The role of Copine A in phototaxis and thermotaxis in developing slugs of *Dictyostelium discoideum*

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Abstract

Copines are a group of calcium-dependent membrane binding proteins found in many organisms, including humans. However, the exact cellular function of these proteins is unknown. We studied the function of copines using *Dictyostelium discoideum*, a single-celled eukaryotic model organism. We focused on one of six copine genes, copine A (*cpnA)*. When under starvation conditions, *Dictyostelium* cells aggregate into mounds that become migrating slugs, which move toward optimal areas of light and heat. To study the role of *cpnA* in development, we compared the slug behavior of cells lacking the *cpnA* gene (*cpnA*- cells) to the slug behavior of wildtype cells. First, we captured images of slugs and then after measuring slug size, we found that the *cpnA*- slugs were much larger than wildtype slugs. In addition, we carried out slug phototaxis and thermotaxis assays and found that *cpnA*- slugs exhibited no phototaxis and negative thermotaxis, while wildtype cells exhibited positive phototaxis and thermotaxis. Mixing a small percentage of wildtype cells with *cpnA*- cells rescued slug size, thermotaxis, and phototaxis defects. Expression of GFP tagged CpnA in *cpnA*- cells under the Actin 15 or CpnA promoter did not rescue these defects. Our results indicate that CpnA has a role in intercellular signaling important in regulating the size, motility, phototaxis, and thermotaxis of slugs in developing *Dictyostelium*.

Background

Copines are a group of newly-discovered soluble, calcium-dependent, membrane binding proteins found in many eukaryotic organisms, ranging from single-celled organisms to humans (Creutz et al., 1998). The presence of copines in many diverse organisms suggests that they play an unknown fundamental role important to all eukaryotic organisms. We are studying the function of copines using the model organism, *Dictyostelium discoideum,* which has six copine

genes, *cpnA* through *cpnF* (Damer et al., 2005). We are focusing on one of the copine genes, *cpnA*. Copines consist of two C2 domains at the N terminus and an "A" domain at the C terminus. C2 domains confer calcium-dependent membrane binding activity and are found in many other eukaryotic proteins involved in either cell signaling or membrane trafficking, whilst the "A" domain is believed to be a protein binding domain (Cruetz et al., 1998; Whittaker and Hynes, 2002).

Dictyostelium discoideum are highly motile social amoebae possessing organelles and membrane trafficking pathways similar to mammalian cells. *D. discoideum* live in the soil and feed on bacteria. When the bacteria are consumed and starvation conditions arise, the amoebae aggregate into mounds that become migrating slugs. Slug movement toward areas of optimal light and heat, called phototaxis and thermotaxis respectively, ends with the slugs culminating into fruiting bodies. Previous studies found that *cpnA* knockout cells (*cpnA*- cells*)* are arrested in the slug stage and do not develop into fruiting bodies (Damer et al., 2007). Time-lapse imaging has also revealed that *cpnA*- cells aggregate into mounds that form into fewer, larger slugs when compared to wildtype slugs (Smith, personal communication). To study the role of CpnA in development further, we are studying the slug behavior of cells lacking the *cpnA* gene (*cpnA*- cells) and comparing their behavior to the slugs of wildtype cells.

Methods

CpnA knockouts cells (*cpnA*-) were created previously (Damer et al., 2007). *cpnA*- cells were transformed to express green fluorescent protein (GFP) tagged CpnA under the *actin 15* (*cpnA-act15:*GFP/CpnA) and *cpnA (cpnA-cpnA:*GFP/CpnA) promoters. A Western blot using an antibody to GFP was used to verify the expression of GFP tagged CpnA in the *cpnA-act15:*GFP/CpnA and *cpnA-cpnA:*GFP/CpnA cells. Cells at 2 x 10^6 cells/mL were used for the

Western blot, with primary antibody to GFP at 1:1000 dilution, and secondary antibody at 1:5000 dilution. The *Dictyostelium discoideum* wildtype, *cpnA-*, *cpnA-act15:*GFP/CpnA, and *cpnA-cpnA:*GFP/CpnA cells were grown on plastic Petri dishes in HL-5 media (0.75% proteose peptone, 0.75% thiotone E peptone, 0.5% Oxoid yeast extract, 1% glucose, 2.5mM Na_2HPO_4, and 8.8mM KH_2PO_4, pH 6.5) supplemented with penicillin-streptomycin at 60 U/mL. The cells were counted using a hemocytometer, pelleted at 1500rpm for 5 minutes at 4^oC, and then washed three times in ice cold 13mL of development buffer (5mM Na_2HPO_4, 5mM KH_2PO_4, 1mM $CaCl_2$, 2mM $MgCl_2$ with approximate pH 6.5). The cells were then brought up in enough development buffer (DB) to make the final concentration equal to $5x10^8$cells/mL. During the washing process, previously prepared DB agar plates (5.9mM Na_2HPO_4, 3.9mM KH_2PO_4, 1mM $CaCl_2$, and 2mM $MgCl_2$ in 1.5% agarose gel) stored at 4^oC were warmed for approximately 30 minutes in a 20^oC incubator. Once the cells were brought up in the DB, 10μL of the appropriate cell type was placed in the center of each DB agar plate. The plates were wrapped in dark paper according to whether heat or light was being applied. For thermotaxis assays, three layers of dark paper were wrapped around the plates and taped to hold the sheets in place. Approximately one-third of each plate was set on a 25^oC slide warmer in a 20^oC incubator for 48 hours. The plates were gently unwrapped and the cells imaged with a Leica MZ 16F dissecting microscope and Leica imaging software. For phototaxis assays, instead of three layers of dark paper, only one layer of the dark paper was wrapped around the plate, then a layer of aluminum foil was wrapped around the plate. The cells were then exposed to light via a single hole approximately 1mm in diameter aligned in both the paper and foil. The light (60W 130V) source was placed 11cm from the bench top to the bottom of the bulb and was approximately 30cm in front of the pipetted cell dots for 48 hours. Again, the plates were gently unwrapped and the cells imaged

with a Leica MZ 16F dissecting microscope and Leica imaging software. For the *cpnA*- and wildtype mixing experiment, the same protocol was used except the cells were mixed to contain 0, 1, 5, 10, 20, 50, 100% wildtype before being placed on the agar.

After imaging the slugs with the dissecting scope, a 0.43μm white nitrocellulose membrane (Millipore, cat. # HAWP04700) was placed on each agar plate's slug trails for two hours in order to transfer the trails from the agar to the filter. The trails on the filters were stained for 10 minutes in 0.1% Amido Black in 30% methanol and 10% Acetic acid. Once removed from the staining solution, the filters were placed twice in destaining solution (30% methanol and 10% Acetic acid) for 10-15 minutes until the filters themselves were only slightly tinted blue. The filters were then rinsed in distilled water baths and allowed to thoroughly dry. The trails on the filters were imaged using a Gel Logic 2200 Imaging System and Kodak Molecular Imaging software. The areas of the slugs imaged with the Leica software and dissecting microscope, and the linear distance the slugs moved (using images of slug trails on filters) from the middle of the initial drop area of the cells on the agar were measured in arbitrary units with Image Pro-Plus software.

Results

Copine A (CpnA) is a newly discovered protein whose function is currently unknown. To further elucidate the role of CpnA in the development of *D. discoideum*, a *cpnA* knockout (*cpnA*-) was developed (Damer et al, 2007). *D. discoideum* cells go through a development cycle under starvation conditions, aggregating together to form mounds, which then form finger-like structures that tip over into mobile slugs. The slugs move in response to light and heat, then culminate into fruiting bodies consisting of a stalk and spore head. Initial comparison between

wildtype and *cpnA*- slugs revealed that *cpnA*- slugs had a developmental defect in which they were arrested in the slug stage and unable to develop into fruiting bodies (Damer et al., 2007).

To study the slug behavior and morphology of *cpnA*- cells, wildtype and *cpnA*- cells were plated at 5 x 10^8 cells/mL as 10μL drops on agar plates and developed for 48 hours. At the same magnifications, wildtype slugs were smaller in area and greater in number compared to *cpnA*- slugs (Fig. 1). These findings suggest that the lack of *cpnA* in the knockouts resulted in fewer but larger slugs when compared to wildtype slugs.

Slug images obtained with the Leica dissecting microscope and software were used to approximate slug areas with Image Pro-Plus software. Measuring the slug areas confirmed that on average, wildtype slugs were smaller than *cpnA*- slugs (Fig. 2A). A distribution graph of slug sizes showed wildtype cells forming a greater number of slugs with smaller areas, whereas *cpnA*- slugs were fewer in number and larger in area (Fig. 2B).

Wildtype slugs move toward optimal areas of heat and light, called thermotaxis and phototaxis respectively, before culminating into fruiting bodies. Though wildtype slugs move at an angle toward a light source, they can be generally characterized as exhibiting positive phototaxis. Slugs move toward heat sources at temperatures close to the growth temperature (21^oC), with thermotaxis accuracy decreasing at higher and lower temperatures until a critical temperature is reached and slugs switch from positive to negative thermotaxis (Fisher and Annesley, 2006). The same concentration of cells were dropped on agar plates, but then exposed to heat or light from one direction. Migration behaviors of slugs were recorded by transferring the slugs and trails to nitrocellulose filters and using amido black to stain the protein in the trails (Khaire et al, 2007). Comparison of slug behaviors revealed that c*pnA*- cells exhibited negative thermotaxis and no phototaxis under the same conditions in which the wildtype cells exhibited

positive thermotaxis and phototaxis (Fig. 3). Unlike the wildtype slugs, the *cpnA*- slugs did not respond to light and moved away from the heat source, suggesting that CpnA is critical for slug phototaxis and thermotaxis.

Slug trails on filters like those in Figure 3 were used to determine the linear slug migration distances of the wildtype and knockout slugs under different developmental conditions. Image Pro-Plus software was utilized to measure the migration distance of the wildtype and *cpnA*- cells under no taxis, phototaxis, and thermotaxis (Fig. 4). The mean linear slug migration distances of the two cell types were similar when under no taxis, but wildtype slugs moved much further than the knockouts when exposed to light and heat (Fig. 4). Wildtype slugs moved more than twice the distance of the *cpnA*- slugs when exposed to light, with a smaller difference evident between the cell types when under thermotaxis conditions. *cpnA*- slugs are able to move, suggesting that CpnA is not involved in motility but is involved in light and heat sensory pathways.

Wildtype cells are able to develop into fruiting bodies within 24 hours when under no taxis, whereas *cpnA*- cells are arrested in the slug stage and therefore unable to develop into fruiting bodies (Damer et al. 2007). Previous studies have shown a rescue of the developmental defect of *cpnA*- cells when mixed at 5% and greater percentages of wildtype cells (Smith, personal communication). These studies showed that mixing wildtype cells expressing GFP with *cpnA*- cells resulted in chimeric slugs with wildtype cells distributed throughout the slug (Smith, personal communication). These results indicated that the slugs were true chimerics and not made up of one cell type.

In this study, wildtype cells were mixed with *cpnA*- cells at varying percentages and chimeric slugs were formed during phototaxis and thermotaxis assays. As the percentage of

wildtype cells in a slug increased, the size of the slugs decreased, in (Fig. 5). Comparison of slug behaviors revealed that slugs consisting of greater than or equal to ten percent wildtype, exhibited positive thermotaxis under the same conditions in which the wildtype cells positively thermotaxed (Fig. 6). Though the trails shown on the filter did not show it, there were slugs that moved toward the heat source at as low as five percent wildtype. Therefore, the *cpnA*- slug negative thermotaxis was reverted back to positive thermotaxis through mixing *cpnA*- cells with as little as five percent wildtype cells. When five percent or more wildtype cells were mixed with *cpnA*- cells, positively phototaxing slugs were formed (Fig. 7). The presence of as little as five percent of wildtype cells enabled *cpnA*- cells to form slugs that properly phototaxed.

In an effort to verify that it was the lack of *cpnA* causing the differences in slug behavior, *cpnA*- cells were transformed with plasmids to express GFP tagged CpnA under the *actin 15* (*cpnA-/act15*:GFP-CpnA) or *cpnA* (*cpnA-/cpnA*:GFP-CpnA) promoters. To verify the expression of GFP tagged CpnA in the transformed *cpnA*- cells, a Western blot using an antibody to GFP was used (Fig. 8). All the cells transformed to express GFP tagged CpnA had a protein band close to the 100kD marker, confirming that the cells were successfully transformed to express GFP-CpnA. The *cpnA-/act15*:GFP-CpnA cells had darker bands than those transformed under the CpnA promoter, signifying a greater production of GFP under the *actin 15* promoter. The dramatically lighter band at 100kD for the wildtype cells transformed under the CpnA promoter, signified a lower production level of GFP in those cells compared to the other cells transformed with GFP tagged CpnA.

In thermotaxis and phototaxis assays, the *cpnA*- cells transformed to express GFP tagged CpnA formed slugs which behaved similarly to the *cpnA*- slugs, except the *cpnA-/act15:*GFP-CpnA slugs exhibited positive phototaxis (Fig. 9). However expression of GFP-CpnA did not

completely rescue the phototaxis defect as non-angular direct light was required for the phototaxis rescue. Preliminary data suggest that expression of GFP-CpnA does not rescue the slug area or slug migration distance defects of *cpnA*- cells (Fig. 10). The slug area data were highly variable and consequently must be repeated for more conclusive results.

Discussion

Our assays revealed that CpnA had specific roles in the regulation of slug size, slug phototaxis, and slug thermotaxis. The lack of *cpnA* in the knockouts resulted in fewer but larger slugs when compared to wildtype slugs. Previous studies have shown that mutant cells with increased cell adhesion form larger slugs, suggesting that CpnA may have a role in cell adhesion (Roisin-Bouffay, 2000).

cpnA- slugs exhibited negative thermotaxis and no phototaxis under the same conditions that wildtype slugs exhibited positive thermotaxis and phototaxis. The differences between the *cpnA*- and the wildtype slug behaviors when exposed to light or heat, indicate that CpnA plays a significant role in slug phototaxis and thermotaxis. Without the expression of CpnA, the *cpnA*- slugs were defective in their movement in response to light and heat, but were still capable of slug movement. The defects in slug behavior suggested that CpnA is involved in the photosensory and thermosensory signaling pathways after the two pathways converge.

cpnA- cells were able to properly respond to light or heat when mixed at proportions of five percent or more wildtype cells. The ability of a small amount of wildtype cells to rescue the slug behavior defects of *cpnA*- cells, suggests that *cpnA*- cells are unable to release or produce a signaling molecule, but are able to respond to the signaling molecule released by the wildtype cells. Therefore our data suggests that CpnA has a role in intercellular communication in development.

Transformation of *cpnA*- cells with GFP tagged CpnA did not completely rescue any of the *cpnA*- slug defects. This may be due to the CpnA being fused to GFP and/or due to the ectopic expression of CpnA. The phototaxis defect of the *cpnA*- slugs was partially rescued by transforming CpnA knockouts with GFP tagged CpnA under the Actin 15 promoter. The partial rescue of the phototaxis defect and not the thermotaxis defect suggested that tagging the CpnA with GFP affected the protein's ability to perform its role in the thermosensory pathway before the light and heat sensory pathways converged. The slug area defect of *cpnA*- slugs was not rescued through expression of GFP tagged CpnA under the Actin 15 promoter, but may have been partially rescued when expressed under the CpnA promoter. Future studies using CpnA by itself would be necessary to determine if GFP fusion interferes with the normal function of CpnA. In the future, we plan to test the hypotheses that CpnA has roles in cell adhesion and/or the release or production of a signaling molecule.

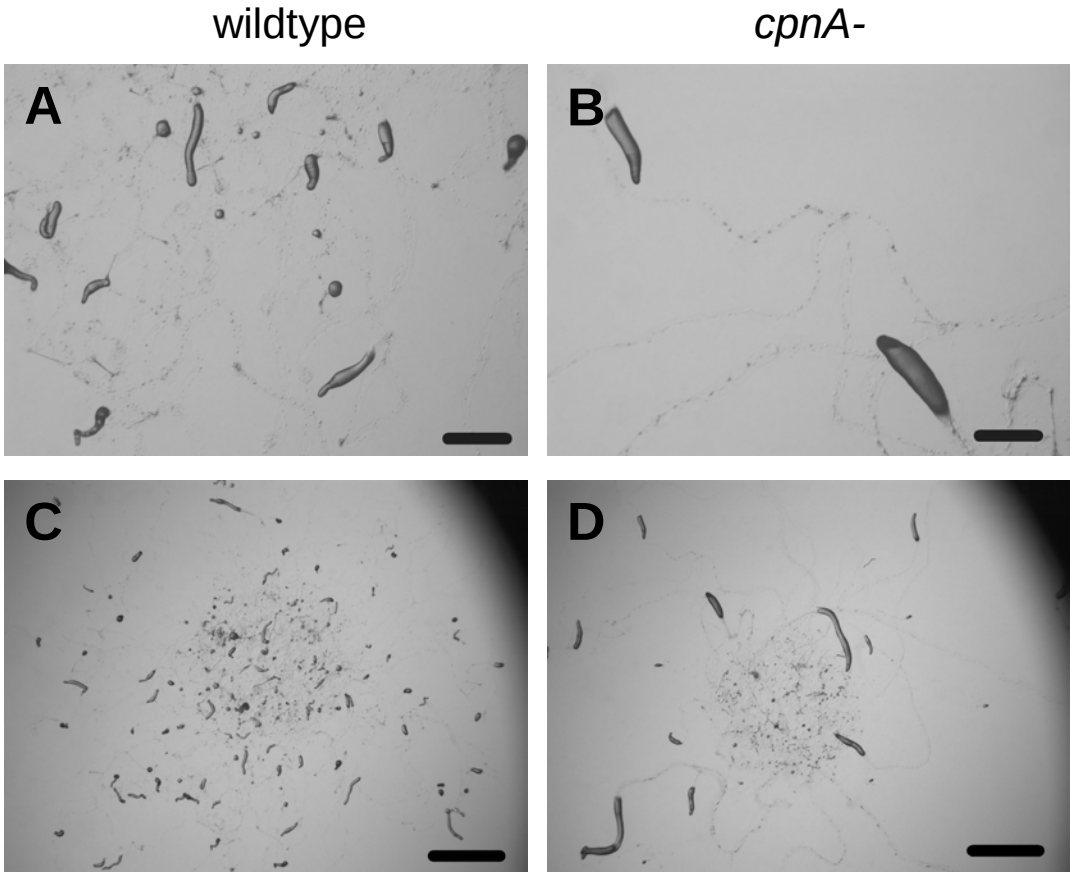


FIG. 1. Wildtype and *cpnA*- cells (5×10^8) were plated on agar and allowed to develop. At 48 hours, images of slugs were taken with a Leica dissecting microscope and analyzed with Image Pro-Plus software. A and B scale bar = 0.5mm. C and D scale bar = 2mm.

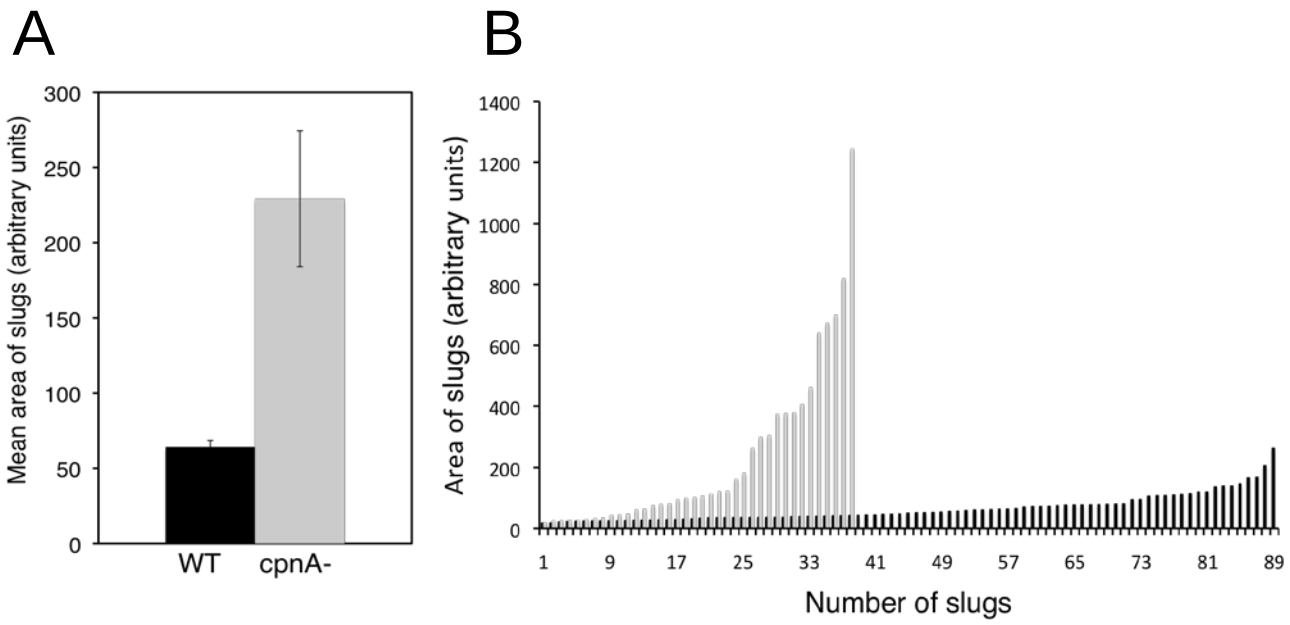


FIG. 2. Wildtype and *cpnA*- cells (5×10^8) were plated on agar and allowed to develop. At 48 hours, images of slugs were taken with a Leica dissecting microscope. Slug area was estimated from these images using Image Pro-Plus software. A) average slug size and B) all slug area data collected from two plates each.

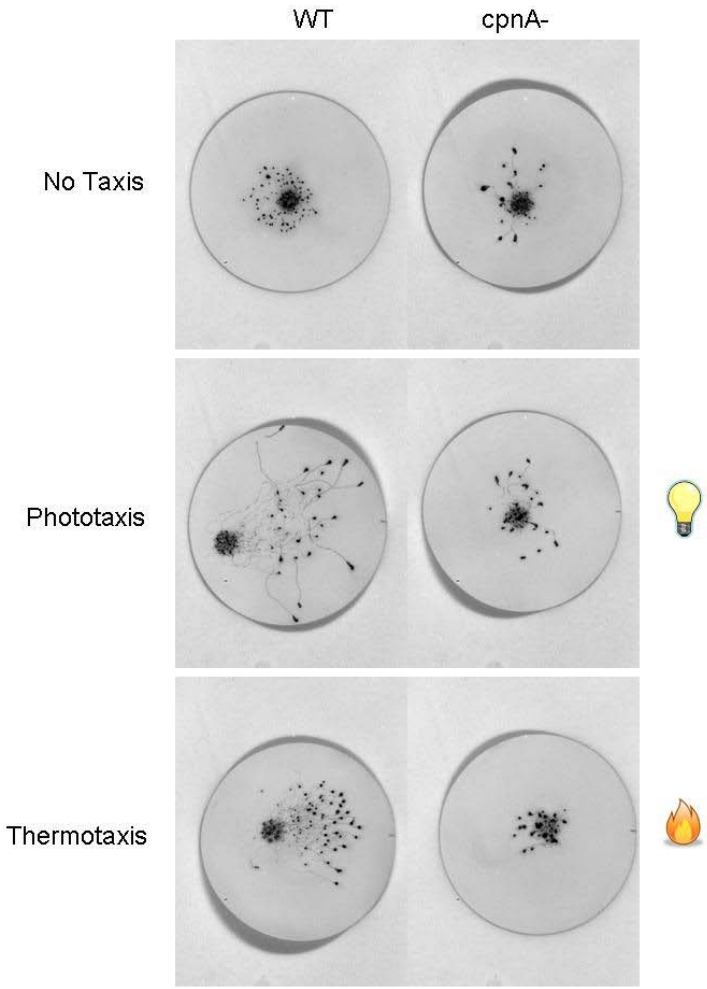


FIG. 3. Wildtype and *cpnA*- cells (5×10^8) were plated on agar and allowed to develop. At 48 hours, filters were placed on top of the slugs for 2 hours. The filters were then stained with amido black. For phototaxis, plates were wrapped in dark paper with a small hole turned toward a light source. For thermotaxis, plates were wrapped in dark paper, placed with one edge on a slide warmer set at 25°C, in an incubator set at 20°C.

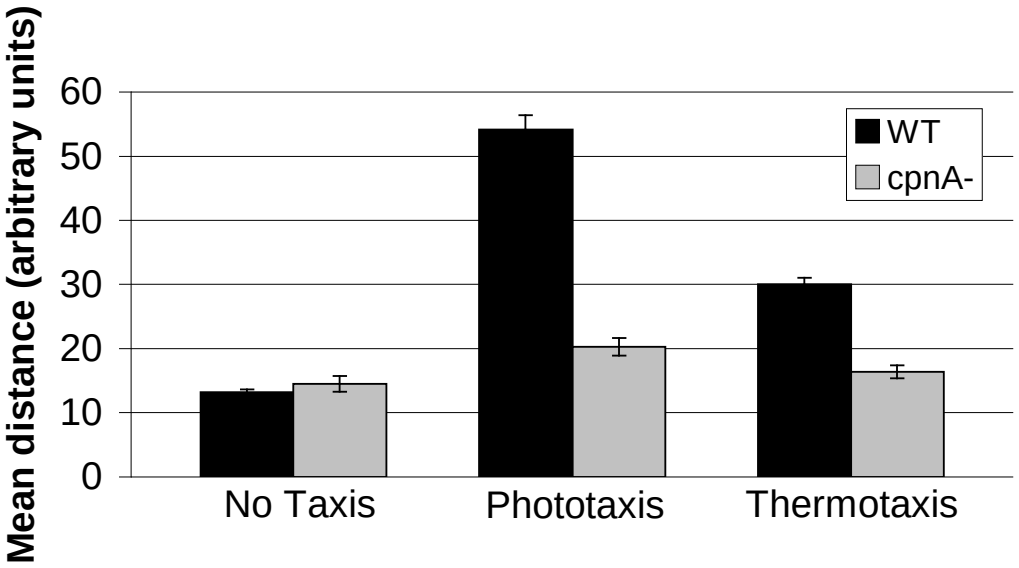


FIG. 4. The linear distance between the spot where cells were plated and each slug after 48 hours was measured using Image Pro-Plus software.

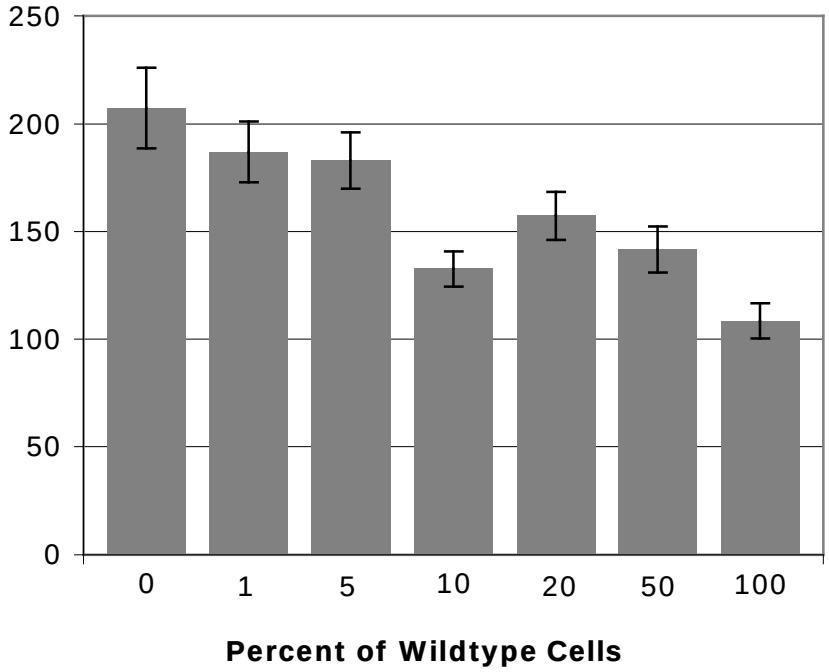


FIG. 5. Wildtype and *cpnA*- cells (5×10^8) were mixed together and plated on agar. The cells were allowed to develop under no taxis, phototaxis, and thermotaxis conditions. At 48 hours, images of slugs were taken with a Leica dissecting microscope and Image Pro-Plus software was used to estimate slug area.

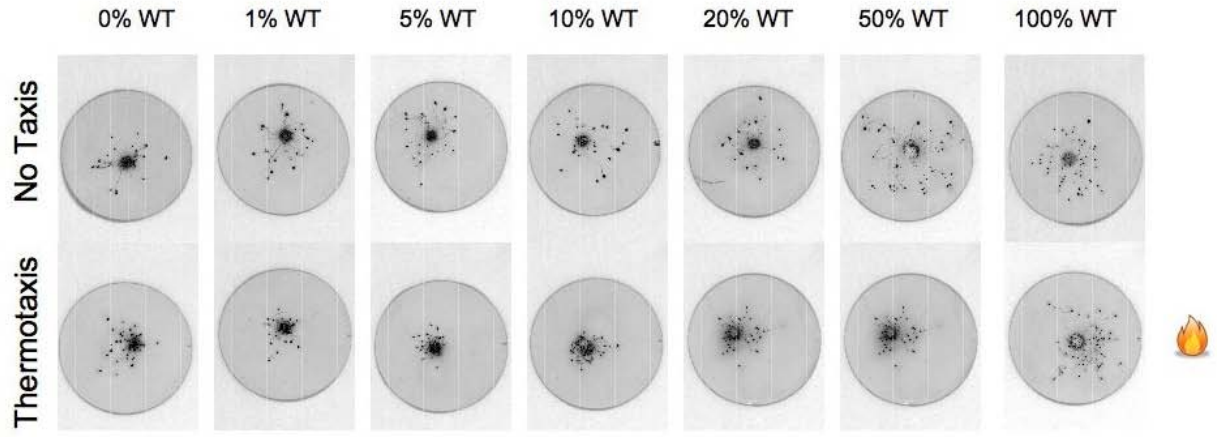


FIG. 6. Wildtype and *cpnA*- cells (5×10^8) were mixed, then plated on agar and allowed to develop. At 48 hours, filters were placed on top of the slugs for 2 hours. The filters were then stained with amido black. The plates were wrapped in dark paper with one edge on a slide warmer set at 25°C, in an incubator set at 20°C.

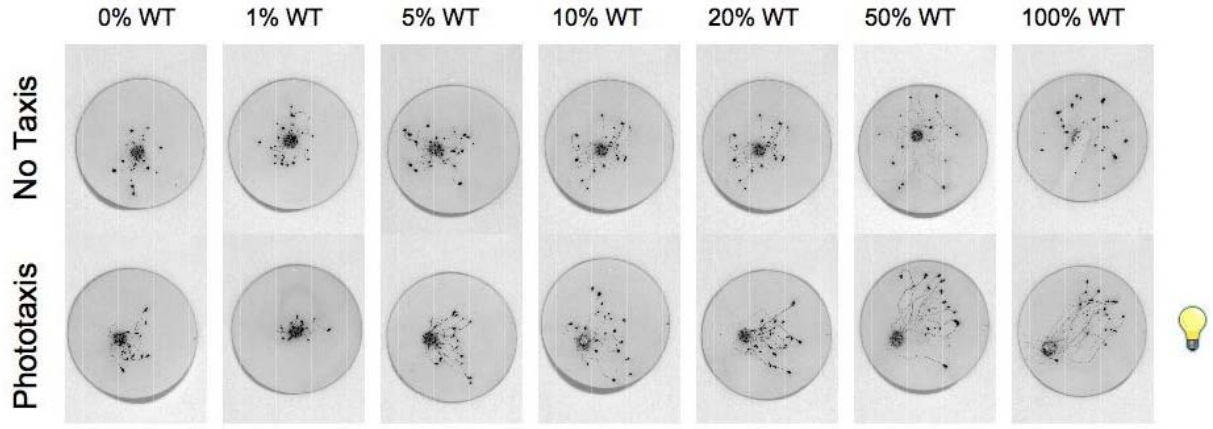


FIG. 7. Wildtype and *cpnA*- cells (5×10^8) were mixed, then plated on agar and allowed to develop. At 48 hours, filters were placed on top of the slugs for 2 hours. The filters were then stained with amido black. The plates were wrapped in dark paper with a small hole turned toward a light source.

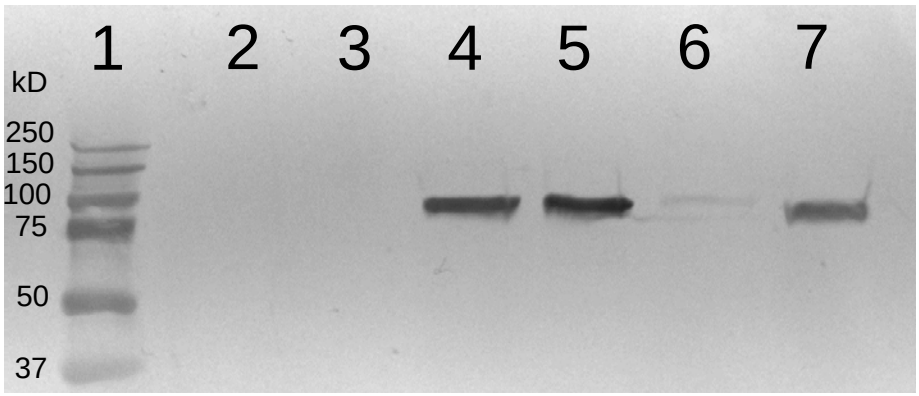


FIG. 8. Western blot using antibody against GFP. Lane 1: ladder, lane 2: wildtype cells, lane 3: *cpnA*- cells, lane 4: wildtype/*act15:*GFP-CpnA, lane 5:*cpnA*-/*act15:*GFP-CpnA, lane 6: wildtype /*cpnA:*GFP-CpnA, and lane 7: *cpnA*-/*cpnA:*GFP-CpnA

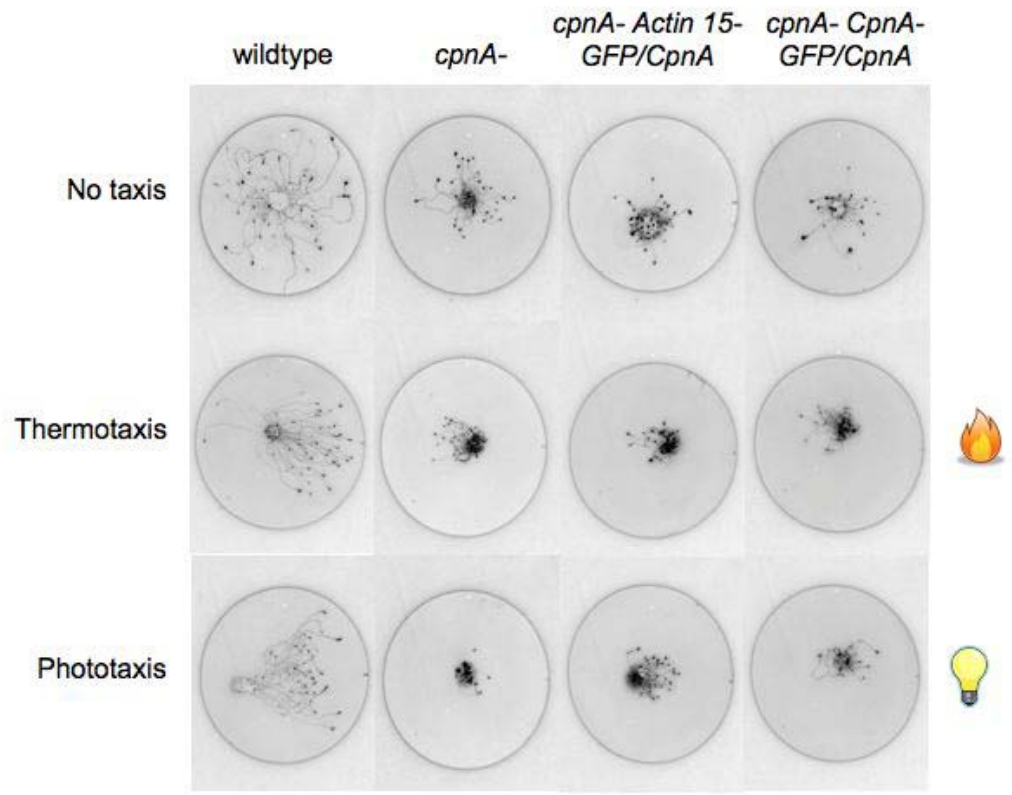
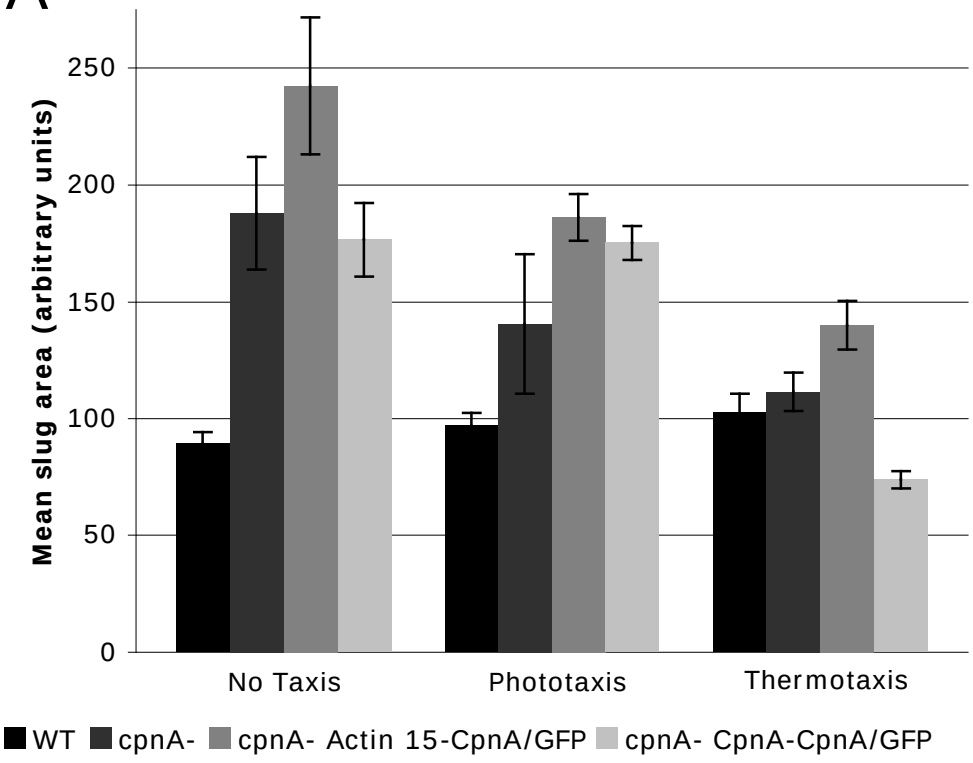


FIG 9. Cells (5 x 10^8) were plated on agar and allowed to develop. At 48 hours, filters were placed on top of the slugs for 2 hours. The filters were then stained with amido black. For phototaxis, plates were wrapped in dark paper with a small hole turned toward a light source. For thermotaxis, plates were wrapped in dark paper, placed with one edge on a slide warmer set at 25^{o}C, in an incubator set at 20^{o}C.

A


Mean slug area (arbitrary units)
250
200
150
100
50
0
No Taxis
Phototaxis
Thermotaxis
WT
cpnA-
cpnA- Actin 15-CpnA/GFP
cpnA- CpnA-CpnA/GFP

B

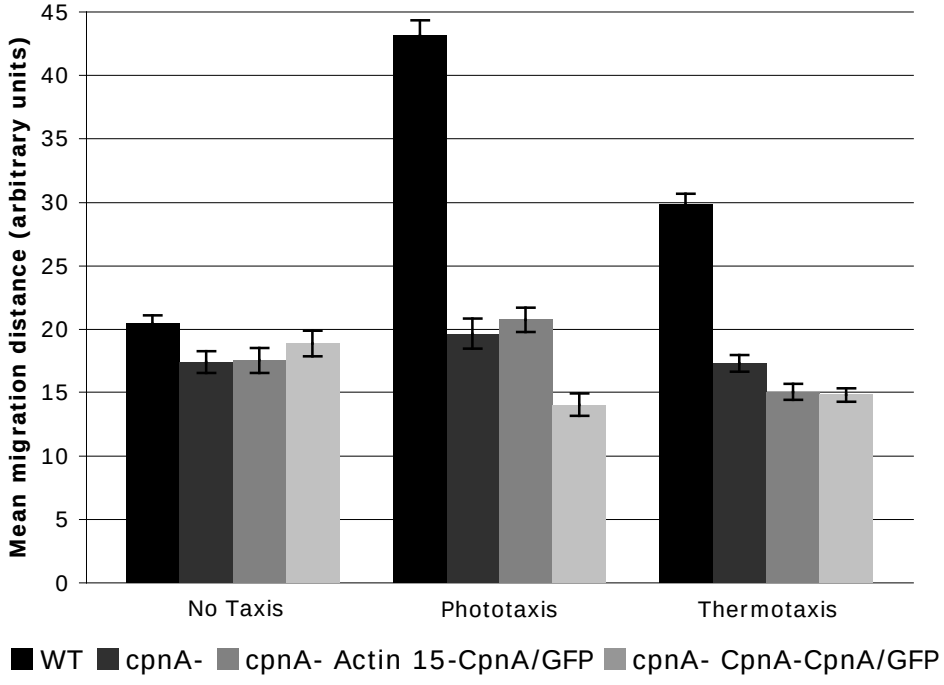

Mean migration distance (arbitrary units)
45
40
35
30
25
20
15
10
5
0
No Taxis
Phototaxis
Thermotaxis
WT
cpnA-
cpnA- Actin 15-CpnA/GFP
cpnA- CpnA-CpnA/GFP

FIG. 10. The cells were allowed to develop under no taxis, phototaxis, and thermotaxis conditions. At 48 hours, images of slugs were taken with a Leica dissecting microscope and Image Pro-Plus software was used to estimate slug area and slug migration distance. (A) average area of slugs (B) mean migration distance of slugs.

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