

CLIC MODULATES FILOPODDIA FORMATION
DOWNSTREAM OF CDC42 AND ITS EFFECTORS IN
DROSOPHILA HEMOCYTES

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Abstract

Cell migration is an essential biological process. In addition to being required for development and maintenance, cell motility also contributes to human disease. For example cancer metastasis is dependent on tumor cells gaining motility and traveling to secondary sites within the body. Cell motility requires the formation of protrusive structures including filopodia and lamellipodia. The formation of these structures requires rearrangement and coordinated organization of the actin cytoskeleton.

Here I show that *Drosophila* Clic, a member of the CLIC family, contributes to the formation of filopodia by regulating the nucleation and elongation of these structures. This is done by examining the localization of Clic and genetic interactions with known cytoskeleton regulators. I show that there are three phases of filopodia formation, supported by the observed genetic interactions. I provide evidence that Clic is involved in all three phases of filopodia formation and interacts with Cell division cycle 42 (Cdc42), Diaphanous (Dia), Wiskott - Aldrich syndrome protein (WASp), the Actin related protein (Arp) 2/3 complex, Moesin (Moe), and Enabled (Ena). I also show that Clic may contribute to *in vivo* cell migration through the study of hemocyte migration in *Drosophila* embryos.

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INTRODUCTION

I. Background

Cancer is a disease that results in the uncontrolled growth of cells within the body.¹ It can affect any body tissues including: breast, lung, prostate, colon, and skin. Some of the factors that increase the risk of cancer are: smoking, viruses, genetic predispositions, alcohol consumption, and exposure to UV or other radiation.¹ Cancer results in an estimated 1,500 deaths per day in the United States, the second most common cause of death.¹ In general, cells must undergo multiple transitions to become cancer cells. Cancer cells then undergo further changes that allow them to spread throughout the body. These changes may include a loss of adhesion and gain of motility, through changes in the actin cytoskeleton.² Cancer cells that spread to secondary sites in the body and form new tumors are known as metastases.³ Up to 90% of the deaths caused by cancer are the result of the spread of malignant cells to secondary sites.² Halting the process of metastasis could be an invaluable tool for treating cancer patients. In order to achieve this goal, we must understand motility and adhesion.

A new facet of cancer research is the search for biomarkers of cancer. Biomarkers are genes that show abnormal expression patterns that correlate with the progression of a disease. Cancer biomarkers can be used to diagnose, to determine the prognosis, and sometime to decide the most appropriate treatment.⁴ Recent research suggests

chloride intracellular channels (CLICs) can be used as biomarkers of cancer. In other words, the expression levels of CLICs can be used to predict the evolution of specific cancers, and the probability of survival. This also suggests that the CLIC family of proteins may be involved in the progression of cancer. Pursuing research on the functions of this protein family is necessary in order to determine whether it contributes to the progression of cancer and could lead to the development of new cancer treatments.

My thesis will investigate the interaction between CLICs and the actin cytoskeleton. I will focus on the interaction between CLICs, Cdc42, an actin cytoskeleton regulator, and its downstream effectors. If there is an interaction between CLICs and the actin cytoskeleton this may provide a model for how CLICs are influencing the progression of cancer.

II. Chloride Intracellular Channels

The first member of the CLIC family was identified in 1987 from bovine kidney and trachea tissues. Since then, CLIC members have been found in humans, mice, fruit flies, worms, plants, and other organisms.⁵ The number of CLIC family members in each organism varies; six have been identified in mammals (CLIC1-CLIC6).

Drosophila melanogaster has one CLIC gene (Clic). The first member of this family, p64, was shown to allow the passage of chloride ions through artificial vesicles. It has not been shown that this occurs *in vivo*, and so the name may be misleading. The

CLIC family is a unique group of channels because they can exist as either soluble proteins in the cytoplasm, or as membrane bound proteins.⁶ Specific CLICs have been shown to localize to intracellular organelles, such as the nucleus and mitochondria as well as the plasma membrane. Changes in conductance have been observed by patch clamp recording when CLICs are associated with a membrane.⁶ CLIC1-dependent currents were anionic specific.⁵ Other members of the CLIC family have been shown to insert into the membrane and produce currents, however, how this occurs is poorly understood.

In addition to possible ion channel activity, CLICs may function in oxidation-reduction (redox) reactions, as a protein chaperone, or as a regulator of the actin cytoskeleton. CLICs have been shown to have structural homology to a family of proteins known as glutathione-S-transferases (GSTs) and to have a thioredoxin motif.⁶ This protein family is involved in redox reactions and helps reduce oxidative stress in the cell.⁷ Although CLICs are similar in structure to GSTs it has not been shown that they function similarly. A CLIC null mutant is available in *Drosophila*, as this organism has only one CLIC protein, called Clic. If there are multiple proteins from the same family, as in mammals, family members may compensate for a lack of function in one member. This makes it difficult to study the role of the protein family by eliminating their function. *Drosophila* also allows us to use a variety of techniques and sophisticated genetic tools. A study of *Drosophila* Clic, showed that flies null for Clic activity, are fully viable at 22°C, but have decreased viability at 25°C. Both of these temperatures are normally permissive and would be encountered in the wild.

These null mutant animals showed an additional decrease in viability when heat shock proteins (HSPs) were disrupted, even at 22°C. HSPs are protein chaperones that help maintain proper protein activity when exposed to high temperatures and other environmental stressors. These two observations suggest that CLIC may act as, or with protein chaperones.⁷ Finally, it has also been proposed that CLICs interact with and regulate the actin cytoskeleton.

III. The Actin Cytoskeleton

The actin cytoskeleton, conserved across eukaryotes, is a dynamic protein meshwork that lies underneath the cell membrane. This scaffold, made through the organization of actin, gives the cell support and allows the completion of cellular processes such as motility, cell division, and phagocytosis (the uptake of foreign objects and dead cells). Actin exists in two forms within the cell, globular-actin and filamentous-actin. Globular actin, G-actin, are small, single actin proteins. These can join together and polymerize to form filamentous actin or F-actin (Fig. 1).⁸ These filaments can be used to construct complex cellular surface structures such as microvilli, stereocilia, filopodia, and lamellipodia. Microvilli are structures that project from epithelial cells in order to increase surface area and thus the efficiency of absorption. Stereocilia are found in the hair cells in the inner ear and are used to transmit sensory information.⁹ Filopodia and lamellipodia are transitory structures that provide the force for cell motility.¹⁰

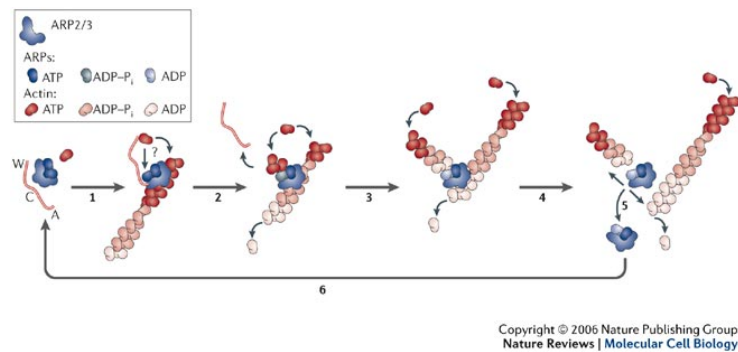


Figure 1.¹¹ Polymerization of G-actin to form F-actin, and filament branching induced by the Arp 2/3 complex. This branching is required for the formation of the webbed network seen in lamellipodia.

Cell motility is regulated through small GTPases including Rho, Rac, and Cdc42. These signaling molecules activate actin remodeling proteins, typically by protein binding.¹² For example, Cdc42 activates Wiskott - Aldrich syndrome protein (WASp), Diaphanous (Dia), and Moesin (Moe) (Fig. 2). Cdc42 binds and activates WASp, which in turn activates the actin related protein 2/3 (Arp2/3) complex. Arp2/3 attaches to actin filaments and promotes branching (Fig. 1), a process necessary for the formation of lamellipodia. Cdc42 also binds and activates Diaphanous, a formin that polymerizes unbranched actin filaments. This is necessary for the formation of filopodia.¹² Finally, Cdc42 activates a protein kinase that, in turn, phosphorylates Moe.¹³ This activates Moe so that it binds to actin and to membrane bound proteins and stabilizes actin filaments.¹⁰

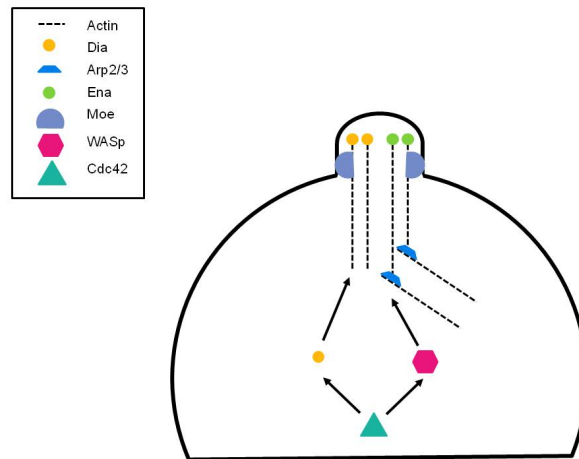


Figure 2. Cdc42 signaling in filopodia formation. This is a model of proteins involved in the formation of filopodia, and how they interact with actin. Black arrows indicate specific targets of the small GTPase, Cdc42: Dia, the Arp2/3 complex, and Moe. Homologs of these proteins can be found in mammals, where they also contribute to filopodia formation and cellular motility in these organisms.

IV. CLICs and Actin

My research has been focused on determining how CLICs interact with the actin cytoskeleton, and how the loss of CLICs affects actin-dependent processes. My research builds on previous work by others that supports that CLICs interact with the actin cytoskeleton, some of which is outlined below.

Berryman and Bretscher identified CLIC5 in human placental microvilli.¹⁴ CLIC5 was found as part of a protein complex that included actin and actin associated proteins: ezrin, α -actinin, gelsolin, and IQGAP1. The localization of CLIC5 was compared to those of other CLICs in placenta tissue. CLIC4 and CLIC5 were in the microvilli and CLIC5 was associated with the actin cytoskeleton. CLIC1 was in the

cytoplasm and did not appear to associate with the actin cytoskeleton. This suggests the members of this protein family may have diverse functions and interactions.¹⁴

Continued research on a splice variant of CLIC5, CLIC5A, showed this protein was capable of inserting into an artificial membrane and acting as a chloride channel *in vitro*. However, channel activity was not observed *in vivo*.¹⁵ This study also showed that actin polymerization is required for the formation of the protein complexes previously described.¹⁴ CLIC5A was able to interact with ezrin but this interaction was prevented with treatment by latrunculin B. This research demonstrated that a member of the CLIC family was closely associated with actin-binding proteins, and its activity was linked to actin remodeling.

CLIC5 is required for the proper formation and function of stereocilia in the inner ear.¹⁶ Mice deficient for CLIC5 were deaf, and closer observation of the cochlea revealed deformed and disorganized stereocilia (Fig. 3). When sound travels into the ear the highly organized stereocilia of the hair cells are bent. This deformation of the membrane results leads to opening of ion channels causing a depolarization in the hair cells. This depolarization leads to signal transduction to the brain. In normal stereocilia, CLIC5 was found localized at the base of stereocilia. In CLIC5 null mice, levels of the actin-binding protein radixin, (related to ezrin) and moesin were reduced. Decreases in radixin might cause destabilization of the actin core in stereocilia, this might underlie the observed deformities.¹⁶ This study gave further evidence that CLICs are involved in the stability of the actin cytoskeleton, possibly through the interactions with known actin-binding proteins such as radixin.

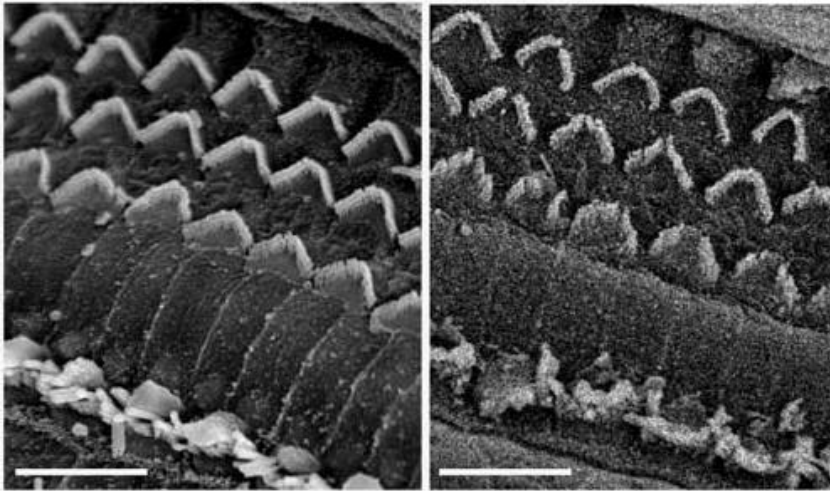


Figure 3.¹⁶ Stereocilia in the inner ear of the mouse. Left: Normal stereocilia. Right: Stereocilia in the CLIC5 mutant mouse are deformed.

Recent research linking CLICs with the actin cytoskeleton has been performed using *Drosophila* as a model organism. This research has shown that *Clic* is necessary for the formation of filopodia in larval hemocytes, or blood cells. The *Clic* null mutant produced fewer hemocytes with filopodia. By combining the *Clic* null mutation with mutations in actin regulatory molecules, it was shown that *Clic* acts downstream of *Cdc42*, the small GTPase required for normal filopodia and lamellipodia discussed above.¹⁷ The genetic interaction of *Clic* and *Cdc42* supports the hypothesis that CLICs are involved in the remodeling of the actin cytoskeleton and, in particular, in cellular motility.

V. CLICs and Cancer

In humans, members of the CLIC family have been linked to nasopharyngeal carcinoma, gastric cancer, hepatocarcinoma, colorectal cancer, gallbladder carcinoma, and breast cancer. The studies linking changes in expression of CLICs to cancer have shown that this protein family can be used as a biomarker. This suggests that CLICs may function directly in cancer progression, but how this occurs is not currently known.

For example, Chen et al. took tissues from gastric tumors and from normal tissues in patients with gastric carcinoma and found that CLIC1 levels were elevated in the tumor tissue. This study continued for five years and showed the patients with higher levels of CLIC1 had a lower survival rate.⁴ CLIC1 was also shown to be up-regulated in nasopharyngeal carcinoma (NPC). Cells were cultured from tumors and the proteins found in the culture media were measured. CLIC1 has been shown to be secreted by dendritic cells and fibroblasts and appears at detectable levels in the plasma of healthy patients. CLIC1 was found in significantly higher levels in patients with NPC than in control patients and these elevated levels were seen during early stages of cancer. This suggests that CLIC1 could be used as a biomarker for NPC as there are very few early symptoms and CLIC1 plasma levels were easily detected as being elevated in NPC patients.¹⁸ CLIC1 over-expression was also observed in hepatocellular carcinoma and colorectal cancer.^{19, 20}

Another study looked for biomarkers to help physicians design better treatment plans for patients. Researchers focused on hormone receptor-negative breast cancer, and looked for markers that indicated whether the cancer was likely to metastasize or not. If the cancer was unlikely to metastasize the patient could have the tumor surgically removed and not have to undergo systemic treatments, such as chemotherapy. Yau et al. showed that increased levels of CLIC5 correlated with decreased proliferation and could be used as a valuable marker for this type of cancer.²¹

VI. Significance

In addition to being a valuable biomarker for cancer, it is possible that CLICs could be a target for anticancer drugs. Before any drug could be developed, it is necessary to determine whether CLICs are contributing to cancer progression. There are two main processes involved in cancer: proliferation, and metastasis. It is possible that CLICs are involved in either, or both, of these processes. As evidence points to an interaction between CLICs and the actin cytoskeleton, this is a good starting point in determining whether CLIC contributes to cancer. The actin cytoskeleton is necessary for the increased cellular motility and the decreased maintenance of cellular attachments which contribute to cancer progression. Further study will illuminate if and how CLIC is acting in these processes.

EXPERIMENTAL DESIGN

I. Overview

The main focus of my research was to determine how CLICs contribute to cell motility and, therefore, metastasis. As discussed previously, metastasis depends on cytoskeletal changes that increase cellular motility. Cellular motility has four stages, all of which are characterized by cytoskeletal changes: protrusion, adhesion, translocation, and retraction (Fig. 4). My research has focused on the first step of motility, protrusion. Protrusion occurs through the formation of two actin-based surface structures, lamellipodia and filopodia. Lamellipodia are made of thin sheets of plasma membrane that overlie a webbed actin network that extend in the direction of movement. Filopodia are thin finger-like projections of membrane overlying core bundles of actin filaments. These structures are used to sense the external environment in order to determine the direction of motility. The formation of filopodia is regulated by the protein Cdc42 and its downstream effectors (Fig. 2).

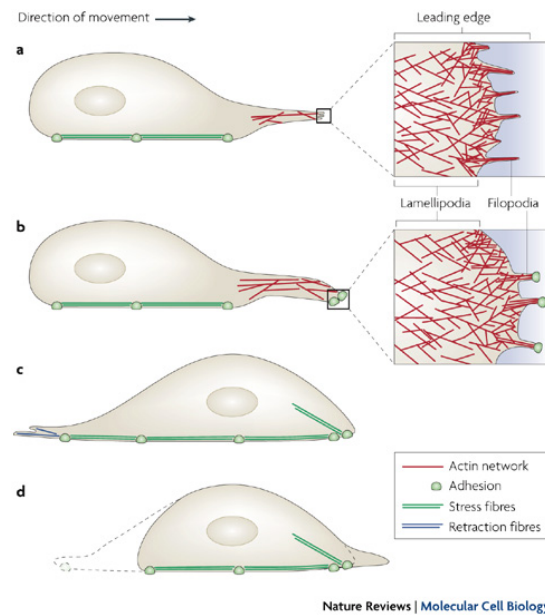


Figure 4.²⁴ A model of cellular motility. Motility consists of four stages: protrusion (a), adhesion (b), translocation (c), and retraction (d). The actin underlying filopodia and lamellipodia is the protrusive force of the cell. Additionally, filopodia aid in sensing the external environment for directional motility. These structures are actin-based and require special organization such as branching and bundling of filaments.^{22, 23}

II. *Drosophila* as a model system

Drosophila is a very tractable model system. This organism has less redundancy in genes than the mammalian systems. For example, *Drosophila* has only one CLIC protein, called Clic, which allows researchers to study a CLIC null mutant. If there are multiple proteins from the same family, as in mammals, one or more family members may compensate for a lack of function in another member. This makes it difficult to study the role of the protein family by eliminating the function of just one member of the family. *Drosophila* also allows scientists to use a variety of

techniques and sophisticated genetic tools. There are available mutations for nearly every gene, and the GAL4/UAS system and RNAi constructs described below allow careful manipulation of gene dosages.

My studies have used *Drosophila* hemocytes (blood cells) as a system to measure cell motility. *Drosophila* has four different lineages of blood cells that perform specific functions: prohemocytes, plasmatocytes, lamellocytes, and crystal cells.²⁵ My studies will use the plasmatocytes, since they are the most motile and most comparable to mammalian white blood cells.²⁵ Plasmatocytes make up most of the hemocyte population, and are easily isolated from the animal, for study *ex vivo*. These cells also travel in well studied routes in the embryo during development, providing an *in vivo* system. Clic is present and can be easily observed in these cells, allowing me to address how this protein is contributing to cellular motility.

III. Research questions and hypotheses

The main focus of my research is on how Clic contributes to and regulates cellular motility. I address this question by focusing on the role that Clic plays in the first step of motility, the formation of protrusive structures including filopodia and lamellipodia. I use three different approaches, studying Clic localization, genetic interactions, and *in vivo* observations. Each of these approaches focuses on a specific aspect of filopodia formation and cellular motility.

The localization of a protein can give some indication of the function of that protein and can help support a hypothesized function. It is for this reason that I will observe the localization of Clic within *Drosophila* hemocytes. Previous research has shown that Clic localizes to other actin based surface projections such as microvilli and stereocilia in mice. It has not been shown in any animal whether Clic is present in filopodia and lamellipodia. One of my hypotheses is that Clic will localize at the cell margin where lamellipodia form and concentrate in the filopodia.

Another one of my hypotheses is that Clic is involved in filopodia formation. I predict that Clic works in the formation of these surface structures through interactions with Cdc42 and its downstream effectors. In order to test this, I will study genetic interactions by observing filopodia formation in the presence and absence of Clic while manipulating the gene dosage of Cdc42 and its effectors.

If Clic is involved in filopodia formation, I predict that this would result in an effect on cell motility and migration. In order to observe migration, I will use the studied routes of plasmacyte migration during embryogenesis. If Clic promotes filopodia formation, I hypothesize that this would translate into a delay in migration in the Clic null mutant. If Clic inhibits filopodia formation, I hypothesize that this would translate into an increase in migration in the Clic null mutant.

MATERIALS AND METHODS

I. Pretreatment and coating of coverslips

Because my research required observing surface structures of larval hemocytes *ex vivo*, it was necessary to allow the cells to form these projections as they would *in vivo* with as few artifacts as possible. This was done by using poly-L-lysine coated coverslips.²⁶ Poly-L-Lysine is a polymerized amino acid that allows adhesion of cells to the substrate. 22mm x 22mm coverslips were cleaned by incubation in 12N HCl for twenty minutes and washed with ten changes of distilled, de-ionized water (ddH₂O) for one minute each. They were then dipped in 100% ethanol and allowed to dry. The acid-treated coverslips were then coated with 1mg/mL poly-L-lysine (all brands for reagents are listed in the Materials section below) for ten minutes and washed with ddH₂O five times for one minute each time. It is necessary to wash the coverslips extensively as free poly-L-Lysine is cytotoxic. Coverslips were then allowed to dry.²⁶

II. Manipulation of gene expression levels

In order to increase or decrease the expression levels of the candidate proteins I utilized the GAL4-UAS system.²⁷ GAL4 is a transcription activator found in yeast. Upon binding to specific DNA sequences, termed upstream activating sequences

(UAS), the downstream gene of interest will be transcribed and translated. A pictorial representation of this is shown below in Fig. 5.

This system is useful in *Drosophila* because there are no endogenous UAS sequences, nor GAL4 proteins. This allows us to pair the gene of interest with the UAS ensuring that we are altering the levels of the protein of interest and not other proteins. With this system, we can limit the alteration of gene expression to specific tissues in the fly by using tissue-specific promoters of GAL4 expression. This is an important tool, since ubiquitous changes in expression of some genes can cause lethality if the dose is altered ubiquitously. The combination of the GAL4 gene and tissue specific promoters is called GAL4 drivers. In my study I used three GAL4 drivers, shown below in Table 1. Only when both the GAL4 driver and the UAS sequence-gene of interest transgene are present will gene expression be increased.

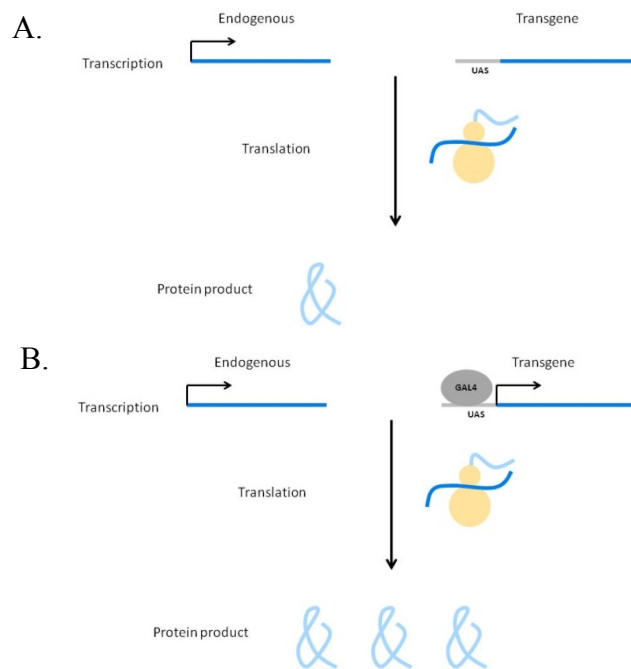


Figure 5. GAL4-UAS System. Panel A shows transcription in the absence of the GAL4 driver. The endogenous gene is transcribed to produce a protein product. However, the transgene is not transcribed. Panel B shows transcription in the presence of the GAL4 driver. Both the endogenous gene and the transgene are expressed, leading to an elevated level of the protein of interest.

Table 1. GAL4 drivers and expression patterns

GAL4 Driver	Abbreviation	Expression Pattern
Collagen type IV GAL4	CgGAL4	Expressed in larval hemocytes and fat bodies
Serpent GAL4	SrpGAL4	Expressed in embryonic hemocytes
Croquemort GAL4	CrqGAL4	Expressed in embryonic hemocytes

Another use of the GAL4-UAS system is to decrease protein levels by using the RNA interference (RNAi) pathway. This pathway uses small interfering RNA (siRNA) that is complementary to the mRNA of a gene. Thus, the siRNA binds to the complementary mRNA and targets it for degradation by the RNA induced silencing complex (RISC). This decreases the level of RNA made and therefore decreases the amount of protein that is translated and present within the cell. The GAL4-UAS system can be used to generate siRNA by producing RNA that is complementary to itself. This results in the formation of a hairpin structure that is recognized by the protein Dicer (Dcr) and cut to form the siRNA. Additional knockdown of a gene can be induced by overexpressing proteins involved in the RNAi pathway, such as Dicer (Dcr). A pictorial representation of the RNA pathway is shown below in Figure 6. The dosage of the following genes was manipulated using the procedure described above: Cdc42, Dia, WASp, the Arp2/3 complex, Moe, Ena, and Clic. A complete list of the fly stocks that were used in my experiments is included in Table 3 in the Materials section.

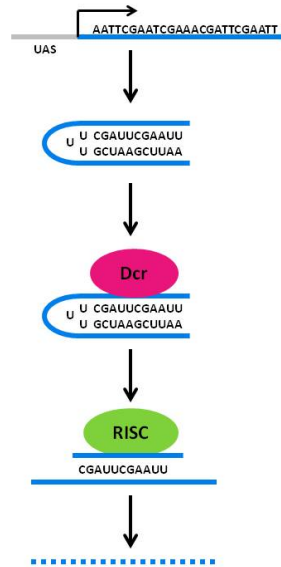


Figure 6. RNAi pathway mediated by the GAL4-UAS system. The UAS sequence is transcribed and the RNA hairpin forms due to complementary base pairing. This is recognized by the protein Dicer and cleaved to form and siRNA. The siRNA interacts with the RISC complex to degrade mRNA and therefore protein product.

III. Maintenance of Fly Cultures

Flies were cultured at 22°C. Food was prepared using 2280g Jazz mix, 360g yeast, 120g soy powder, and 12L of water. Subcultures were made daily to allow the larvae to be aged accurately. Feeding third-instar larvae were picked up at day 6 or 7.

All experiments were done in the same genetic background (carry marker mutations of the *white* and *yellow* (*y*) genes on the X chromosome) and balanced with the *CyO*, *y*⁺ (second chromosome) and *TM6*, *y*⁺ (third chromosome) balancer chromosomes. The *yellow* gene encodes for the body pigment protein. When mutated, the adult fly has a yellowish body, and the larva has yellowish mouthparts due to less pigmentation (Fig. 7). The presence of a wild-type copy of the *yellow* gene (*y*⁺) on the balancer chromosome, allows me to select larvae with the proper constructs by selecting those with yellowish mouthparts.

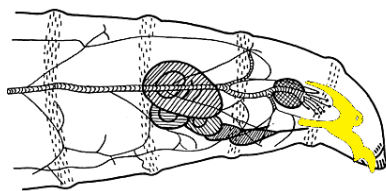


Figure 7.²⁸ The mouthparts in larvae that are affected by the *yellow* mutation. This marker can be used to easily identify larvae carrying the genes of interest.

IV. Isolation, fixation, and staining of hemocytes

Hemocytes (blood cells) were isolated from third instar larvae by gently cutting the cuticle on the posterior end of the larvae while submerged in tissue culture media. The hemocytes were allowed to attach to the poly-L-lysine coated coverslips for one hour in a humid chamber in tissue culture media. They were fixed for ten minutes with 3.7% electron microscopy-grade formaldehyde in Phosphate Buffered Saline (PBS). The coverslips were then washed in PBS for five minutes, and permeabilized in 0.1% Triton X in PBS for ninety seconds. The coverslips were washed twice in fresh PBS for five minutes each. Filamentous actin was stained with fluorescent Phalloidin using a 1:50 dilution (4 units/mL) in PBS. Phalloidin is a compound that is found in the death cap mushroom, *Amanita phalloides*, which binds to actin and prevents its depolymerization. By conjugating this compound to a fluorescent probe, we can use it to mark the actin cytoskeleton. Cells were incubated with phalloidin in a humid chamber for thirty minutes. They were then washed for five minutes in PBS and mounted on slides using 15 μ L Prolong with 4,6-diamidino-2-phenylindole (DAPI), a

fluorescent dye that binds to DNA. The next day the slides were sealed with clear nail polish.

V. Localization studies

Clic was localized using fluorescent tags and antibody staining. I used three fly lines with fluorescently marked Clic. The first line was a protein trap line with the yellow fluorescent protein (YFP) gene and a FLAG epitope sites inserted into the first intron of the Clic gene. This allowed me to visualize endogenous Clic protein using fluorescent microscopy. I also used two other fly lines expressing UAS-Clic with the green fluorescent protein (GFP) fused to the beginning or end of the Clic protein. These lines allowed me to observe Clic localization in living cells using fluorescence microscopy directly or indirectly using fixed cells with antibody staining. Antibody staining against the FLAG epitope, YFP, and GFP has several benefits including a brighter and more stable signal.

Antibody staining was performed using isolated hemocytes prepared as described above. In some cases the cells were stained with phalloidin, as described above. After fixing the cells, permeabilizing, and staining with phalloidin (if done), the cells were blocked to prevent non-specific protein binding using 10% heat-inactivated normal goat serum (HI-NGS) for thirty minutes in a humid chamber. The goat serum was removed and the primary antibody, diluted in PBS/1% HI-NGS was applied for forty-five minutes. The anti-FLAG antibody was diluted in a 1:250 ratio, and the anti-GFP

antibody was diluted in a 1:1000 ratio. After the cells were incubated in the primary antibody they were washed in 1X PBS for five minutes and the secondary antibody with a fluorescent label was applied for forty-five minutes. The secondary antibodies were diluted in a 1:1000 (2 μ g/mL) ratio in PBS/1% HI-NGS. The coverslips were then washed in PBS for five minutes and mounted in Prolong with DAPI.

VI. Quantification of filopodia formation

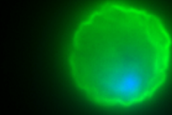
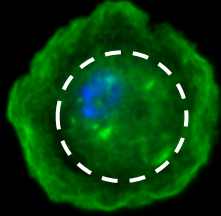
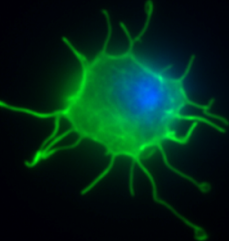
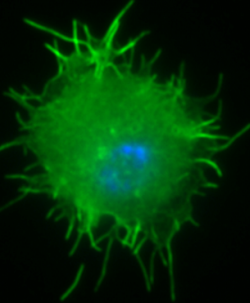
In order to study genetic interactions between proteins involved in filopodia formation, three parameters were measured: the percentage of cells forming filopodia, the number of filopodia formed per cell, and the length of the filopodia formed.

In order to measure the percentage of cells forming filopodia, cells were counted and divided into four categories based on the formation or absence of surface structures. A description and example of each of the categories is shown in Table 2. The distribution of cells into these categories was compared between different genotypes in order to determine if Clic affected filopodia priming. For each genotype at least four larvae were bled, though in most cases eight or more larvae were bled. Each larva was bled on a separated coverslip, and 100 cells from each coverslip were classified into the four categories described below.

Finally, in order to better quantify how Clic is affecting filopodia formation the number of filopodia per cell and the length of filopodia were measured and compared. Pictures of twenty double positive cells (forming both filopodia and lamellipodia)

were taken for each of the genotypes. The cells were approximately the same size and shape to control for cell and membrane variability. I used the computer program ImageJ to measure the number of filopodia per cell and their length. The average was calculated and compared across genotypes.

Table 2. Categorization of hemocytes based on the formation of surface structures.

Category	Description	Phalloidin Staining
Double Negative Cells	These cells are spherical and show fewer than three filopodia and minimal spreading.	
Cells with Lamellipodia	These cells are relatively symmetrical and show a thin lamella that extends at least one-third of the diameter of the cell body (dashed circle) past the cell body. Since the lamella is very thin, it can be differentiated from the cell body.	
Cells with Filopodia	These cells show three or more finger-like projections that extend one-third or more of the diameter of the cell body past the cell membrane.	
Double Positive Cells	These cells meet the requirements for both categories “cells with lamellipodia” and “cells with filopodia”	

VII. Embryo Collection

Adult flies were placed in egg collection chambers with molasses plates and apple cider vinegar (which stimulates egg laying) yeast paste and allowed to lay eggs. Flies were allowed to lay eggs for two to three hours and the eggs developed for approximately twelve hours at 22°C or until they were at stage 12 to 14 in development.²⁹ Embryos were washed from the plates into an egg collection basket and rinsed with ddH₂O. It is necessary to remove the outermost extraembryonic membrane of the embryo, the chorion. Embryos were dechorionated in 50% bleach for two minutes with constant agitation, and then thoroughly rinsed with ddH₂O.

The molasses plates were prepared by mixing 145mL ddH₂O, 16.2g agar, and 72mL molasses. The mixture was heated for six minutes, or until the mixture boiled steadily, in the microwave with occasional swirling. Once the solution boiled steadily, the mixture heated for four more minutes in the microwave. Once it cooled, 3-4mL of glacial acetic acid was added, and the solution was poured into petri dishes.

VIII. Fixation of embryos

The dechorionated embryos were transferred to a 10mL glass beaker using 3mL of heptane to wash the embryos from the mesh of the collection basket. The embryos and heptanes were then transferred to a glass vial using a Pasteur pipette. 3mL of 3.7% formaldehyde in PBS were added to the vial and shaken for twenty seconds. The

solution was then allowed to settle for twenty minutes; the embryos settled between the upper heptane layer and the lower formaldehyde layer. The formaldehyde was removed and 3mL of methanol was added to the vial. The vial was shaken for twenty seconds and allowed to stand for one minute. The upper heptane layer was removed along with any vitellinized embryos (devitellinized embryos will sink to the bottom of the vial). Retention of the vitelline membrane will prevent the antibodies from reaching the underlying tissues, so only devitellinized embryos are of interest. 2mL of methanol was added and mixed. After sitting for one minute most of the methanol was removed and fresh methanol was added. The embryos remained in the methanol for two hours. Next, the embryos were transferred to a microcentrifuge tube. The methanol was removed except for 300 μ L. 300 μ L of PBTA (1X PBS, 1%BSA, 0.05% Triton-X 100, 0.02% sodium azide) was added. After the embryos settled, another 800 μ L of PBTA was added and incubated for twenty minutes on a rotator. Most of the solution was removed and 1.2mL of fresh PBTA was added. After the embryos settled, the PBTA was removed and 1.2mL of fresh PBTA was added. The embryos were incubated for two hours on a rotator to block non-specific protein binding. After blocking the embryos were transferred to a fresh microcentrifuge tube, the PBTA was removed and the primary antibody was added. The antibodies used were anti-GFP (1:800 dilution of whole antiserum) and anti-engrailed (7.6 μ g/mL) diluted in PBTA. The embryos were incubated in the primary antibody at 4°C overnight. After incubating, the primary antibody was removed and the embryos were rinsed in fresh PBTA three times for five minutes each and then four times at fifteen minutes each on

a rotator. The secondary antibodies were added (diluted 1 μ g/mL in PBTA) and incubated for one hour on a rotator. After incubating, the embryos were rinsed briefly four times with PBTA and then four times for fifteen minutes on a rotator. They were then washed briefly three times with PBS/0.2% sodium azide. The embryos were mounted on cleaned slides using Prolong with DAPI.³⁰

IX. Quantification of hemocyte migration

Images of the ventral side of the embryos were taken using a fluorescent microscope. Stage 11 and 12 embryos were selected for quantification.²⁹ Engrailed staining, as well as developmental markers including germ band retraction and head involution, was used to stage embryos. Stage 11 hemocytes emerge from the head and travel posteriorly a variable number of segments along the ventral midline. The number of segments the cells had migrated was noted. For stage 12 embryos, I counted the number of embryos with hemocytes that had traveled past segment A2. For stage 13 embryos, I counted the number of embryos that had completed migration along the ventral midline. A chi-Square test was used to determine the significance of differences between genotypes.

X. Materials

A. Fly Stocks

Below is a table of the stocks used in my experiments. In some cases these stocks were combined to generate the correct genetic background and to allow for genotyping the larvae. Additionally, stocks were combined to generate the appropriate genotype of interest.

Stock Genotype	Source
$y^1 v^1$; P{TRiP.JFo2785}atp2/TM3, Sb ^r	Bloomington Drosophila Stock Center
w*; P{UASp-Arp66B.GFP}	Lynn Cooley
w ¹¹¹⁸ ; P{UAS-Moe.IR.327-775}3	Bloomington Drosophila Stock Center
w ¹¹¹⁸ ; P{UAS-Moe.Myc.K}2	Bloomington Drosophila Stock Center
w*; P{UAS-WASp.B}	Barry Ganetsky, Eyal Schejter
$y^1 v^1$; P{TRiP.JF01975}atp2	Bloomington Drosophila Stock Center
$y^1 v^1$; P{TRiP.HM05027}atp2	Bloomington Drosophila Stock Center
w*; P{UAS-dia.ca}3	Bloomington Drosophila Stock Center
w*; P{UAS-dia.EGFP}	Mark Peifer
$y^1 w^*$; P{UAS-ena.AD}3	Bloomington Drosophila Stock Center
$y^1 w^*$; P{TRiP.JF01155}atp2	Bloomington Drosophila Stock Center
w ¹¹¹⁸ ; P{UAS-Cdc42.V12}LL1	Bloomington Drosophila Stock Center
w ¹¹¹⁸ ; P{CgGAL4.A}2	Charles Dearolf
w*; P{SrpHEMO-GAL4}	Richard Binari
y* w*; P{UAS-GFP}	Bloomington Drosophila Stock Center
w*; P{UAS-Dcr-2.D}	Cheng Yu Lee
w*; P{UAS-Clic}	Bloomington Drosophila Stock Center
w ¹¹¹⁸ PBac{754.P.FSUS-O}Clic ^{CPTI003213}	Drosophila Genetic Resource Center

w ¹¹¹⁸ ; P{UAS-GFP.Clic}	Bloomington Drosophila Stock Center
w ¹¹¹⁸ ; P{UAS-Clic.GFP}	Bloomington Drosophila Stock Center
w*; P{UAS-LA::mEGFP}	Bloomington Drosophila Stock Center

B. Chemical Reagents

Reagent	Source
Poly-L-lysine	Sigma
Agar	Genesee Scientific
Jazz Mix	Fisher Scientific
Express Five serum free medium + 2mM L-glutamine	Gibco
10% Methanol Free EM grade Formaldehyde	Polysciences
10X PBS	Fisher Scientific
Triton-X	Fisher Scientific
Normal goat serum	Sigma
Alexa Fluor 546 Phalloidin	Invitrogen/Molecular Probes
Anti-FLAG antibody	Sigma
Anti-GFP antibody	Abcam
Anti-engrailed	Developmental Hybridoma Center
Alexa Fluor 488 Goat Anti-Rabbit secondary antibody	Invitrogen/Molecular Probes
Alexa Fluor 546 Goat Anti-Mouse secondary antibody	Invitrogen/Molecular Probes
Prolong with DAPI	Invitrogen/Molecular Probes
Heptane	Fisher Scientific
Methanol	Fisher Scientific
Bovine Serum Albumin	Sigma
Sodium Azide	Fisher Scientific

RESULTS

I. Clic localizes to actin based surface projections

Although there has been evidence that CLICs interact with the actin cytoskeleton in mammals, it was necessary to show that this was also occurring with *Drosophila* Clic. I used *Drosophila* larval hemocytes (blood cells) as a model of cells capable of migration. Embryonic hemocytes migrate during development. They then lose motility, while maintaining a dynamic actin cytoskeleton. In order to determine if Clic is present and localizing in surface projections I initially used antibody staining. Antibodies made against Clic did stain hemocytes. Unfortunately, the Clic antibody only worked on cells fixed in methanol, which destroyed the actin cytoskeleton. Therefore, I could not determine whether Clic was present in actin-based projections without using a transgenic strain.

To determine if Clic was present in the surface projections, we obtained three *Drosophila* lines that express Clic fusion proteins. The first line was a protein trap line that had YFP and a FLAG epitope attached to Clic. The two remaining lines were transgenic lines overexpressing Clic fused to GFP. One construct fused GFP at the N-terminus and the other at the C-terminus. Overexpression was achieved using the yeast GAL4-UAS system.²⁷

In one set of experiments, antibody staining against FLAG in the protein-trap line was used to visualize Clic. I observed Clic in the nucleus and in actin-based structures

including filopodia, lamella, membrane ruffles, and in the area of the cleavage furrow. Unfortunately, the staining conditions were not compatible with visualizing actin using phalloidin. In order to decrease non-specific binding with the FLAG antibody, I had to use 100% heat-inactivated normal goat serum (HI-NGS) which prevented phalloidin staining. It was therefore necessary to use staining using the anti-GFP antibody that also recognizes YFP in order to determine if Clic colocalizes with actin. The anti-GFP antibody produced less background, allowing for normal blocking and staining procedures. This allowed co-staining for the actin cytoskeleton (Fig. 8 F-J). This experiment showed that Clic does co-localize with the actin cytoskeleton in surface projections including filopodia and the edge of the lamella. The results from these experiments are shown in Figure 8. Live observation of the lines overexpressing the GFP-Clic fusions also showed similar localization to the protein-trap line (Fig. 8 A-E).

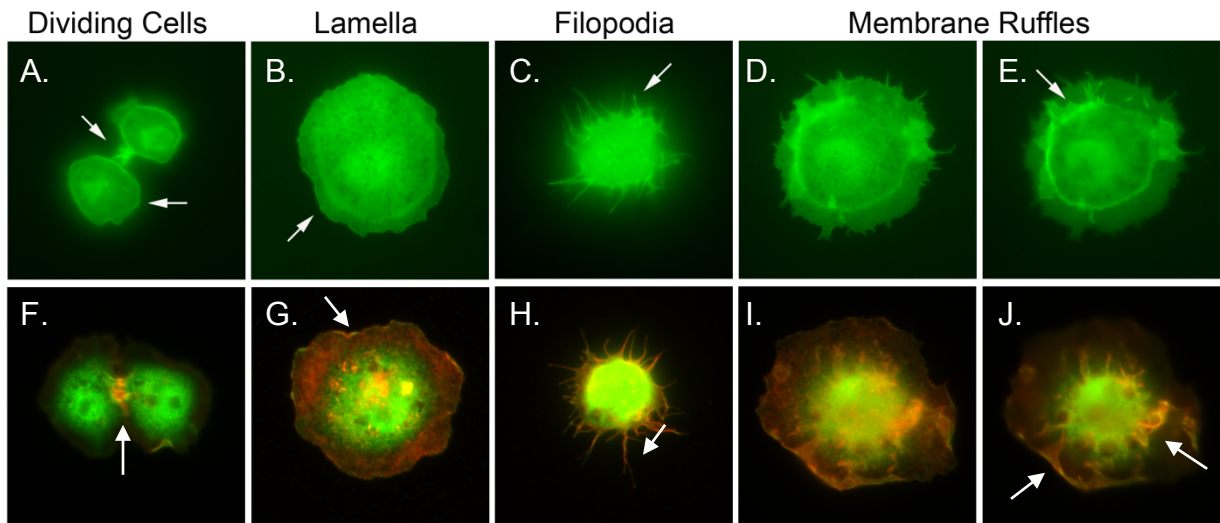


Figure 8. Clic localizes to actin based structures in larval hemocytes. Panels A-E show live hemocytes expressing UAS-GFP.Clic. Panels F-J show hemocytes isolated from the protein trap line stained with anti GFP to mark Clic and with phalloidin to mark the actin cytoskeleton. Panel A shows that Clic localizes to the area of the cleavage furrow and the cell cortex. Note that localization at the cortex was not well preserved with antibody staining. Panel B shows that Clic also localizes at the cell margin in the area of the lamella. Panel C shows that Clic localizes in filopodia. Panel D and E show two focal planes, in which Clic localizes in membrane ruffles. Panel F shows that Clic colocalizes with actin in the area of the cleavage furrow. Panel G shows Clic colocalizes with actin at the cell margin. Panel H shows Clic colocalizing with actin in the filopodia. Panel I and J show two focal planes, with Clic colocalizing with actin in the membrane ruffles.

II. Genetic interactions reveal a role for Clic in filopodia formation in larval hemocytes.

In order to determine if Clic is functioning in filopodia formation, I measured three parameters, representing different phases of filopodia formation. The first measurement was the percentage of cells in a given population that form filopodia. This indicates a cellular environment that favors initiation of filopodia formation. The second measurement was the number of filopodia formed per cell, which represents

the efficiency of nucleation of filopodia. The third measurement was the average length of filopodia, which represents the efficiency of elongation and/or stability of the filopodia formed.

I observed the phenotypes of each genotype separately, and when gene dosages were manipulated in different genetic backgrounds. This allowed me to generate a potential pathway for filopodia formation. Flies wild-type for *Clic* had an average of 27.4% of hemocytes forming filopodia. As shown in Fig.10, the *Clic* null mutant (*Clic*¹⁰⁹) had an average of 15.8% of hemocytes forming filopodia. This was a 42% reduction in the total number of cells forming filopodia when compared to *w*¹¹¹⁸, the wild-type control ($p < 0.01$). Overexpression of two copies of *Clic* resulted in a 65% increase in the total number of cells forming filopodia ($p < 0.05$). On the other hand, the average filopodia length increased in *Clic*¹⁰⁹ (61%, $p < 0.001$) and *Clic*(2X) (34%, $p < 0.001$), as you can see in Fig. 13. Finally, the morphology of the filopodia was altered with the manipulation of *Clic* dosage. Filopodia are usually straight with a pointed tip, but can be branched or curled. Curled filopodia were never observed in *Clic*¹⁰⁹, however overexpression of two copies of wild-type *Clic* resulted in many large, bubbled filopodia tips and many curled filopodia, as shown in Fig. 9. Together, these results suggest that *Clic* functions by promoting filopodia formation, and regulating elongation either through influencing the growth or stability of the filopodia. I was able to generate a potential pathway for *Clic* activity in filopodia formation by observing genetic interactions with known actin regulators. A table with a complete set of my data can be found in the appendix.

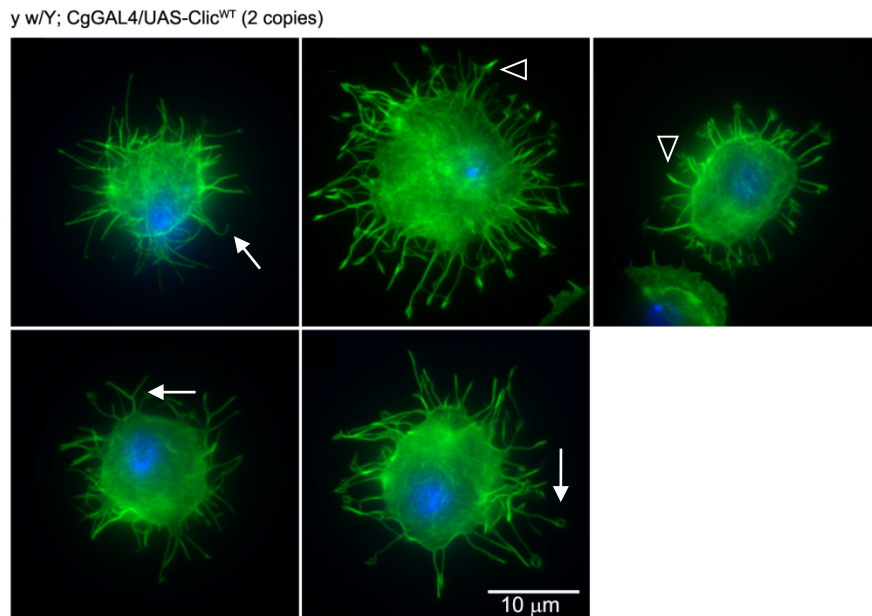


Figure 9. Overexpression of *Clic* leads to increased incidence of filopodia that are curly and branched (arrows) or with actin enrichment at the tip (arrowheads).

A. *Clic* functions with *Cdc42* and *Dia* to prime cells for filopodia formation

I first examined an interaction between *Cdc42* and *Clic*. Rogers et al. (2003) showed that overexpression of a constitutively active form of *Cdc42* (*Cdc42*^{V12}) produced proliferation of filopodia.³¹ This was confirmed in my study. In my control strains (y w, CgGAL4/UAS-GFP, y w *Clic*¹⁰⁹ CgGAL4/UAS-GFP) 10% of cells form only filopodia only, and 50% of the cells form filopodia or filopodia + lamella (Fig. 10). These averages are represented by the horizontal black lines in Fig. 10. To determine if a gene is involved in the formation of filopodia, the number of cells forming filopodia only and the number forming filopodia were compared to two controls: the CgGAL4/UAS-GFP control, and the UAS control

for the gene of interest. These controls should show the effect of insertion of the GAL4 driver and the UAS construct in order to better determine if changes are due to gene overexpression

Expression of $Cdc42^{V12}$ led to an increase in the number of cells forming filopodia only when compared to the controls. There was a 3.6 fold increase when compared to the CgGAL4/UAS-GFP control ($p < 0.01$). There was no significant change in the percentage of cells forming filopodia only when compared to the UAS- $Cdc42^{V12}$ control ($p > 0.05$). Overexpression of $Cdc42^{V12}$ also led to an increase in the total number of cells with filopodia; there was a 1.8 fold increase when compared to the CgGAL4/UAS-GFP control ($p < 0.001$) and a 1.3 fold increase when compared to the UAS- $Cdc42^{V12}$ control ($p < 0.001$). This indicates that $Cdc42$ plays a role in priming the cell to make filopodia. When $Cdc42^{V12}$ was expressed in the $Clic$ null mutant background ($Clic^{109}$), the number of cells forming filopodia only decreased 63%, and the total number of cells forming filopodia decreased 18% when compared to $Cdc42$ overexpression by itself ($p < 0.001$ and $p < 0.001$ respectively). Together these results suggest that $Clic$ is involved in filopodia priming and functions downstream of $Cdc42$.

This led me to explore interactions between $Clic$ and three downstream effectors of $Cdc42$: Dia, WASp, and the Arp2/3 complex. I used two UAS constructs to overexpress Dia: one overexpressed a constitutively active form of Dia (dia^{CA}), and the other overexpressed wild-type Dia tagged with GFP (dia^{WT}).

Note that Dia usually exists in an auto-inhibited form and is only active when bound to the active form of Cdc42 (Fig. 11)

Expression of dia^{CA} led to an increase in the number of cells forming filopodia, suggesting that like Cdc42, Dia is involved in the priming phase. When the number of cells forming filopodia only were compared to the controls there was a 4.0 fold increase when compared to the CgGAL4/UAS-GFP control and a 3.3 fold increase when compared to the UAS-dia^{CA} control ($p < 0.001$, and $p < 0.001$, respectively). There was also a significant increase in the total number of cells forming filopodia. There was a 1.9 fold increase when compared to the CgGAL4/UAS-GFP control and a 1.6 fold increase when compared to the UAS-dia^{CA} control ($p < 0.001$ and $p < 0.001$, respectively). When dia^{CA} was expressed in Clic¹⁰⁹ the number of cells forming filopodia only did not significantly change when compared to dia^{CA} by itself. There was a 9% decrease in the total number of cells forming filopodia when dia^{CA} was expressed in Clic¹⁰⁹ ($p < 0.05$) and no significant difference in the number of cells forming filopodia only. This suggests that Clic is functioning upstream of the activated form of Dia. Alternatively, there may be no functional interaction of Clic the the activated form of Dia.

As shown in Fig. 10, expression of dia^{WT} did not significantly affect the percentage of cells forming filopodia. When dia^{WT} was expressed in Clic¹⁰⁹, there was a 40% decrease in the number of cells forming filopodia only ($p > 0.05$) and a 55% decrease in the total number of cells forming filopodia ($p < 0.01$) when compared to dia^{WT} by itself. This suggests that Clic functions in the activation of

Dia by Cdc42 and influences its activity in the priming phase of filopodia formation.

I also examined an interaction between WASp and Clic, by overexpressing a wild-type copy of WASp (WASp^{WT}). Note that WASp is normally in an auto-inhibited state that requires activation by Cdc42 (Fig. 11). Overexpression of WASp^{WT} led to a 1.3 fold increase in the total number of cells forming filopodia ($p < 0.01$). However, it did not affect the number of cells forming filopodia only. This may be due to WASp's involvement in the formation of lamellipodia in addition to its role in filopodia formation. Therefore, like Cdc42 and Dia, WASp is involved in the priming phase of filopodia formation. Unlike Cdc42 and Dia, this role was unaltered in Clic¹⁰⁹ ($p > 0.05$). Overexpression of WASp^{WT} with Clic overexpression did not significantly alter the total number of cells forming filopodia ($p > 0.05$). However there was a 2.0 fold increase in the number of cells forming filopodia only ($p < 0.05$). This suggests that Clic is not necessary for WASp priming activity but enhances the function of WASp.

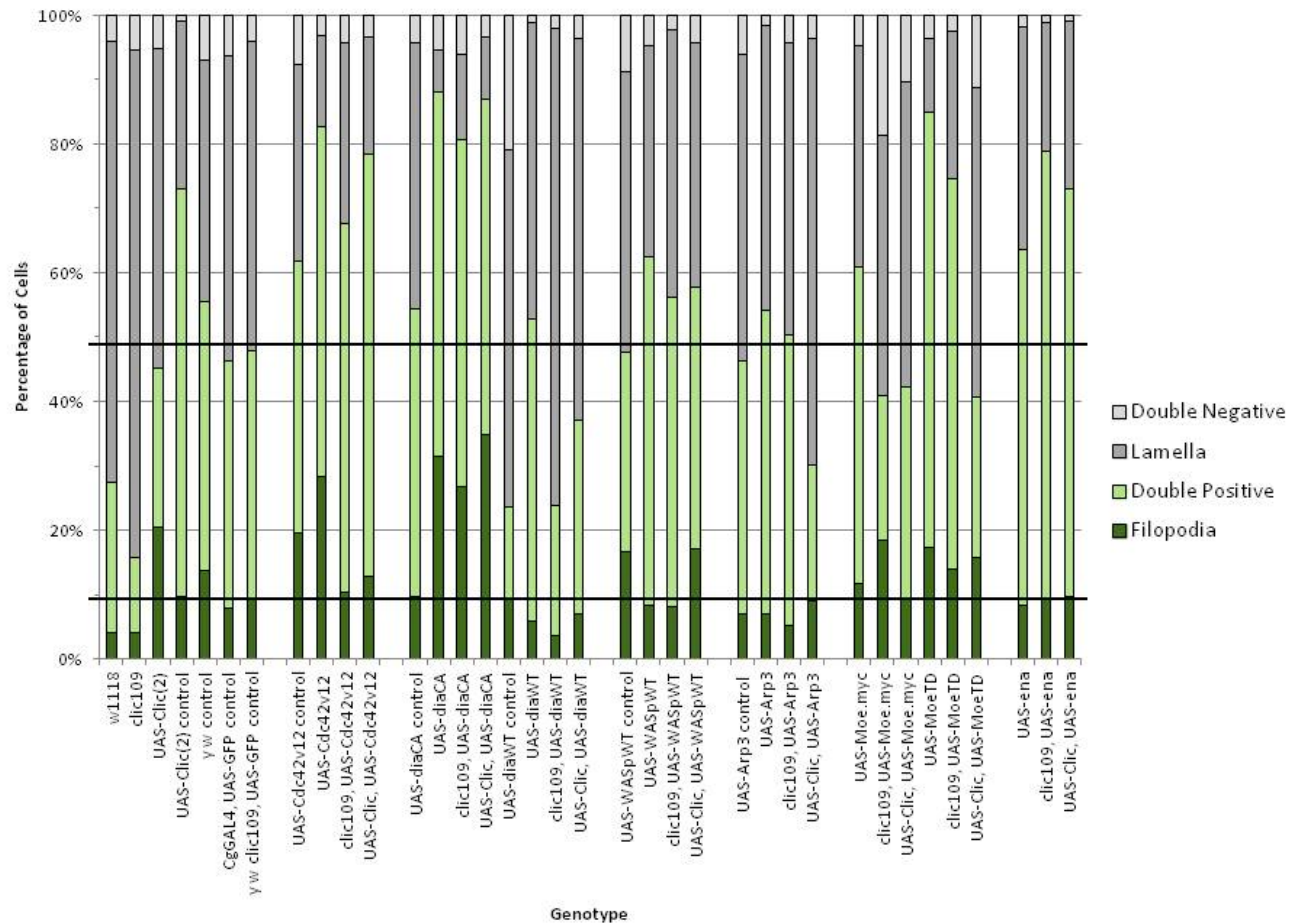


Figure 10. The percentage of hemocytes forming filopodia. This figure shows how genotype affects the surface projections of hemocytes. For control genotypes, 10% of cells form only filopodia (no lamella), shown by a horizontal black line. The percent of cells with only filopodia (dark green) plus those with both filopodia and lamella (light green) was 50%, shown by a second horizontal black line.

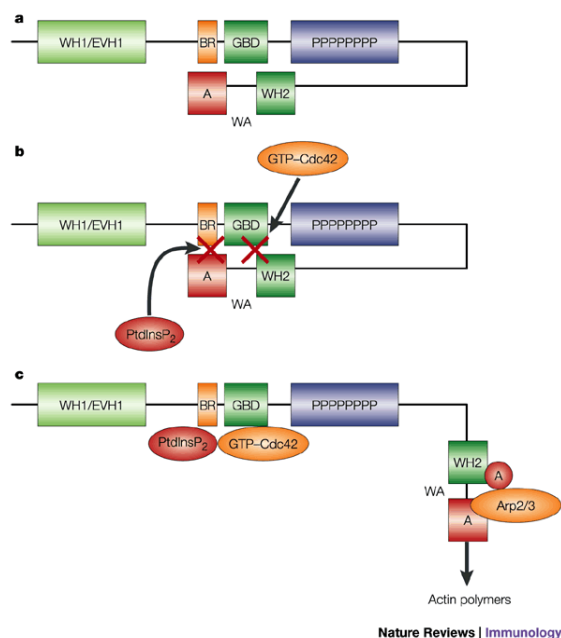


Figure 11.³² Autoinhibition of WASp. a) Auto-inhibited state b) Cdc42 binds to the CRIB domain of WASp, represented in this figure as the GTPase binding domain (GBD) c) Activated WASp can bind the Arp2/3 complex and initiate actin nucleation.

The Arp2/3 complex functions downstream of WASp, but can also be activated by other proteins. Because this complex functions downstream of WASp, I would expect that overexpression of the Arp2/3 complex would increase the number of cells forming filopodia, but that this would be independent of Clic. However, I did not observe a significant difference in the number of cells forming filopodia when Arp3 was overexpressed. Overexpression of Arp3 in the *Clic*¹⁰⁹ background did not alter the phenotype ($p > 0.05$). These results suggest that Arp2/3 does not function in filopodia priming and does not interact with *Clic*. Note that the Arp2/3 complex is made up of several proteins, and overexpressing one component of that complex may not accurately represent overexpression of the complex as a whole.

I also examined the role of two other proteins in the priming phase of filopodia formation: Moe and Moe^{TD}. Ezrin, Radixin, and Moesin make up a family of

proteins, called ERM proteins, that links the actin cytoskeleton to the plasma membrane. CLIC5 physically interacts with Ezrin, and regulates the localization of Radixin in mammals.^{14,15, 16} Moe is thought to be indirectly regulated by Cdc42 through another kinase.³³ Clic was shown to physically interact with a related protein Ezrin, and to regulate the localization of Radixin.^{15,16} Recently, there has been evidence that Clic is binding directly or indirectly to Moe, as demonstrated by co-immunoprecipitation. It is therefore possible that Clic functions with Moe to tether the actin cytoskeleton to the plasma membrane. Overexpression of wild-type moesin led to a 1.3 fold increase in the total number of cells forming filopodia when compared to the CgGAL4/UAS-GFP control ($p < 0.05$). When Moe was overexpressed in the Clic¹⁰⁹ background the total number of cells forming filopodia decreased by 33% ($p < 0.001$) compared to Moe overexpression by itself (Fig. 10).

Moe also exists in a closed auto-inhibited state, that is opened by phosphorylation. This can be mimicked by substituting the phosphorylatable amino acid, threonine 559 in *Drosophila*, for a negatively charged amino acid, in this case aspartic acid. This amino acid is conserved across species and has been shown to be necessary for the proper regulation of Moe. The phosphomimetic form of Moe is one way to study the effects of a constitutively active form of Moe. Overexpression of Moe^{TD} resulted in an 2.2 fold increase in the number of cells forming filopodia only when compared to the CgGAL4/UAS-GFP control ($p < 0.01$) and the total number of cells forming filopodia also increased 1.8 fold ($p <$

0.001). When Moe^{TD} was overexpressed in the Clic¹⁰⁹ background, there was a 12% decrease in the total number of cells forming filopodia ($p < 0.05$) when compared to Moe^{TD} expression alone. This reduction was not as large as the reduction seen with the wild-type form of Moe. The data for both wild-type Moe and Moe^{TD} suggests that Clic interacts with Moe in priming filopodia.

I also wanted to examine whether there was an interaction of Clic with the protein Enabled (Ena). Ena is a protein that nucleates actin filaments and stimulates filopodia formation.³³ Its function is similar to Dia, however it is not directly regulated by Cdc42. This interaction was of interest in order to determine if Clic acts broadly on actin nucleators, or only on nucleators downstream of Cdc42. Overexpression of Ena led to a 1.4 fold increase in the total number of cells forming filopodia ($p < 0.05$). Overexpression of Ena in the Clic¹⁰⁹ background led to a 1.2 fold increase in the number of cells forming filopodia; however this was not statistically significant ($p > 0.05$). This suggests that these proteins are not working together to prime cells for filopodia formation.

B. Clic functions with Cdc42, WASp, and the Arp2/3 complex to nucleate filopodia

Once the cell is primed to form filopodia, the signal must be translated into the formation of filopodia. This requires the proteins to physically interact (though not necessarily directly) with actin and for the filaments to be organized. In order to

determine which proteins were involved in this phase of filopodia formation, I examined how many filopodia formed per cell, for each of the genotypes described above. This was done by counting the number of filopodia found on twenty double positive hemocytes for each genotype. Because these cells are forming filopodia, I will consider them already primed for filopodia formation. Fig. 12 shows the results of this experiment.

Overexpression of $Cdc42^{V12}$ resulted in a 1.6 fold increase in number of filopodia per cell when compared to the $CgGAL4/UAS-GFP$ control ($p < 0.001$). The phenotype of $Cdc42^{V12}$ overexpression was decreased in the $Clic^{109}$ background, resulting in 20% less filopodia per cell ($p < 0.01$) when compared to $Cdc42^{V12}$ alone. This suggests that $Cdc42$ functions in the nucleation of filopodia and that $Clic$ functions downstream in this process.

Overexpression of either Dia construct also resulted in the production of more filopodia per cell, suggesting that Dia functions in the nucleation phase. Overexpression of dia^{CA} led to an insignificant increase in the number of filopodia per cell when compared to the $CgGAL4/UAS-GFP$ control and a 1.6 fold increase in the the $UAS-dia^{CA}$ control ($p > 0.05$ and $p < 0.01$, respectively). When dia^{CA} was expressed in $Clic^{109}$, the number of filopodia increased 1.4 fold ($p < 0.05$) when compared to dia^{CA} alone, although the same increase also occurred when a wild-type copy of $Clic$ was expressed ($p < 0.05$). The expression of dia^{WT} also resulted in a 1.5 fold increase in the number of filopodia per cell when compared to the $CgGAL4/UAS-GFP$ control and the $UAS-dia^{WT}$ control ($p < 0.01$ and $p <$

0.01, respectively). The expression of dia^{WT} in Clic^{109} did not significantly affect the number of filopodia per cell. Together these results suggest that the role of Dia in the nucleation phase of filopodia formation is independent of Clic.

Overexpression of WASp^{WT} also led to a 2.4 fold increase in the number of filopodia formed per cell when compared to the CgGAL4/UAS-GFP control and a 1.5 fold increase when compared to the $\text{UAS-WASp}^{\text{WT}}$ control ($p < 0.001$ and $p < 0.001$, respectively). Expression of WASp^{WT} in Clic^{109} resulted in a 26% decrease in the number of filopodia formed per cell ($p < 0.01$) when compared to WASp^{WT} alone. This suggests that Clic works with WASp in the nucleation phase, though an interaction between WASp and Clic was not observed in the priming phase.

Though overexpression of Arp3 did not appear to be involved in the priming phase, it did appear to be involved in the nucleation phase of filopodia formation. Overexpression of Arp3 led to a 1.9 fold increase in the number of filopodia formed per cell when compared to the CgGAL4/UAS-GFP control and a 2.5 fold increase when compared to the UAS-Arp3 control ($p < 0.01$ and $p < 0.001$, respectively). Overexpression of Arp3 in the Clic^{109} background led to an insignificant change in the number of filopodia per cell ($p > 0.05$) when compared to overexpression of Arp3 alone. Overexpression of Clic in conjunction with overexpression of Arp3 resulted in a 43% decrease in the number of filopodia per cell ($p < 0.01$). This suggests that overexpression of Arp3 is able to cause an effect despite only being a portion of the Arp2/3 complex. Additionally, these

results suggest that Clic interacts with the Arp2/3 complex, and may inhibit or prevent nucleation activity of the Arp2/3 complex.

Overexpression of wild-type Moe led to a 1.3 fold increase in the number of filopodia per cell when compared to the CgGAL4/UAS-GFP control ($p < 0.05$). When wild-type Moe was overexpressed in the Clic¹⁰⁹ background the number of filopodia per cell decreased by 24% ($p < 0.01$). Overexpression of Moe^{TD} also led to a 1.3 fold increase in the number of filopodia per cell, though this was not significant ($p > 0.05$). Overexpression of Moe^{TD} in the Clic¹⁰⁹ background led to a 1.3 fold increase in the number of filopodia per cell ($p < 0.05$) when compared to overexpression of Moe^{TD} alone. This was opposite to the effect seen with overexpression of wild-type Moe. This suggests that Clic may interact with Moe differently based on its activation state.

Overexpression of Ena led to a 2.4 fold increase in the number of filopodia per cell when compared to the CgGAL4/UAS-GFP control ($p < 0.001$). When Ena was overexpressed in the Clic¹⁰⁹ background the number of filopodia per cell was unchanged ($p > 0.05$). When Ena was overexpressed in conjunction with overexpression of Clic the number of filopodia per cell decreased 20% ($p < 0.01$). This was similar to the interaction observed with Arp3, suggesting that the levels of Clic may work antagonistically to the nucleating activity of Ena.

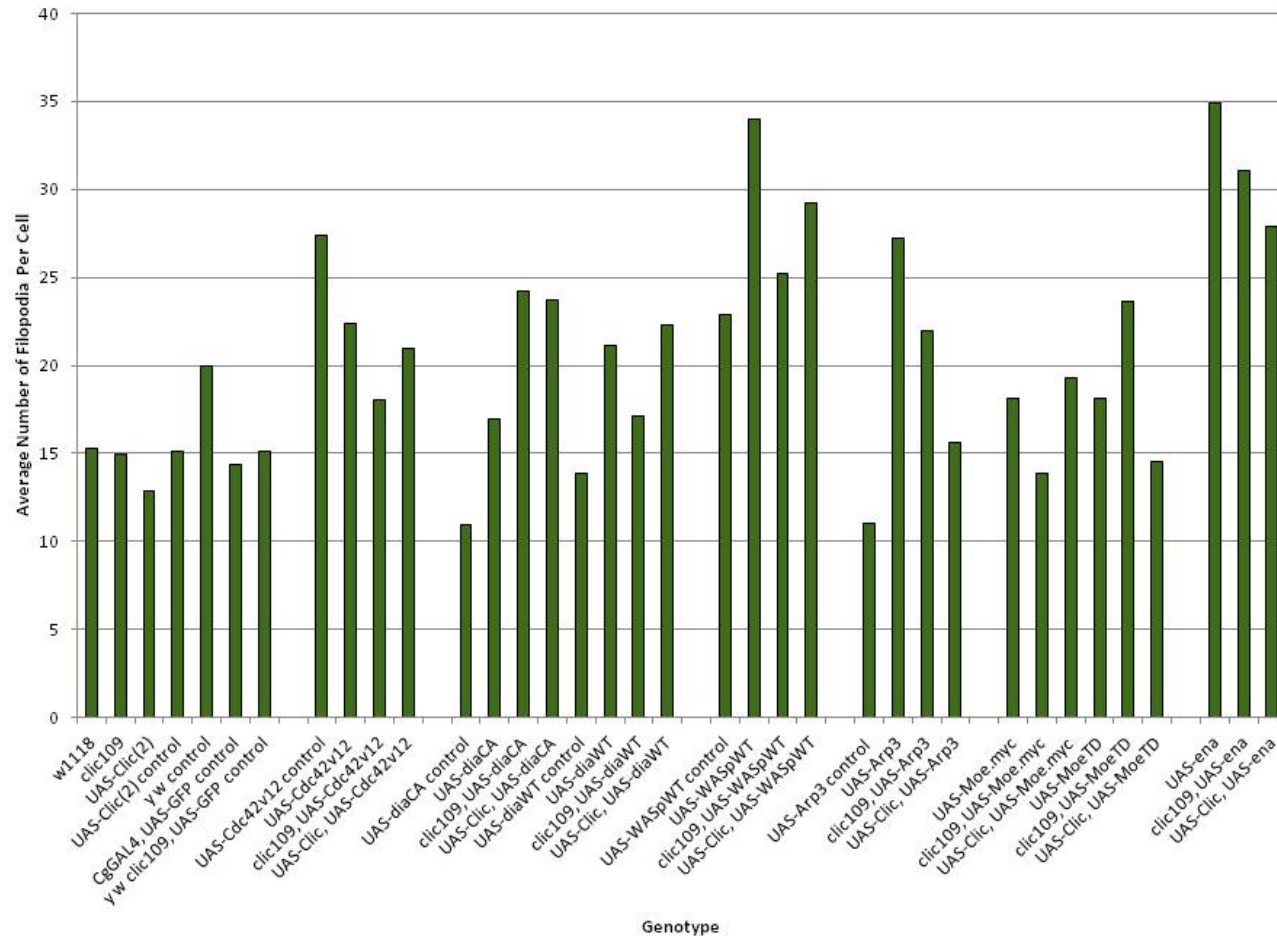


Figure 12. The average number of filopodia per cell for each genotype. $n = 20$ for all genotypes except those stated below. yw Clic¹⁰⁹, CgGAL4 control ($n = 40$), UAS-Cdc42^{V12} ($n = 60$), Clic¹⁰⁹, UAS-Cdc42^{V12} ($n = 50$), UAS-Clic, UAS-Cdc42^{V12} ($n = 40$), UAS-Clic, UAS-dia^{WT} ($n = 40$).

C. Clic functions with Dia, Moe, and Ena in the elongation of filopodia

After the filopodia forms, many factors contribute to its continued growth and stability. This phase makes up the elongation phase of filopodia formation. I measured the length of the filopodia counted in the previous section and calculated the average filopodia length for each of the previously mentioned genotypes. Proteins that I found to significantly affect filopodia length were Dia, WASp, Moe, and Ena. Proteins that did not affect filopodia length, but did show a difference in filopodia length when levels of Clic were changed included Cdc42 and the Arp2/3 complex.

Overexpression of Cdc42^{V12} did not significantly change filopodia length when compared to the CgGAL4/UAS-GFP control ($p > 0.05$). Overexpression of Cdc42^{V12} in the Clic¹⁰⁹ background was not significantly different from the Clic¹⁰⁹; CgGAL4/UAS-GFP control ($p > 0.05$). There was a 1.1 fold increase in filopodia length when Cdc42 was overexpressed in combination with Clic overexpression when compared to the CgGAL4/UAS-GFP control ($p < 0.05$) and a 1.1 fold increase when compared to the overexpression of Clic ($p < 0.001$). This suggests that Cdc42 and Clic may function synergistically to promote filopodia elongation.

Overexpression of dia^{CA} did not significantly affect filopodia length when compared to the CgGAL4/UAS control ($p > 0.05$). Overexpression of dia^{WT} led to a 33% decrease in the average length of the filopodia ($p < 0.001$). Expression of dia^{WT} in the Clic¹⁰⁹ background led to a 1.2 fold increase in filopodia length ($p <$

0.001), though the filopodia were still shorter than the CgGAL4/UAS-GFP control ($p < 0.001$). Overexpression of *dia*^{WT} with overexpression of *Clic* also resulted in a 1.2 fold increase in filopodia length ($p < 0.001$) when compared to *dia*^{WT} overexpression alone.

WASp overexpression also decreased filopodia length 12% when compared to the CgGAL4/UAS-GFP control ($p < 0.05$). There was an 8% decrease in filopodia length when WASp was overexpressed in the *Clic*¹⁰⁹ background ($p < 0.05$). When WASp and *Clic* were overexpressed simultaneously there was an 18% decrease in filopodia length ($p < 0.001$) when compared to overexpression of WASp^{WT} alone.

Overexpression of *Arp3* did not significantly influence filopodia length when compared to the CgGAL4/UAS-GFP control ($p > 0.05$). However, when *Arp3* was overexpressed in the *Clic*¹⁰⁹ background there was an 18% decrease in filopodia length when compared to the *Clic*¹⁰⁹; CgGAL4/UAS-GFP control ($p < 0.001$) and a 13% decrease when compared to overexpression of *Arp3* alone ($p < 0.01$). Overexpression of *Arp3* in conjunction with overexpression of *Clic* was not significantly different from overexpression of *Arp3* by itself or from the CgGAL4/UAS-GFP control ($p > 0.05$, and $p > 0.05$ respectively).

Overexpression of wild-type *Moe* led to a 26% decrease in filopodia length compared to the CgGAL4/UAS-GFP control ($p < 0.001$). Overexpression of wild-type *Moe* in the *Clic*¹⁰⁹ background led to a 1.1 fold increase in filopodia length ($p < 0.001$), as seen with *dia*^{WT}. Similarly, overexpression of wild-type *Moe* in conjunction with overexpression of *Clic* also led to a 1.2 fold increase in filopodia

length ($p < 0.001$). Overexpression of Moe^{TD} did not significantly affect filopodia length ($p > 0.05$). It was interesting to note, however, that combining overexpression of Moe^{TD} and Clic led to a 1.4 fold increase in length of filopodia compared to the CgGAL4/UAS-GFP control ($p < 0.001$).

The last protein that affected filopodia length was Ena. Overexpression of Ena led to a 12% decrease in filopodia length when compared to the CgGAL4/UAS-GFP control ($p < 0.01$). Expression in the Clic¹⁰⁹ background was not significantly different from overexpression of Ena by itself ($p > 0.05$). Combining overexpression of Ena with overexpression of Clic led to a 10% decrease in filopodia length ($p < 0.01$).

Together this data suggests that the level of Clic is very important in filopodia length, as both the removal and addition of Clic led to decreases in filopodia length. Also, the data suggests that there is an interaction between activated Moe and Clic due to the synergistic effect on increasing filopodia length.

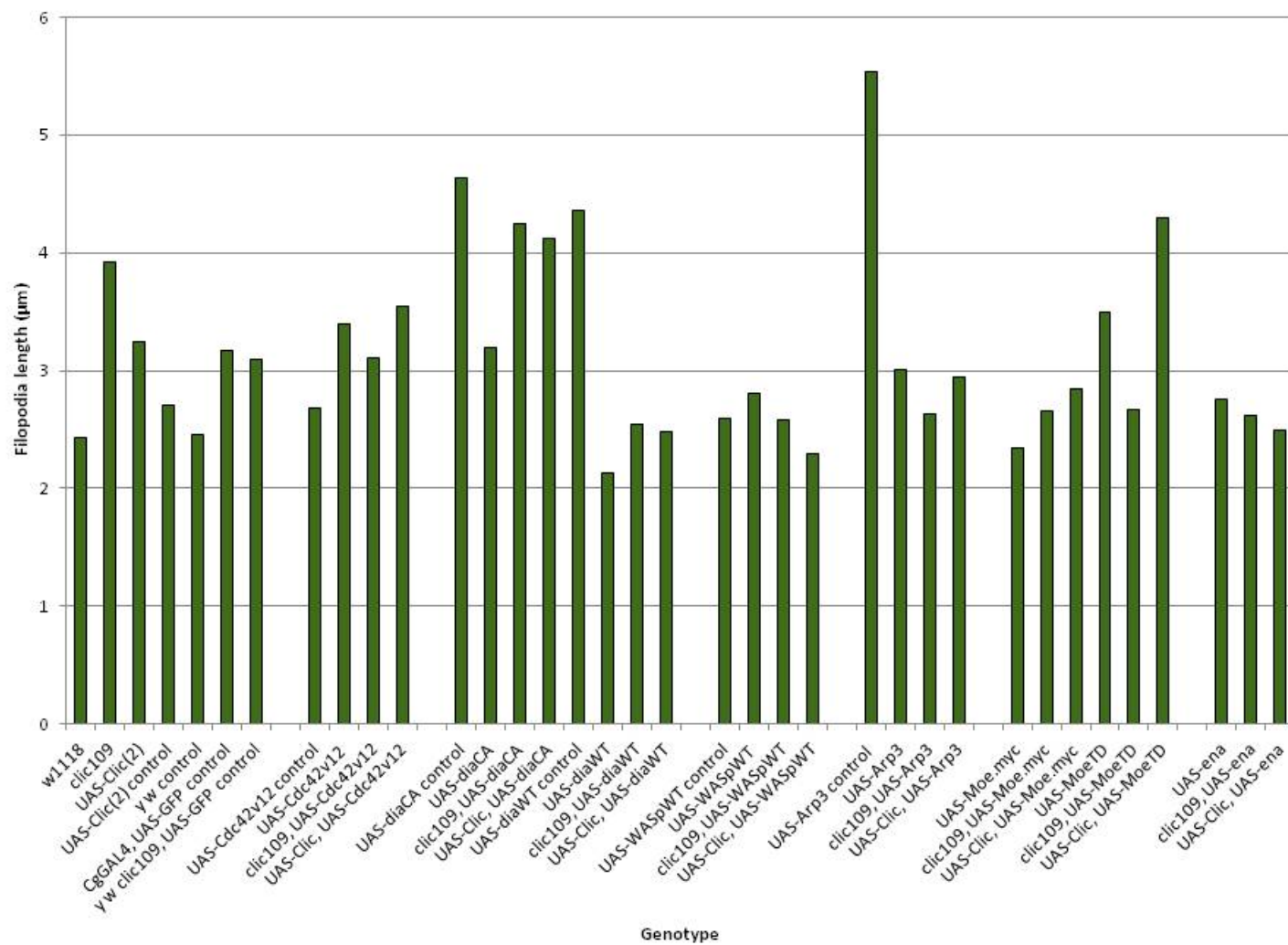


Figure 13. The average length of filopodia for each genotype. $n \geq 168$ filopodia

III. In vivo studies reveal that Clic influences filopodia dynamics in vivo which could contribute to hemocyte migration

I have shown that Clic is involved in filopodia formation in larval hemocytes and that it functions with known actin cytoskeleton regulators. It is important to determine if Clic has a biological significance in cell migration or other biological processes *in vivo*. In order to test this, a three dimensional, *in vivo* system was ideal. I chose to use embryonic hemocyte migration. This system is well studied, and it is easy to recognize migration deficiencies. Hemocyte development in *Drosophila* occurs in two waves, embryonic wave and a larval wave.²⁵ The previous experiments used hemocytes isolated from larvae, as they are easy to remove from the animal. Embryonic hemocytes are more motile, as they must disperse through the animal. The first migration event occurs at stage 10 when plasmatocytes migrate from the head region into the germ band at the posterior end of the embryo, as shown in Fig 14 A-B. As the germ band retracts, these hemocytes are carried with it towards the posterior of the embryo. Plasmatocytes also migrate from the head region along the ventral midline (Fig 14 C). At stage 13 most hemocytes will have completed migration along the ventral midline with cells from the posterior end meeting those traveling from the head region (Fig 14 A).³⁴ Because the embryonic hemocytes are more motile we will use this system to determine if Clic influences the motility of these cells. Images of the path taken by hemocytes during development are shown in Fig. 14 below.

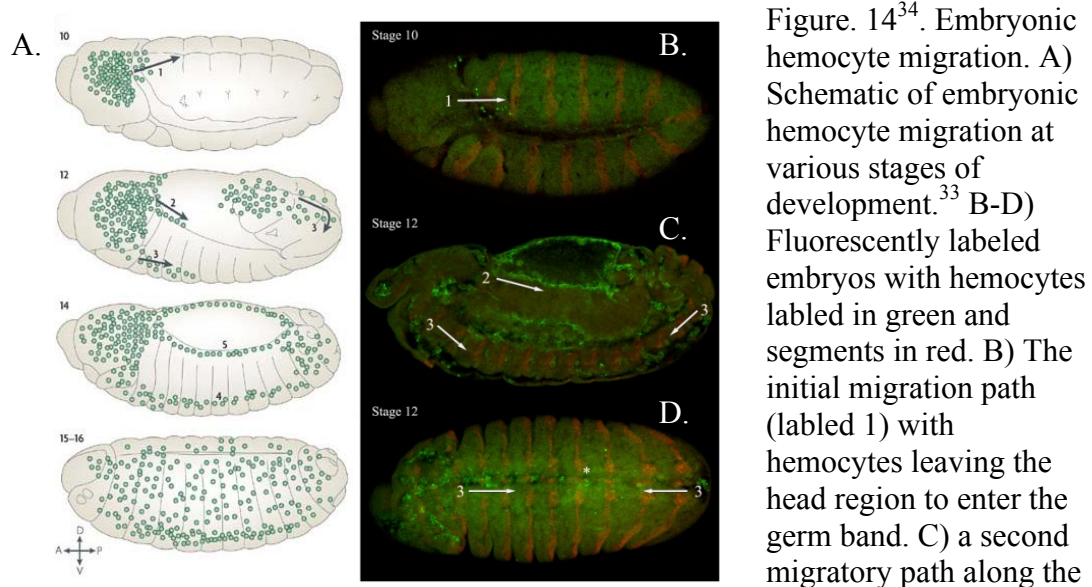


Figure 14³⁴. Embryonic hemocyte migration. A) Schematic of embryonic hemocyte migration at various stages of development.³³ B-D) Fluorescently labeled embryos with hemocytes labeled in green and segments in red. B) The initial migration path (labeled 1) with hemocytes leaving the head region to enter the germ band. C) a second migratory path along the

dorsal side of the embryo from the head region along the edge of the dorsal epithelium. D) third migration path along the ventral midline. Hemocytes travel from the head region towards the posterior and meet up with hemocytes traveling from the germ band toward the anterior end of the embryo. By stage 15-16 the hemocytes are distributed throughout the embryo.

In order to quantify hemocyte migration, I fixed embryos with fluorescently labeled hemocytes. The embryos were imaged and staged, and the farthest segment that the hemocytes had traveled to was measured and marked. I compared wild-type and *Clic*¹⁰⁹ genotypes for this experiment. For stage 12 embryos, I compared the number of embryos that had hemocytes that had migrated to abdominal segment 2 or beyond. The data is shown below in Table 5. The χ^2 -test showed that there was not a significant difference in migration between WT and *Clic*¹⁰⁹ embryos.

Table 5. Stage 12 embryo hemocyte migration

	Wild-type (n = 5)	Clic ¹⁰⁹ (n = 15)
Hemocytes had traveled up to segment A2	3	8
Hemocytes had traveled to segment A2 or beyond	2	7
p > 0.05		

I also examined stage 13 embryos. In this case, I compared the number of embryos with hemocytes completely distributed along the ventral midline. This data is shown in Table 6 below. Again, a χ^2 -test showed no significant differences in migration between these genotypes. Clic¹⁰⁹ stage 13 embryos trend toward delayed migration, and it would be beneficial to repeat this experiment to increase the sample sizes. The ratio of stage 13 embryos with incomplete hemocyte migration to complete hemocyte migration in wild-type embryos was 0.67. In Clic¹⁰⁹ the ratio was 1.17.

Table 6. Stage 13 embryo hemocyte migration

	Wild-type (n = 10)	Clic ¹⁰⁹ (n = 26)
Incomplete migration along ventral midline	4	14
Complete migration along ventral midline	6	12
p > 0.05		

Discussion

I. Overview

I have shown that Clic is involved in filopodia formation. My results showed that Clic promotes filopodia formation as removal of Clic resulted in fewer cells forming filopodia and overexpression of Clic resulted in more cells forming filopodia. Localization studies showed that Clic co-localizes with actin-based surface structures

including, but not limited to, filopodia. Genetic interactions suggest that Clic interacts with Cdc42, Dia, WASp, the Arp2/3 complex, Moe, and Ena. These interactions are context dependent and influence the propensity of a population of cells to form filopodia, the nucleation of filopodia, and the elongation of filopodia. Although there was not a significant difference in embryonic hemocyte migration, the data trend towards showing a delay in the Clic¹⁰⁹ mutant.

II. The role of Clic in priming, nucleating, and elongating filopodia

For multiple genotypes, regression analysis showed that the percentage of cells forming filopodia did not correlate with the number of filopodia per cell, or with the length of filopodia. This suggests that there are multiple phases to filopodia formation. Here I propose that there are three phases to filopodia formation: priming, nucleation, and elongation. This classification is supported by the observation that the number of cells forming filopodia, the number of filopodia formed per cell, and the length of filopodia did not correlate with one another, as shown in Fig. S1 in the Appendix. Additionally, I observed different genetic interactions when using these different measurements, suggesting that proteins function together in different ways during these phases. The different protein interactions could be regulated temporally and/or spatially. For example the timing of the activation of these different proteins could allow them to interact with one another at specific times. It is also possible that the localization of these proteins limits them to interact with a specific subset of proteins.

Further experiments would be needed to determine if and which of these cases is an accurate representation of actin dynamics in filopodia formation. Below is a description of each of the proposed phases, based on the results from the above experiments.

In the priming phase, the cell creates an environment conducive to forming filopodia. Proteins that were shown to be important in the priming phase included Cdc42, Dia, WASp, Moe, and Ena. Most of these molecules are capable of relaying signals to remodel the actin cytoskeleton. It has been shown that Cdc42 signals to Dia and WASp in *Drosophila* and in mammals, and signals Moe in mammalian systems.^{35,13} It has been shown that Moe functions to inhibit Rho signaling; Rho has been shown to activate Dia.^{36,35} Therefore, through this pathway Moe activity would inhibit Dia activity. Activation of Cdc42, Dia, WASp, Moe, and Ena would provide all of the signals necessary throughout filopodia formation. However, it appears that the presence of this signal does not necessarily lead to nucleation, as shown in Fig S1.

In the nucleation phase the cell sums the available signals and translates them into the organization of actin filaments and formation of filopodia. Proteins that appear to be involved in this phase of filopodia formation include Cdc42, Dia, WASp, the Arp2/3 complex, Moe, and Ena. The signaling molecules shown to be acting in the priming phase are also present. However, there are also proteins shown to nucleate new actin filaments and to promote growth of these filaments. The generation of new actin filaments is a necessary first step in the formation of filopodia. These filaments are then grouped together and begin to protrude from the membrane.

Once the filaments are organized, they continue to grow and are stabilized. This is the elongation phase. Growth occurs through the prevention of capping and severing of actin filaments, and the addition of actin monomers to the end of actin filaments. Stability occurs through bundling and crosslinking. Bundling proteins group together actin filaments to maintain the core of the filopodia. Crosslinking proteins link the actin core to the plasma membrane to allow the stereotypical filopodia structure. Proteins shown to be important in the elongation phase include Dia, WASp, Moe, and Ena. Dia and Ena have been shown to localize to filopodia tips and are shown to have anti-capping properties to allow continued growth of the actin filaments. Moe is a crosslinking protein, in addition to its signaling abilities.

A. Clic in the priming phase of filopodia formation

By comparing the percentage of cells forming filopodia in a given population of cells with the same genotype, I found that Clic interacts with Cdc42 and Dia to prime cells for filopodia formation (Fig. 10). The Clic null mutation masked the effect of Cdc42 on priming, suggesting that Clic functions downstream of Cdc42. The Clic null mutation also masked the effect of overexpression of the wild-type form of Dia, but not, however, the effect of the constitutively active form of Dia. This suggests that Clic functions prior to the activation of Dia. Here I propose that Clic functions with Cdc42 to activate Dia in order to activate the signals necessary for filopodia formation, resulting in the priming of the hemocytes (Fig. 15).

Additional evidence for the interaction between Cdc42 activating Dia was found when I overexpressed Cdc42^{V12} and simultaneously knocked down WASp. This genotype did not produce as many cells with filopodia as the overexpression of dia^{CA}; however, I did observe similar cellular morphologies as seen with overexpression of dia^{CA}. Dia^{CA} overexpression was characterized by the production of membrane ruffles and more lamellocytes (another class of blood cell). This was also seen with knockdown of WASp while overexpressing Cdc42^{V12}. By removing WASp more Cdc42 should be available to activate Dia, leading to a phenotype similar to Dia^{CA}. This was observed with the increased numbers of lamellocytes. Fig. 15 shows, is my proposed model for how Clic, Cdc42, and Dia interact in the priming of cells for filopodia formation.

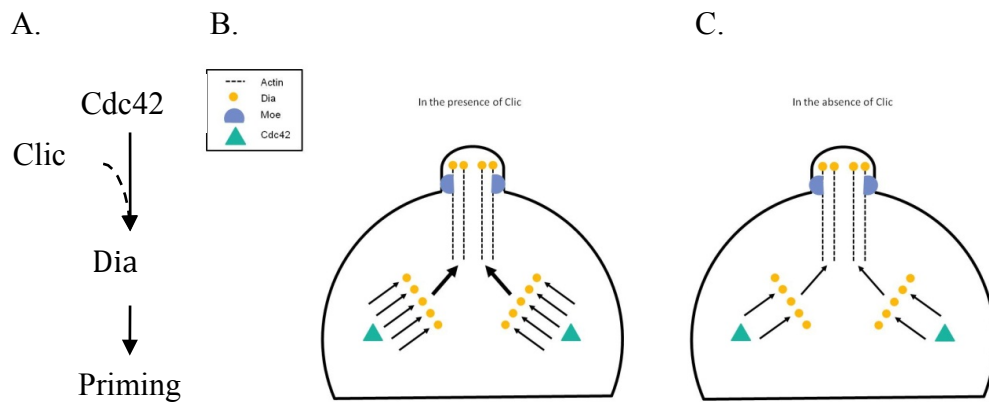


Figure 15. Clic functions with Cdc42 to activate Dia and promote priming. Panel A shows the signaling pathway. Panel B and C show a schematic of signaling pathway. Panel B shows signaling in a wild-type setting. Panel C shows that in the absence of Clic there would be less signal resulting in less priming.

An interesting result of this experiment was the role of Moe in promoting more cells to form filopodia. This protein has typically been thought of as a crosslinker between the actin cytoskeleton and the plasma membrane, rather than a signaling molecule in filopodia formation.³⁷ However, as shown in Fig 16, Moe can play a role in signaling. I used the Moe^{TD} allele as a way to observe the effect of a constitutively active Moe. It is important to note that in mammalian systems, Ezrin (closely related to Moe) activity is dependent on its ability to switch between its active and inactive states, therefore the Moe^{TD} allele may not be an accurate representation of increased Moe activity.³⁸ Another study showed that Moe^{TD} has a dominant negative effect in *Drosophila* oogenesis.³⁹ Overexpression of Moe, both wild-type and constitutively active, led to an increase in filopodia formation (Fig 10). This data contradicts the current model (Fig 16A) and suggests that Moe promotes filopodia formation. Similar inconsistencies can be found in the mammalian system where Moesin (and other ERM proteins) can activate Rho through interactions with other proteins.⁴⁰ Therefore further study is required to determine how Moe is functioning with Rho in this context.

The phenotype of Moe overexpression was reduced in the Clic¹⁰⁹ background. The phenotype of Moe^{TD} was also reduced, though to a lesser extent. Western blot analysis showed that there was more phosphorylated Moe in the Clic¹⁰⁹ background (unpublished data). If Clic is involved in the regulation of the phosphorylation of Moe, this may explain the difference in the effect of Clic¹⁰⁹ on the different forms of Moe (Fig. 16B). Wild-type Moe would become hyper-

phosphorylated in the *Clic*¹⁰⁹ background which could be the cause for the reduced effect of Moe. Moe^{TD} would not be affected by the dephosphorylation in the *Clic*¹⁰⁹ background, however, the endogenous Moe would be. This could explain the less drastic reduction seen with Moe^{TD} in the *Clic*¹⁰⁹ background.

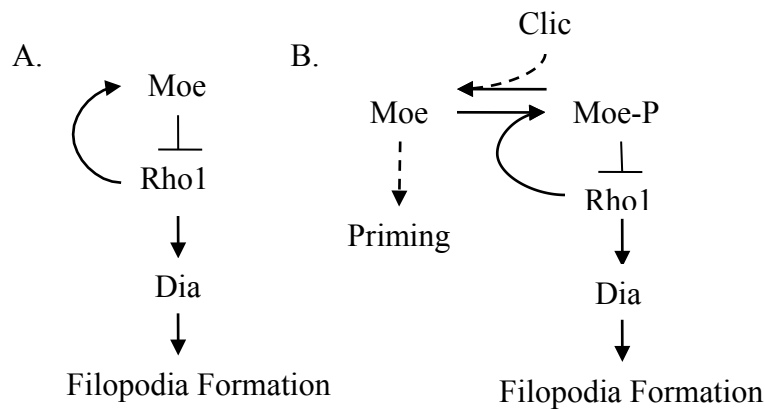


Figure 16. Moe signaling pathway in filopodia formation. Panel A shows a simplified pathway leading to inhibition of Dia activity via Moe. Panel B shows the switch between non-phosphorylated (inactive) Moe and phosphorylated (active) Moe. A western blot showed increased phosphorylated Moe in the *Clic* mutant background, suggesting that *Clic* promotes dephosphorylation of Moe. Dashed lines represent the proposed mechanism based on my data.

In order to determine a more comprehensive idea of how these proteins interact, it would be beneficial to study the protein interactions. This can be done in two ways. Localization assays would show if Cdc42, Dia and *Clic* are within the same subcellular location in order for them to physically interact. A co-immunoprecipitation (co-IP) experiment would also show if these proteins are directly or indirectly binding to one another. A recent co-IP experiment was able

to pull down Moe with Clic, showing that these proteins likely bind directly or indirectly to one another (unpublished results).

B. Clic in the nucleation phase of filopodia formation

The number of filopodia formed per cell was used as a measurement of how well the signal generated in the priming phase was translated into the nucleation of filopodia. I observed that Cdc42, WASp, the Arp2/3 complex, Moe, and Ena functioned with Clic in the nucleation phase of filopodia formation. WASp and the Arp2/3 complex have long been thought to promote lamellipodia formation. Yang et al. proposed a model in 2007 in which the branched filaments generated by the Arp2/3 complex could be incorporated into filopodia, in addition to the unbranched filaments generated by Dia and Ena.⁴¹ Clic appears to function downstream of WASp as the removal of Clic results in the masking of the effect of WASp on nucleation. Clic also appears to function upstream of the Arp 2/3 complex as there was not a significant difference in nucleation between Arp3 overexpression and Arp3 overexpression in the Clic¹⁰⁹ background. When Arp3 was overexpressed in conjunction with Clic there was a decrease in the number of filopodia per cell. This suggests that Clic functions downstream of Arp3 activation to prevent nucleation. Below is a model that shows the decrease in WASp activity and therefore in filopodia nucleation in the absence of Clic (Fig.17).

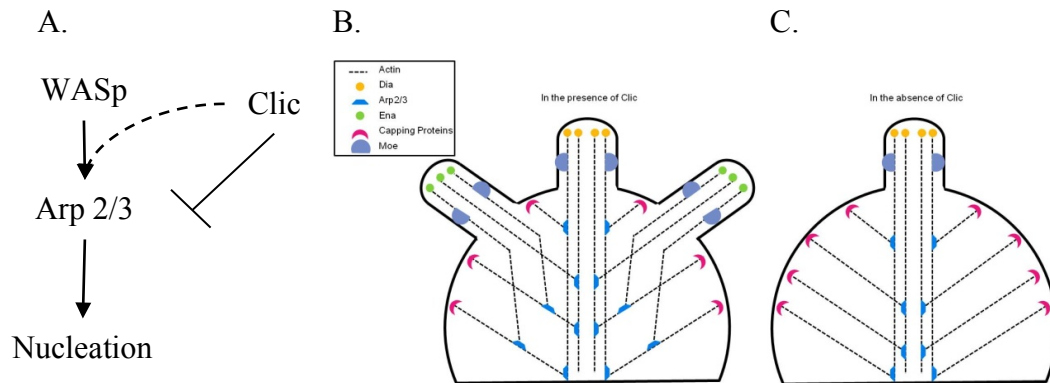


Figure 17. Model for the interactions between WASp, the Arp2/3 complex, and Clic in the nucleation phase of filopodia formation. Panel A shows the proposed signaling pathway based on genetic interactions. Panel B shows nucleation in the wild-type cell. Panel C shows that in the absence of Clic there is less activation of the Arp 2/3 complex via WASp, leading to a reduction in the number of filopodia formed per cell.

It is possible that the increased number of filopodia per cell with Moe overexpression is due to signaling activity of Moe through Rho and Dia (Fig 16). Moe^{TD} overexpression showed an opposite interaction with Clic in the nucleation of filopodia to wild-type Moe overexpression. This may be due to the effect of the Moe^{TD} allele. In the developing oocyte, Moe^{TD} overexpression resulted in all of the Moe localizing to the cell cortex. Therefore, the effect of Moe^{TD} may be due to increased stability of the filopodia. If there is more phosphorylation of Moe in the Clic¹⁰⁹ mutant, endogenous Moe could become phosphorylated and recruited to the membrane. This could explain the increase in the number of filopodia per cell when Moe^{TD} is overexpressed in the Clic¹⁰⁹ background. Priming data suggests that Moe and Clic function together with Clic promoting Moe activity. This was also seen in the nucleating data. Below I propose a model where unphosphorylated Moe interacts with Dia at the plasma membrane to help promote

the nucleation of filopodia (Fig 18). Further study is required to determine if this is a plausible mechanism.

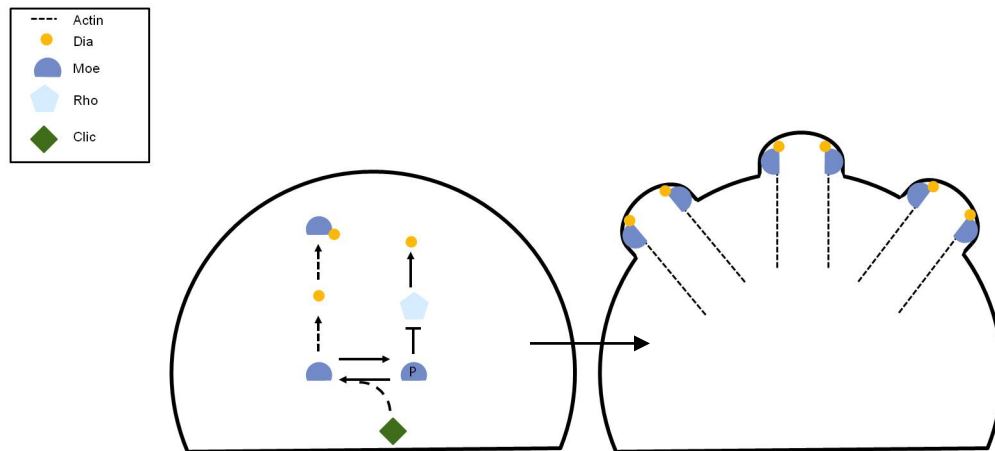


Figure 18. Moe activity in priming and nucleating. Left: Moe signaling in the priming phase Right: Proposed interaction between Dia and Moe would promote filopodia formation by providing stability and elongation of actin filaments.

C. Clic in the elongation phase of filopodia formation

The length of the filopodia was used to measure the elongation phase of filopodia formation. This phase is not only dependent on the continued growth of the filopodia, but also on the stability of the actin within, and the retraction of the filopodia. This would incorporate severing proteins, cross-linking proteins, bundling proteins, and capping proteins, in addition to the proteins that I examined. It was not surprising that Dia and Ena were shown to contribute to the elongation phase of filopodia formation, as both of these are known to promote

growth of actin filaments and prevent capping. However, it was unexpected that overexpression of these proteins resulted in shorter filopodia.

Overexpression of the constitutively active form of Dia did not significantly affect the length of the filopodia. When dia^{CA} was overexpressed in the Clic^{109} background the length of filopodia increased when compared to dia^{CA} by itself, however this was not significantly different from the Clic^{109} mutant. The length also increased when dia^{CA} was overexpressed with overexpression of Clic. Overexpression of dia^{WT} led to a decrease in filopodia length. This was partially rescued by both the removal and addition of Clic. Because dia^{CA} did not affect filopodia length but dia^{WT} did, this suggests that the ability to switch between active and inactive states inhibits elongation. This is in agreement with the current model, in which Dia allows elongation while active and attached to the actin. The elongation and anti-capping activity halts when Dia becomes inactive and removed from tip of the actin filament. Because Clic interacted in the same manner with both dia^{CA} and dia^{WT} , albeit to a lesser extent in dia^{WT} , this suggests that Clic is functioning downstream or independently of Dia.

Overexpression of wild-type Moe led to a decrease in filopodia length. This could be due to increased tethering of the actin core of the filopodia to the plasma membrane. Actin filaments naturally treadmill in filopodia; increased tethering could decrease the treadmill activity, resulting in the decreased length. Increased treading promotes longer filopodia. In contrast, if there is not enough stability due to linking of actin to the plasma membrane, the actin filaments will not be able

to extend with the plasma membrane, resulting in shorter protrusions. This was observed in the knockdown of Moe, and pictured in Fig. S2. This information in conjunction with the evidence that Clic promotes the dephosphorylation of Moe physically interacts with Moe, led to the model below (Figure 19).

I hypothesize that there is a delicate balance required in the amount of cross-linking in filopodia formation, and that this crosslinking is mediated by physical interactions and phosphorylation of Moe. The physical interaction between Moe and the actin core can add stability to the filopodia by promoting protrusion. However, too much Moe can lead to tethering of the actin to the membrane resulting in reduced treadmilling. In the model below, I hypothesize that by promoting the switch between phosphorylated Moe and unphosphorylated Moe this will lead to stability but still allow treadmilling, leading to increased length (Fig. 19A). If Moe remains phosphorylated, it will remain bound to actin resulting in stronger tethering (Fig 19B). Reduction of Moe by RNAi led to decreased protrusion and length, suggesting that crosslinking of actin to the membrane is required for stability (Fig. 19E). Expression of Moe RNAi in the Clic¹⁰⁹ background would increase the phosphorylation, helping the filopodia to protrude and increasing filopodia length (Fig.19F), as seen in the appendix (Fig. S2). Overexpression of Moe^{TD} did not result in a significant change in filopodia length. However, there was a significant increase in length when Moe^{TD} was overexpressed with the overexpression of Clic. There was also an increase in filopodia length when wild-type Moe and Clic were overexpressed. If Clic is

suppressing the phosphorylation of Moe, overexpression of Clic would lead to less Moe activity, and therefore less interaction with the actin cytoskeleton. This would reduce the stabilizing effect of Moe and allow the filopodia to elongate (Fig.18C). This model was supported by the increase in filopodia length when both wild-type Moe and Moe^{TD} were overexpressed with additional Clic.

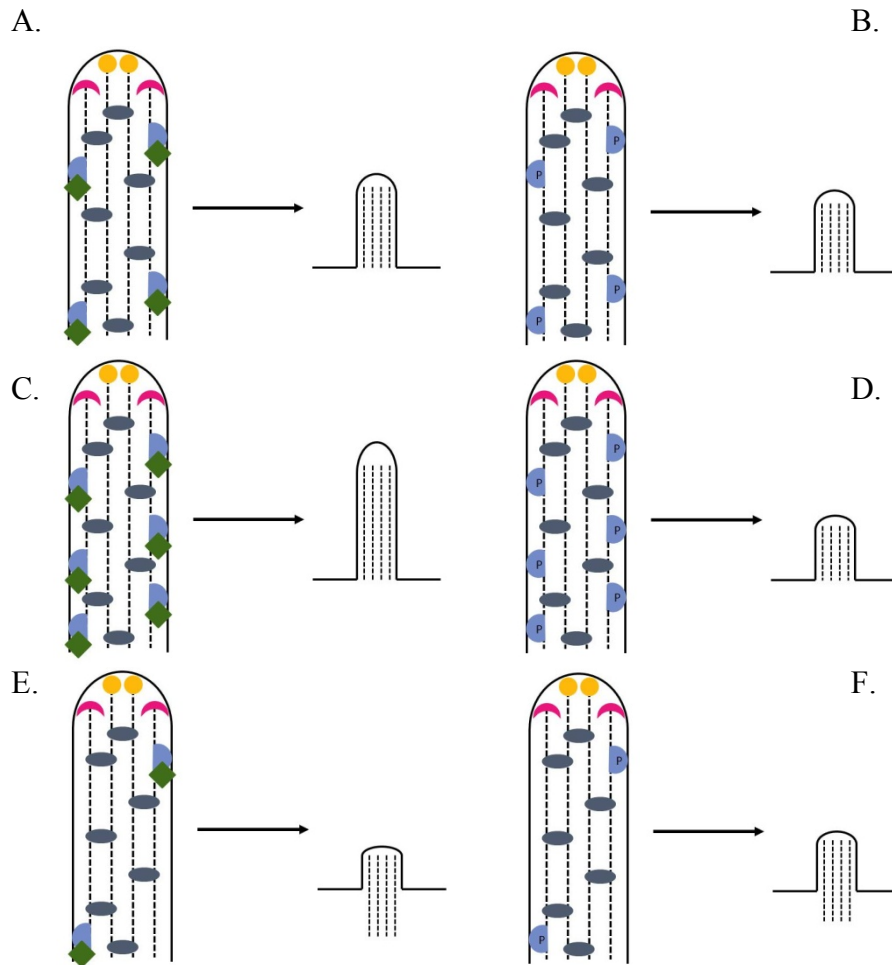


Figure 19. Model of Moe and Clic interactions in filopodia elongation. Panel A shows the wild-type condition. Moe and Clic physically interact allowing the filopodia to be stabilized by crosslinking. The close proximity of Clic to Moe allows dephosphorylation of Moe to promote treadmilling. Panel B shows that without the interaction with Clic, Moe would remain phosphorylated and would lead to decreased length. Panel C shows the effect of Moe overexpression with overexpression of Clic. This maintains the balance of phosphorylated Moe, while increasing the stability of the filopodia. Panel D shows the effect of Moe overexpression in the *Clic¹⁰⁹* background, which results in tethering of the actin because Moe remains phosphorylated. Panel E shows the effect of Moe RNAi. Decreased levels of Moe prevent protrusion. Panel F shows the rescue of Moe RNAi by expression in the *Clic¹⁰⁹* background. In this context Moe phosphorylation allows actin to maintain contact with the membrane in order to protrude.

III. Clic in hemocyte migration

Although the differences in embryonic hemocyte migration were not significant, there was a trend in the stage 13 embryos showing that Clic¹⁰⁹ may lead to a migration deficiency. The sample sizes for this experiment were very small, so increasing the sample size may lead to a significant difference. It is also possible to use this system to determine if the other altered levels of proteins shown in this study to contribute to filopodia formation would lead to a migration deficiency. Both Moe and Ena have been shown to be important for invasive behavior, and Ena has already been shown to alter embryonic hemocyte migration.^{42,43}

Future Directions

My research focused on Clic's interactions with Cdc42 and its downstream effectors. This leaves a broad spectrum of actin regulators still to be tested. Some proteins of interest include Fascin, an actin bundling protein shown to be involved in cell motility, and Rho a small GTPase capable of activating Dia. It would also be interesting to look at another allele of Moe. The Moe^{TA} allele is similar in construct to the Moe^{TD} allele, however this form is not able to be phosphorylated. This would perhaps illuminate how the phosphorylation of Moe allows it to interact with different proteins in the different phases of filopodia formation.

This research has led to the proposal of several new mechanisms for filopodia formation. In order to determine if these proposed mechanisms are occurring further testing is required. It is necessary to determine how proteins are interacting. My research mainly used genetic interactions to determine which proteins were interacting. Determining how proteins are physically interacting would help explain the genetic interactions that I observed. For example, I proposed that Dia and Moe physically interact when Moe is unphosphorylated to promote nucleation of filopodia. Co-immunoprecipitation would show if these proteins are physically interacting and antibodies specific to phosphorylated ERMs would be able to determine if Moe is phosphorylated or not.

It would be beneficial to repeat the experiments with fixed embryos. The data trends toward a migration delay in *Clic*¹⁰⁹, but did not show a significant difference. The sample sizes for this experiment were small, and if increased a statistical difference may be observed. I also wanted to observe live hemocyte migration in wild-type and *Clic*¹⁰⁹ embryos. Unfortunately, time did not permit me to finish this experiment. Therefore, this is a project that would be of interest for further study. Live observation would give insight into if there is a migration delay, and if so why this is occurring.

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Appendix

I. Complete Data Set

Genotype	Number of cells forming lamella only	Total number of cells forming lamella	Number of cells forming filopodia Only	Total number of cells forming filopodia	Number of filopodia formed per cell	Length of Filopodia
W ¹¹¹⁸ /Y	68.4 ± 8.7	91.9 ± 3.4	4.0 ± 3.1	27.4 ± 8.4	15.3 ± 4.0	2.4 ± 1.6
Clic ¹⁰⁹ /Y	78.9 ± 7.9	90.6 ± 5.6	4.1 ± 1.7	15.8 ± 6.0	15.0 ± 9.7	3.9 ± 2.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(2)}	49.5 ± 17.6	74.3 ± 14.0	20.5 ± 10.7	45.3 ± 17.7	12.9 ± 5.4	3.2 ± 2.0
y w/Y; UAS-Clic ^{WT(2)} /+	50.3 ± 15.3	70.5 ± 15.9	15.9 ± 6.2	36.1 ± 7.8	15.1 ± 7.7	2.7 ± 1.8
y w/Y	37.6 ± 10.6	79.5 ± 6.4	13.6 ± 4.5	55.5 ± 11.1	20.0 ± 6.1	2.5 ± 1.6
y w/Y; CgGAL4, UAS-GFP/+	47.4 ± 10.2	85.9 ± 7.2	7.9 ± 4.6	46.4 ± 11.9	14.4 ± 6.0	3.2 ± 3.1
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/+	45.5 ± 9.3	86.0 ± 5.2	9.3 ± 4.2	49.8 ± 10.4	15.2 ± 5.7	3.1 ± 2.0
y w/Y; CgGAL4, UAS-dcr2/+	51.4 ± 10.6	83.1 ± 8.4	12.4 ± 5.0	44.1 ± 7.4	23.8 ± 7.9	2.8 ± 1.8
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-dcr2/+	53.1 ± 6.8	77.1 ± 4.7	11.4 ± 3.2	35.4 ± 6.1	24.7 ± 10.6	3.1 ± 2.1
W ¹¹¹⁸ /Y	68.4 ± 8.7	91.9 ± 3.4	4.0 ± 3.1	27.4 ± 8.4	15.3 ± 4.0	2.4 ± 1.6
Clic ¹⁰⁹ /Y	78.9 ± 7.9	90.6 ± 5.6	4.1 ± 1.7	15.8 ± 6.0	15.0 ± 9.7	3.9 ± 2.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(2)}	49.5 ± 17.6	74.3 ± 14.0	20.5 ± 10.7	45.3 ± 17.7	12.9 ± 5.4	3.2 ± 2.0
y w/Y; UAS-Cdc42 ^{V12} /+	30.6 ± 6.5	72.9 ± 9.9	19.6 ± 7.0	61.9 ± 3.6	27.4 ± 11.1	2.7 ± 2.0
y w/Y; CgGAL4, UAS-GFP/UAS-Cdc42 ^{V12}	6.6 ± 2.9	53.6 ± 17.0	42.6 ± 16.8	89.6 ± 4.3	22.4 ± 9.4	3.4 ± 3.1
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/UAS-Cdc42 ^{V12}	34.0 ± 8.7	88.9 ± 3.1	9.3 ± 2.3	64.1 ± 8.2	18.0 ± 8.0	3.1 ± 1.9
y w/Y; CgGAL4, UAS-GFP/UAS-Cdc42 ^{V12} , UAS-Clic ^{WT(7-7)}	23.2 ± 10.2	86.0 ± 3.8	10.9 ± 3.4	73.7 ± 10.2	21.0 ± 10.7	3.5 ± 2.4
y w/Y; CgGAL4, UAS-dcr2/UAS-Cdc42v12; UAS-WASpRNAi/+	29.6 ± 11.0	78.0 ± 7.4	13.8 ± 5.8	62.1 ± 11.7	22.6 ± 6.5	3.2 ± 2.4
y w/Y; CgGAL4, UAS-dcr2/UAS-Cdc42v12; UAS-diaRNAi/+	24.3 ± 11.3	77.9 ± 5.9	15.0 ± 5.0	68.6 ± 10.9	25.6 ± 8.1	2.4 ± 1.7
W ¹¹¹⁸ /Y	68.4 ± 8.7	91.9 ± 3.4	4.0 ± 3.1	27.4 ± 8.4	15.3 ± 4.0	2.4 ± 1.6
Clic ¹⁰⁹ /Y	78.9 ± 7.9	90.6 ± 5.6	4.1 ± 1.7	15.8 ± 6.0	15.0 ± 9.7	3.9 ± 2.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(2)}	49.5 ± 17.6	74.3 ± 14.0	20.5 ± 10.7	45.3 ± 17.7	12.9 ± 5.4	3.2 ± 2.0
y w/Y; +/+; UAS-Dia ^{CA} /+	41.4 ± 14.4	86.1 ± 4.8	9.6 ± 4.3	54.3 ± 14.1	10.9 ± 5.0	4.6 ± 3.1
y w/Y; CgGAL4, UAS-GFP/+; UAS-dia ^{CA} /+	6.5 ± 2.8	63.0 ± 10.0	31.5 ± 6.9	88.0 ± 5.9	17.0 ± 8.4	3.2 ± 2.3
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/+; UAS-dia ^{CA} /+	13.4 ± 7.4	67.3 ± 14.9	26.7 ± 11.1	80.6 ± 5.9	24.2 ± 12.1	4.3 ± 2.9
y w/Y; CgGAL4, UAS-GFP/ UAS-Clic ^{WT(7-7)} ; UAS-dia ^{CA} /+	9.67 ± 12.9	61.89 ± 9.5	34.8 ± 8.4	87.0 ± 11.6	23.8 ± 9.4	4.1 ± 3.3
y w/Y; +/+; UAS-Dia ^{WT} /+	45.0 ± 13.6	84.7 ± 12.5	9.4 ± 7.8	49.1 ± 10.7	13.9 ± 5.1	4.4 ± 3.0
y w/Y; CgGAL4, UAS-GFP/+; UAS-GFP-dia ^{WT} /+	46.0 ± 18.6	93.0 ± 3.9	5.9 ± 2.9	52.9 ± 18.0	21.2 ± 7.4	2.1 ± 1.3
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/+; UAS-GFP-dia ^{WT} /+	74.1 ± 7.1	94.3 ± 3.2	3.67 ± 2.2	23.9 ± 6.4	17.1 ± 7.9	2.5 ± 1.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(7-7)} ; UAS-dia ^{WT} /+	59.2 ± 9.0	89.3 ± 3.3	7.0 ± 3.0	37.2 ± 8.9	22.3 ± 7.6	2.5 ± 1.7
y w/Y; CgGAL4, UAS-dcr2/+; UAS-diaRNAi/+	39.6 ± 13.2	89.6 ± 3.6	6.2 ± 2.5	56.2 ± 12.5	26.0 ± 8.8	2.3 ± 1.5
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-dcr2/+; UAS-diaRNAi/+	67.3 ± 17.2	90.50 ± 2.8	4.9 ± 3.8	28.1 ± 18.9	18.8 ± 7.4	3.0 ± 2.0
W ¹¹¹⁸ /Y	68.4 ± 8.7	91.9 ± 3.4	4.0 ± 3.1	27.4 ± 8.4	15.3 ± 4.0	2.4 ± 1.6
Clic ¹⁰⁹ /Y	78.9 ± 7.9	90.6 ± 5.6	4.1 ± 1.7	15.8 ± 6.0	15.0 ± 9.7	3.9 ± 2.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(2)}	49.5 ± 17.6	74.3 ± 14.0	20.5 ± 10.7	45.3 ± 17.7	12.9 ± 5.4	3.2 ± 2.0
y w/Y; CgGAL4, UAS-GFP/+; UAS-Moe.myc/+	34.3 ± 10.9	83.7 ± 8.6	11.7 ± 6.9	61.0 ± 10.0	18.2 ± 5.1	2.3 ± 1.4
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/+; UAS-Moe.myc/+	40.4 ± 15.5	62.8 ± 15.3	18.5 ± 8.8	40.9 ± 10.0	14.0 ± 4.7	2.7 ± 1.7
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(7-7)} ; UAS-Moe.myc/+	47.4 ± 15.6	80.2 ± 11.7	9.5 ± 4.9	42.3 ± 21.4	19.3 ± 10.6	2.8 ± 1.8
y w/Y; CgGAL4, UAS-dcr2/+; UAS-MoeIR/+	57.4 ± 15.6	78.3 ± 11.4	10.0 ± 3.9	30.9 ± 13.5	14.0 ± 6.5	2.6 ± 1.7
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-dcr2/+; UAS-MoeIR/+	55.4 ± 15.0	69.6 ± 17.4	9.4 ± 3.0	23.6 ± 7.7	8.4 ± 2.9	3.0 ± 1.6

y w/Y; CgGAL4, UAS-dcr2/UAS-Clic ^{WT(7-7)} ; UAS-MoeRNAi/+	56.6 ± 6.3	82.1 ± 7.9	7.9 ± 1.8	33.4 ± 8.5	12.1 ± 4.0	4.0 ± 3.3
y w/Y; CgGAL4, UAS-GFP/UAS-Moe ^{T559D}	11.6 ± 7.5	79.2 ± 6.4	17.2 ± 5.0	84.9 ± 8.7	18.2 ± 8.4	3.5 ± 2.2
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/UAS-Moe ^{T559D}	23.0 ± 5.6	83.5 ± 4.0	14.0 ± 4.1	74.5 ± 5.5	23.7 ± 8.7	2.7 ± 1.9
y w/Y; CgGAL4, UAS-GFP/ UAS-Moe ^{T559D} , UAS-Clic ^{WT(7-7)}	47.9 ± 10.0	73.0 ± 12.0	15.7 ± 9.8	40.8 ± 9.6	14.5 ± 4.1	4.3 ± 3.1
W ¹¹¹⁸ /Y	68.4 ± 8.7	91.9 ± 3.4	4.0 ± 3.1	27.4 ± 8.4	15.3 ± 4.0	2.4 ± 1.6
Clic ¹⁰⁹ /Y	78.9 ± 7.9	90.6 ± 5.6	4.1 ± 1.7	15.8 ± 6.0	15.0 ± 9.7	3.9 ± 2.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(2)}	49.5 ± 17.6	74.3 ± 14.0	20.5 ± 10.7	45.3 ± 17.7	12.9 ± 5.4	3.2 ± 2.0
y w/Y; +/-; UAS-WASp ^{WT/+}	43.6 ± 15.9	74.6 ± 13.3	16.7 ± 5.9	47.7 ± 9.6	22.9 ± 6.8	2.6 ± 1.8
y w/Y; CgGAL4, UAS-GFP/+; UAS-WASp ^{WT/+}	33.0 ± 9.5	87.0 ± 4.9	8.4 ± 2.7	62.4 ± 8.2	34.0 ± 9.1	2.8 ± 1.9
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/+; UAS-WASp ^{WT/+}	41.6 ± 11.1	89.6 ± 2.8	8.1 ± 2.9	56.1 ± 11.1	25.2 ± 6.1	2.6 ± 1.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(7-7)} ; UAS-WASp ^{WT/+}	37.9 ± 8.5	78.6 ± 9.5	17.0 ± 8.5	57.8 ± 8.8	29.3 ± 9.9	2.3 ± 1.5
y w/Y; CgGAL4, UAS-dcr2/+; UAS-WASpRNAi/+	30.7 ± 13.6	80.0 ± 10.4	6.3 ± 3.3	55.5 ± 8.8	24.6 ± 7.7	2.8 ± 1.9
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-dcr2/+; UAS-WASpRNAi/+	60.6 ± 10.5	82.3 ± 8.3	3.6 ± 1.9	25.3 ± 8.6	14.2 ± 6.0	2.9 ± 2.0
W ¹¹¹⁸ /Y	68.4 ± 8.7	91.9 ± 3.4	4.0 ± 3.1	27.4 ± 8.4	15.3 ± 4.0	2.4 ± 1.6
Clic ¹⁰⁹ /Y	78.9 ± 7.9	90.6 ± 5.6	4.1 ± 1.7	15.8 ± 6.0	15.0 ± 9.7	3.9 ± 2.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(2)}	49.5 ± 17.6	74.3 ± 14.0	20.5 ± 10.7	45.3 ± 17.7	12.9 ± 5.4	3.2 ± 2.0
y w/Y; CgGAL4, UAS-GFP/+; UAS-ena/+	34.6 ± 16.6	90.0 ± 5.2	8.3 ± 4.4	63.6 ± 17.1	34.9 ± 7.4	2.8 ± 1.5
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/+; UAS-ena/+	20.0 ± 11.0	89.8 ± 4.5	9.1 ± 3.4	78.9 ± 10.9	31.1 ± 9.6	2.6 ± 1.8
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(7-7)} ; UAS-ena/+	26.0 ± 11.6	89.4 ± 4.6	9.8 ± 5.0	73.1 ± 12.1	27.9 ± 5.7	2.5 ± 1.6
y w/Y; CgGAL4, UAS-dcr2/+; UAS-enaRNAi/+	67.3 ± 19.0	89.5 ± 6.2	8.0 ± 5.8	30.3 ± 19.1	26.3 ± 8.9	3.2 ± 2.3
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-dcr2/+; UAS-enaRNAi/+	52.9 ± 6.6	89.1 ± 10.8	8.4 ± 6.6	44.6 ± 5.9	18.7 ± 6.9	3.3 ± 2.0
W ¹¹¹⁸ /Y	68.4 ± 8.7	91.9 ± 3.4	4.0 ± 3.1	27.4 ± 8.4	15.3 ± 4.0	2.4 ± 1.6
Clic ¹⁰⁹ /Y	78.9 ± 7.9	90.6 ± 5.6	4.1 ± 1.7	15.8 ± 6.0	15.0 ± 9.7	3.9 ± 2.6
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(2)}	49.5 ± 17.6	74.3 ± 14.0	20.5 ± 10.7	45.3 ± 17.7	12.9 ± 5.4	3.2 ± 2.0
y w/Y; pUASp-GFP-Arp3/+	42.4 ± 14.4	85.6 ± 9.0	7.8 ± 4.7	51.0 ± 14.5	11.0 ± 5.2	5.5 ± 3.9
y w/Y; CgGAL4, UAS-GFP/pUASp-GFP-Arp3	44.3 ± 12.2	91.5 ± 1.9	7.0 ± 0.0	54.3 ± 13.7	27.3 ± 16.0	3.0 ± 2.1
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-GFP/pUASp-GFP-Arp3	45.3 ± 7.3	90.4 ± 4.1	5.3 ± 1.8	50.4 ± 7.4	22.0 ± 9.8	2.6 ± 1.8
y w/Y; CgGAL4, UAS-GFP/UAS-Clic ^{WT(7-7)} ; pUASp-GFP-Arp3	66.3 ± 10.4	87.4 ± 2.4	9.0 ± 2.6	30.1 ± 10.8	15.7 ± 7.8	2.9 ± 2.0
y w/Y; CgGAL4, UAS-dcr2/+; UAS-Arp14DRNAi/+	24.8 ± 17.8	61.9 ± 10.8	23.6 ± 7.3	60.8 ± 16.8	27.1 ± 9.1	2.4 ± 1.9
y w clic ¹⁰⁹ /Y; CgGAL4, UAS-dcr2/+; UAS-Arp14DRNAi/+	30.0 ± 12.7	49.5 ± 9.5	33.5 ± 9.8	53.0 ± 12.8	21.5 ± 8.6	3.9 ± 2.8

II. Supplementary figures

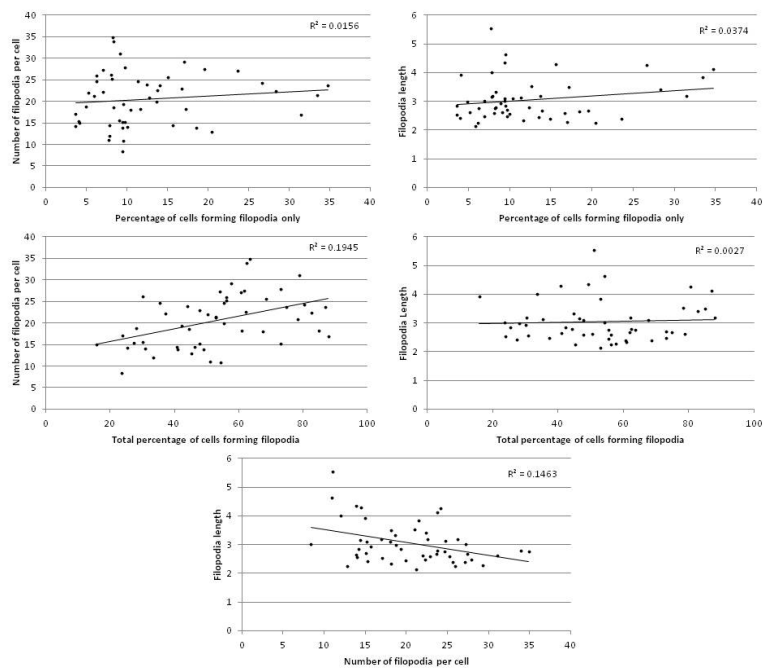


Figure S1. Regression analysis of filopodia measurements. These measurements did not correlate to one another, suggesting that these measurements are independent of one another and that filopodia formation may be divided into discrete phases.

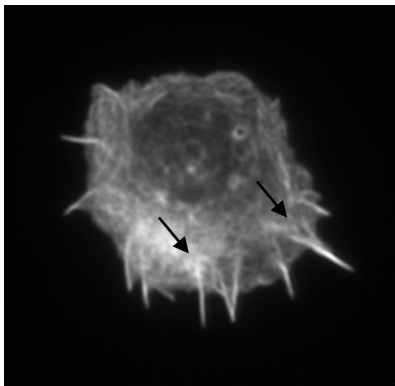


Figure S2. Decreased levels of Moe lead to lack of protrusion. This hemocyte has decreased levels of Moe due to RNAi. Filopodia remain embedded in the cytoplasm, as shown by the arrow.