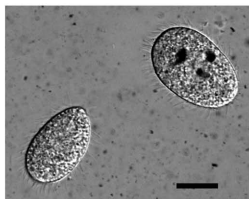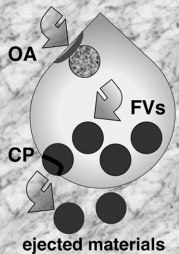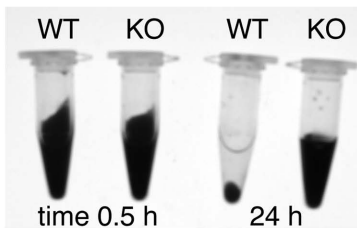
MTs | actin

CU428

adf73KO

OA
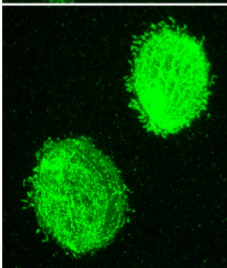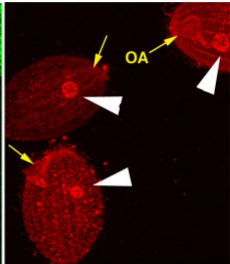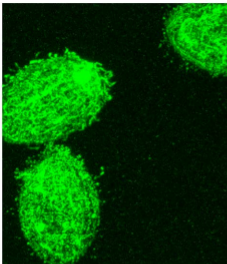
Table 1. Cell motility of *adf73* knockout cells

	Wild-type	*adf73*KO
swimming velocity (μm/s)	123.9± 61.3	108.9±33.1
ciliary beat frequency (Hz)	22.5 ± 3.3	17.3 ± 2.7
average number of turns (s^{-1})	0.14 ± 0.18	0.64 ± 0.31

Swimming velocity is average of 80 cells randomly selected in the microscopic field. Ciliary beat frequency is average of 20 cells randomly selected in the microscopic field. Average number of turns is defined as the frequency of a cell changing the swimming direction during 5 sec (n=100).

Supplemental text

Materials and Methods

Purification of recombinant *T. thermophila* Act1p from *Dictyostelium discoideum*

ACT1 in *T. thermophila* contains two TAA codons in its open reading frame. For the expression of *ACT1* in *D. discoideum*, both TAA codons in *ACT1* were substituted with CAA codons and the obtained DNA fragment, ttact1, was introduced into pTIKLART, a vector for the expression of Act15 fused with thymosin β4 moiety and His-tag, as a replacement. An anti-His-tag antibody reacted with extracts of both wild-type *D. discoideum* (*Ax2*) cells transformed with pTIKLART or pTIKLART-ttact1 while it did not react with extracts of untransformed wild-type cells (Fig. S2A, an upper panel). In addition, an anti-actin antiserum, which recognizes *T. thermophila* Act1p but not *D. discoideum* actin, specifically reacted with the cell extract expressing Act1p (Fig. S2A, lower panel).

Exogenous Act1p was purified as described (1) with some modifications. *D. discoideum* cells (5.5×10^6 cells/ml) expressing *T. thermophila* Act1p fused with thymosin β4 and His-tag were collected by centrifugation at low speed for 5 min at 4°C and were washed with Tris-HCl (pH 7.5). The cells were suspended in lysis buffer containing 40 mM imidazole (pH 7.4), 10 mM HEPES (pH 7.4), 300 mM NaCl, 2 mM $MgCl_2$, 1 mM ATP, 7 mM β-mercaptoethanol, 0.01% NaN_3, 0.625% TritonX-100 and protein inhibitors and were lysed (Fig. S2B, lane 1). Cell homogenates were centrifuged at 36,000 × g for 30 min at 4°C (Fig. S2B, lane 2: pellet). The supernatant was incubated with nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA, USA) for 1 h (Fig. S2B, lane 3: unbound materials). The resin with bound proteins was washed

with buffer containing 40 mM imidazole (pH 7.4), 10 mM HEPES (pH 7.4), 300 mM

NaCl, 0.5 mM $MgCl_2$, 0.1 mM ATP, 7 mM β-mercaptoethanol and 0.01% NaN_3 (Fig.

S2B, lane 4) and then extracted with buffer containing 500 mM imidazole (pH 7.4), 10

mM HEPES (pH 7.4), 300 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM ATP, 7 mM

β-mercaptoethanol and 0.01% NaN_3 (Fig. S2B, lane 5: resin). The extract (Fig. S2B,

lane 6) was dialyzed against G-buffer (2 mM Tris-HCl (pH 7.4), 0.2 mM $CaCl_2$, 0.2 mM

ATP, 0.5 mM DTT and 0.01% NaN_3) overnight at 4°C. Then the fraction was treated

with 1-chloro-3-tosylamido-7-amino-2-heptanone-treated chymotrypsin (Sigma, St.

Louis, MO, USA) at a final concentration of 8.34 μg/ml for 20 min at 25°C (Fig. S2B,

lane 7) and the reaction was stopped by adding 0.4 mM PMSF. The digested

mixture was applied to an Econo-Pac HighQ Cartridge (Bio Rad, Hercules, CA, USA)

pre-equilibrated with G-buffer, and bound proteins were eluted with a linear 0-0.5 M

NaCl gradient in G-buffer (flow velocity: 1 ml/min). Fractions containing Act1p were

collected and dialyzed against G-buffer overnight at 4°C (Fig. S2B, lane 8). Act1p

monomers were polymerized by adding the final 2 mM ATP, 100 mM KCl and 4 mM

$MgCl_2$ for 2 h at 25°C and centrifuged at 300,000 ×g for 30 min at 4°C (Fig. S2B,

lane 9: supernatant). F-actin pellet (Fig. S2B, lane 10) was suspended with G-buffer and

sonicated. After dialyzing against G-buffer overnight at 4°C, monomeric Act1p was

centrifuged at 300,000 × g for 15 min at 4°C (Fig. S2B, lane 12: pellet) and purified

recombinant Act1p was obtained in the supernatant (Fig. S2B, lane 11).

Supplemental references

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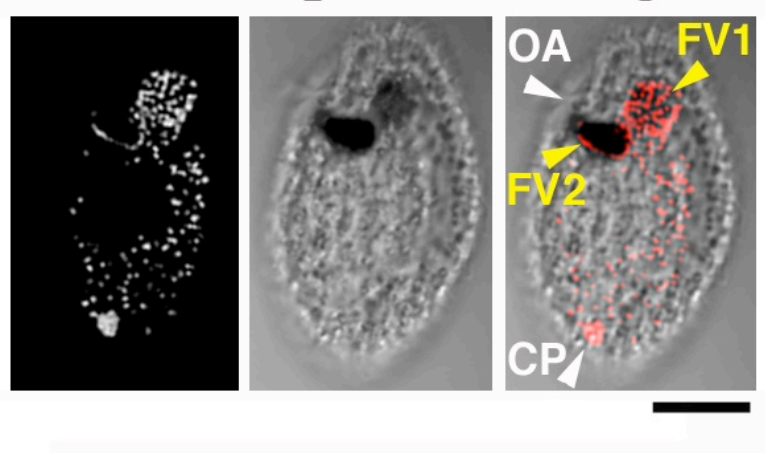
FIG S1 Actin localization in *Tetrahymena* cells undergoing phagocytosis

After 90 s of incubation with 0.1% India ink, cells were fixed and processed for immunofluorescence microscopy with anti-actin serum. This short period of India ink loading made it possible to label a few nascent FVs near the oral apparatus (OA). Judging from the density of India ink in the lumen of FV, FV2 was formed just after India ink had been loaded while the formation of FV1 had probably initiated previously. During FV formation, the cable-like actin structure frequently runs along the boundary of the bottom of FV (see FV2), and the accumulation of actin as dots surrounding the nascent FV is induced just after FV formation (see FV1). Numerous dots dispersed all around the cytoplasm also seemed to associate with maturated FVs that had previously been formed, although the number of dots around FV was smaller than that of FV1, suggesting that actin dots seemed to gradually dissociate from FVs during their maturation. Strong accumulation of actin was also frequently observed near a cytoproct (CP). This actin localization is associated with the ejection of old FV from CP (2).
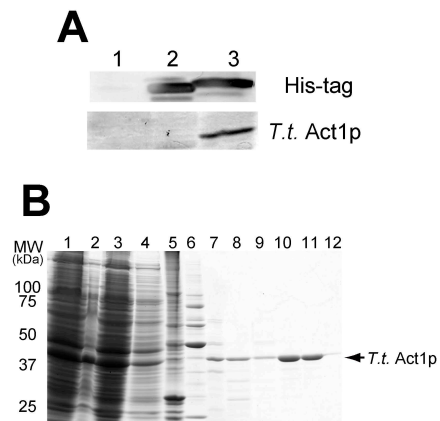
FIG S2 Purification of *T. thermophila* Act1p from *Dictyostelium discoideum*

(A) Wild-type *D. discoideum* cells (lane 1), *D. discoideum* cells transformed with pTIKLART (lane 2) or pTIKLART-ttact (lane 3) were lysed and subjected to Western blotting using an anti-His antibody (upper) or an anti-*Tetrahymena* actin antiserum (lower), respectively. (B) *Tetrahymena* Act1p was purified from *D. discoideum*. The detailed procedure of the purification is described in Supplemental Materials and Methods.
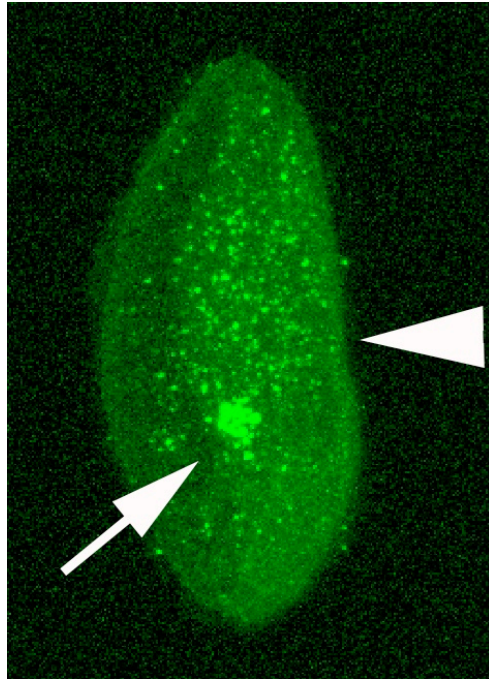
FIG S3 Localization of Adf73p in a mitotic cell

Cells transiently expressing GFP-Adf73p under exogenous promoter of the *MTT1* gene were observed. The dark region with less fluorescence signal seen on the left side of the cell is the macronucleus elongated for division. No marked accumulation of GFP-Adf73p was found in the division furrow initiated in the middle of the mitotic cell, as indicated by an arrowhead, while GFP-Adf73p was dispersed throughout the cytoplasm and several cytoplasmic dots were also observed. These dots were occasionally observed as a cluster, as indicated by an arrow.
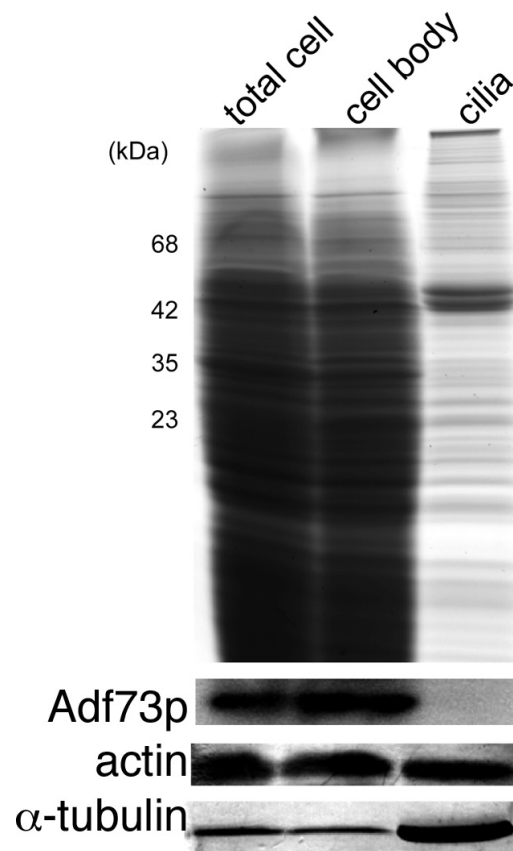
FIG S4 Expression of Adf73p in cell body but not in cilia

T. thermophila total cell extract, cell body fraction, and cilia fraction were subjected to immunoblotting using anti-Adf73p antibody (upper), an anti-*T.thermophila* actin antiserum (middle) or anti-α-tubulin antibody (lower).