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Biochemical Reconstitution of an Actin-Driven Positive Feedback Loop

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Biochemical Reconstitution of an Actin-Driven Positive Feedback Loop

by

Derek Tak-Lai Wong

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Joint Doctor of Philosophy

with the University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jack Taunton, Co-chair
Professor Daniel A. Fletcher, Co-chair
Professor David G. Drubin
Professor Francis C. Szoka

Fall 2010
Biochemical Reconstitution of an Actin-Driven Positive Feedback Loop

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by

Derek Tak-Lai Wong

University of California, Berkeley
Abstract

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Actin polymerization provides the physical force for membrane protrusion and determines the directionality of membrane movement in a variety of cellular processes, such as cell migration, establishment of cell polarity, podosome and invadopodia assembly, and endosome motility. To ensure proper directionality is achieved, actin assembly occurs at distinct regions along the membrane surface. Actin nucleation promoting factors, which include members of the WASP/WAVE family, must first be recruited to the membrane from the cytoplasm and activated at the surface in a localized fashion; the specific location of their activation precisely dictates where along the membrane the actin network is produced.

Theoretical studies suggest that positive feedback mechanisms play an essential role in the spatial regulation of signaling events at the membrane. Furthermore, experimental observations demonstrate that the actin cytoskeleton is involved in feedback loops that locally cluster and amplify signaling components that regulate actin assembly. However, despite our extensive knowledge in the biochemistry of actin polymerization, it remains unclear how molecular interactions between F-actin, actin-binding proteins, membrane-bound proteins, and lipids produce the micron-scale spatial organization of clustered signaling complexes observed in cells.

To dissect the molecular mechanisms of actin-dependent feedback loops, I have reconstituted N-WASP-driven actin assembly at a model membrane using purified components. I find that during actin polymerization along the surface of
lipid-coated microspheres, the local surface density of membrane-bound N-WASP is increased in an actin-dependent manner. While initial localization and activation of N-WASP requires first binding to Cdc42 at the membrane, this interaction is not required to maintain N-WASP at the interface between the actin network and the membrane. N-WASP is released from Cdc42 and remains tethered at the membrane by the actin network, thus allowing Cdc42 to undergo subsequent cycles of N-WASP activation. Catalytic activation by Cdc42 and local amplification by the product of N-WASP's nucleation promoting factor activity, i.e. F-actin, together provide the mechanism for spontaneous clustering of N-WASP and localized actin assembly along the membrane, even in the absence of any asymmetric cues at the surface. This work provides mechanistic insights into how dendritic actin networks are organized into spatially distinct regions, and in turn, how they may exert feedback control on signaling transduction events at the membrane.
To my beloved family.
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As I write this, it still has not quite sunk in that this chapter of my life is coming to a close. It has been a long journey, full of twists and turns, and I would not have made it to the finish line without an enormous group of supporters around me.

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Chapter 1 – Introduction
Overview

Organization of proteins and cellular structures is critical to many cellular processes, including recognition of cells’ immediate environments, establishment of directionality and polarity for cell division and migration, and cell-cell communication. When cells sense changes in the outside environment, usually involving interactions between surface receptors and specific ligands, the signals can be transmitted across the membrane, triggering the activation of downstream signaling cascades within the cell. These signaling events often lead to recruitment of cytosolic signaling factors to the membrane (Kholodenko et al. 2000) (Kholodenko 2003), generating higher-order clustering of proteins at the membrane surface and large-scale rearrangement of subcellular structures, such as the actin cytoskeleton. Macroscopically, a cell that appears symmetrical at rest may break symmetry and polarize by assembly an actin network at the “front” of the cell.

To generate localized or asymmetric protrusive structures, signaling events that regulate actin dynamics must occur in a localized fashion and at specific regions along the membrane. However, the cellular environment is notoriously “noisy”, where signaling molecules are constantly undergoing three-dimensional Brownian motion throughout the cytoplasm (Berg 1993). Biological membranes, on which signal transduction occurs, are also highly dynamic; they exist as two-dimensional fluid structures where integral and membrane-associated proteins are rapidly diffusing. Strikingly, despite the apparent disorder, minute changes in the environment, such as a shallow gradient of signals, are often sufficient to generate micron-scale asymmetric patterns inside the cell. How signal transduction and amplification is spatially organized remains a central question in cell biology.

Rearrangement of the actin cytoskeleton

Many signal transduction pathways trigger changes in the three-dimensional organization and dynamics of the actin cytoskeleton. For instance, upon sensing a chemoattractant, motile cells, such as neutrophils (Xu et al. 2003) and dictyostelium (Janetopoulos et al. 2008), migrate up the signal gradient by assembling a dendritic actin network at the leading edge of the cells. When immune cells, including T cells (Dustin 2009), B cells (Harwood et al. 2010), and natural killer cells (Topham et al. 2009), identify and are attached to their targets, specialized actin structures organize into ring-like patterns at the membrane to form the immunological synapse. For macrophages (J. G. Evans et al. 2006), osteoclasts (Saltel et al. 2008), and certain malignant cancer cells (Vignjevic et al. 2008) (Weaver 2006), actin structures known as invadopodia or podosomes are important for their attachment and invasion through the extracellular matrix. On the subcellular level, actin polymerizes at sites of endocytosis and is thought to aid in the dynamin-driven fission process (Doherty et al. 2009) (Roux et al. 2006). Actin also forms polarized “comet tails” that propel endosomes and vesicles through the cytoplasm (Taunton et al. 2000). Certain intracellular pathogens, such as Listeria, Shigella, and vaccinia, have evolved to hijack the same actin machineries in the host cells for their intracellular invasion and motility (J. M. Stevens et al. 2006).
**Basic building blocks of an actin network**

While the location, size, and precise architecture of the various types of F-actin structures may differ, the fundamental function of the actin cytoskeleton is the same: it provides the pushing or pulling force for membrane protrusion or involution and determines the overall direction of movement. The basic building block of the actin cytoskeleton is the actin monomer, G-actin. Each actin molecule consists of a barbed end and a pointed end (also known as the plus and minus ends, respectively). Actin monomers can polymerize to form a polar actin filament (F-actin), which comprises a twisted double helix, by aligning the pointed end of one actin monomer with the barbed end of another (Pollard et al. 2003). The directionality of actin filament elongation is kinetically biased; the addition of actin monomers occurs more rapidly at the barbed end. This process is enhanced when actin monomers are ATP-bound and are present above the critical concentration (∼0.1 μM) (Carlier and Pantaloni 1997). Shortly after binding to the filament, ATP on the newly incorporated actin subunits is rapidly hydrolyzed to give ADP and inorganic phosphate (P$_i$) (Pollard et al. 2000). However, the P$_i$ release occurs at a slower rate than ATP hydrolysis (Carlier et al. 1986). The end result is that the growing barbed end of an actin filament contains ATP/ADP-P$_i$-bound subunits, whereas the rest of the filament is primarily ADP-bound. The nucleotide states along the filament provide an added layer of polarity, allowing regulators of the actin network (e.g., actin depolymerizer like cofilin) to recognize the “age” of different parts of the structure (Pollard et al. 2000).

The cellular actin concentration is estimated to be in the hundreds of micromolar (Pollard et al. 2000), and therefore, additional controls are in place to prevent spontaneous actin polymerization in the cell. There are many classes of actin regulators, and a comprehensive list can be found in the following references: (Siripala and Welch 2007a) (Siripala and Welch 2007b). Here I will focus on a few that are fundamental to a variety of cellular processes and relevant to my studies.
The assembly of F-actin is promoted by actin nucleators. There are many classes of actin nucleators, each responding to different upstream signals and ultimately generating a different type of actin structure. One of the most critical nucleators is the actin-related protein 2/3 (Arp2/3) complex (H. N. Higgs et al. 2001). The Arp2/3 complex is made up of 7 subunits, of which the Arp2 and Arp3 subunits are particularly crucial, as they are structurally similar to an actin monomer, and they form the “nucleus” on which actin monomers are added during the initial formation of a new actin filament. Nucleation preferentially takes place when the Arp2/3 complex is bound to the side of an existing actin filament such that the newly formed barbed end elongates away from the “mother” filament. The end result is that Arp2/3-dependent nucleation forms Y-branched actin networks (Mullins et al. 1998).

Actin dynamics are regulated by a variety of actin-binding proteins (Le Clainche et al. 2008). One of these is profilin, which plays an important role in promoting nucleotide exchange from ADP to ATP on actin monomers in order to facilitate their addition to the barbed ends of existing actin filaments (Yarmola et al. 2006). Furthermore, by binding to actin monomers, profilin serves as a buffer for G-actin and prevents uncontrolled, spontaneous actin polymerization in the cytoplasm.
Moreover, it has been postulated that profilin may also interact with signaling molecules at the membrane and help to deliver actin monomers to membrane surfaces where actin networks are being assembled (Yarar et al. 2002). Profilin/actin complexes preferentially add to F-actin barbed ends; upon incorporation of the actin monomer into the filament, profilin rapidly dissociates.

Balancing the proteins that promote actin assembly are proteins that take actin structures apart and block filament elongation. Capping protein is a heterodimer of alpha and beta subunits, and it has nanomolar affinity for actin filament barbed ends (Pantaloni et al. 2000). It functions as a “cap” that stops elongation at the growing barbed ends by sterically preventing further addition of actin monomers. Capping protein also helps to prevent spontaneous unregulated actin polymerization in cytoplasm.

Another class of negative actin regulators is the actin depolymerization factors, of which coflin is a member. The primary function of coflin is to sever actin filaments by binding to the older, predominantly ADP-bound, region of actin filaments (Carlier, Laurent, et al. 1997). Upon being severed by coflin, the actin network depolymerizes into actin monomers, which undergo profilin-mediated ATP/ADP exchange, at which point, they are ready to participate in further rounds of nucleation and filament elongation.

There exist other classes of actin-binding proteins, such as cross-linking and bundling proteins (Le Clainche et al. 2008), which can modulate the process of actin assembly and ultimately produce the distinct features of the various types of actin structures observed in cells. Nevertheless, the positive and negative regulators discussed above have been found to be sufficient to form branched (“dendritic”) actin structures in vitro that closely mimic their biological counterparts and capture many physiological features of the actin cytoskeleton (Loisel et al. 1999), including the ability to generate force and move macroscopic objects. All of these regulators work as an ensemble to control the location, dynamics, and physical properties of the actin networks.

**Activation of actin assembly at the membrane**

To initiate the assembly of a force-producing dendritic actin network, the Arp2/3 complex must first be activated at the cytoplasmic surface of cellular membranes by actin nucleation promoting factors (Le Clainche et al. 2008). Wiskott Aldrich Syndrome Protein (WASP) and WASP-family Verprolin-homologous (WAVE) family proteins comprise a major class of nucleation promoting factors that produce a variety of dendritic actin structures found in cells (Mullins 2000) (Takenawa et al. 2007) (Machesky et al. 1999). WASP (primarily expressed in hematopoietic cells) and N-WASP (ubiquitously expressed) contain multiple domains that interact with a variety of membrane and cytoskeletal components.
Shown in Figure 2 is a schematic representing the domain structure of N-WASP – the nucleation promoting factor used throughout this work – and some of the known binding partners of each domain. Note that the wildtype and mutant N-WASP used in this study are based on the ΔEVH1 truncated construct. WASP and WAVE are very similar to N-WASP; both WASP and WAVE have only one WH2 domain, while WAVE has a Scar homology (SH) domain in lieu of the Ena/VASP homology domain 1 (EVH1) at the N-terminus. Although the N-terminal structures are divergent among WASP/WAVE-family proteins, they all possess the WASP homology/verprolin homology domain (denoted by “WH2”, “W”, or “V”), the central hydrophobic/cofilin homology domain (C), and the acid region (A) at the C-terminal end. Together, these three domains form the VCA fragment, which is the constitutively active fragment that activates Arp2/3 and is often used in *in vitro* actin polymerization assays.

In unstimulated cells, WASP-family proteins adopt an auto-inhibited conformation and reside in the cytoplasm. They are recruited to the membrane and activated by multiple upstream signals at the surface, including phosphoinositides, small GTPases of the Rho family, SH3-containing adapter proteins, as well as protein kinases (Figure 2) (Padrick et al. 2010). *In vitro*, N-WASP can be potently activated by binding to phosphoinositol 4,5-bisphosphate (PIP$_2$), and the small GTPase, cell division cycle protein 42 (Cdc42), in a cooperative manner (Prehoda et al. 2000) (H. N. Higgs et al. 2000). The autoinhibition of N-WASP, which is conferred by the intramolecular interactions between the N-terminal B-GBD domains and the C-terminal VCA region, is relieved upon N-WASP binding to PIP$_2$. 

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**Figure 2** – Schematic of domain structure of N-WASP and the major binding partners. The Ena/VASP homology domain 1 (EVH1) binds to WASP-interacting protein (WIP). The basic region binds to acidic phospholipids, such as PIP$_2$. The GTPase Binding Domain (GBD) interacts with Cdc42. Both B and GBD are important for WASP/N-WASP recruitment to the membrane. The proline-rich domain (PRD) interacts with a variety of SH3-containing adapter proteins. The two WASP homology 2 (WH2) domains (denoted be Wa and Wb), also known as the verprolin homology domain (often denoted as V), are actin-binding domains. The cofilin homology/central hydrophobic region (C) and acid region (A) bind and activate Arp2/3. All WASP/WAVE-family proteins contain the WH2 (or V), C, and A domains, which together form the VCA peptide, the constitutively active fragment that is often used in *in vitro* actin polymerization assays.
and Cdc42. This exposes the active fragment, VCA, to activate Arp2/3 near the membrane and catalyze actin nucleation.

On the other hand, unlike WASP and N-WASP, WAVE does not adopt an autoinhibitory conformation. Instead, WAVE is constitutively bound to four other proteins to form a 5-member complex. The precise mechanisms that control activation of the WAVE complex remain unclear. Nevertheless, activation of the WAVE complex is thought to be analogous to WASP proteins; it has been found that WAVE can be activated at the membrane by the phosphoinositide, PIP₃, and the small GTPase, Rac (Chen et al. 2010) (Lebensohn et al. 2009), which relieve the intermolecular interactions within the WAVE complex, exposing the VCA region to activate the Arp2/3 complex and promote actin nucleation and formation of a branched actin network (Chen et al. 2010).

Rho-family GTPases, of which Cdc42 and Rac are members, are proteins whose activity is modulated according to their nucleotide state (GTP is “on” and GDP is “off”) (Etienne-Manneville et al. 2002). They are regulated by three factors: nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). Rho GTPases have a CAAX motif at the C-terminus, which is post-translationally modified by isoprenylation (Casey et al. 1996); Cdc42 and Rac are geranylglycosylated. Because of the lipid modification, Cdc42 and Rac do not exist in isolation. Instead, when in the GDP-bound form, they are in complex with GDI in the cytosol, or partition to the membrane by inserting the geranylgeranyl group into the lipid bilayer. In terms of signal transduction, this allows Rho GTPases to recruit downstream effectors (e.g., WASP/WAVE-family proteins) from to the membrane. Once anchored at the membrane, nucleotide exchange is induced by GEFs, which promote the release of GDP from the Rho GTPases in exchange for GTP, and thereby switching “on” the GTPase (Rossman et al. 2005). Rho GTPases have the intrinsic ability to hydrolyze GTP, but the process is accelerated up to 5 orders of magnitude by GAPs, which switch the GTPase “off” (Lamarche et al. 1994). The ratio between GAPs and GEFs is critical for controlling both the level and the turnover rates of Rho GTPases at the membrane (Goryachev et al. 2006), and in turn could affect the rates and levels of activation for downstream effectors.

Biochemical reconstitution of dendritic actin network assembly

Actin polymerization is classically studied in bulk solution assays in which actin nucleation and elongation are monitored by an increase in fluorescence generated by the alignment of pyrene-labeled actin monomers as they assemble into actin filaments (Mullins et al. 1998). The assay has been very effective for measuring the kinetic parameters for different Arp2/3 complex activators. However, this assay is limited in that little information about the spatial organization of actin networks can be extracted.

Complementary to bulk solution studies of actin polymerization is the reconstitution of actin comet tail formation on “biomimetic” surfaces using cell-free extract systems (Theriot et al. 1994). Unlike pyrene assays, imaging experiments allow us to examine membrane recruitment of signaling components and spatial
organization of actin structures on surfaces. While the system was initially implemented to study intracellular pathogen motility and actin comet tail formation (Theriot et al. 1994) (Egile et al. 1999), these experiments have made possible the identification of membrane-bound signaling components, including PIP$_2$/PIP$_3$-dependent and Cdc42-dependent manner (Ma et al. 1998) and N-WASP (Taunton et al. 2000), that activate the production of actin comet tails during intracellular vesicles movements. In addition, the mechanical forces exerted by the actin comet tails have also been probed by monitoring the morphological changes of vesicles induced by the actin network (Giardini et al. 2003) (Upadhyaya et al. 2003).

When vesicles made up of endosomal membranes or of pure lipids are added to the extract, actin assembly can be stimulated by the addition of phorbol 12-myristate 13-acetate (PMA), a phorbol ester that mimics diacylglycerol and activates protein kinase C (PKC) (Taunton et al. 2000) (Co et al. 2007). Upon stimulation, Cdc42 and N-WASP are recruited to the surface of vesicles, resulting in the assembly of actin comet tails that propel the vesicles forward (Figure 3). Furthermore, the surface distribution of N-WASP and the actin comet tail at the membrane surface are asymmetric and polarized (Co et al. 2007).

![Figure 3 – Time-lapse images of actin assembly at the surface of endosomal membranes in cell-free extract. The vesicle is propelled by the actin network (phase) that is continually polymerizing by adding actin monomers at the membrane-actin interface. N-WASP (green) appears polarized at the surface of the membrane and co-localizes with the actin comet tail. Scale bar: 2 μm.](image)

Recently, our understanding of actin assembly along surfaces has been vastly improved with the ability to reconstitute actin comet tail formation with purified components (Loisel et al. 1999) (Wiesner et al. 2003) (Akin et al. 2008). This platform has allowed us to decipher the molecular mechanisms of actin polymerization with much greater precision, since all of the components and their respective concentrations in the motility reaction can be carefully controlled. The standard assay comprises of plastic microspheres coated with actin nucleation
promoting factors, such as N-WASP or its actin- and Arp2/3-binding fragment, VCA, and it has been shown that a handful of actin regulators – Arp2/3, capping protein, coflin, and profilin – in the motility medium are sufficient to induce branched actin network assembly at the surface of these beads (Wiesner et al. 2003) (Bernheim-Groswasser et al. 2002).

**Stress-induced symmetry breaking of the actin network**

A key question is how actin assembly is spatially regulated to produce polarized actin networks, such as actin comet tails or lamellipodia, which push membrane surfaces forward. Previous studies have shown that when nucleation promoting factors, such as N-WASP or its actin- and Arp2/3-binding fragment, VCA, were immobilized onto polystyrene beads and introduced into the motility medium containing actin polymerization machinery (i.e., actin, Arp2/3, capping protein, coflin, and profilin), the actin structure that is initially formed at the bead surface appears to be a uniform, spherically symmetrical actin shell (Bernheim-Groswasser et al. 2002) (van der Gucht et al. 2005). As actin elongation proceeds and the actin network expands, the symmetry of the actin structure is subsequently broken, thus producing a polarized actin comet tail that protrudes from one side of the beads. Mechanical stress-induced fracture during isotropic expansion of the actin network has been demonstrated to be the primary driving force of actin network symmetry breaking (van der Gucht et al. 2005), at least with plastic bead systems. Whether this mechanism accounts for symmetry breaking on membrane surfaces is not clear.

**Feedback loops and symmetry breaking at the membrane**

In cells, polarized actin networks are formed in distinct regions rather than uniformly across the entire membrane surface. Moreover, signaling molecules are not immobilized at high density at the surface, in contrast to reconstituted motility systems based on nucleation promoting factor-coated plastic beads. Instead, they are dynamically exchanging between the cytoplasm and the membrane, and rapidly diffusing along the membrane. Additionally, it is observed that signaling molecules, such as N-WASP, form clusters around the sites of actin polymerization. Based on these observations, it is clear that the existing in vitro studies have not adequately addressed the physiological mechanisms of actin symmetry breaking in biological systems.

Evidence from in vivo observations (Charest et al. 2006) (Weiner et al. 2007) as well as theoretical studies (Altschuler et al. 2008) suggests that positive feedback loops are essential for the organization of signaling molecules and actin structures into discrete areas at membrane surfaces. In particular, actin-dependent positive feedback loops play a central role in focusing actin assembly at the leading edge of a motile cell and locally amplifying upstream signals at the front end of the cell; it has been found that Rac and WAVE distribution at the leading edge is dependent on continuous actin polymerization (Weiner et al. 2002). Similarly, PIP3 localization at the front of a chemotaxing dictyostelium requires actin assembly (Charest et al. 2006) (Janetopoulos et al. 2008). Nevertheless, it is unclear how, on
a molecular level, interactions between actin and signaling components at the membrane would confer spatial control of these signaling events. Therefore, the goal of my dissertation studies was to elucidate the underlying molecular mechanisms of actin-dependent feedback loops that produce localized actin assembly and membrane signaling.

**A novel reconstituted motility system combining physiological activation of Cdc42/N-WASP and actin assembly**

Despite extensive knowledge that we have garnered from previous *in vitro* studies, how the lipid bilayer and the dynamics of membrane-recruited signaling factors influence actin assembly remain poorly understood. To that end, we have extended the reconstituted motility system by using membrane-coated silica microspheres. Critical to these studies was the introduction of upstream signaling components to recapitulate the physiological mechanisms of membrane recruitment and activation of Cdc42 and N-WASP. When the membrane-coated silica microspheres (lipid-beads) are added to a motility medium containing actin polymerization machinery (actin, Arp2/3, capping protein, profilin, and cofiliin) and signaling components (Cdc42/RhoGDI, N-WASP, GEF, GTP) (Figure 4a), actin comet tails are assembled at the bead surface (Figure 4b). Cdc42 dissociates from RhoGDI and partitions to the membrane (Figure 4a, i), where it is activated by a GEF (the DH-PH fragment of intersectin) and exchanges GDP for GTP. GTP-bound Cdc42 and PIP2 recruit N-WASP to the surface (Figure 4a, ii), where it activates Arp2/3 and promotes actin nucleation (Figure 4a, iii). I used this reconstituted system extensively throughout this work to investigate the interplay between membrane-associated signaling complexes and the actin cytoskeleton.
**Signaling components**

- PIP$_2$: 2 mol%  
- RhoGDI: 250 nM  
- N-WASP: 100 nM  
- GEF (DH-PH): 50 nM

**Actin machinery**

- actin: 6 μM  
- Arp2/3 complex: 75 nM  
- capping protein: 50 nM  
- coflin: 2.5 μM  
- profilin: 3.5 μM
Figure 4 – Reconstitution of Cdc42/N-WASP-dependent actin assembly at the membrane. **a.** Schematic of the purified components in the reconstituted motility system used throughout this work, highlighting the fundamental molecular interactions at the membrane (left) that give rise to the actin assembly observed (right). First, Cdc42 dissociates from RhoGDI, partitions to the membrane and undergoes nucleotide exchange (i). Then GTP-bound Cdc42, along with PIP₂, recruits N-WASP (ii) and promotes actin assembly. The motility system comprises the actin polymerization machinery (actin, Arp2/3, capping protein, coflin, profilin) and signaling components (Cdc42/RhoGDI, N-WASP, GEF, GTP) and membrane-coated silica microspheres that contain PIP₂. The concentrations in the standard motility reaction are listed in the legend. **b.** A typical time-lapse movie of a moving lipid-bead propelled by an actin comet tail in the reconstituted system with purified components. Similar to what has been observed in cell-free extracts, N-WASP (red) is recruited the membrane and produces a polarized actin structure (green) at the surface that can propel lipid-beads forward. In this example, the lipid-bead moves at a speed of ~4 μm/min. Scale bar: 5 μm.
Dissertation outline

In this dissertation, I will discuss my efforts to dissect the molecular mechanisms of an actin-driven feedback loop, which spatially regulates and locally amplifies its upstream signal, N-WASP, at the membrane. I first explore the thermodynamic driving forces that govern N-WASP membrane recruitment and surface density, in order to understand how N-WASP is activated at the membrane in the absence of actin assembly. Then, I investigate the effects of N-WASP density on the mechanism of symmetry breaking during actin polymerization in a reconstituted system containing purified signaling components and actin machinery (Chapter 2). Next, I describe an actin-N-WASP positive feedback loop that I have uncovered in this study, and determine the molecular interactions between actin and N-WASP that are essential for the local amplification of N-WASP by actin. The role of Cdc42 in the actin-N-WASP feedback loop is also examined (Chapter 3). Finally, I summarize the mechanistic insights I have gained from my in vitro studies and discuss their implications for the physiological roles of actin-driven feedback loops in the spatial control and local amplification of signal transduction events at the membrane (Chapter 4).
Chapter 2 – Reconstitution of N-WASP activation and actin assembly at the membrane with purified components
Abstract

Actin assembly pushes membrane surfaces forward and is regulated by upstream signaling components that localize to the membrane. Previous studies of reconstituted motility have shown that, in order to generate a polarized, force-producing actin comet tail at a surface, N-WASP must be present at sufficiently high surface density, and the actin network must undergo mechanical stress-induced symmetry breaking. How this is achieved in the context of a fluid lipid bilayer and diffusible signaling components that cycle between the cytosol and the membrane remains unclear. Here, I will first establish the thermodynamic forces that govern N-WASP membrane recruitment and surface density onto the lipid-beads. I will then examine the effects of N-WASP density on actin assembly and symmetry breaking in our reconstituted motility system.
Introduction

Dendritic actin structures are formed by Arp2/3-dependent actin nucleation and branching. The Arp2/3 complex is recruited and regulated by membrane-associated upstream activators, RhoGTPases and WASP/WAVE-family proteins. The fundamental steps of Cdc42 (a member of the Rho-family of GTPases) and N-WASP (a member of the WASP proteins) activation at the membrane are outlined in Figure 1.

**Cdc42 cycles between the cytoplasm and the membrane by inserting its geranylgeranylated C-terminus either into the lipid bilayer or a cytoplasmic negative regulatory factor, RhoGDI.** (Etienne-Manneville et al. 2002) (Leonard et al. 1992). When bound to GDP, Cdc42 is inactive and preferentially remains in complex with RhoGDI. However, upon translocating to the membrane (Figure 1, step i), Cdc42 can become activated by a guanine nucleotide exchange factor (GEF) (Figure 1, step ii) (Erickson et al. 2004) (Hoffman et al. 2002). PIP$_2$ and GTP-bound Cdc42 activate N-WASP by binding to the N-terminal B-GBD domains and disrupting the autoinhibitory intramolecular interactions between B-GBD and VCA, thus exposing the C-terminal Arp2/3-binding region to promote actin nucleation and branch formation at membrane surfaces (Figure 1, step iii) (Prehoda et al. 2000) (H. N. Higgs et al. 2000).

While most previous studies have focused on the biochemical activities of Cdc42 and N-WASP in solution, little is known about their activities and spatial regulation in the context of a membrane surface. However, N-WASP-dependent actin assembly at a surface has been examined in a reconstituted system with...
purified components. In these experiments, N-WASP was immobilized onto the surface of polystyrene beads, bypassing membrane recruitment and activation by PIP$_2$ and Cdc42. When N-WASP-coated beads are introduced into a reaction mix containing actin polymerization machinery, polarized actin structures are formed at the bead surfaces (Wiesner et al. 2003) (Bernheim-Groswasser et al. 2002) (van der Gucht et al. 2005). These actin structures appear morphologically very similar to actin comet tails that propel pathogens and vesicles in the cytoplasm.

Two important properties were uncovered by using this experimental system. First, there is a strong correlation between N-WASP surface density and actin assembly. To ensure 100% of the N-WASP-coated beads produce actin comet tails, N-WASP must be present at the critical surface density of ~16,000 molecules/µm$^2$ or above (Wiesner et al. 2003). Second, when N-WASP-coated beads are introduced into the motility reaction, the nascent actin structure produced at the surface is a spherically symmetrical shell and must undergo symmetry breaking to produce the polarized actin comet tails observed at steady state.

The mechanism of actin symmetry breaking mechanism in this system has been well characterized in vitro (Bernheim-Groswasser et al. 2002) (van der Gucht et al. 2005) and in silico (Dayel et al. 2009). Because of the highly cross-linked nature of the actin structure generated by Arp2/3-dependent nucleation and branching, mechanical stress is built up as the actin structure expands. When the tangential stress at the outer edge of the actin shell reaches a critical level, the actin network fractures, and the symmetry of the system is broken (van der Gucht et al. 2005) (Akin et al. 2008). Importantly, since tangential stress at the surface of a sphere is directly related to its curvature, which in turn is proportional to the diameter, assuming a constant rate of actin polymerization and actin network expansion, the time to symmetry breaking – i.e. the time it takes from the initial moment of actin assembly until the moment the actin network is fractured – is directly proportional to the diameter of the beads (Bernheim-Groswasser et al. 2002) (van der Gucht et al. 2005).

In the context of a fluid lipid bilayer and diffusible membrane-recruited signaling proteins, it remains unclear how sufficient N-WASP surface density is achieved and how actin symmetry breaking occurs. I extended the reconstituted system by employing lipid-coated silica beads, which retain the biological properties of a lipid bilayer, such as membrane fluidity (Baksh et al. 2004), while allowing us to control precisely the composition of the lipid bilayer and total surface area in the reaction. I will first characterize the relationship between the concentrations of N-WASP and Cdc42 in solution and N-WASP density at the lipid-beads. Then, I will investigate how N-WASP surface density affects actin polymerization and symmetry breaking at the membrane.

Results

Thermodynamic driving forces for N-WASP recruitment to the membrane

First, I explored the relationship between the concentrations of N-WASP and Cdc42 in solution and N-WASP density at the membrane. I developed a quantitative method to reliably monitor the recruitment of fluorescent N-WASP
(Alexa594-labeled) to lipid-beads. By incorporating a fluorescent lipid marker (Alexa488-PE) into the supported membranes, I could use the ratio of N-WASP signal at the surface to the lipid fluorescence as a quantitative readout for N-WASP surface density from different experiments.

Incubation of PIP$_2$-containing lipid-beads with varying amounts of the Cdc42-RhoGDI complex and N-WASP, along with 250 nM of a GEF (DH-PH fragment of intersectin) and 10 µM of GTP, led to the recruitment of N-WASP to the membrane (Figure 2). In the absence of Cdc42-RhoGDI, N-WASP was barely detectable at the membrane surface. N-WASP signal increased approximately linearly with the amount of Cdc42-RhoGDI in the reaction (Figure 2a, b). I estimated the binding affinity of N-WASP to the membrane by fitting a single exponential binding curve (Figure 2b), assuming a single-site binding event. In the presence of Cdc42, N-WASP was recruited to the membrane with an apparent dissociation constant, K$_D$, of ~0.3 µM (Figure 2c). The binding affinity I measured is approximately 4 fold lower than the reported affinity between the isolated GTPase Binding Domain (GBD) of N-WASP and Cdc42 (K$_D$ ~ 70 nM) (Hemsath et al. 2005), but it is consistent with previous studies showing that N-WASP constructs that adopt an autoinhibited conformation generally have a lower affinity for Cdc42 (Leung et al. 2005).
[Cdc42/RhoGDI] (µM) & Kₐ (µM) & maximum N-WASP density (molecules/µm²)
1.25 & 0.34 ± 0.09 & ~19,000
0.25 & 0.31 ± 0.08 & ~4,700
0 & n.d. & < 1,000
Figure 2 – N-WASP recruitment to lipid-coated beads as a function of Cdc42-RhoGDI and N-WASP concentrations in solution. a. Representative images of Alexa594-labeled N-WASP recruited to lipid-coated beads incubated with varying amounts of N-WASP and Cdc42-RhoGDI, in the presence of GTP and a nucleotide exchange factor. b. Quantification of N-WASP fluorescence along the membrane normalized to the fluorescence of the Alexa488-labeled lipid bilayer (left axis). N-WASP:lipid fluorescence ratio is calibrated to N-WASP surface density (right axis). c. Calculated N-WASP binding affinities to the membrane and maximum N-WASP density at the different Cdc42-RhoGDI concentrations. Scale bar: 2 µm.

Estimating N-WASP surface density by fluorescence imaging

In order to quantify the actual amount of N-WASP at the membrane, I set up a standard calibration curve from which I could convert N-WASP:lipid fluorescence ratio to N-WASP density at the membrane. First, I incubated varying amounts of fluorescent N-WASP with silica microspheres (the same beads typically used for making lipid-beads except without the lipid bilayer coating) and allowed N-WASP to be adsorbed non-specifically onto the glass surface. I then imaged these N-WASP-coated silica beads under the microscope and measured the fluorescence intensities as a function of total amount of N-WASP input, ranging from 25 to 250 pmol (Figure 3a). N-WASP intensity at the silica surface increased linearly with N-WASP input until saturation was reached at approximately at 150 pmol input (Figure 3c). Presumably, at the saturation point, the entire surface of the silica beads was covered by N-WASP. The linearity and saturation point of N-WASP adsorption were independently verified by eluting the silica beads with SDS and quantifying the eluted N-WASP by SDS-PAGE with Coomassie staining and fluorescence scanning (Figure 3b); notably, below the saturation point, input N-WASP was quantitatively adsorbed to the silica beads.

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<th>a</th>
<th>N-WASP (pmol)</th>
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![Representative images of Alexa594-labeled N-WASP recruited to lipid-coated beads](image1)

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<tr>
<th>b</th>
<th>N-WASP (pmol)</th>
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![Quantification of N-WASP fluorescence along the membrane](image2)
Figure 3 – N-WASP:lipid ratio to surface density calibration. a. Images of fluorescent N-WASP absorbed to silica beads. b. Coomassie staining (top) and fluorescence scanning (bottom) of SDS-PAGE of N-WASP adsorbed onto silica bead surface. Unbound N-WASP was separated by centrifugation. N-WASP bound to the silica beads is in the pellet fractions. c. Fluorescence intensity measurement of N-WASP absorbed onto silica beads in a. d. Correlation between N-WASP surface density and N-WASP fluorescence based on the linear region of the absorption curve in c. e. N-WASP membrane recruitment assay performed under identification conditions as in Figure 2b. N-WASP:lipid fluorescence ratio is plotted on the left y-axis, and the absolute N-WASP fluorescence is plotted on the right x-axis. The absolute fluorescence was then converted to surface density using the standard curve from d and used in Figure 2b.

Using the linear range of the N-WASP intensity vs. N-WASP input curve, and by assuming that every molecule of N-WASP in solution was bound to the surface under these conditions (Figure 3b), I generated a calibration curve that correlates N-WASP fluorescence intensity to surface density (Figure 3d). Concurrently, I performed a Cdc42-dependent N-WASP recruitment assay as described in the previous section, using the Alexa488-labeled lipid bilayer, and imaged the samples under the same epi-fluorescence settings (Figure 3e). By correlating N-WASP:lipid ratio to absolute N-WASP fluorescence, which was then converted to surface density, I could calibrate N-WASP:lipid fluorescence ratio to N-WASP surface density (Figure 2b).

Based on the calibration curves, N-WASP recruitment to the lipid-beads reached saturating surface densities of approximately 19,000 and 4,700 molecules/µm² in the presence of 1.25 µM and 0.25 µM of Cdc42-RhoGDI in solution, respectively (Figure 2b). By contrast, in the absence of Cdc42, the maximum N-WASP density achieved was only ~800 molecules/µm² (Figure 2b). The results clearly demonstrate that even in the presence of very high concentrations of N-WASP, its maximum surface density is determined and limited by the amount Cdc42 at the membrane, which is in turn determined by the amount of Cdc42/RhoRhoGDI complex in solution. Control experiments showed that N-WASP recruitment was negligible in the absence of DH-PH or GTP (Co et al. 2007) (data not shown).

Reconstitution of actin-based motility with lipid-beads

One of the characteristics uncovered by previous studies of reconstituted actin-based motility is that the nucleation promoting factors must be present at sufficiently high surface density in order to promote the assembly of the actin network. For example, an N-WASP threshold density of ~16,000 molecules/µm² is necessary for generating cohesive actin comet tails capable of propelling polystyrene microspheres (Wiesner et al. 2003). This density of N-WASP was typically achieved by coupling N-WASP or its active fragment VCA to the surface of a polystyrene microsphere by non-specific adsorption, or by binding His-tagged N-WASP fragments to liposomes containing 10 mol% of Ni-NTA lipids (Delatour et al. 2008).
In my lipid-bead system, the previously reported threshold density (> 16,000 N-WASP molecules/µm²) was achieved by incubating lipid-beads with a high concentration of N-WASP (> 0.5 µM) and Cdc42-RhoGDI (> 1.25 µM) in the presence of GTP and a GEF (Figure 2b). In line with the results from previous studies, when lipid-beads pre-loaded with N-WASP were introduced into the motility medium containing actin polymerization machinery (Figure 5a), an actin comet tail formed on the beads’ surface (Figure 4).

Figure 4 – Representative image of an actin structure (Alexa488-labeled) protruding from the lipid-bead at t ~ 10 min after the motility reaction has begun. When lipid-beads pre-loaded with a high density of N-WASP were introduced into the motility reaction, actin comet tails appeared on the surface of the lipid-beads. Scale bar: 5 µm.
a

Lipid-bead pre-activation

N-WASP (500nM)

N-WASP (100nM)

Actin

Formation of symmetrical actin shell

5X dilution into motility medium

Stress-induced actin symmetry-breaking

b

Phase

Actin

N-WASP

2 min 3 min 4 min 5 min 8 min
Figure 5 – Actin assembly and symmetry breaking under conditions where lipid-beads were pre-loaded with a high density of N-WASP. a. Schematic of the motility reaction. N-WASP is pre-loaded onto the lipid-beads by first incubating the membranes with a high concentration of N-WASP (500 nM) and Cdc42-RhoGDI (1.25 µM), in the presence of DH-PH (250 nM), and GTP (10 µM). Pre-loaded beads were then diluted 5X into the motility reaction containing actin machinery (actin, Arp2/3, capping protein, cofillin, and profilin). b. Time-lapse images of actin assembly at the N-WASP-pre-loaded lipid-beads, with fluorescent actin (Alexa488-labeled) and N-WASP (Alexa594-labeled). The yellow arrow indicates where the fracture of the actin network caused symmetry breaking. Scale bar: 3 µm

Symmetry breaking during actin assembly at the lipid-beads

I initially hypothesized that in the context of a fluid lipid bilayer and diffusible signaling components (Cdc42, N-WASP, and PIP₂), actin symmetry breaking would occur in a manner different from a system where nucleation promoting factors are immobilized onto the surface. Contrary to this hypothesis, nascent actin structures produced by N-WASP pre-loaded lipid-beads were spherically symmetrical (Figure 5b). As the actin shell expanded during actin polymerization, a fracture appeared at the outer edge of the symmetrical actin network (Figure 5b, arrow). The symmetry breaking event occurred in a manner that was similar to the stress-induced mechanism characterized in previous studies. As the actin network transformed into a mature comet tail, N-WASP redistributed and polarized at the membrane near the sites of actin polymerization (Figure 5b). A recent study with N-WASP VCA adsorbed at high density to the surface of Ni-NTA liposomes revealed symmetry breaking behavior consistent with my observation (Delatour et al. 2008).

In cells, nucleation promoting factors are not activated uniformly at high densities across the membrane surface, and actin assembly occurs at discrete regions. In fact, the cytoplasmic concentration of Cdc42, estimated to be in the 150 nM range (Zigmond et al. 1997) (Roos et al. 1983) (Schmid-Schonbein et al. 1980), would severely limit the amount of N-WASP that could be recruited to the surface (Figure 2b) such that a critical density could not be achieved (see below). Ostensibly, additional mechanisms are needed to recruit and organize N-WASP at a high local density at the surface to generate localized actin structures.

Actin-dependent feedback loops have been found to be critical for symmetry breaking during cell motility (Weiner et al. 2002). They are essential for localizing and amplifying membrane signals at the leading edge of a moving cell. I hypothesized that an actin-driven feedback loop could overcome the intrinsically low concentrations of Cdc42 in the cytoplasm or solution and provide a mechanism for achieving a sufficiently high local density of N-WASP at the membrane. To test the hypothesis that actin assembly can locally amplify N-WASP density at the surface in vitro, I used sub-threshold levels of Cdc42 and N-WASP, and monitored the evolution of actin assembly and N-WASP recruitment at the membrane (Figure 6a).
Figure 6 – Symmetry breaking of the actin structure without pre-loading lipid-beads with N-WASP. a. Schematic of the motility reaction. Lipid-beads (without pre-incubation with Cdc42 and N-WASP) were introduced into the motility medium supplemented with signaling components (100 nM N-WASP, 250 nM Cdc42/RhoGDI, 50 nM DH-PH, 2 µM GTP). Note that the final concentrations of all components in the motility reaction were identical to the experiment shown in Figure 4. b, c. Time-lapse images of actin assembly at the lipid-beads with Alexa488-actin (green) and Alexa594-N-WASP (red). The yellow arrows indicate the first visible actin structures at the membrane surface. c. Multiple nascent actin structures attached to different regions of the membrane were occasionally observed (yellow arrows). Scale bar: 3 µm

Remarkably, as little as 250 nM Cdc42 and 100 nM N-WASP in the motility medium was sufficient to generate force-producing actin comet tails that resulted in lipid-bead propulsion (Figure 6b, c). Two key features were observed. First, an actin comet tail was generated in the absence of a high initial density of N-WASP at the membrane. Based on the binding data in Figure 2, with identical concentrations of N-WASP and Cdc42, N-WASP recruitment to the membrane could only reach a density of ~1,000 molecules/µm², one order of magnitude below the reported threshold density for actin assembly (Wiesner et al. 2003). Second, the actin structure produced at the surface appeared at discrete regions of the lipid-beads, rather than uniformly over the entire bead surface (Figure 6b, c; arrows). Thus, the actin symmetry was broken spontaneously without undergoing the stress-induced fracture. These two observations – (1) ability to undergo actin assembly with sub-threshold levels of nucleation promoting factors and (2) a “spontaneous” symmetry-breaking mechanism that differs from the classical mechanical fracture mechanism – are unexpected based on our current understanding of how actin assembly and symmetry breaking occurs at a bead surface in reconstituted systems.
Multiple actin structures attached to a single lipid-bead were occasionally observed at the early time points (Figure 6c, Figure 7). This effect was especially pronounced with lipid-beads of larger diameters (> 5 µm), where multiple comet tails often appeared at the surface. These actin structures persisted for several minutes and grew several microns in length. At later time points, they usually coalesced into one cohesive comet tail (Figure 7b). By contrast, the stress-induced symmetry breaking mechanism observed with N-WASP pre-loaded lipid-beads produced one actin comet tail at the surface, regardless of bead size.

The results clearly demonstrate that actin assembly and symmetry breaking can proceed without an initially high density of N-WASP (~1,000 molecules/µm², according to Figure 3 with 250 nM Cdc42/RhoGDI and 100 nM N-WASP), limited by N-WASP and Cdc42 concentrations; actually, since only a small fraction of the total is recruited, neither Cdc42 nor N-WASP is stoichiometrically “limiting.” Rather, the maximum N-WASP density is limited by its concentration and the Cdc42/RhoGDI concentration because of the various coupled equilibria and the associated equilibrium and kinetic constants. Furthermore, the symmetry breaking event appears to involve a distinct mechanism compared to lipid-beads that were pre-
loaded with N-WASP. To further distinguish the two actin symmetry-breaking mechanisms, I examined the effects of bead diameter on the time to symmetry breaking. Using time-lapse imaging, I monitored the initial moments of actin comet tail assembly and measured the time from the moment lipid-beads were added to the motility reaction (t = 0) to the moment at which the appearance of asymmetry in the actin structure was first visible. For lipid-beads pre-loaded with N-WASP, the symmetry breaking occurs when the initial uniform actin shell structure fractures (Figure 5b). Without N-WASP pre-loading, the first appearance of a localized actin structure is asymmetric (Figure 6b, c).

Figure 8 – Time to symmetry breaking under conditions with or without N-WASP pre-loading onto the lipid-beads. Under pre-loading conditions, time to symmetry breaking increases with bead diameter, but it remains unchanged without initial N-WASP pre-loading at the membrane. n = 8 lipid-beads per condition. error bar = s.d.

Under pre-loading conditions (initial N-WASP density at 15,000 molecules/µm²), the time to symmetry breaking is significantly higher for the 6.8 µm beads compared to 2.3 µm beads (Figure 8). This result is consistent with the stress-induced symmetry breaking model, where time to symmetry breaking is proportional to bead diameter (Bernheim-Groswasser et al. 2002) (van der Gucht et al. 2005). By contrast, without N-WASP pre-loading, the time to symmetry breaking – defined by the frame in a time-lapse series at which a polarized actin structure appears on the bead surface (Figure 6b, c; arrows) – was unaffected by bead diameter (Figure 8).

Collectively, these results demonstrate that there are two distinct actin symmetry-breaking mechanisms that produce polarized actin structures at the membrane, depending on whether or not the lipid-beads were pre-saturated with a high surface density of N-WASP. Under pre-loading conditions, actin symmetry breaking occurs via the stress-induced mechanism. By contrast, the imaging and time-to-symmetry-breaking data in the absence of pre-loading are inconsistent with this model. I therefore hypothesized that an actin-driven positive feedback loop,
which locally elevates N-WASP density and spatially restricts new nucleation events to existing sites of actin assembly at the membrane, underlies spontaneous symmetry breaking without a symmetrical actin shell.

Discussion
A novel symmetry breaking mechanism
A major finding from my study is that actin symmetry breaking at spherical lipid-beads does not always follow the well-established stress-induced mechanism. I initially predicted that in the presence of a fluid membrane and in the context of signaling components (Cdc42/RhoGDI and N-WASP) that are diffusible both in solution and along the membrane, symmetry breaking would occur in a manner resembling what is observed in cells – spontaneously localized to distinct regions along the membrane. However, I discovered that those properties alone are not sufficient for localized actin assembly. Rather, the mechanism of symmetry breaking critically depends on the initial surface density of membrane-localized signaling components. When the lipid-beads were pre-charged with a high initial surface density of N-WASP, stress-induced fracture remains the driving force for producing polarized actin comet tails. By contrast, actin assembly is spontaneously localized and polarized without N-WASP pre-activation.

An actin-driven positive feedback mechanism could explain how actin assembly is spontaneously localized to discrete regions, even when the membrane appears uniform. Under this mechanism, initial actin nucleation would occur randomly at the surface. However, once a nascent actin structure appears, the subsequent rounds of actin nucleation would preferentially take place where existing actin protrusions already exist. This could be achieved when N-WASP surface density is elevated preferentially at the same regions of the membrane in an actin-dependent manner. In turn, N-WASP will further focus actin nucleation by locally activating Arp2/3 at these regions. In addition, autocatalysis is also built into the Arp2/3 activation (Pantaloni et al. 2000), where newly formed filaments can activate additional Arp2/3 molecules, further promoting the creation of branched filaments and barbed ends at the surface. Indeed, localized actin assembly is especially apparent when I used larger lipid surfaces, where I often observed multiple cohesive actin structures independently protruding from different regions along the membrane. Sometimes these actin protrusions would remain independent for an extended period of time, growing over ten microns in length before they finally coalesce into a single actin network. However, when the surface is saturated with the nucleation promoting factor, the autocatalysis/feedback effect is masked, because nascent actin nucleation is occurring over the entire surface. Therefore, the mechanism of symmetry breaking depends on the surface density of the activators.

Controlling N-WASP density at the surface, with and without actin polymerization
It is clear from this study that N-WASP surface density determines whether actin assembly could be initiated and which symmetry breaking mechanism would
produce the polarized actin structure. Results from the N-WASP recruitment studies show that Cdc42 is the critical determinant of N-WASP levels at the membrane (Figure 2). While it has been known that Cdc42 can tightly bind to WASP proteins in solution (Owen et al. 2000), little is known about how N-WASP surface density at the membrane is controlled by its upstream activator; it is simply assumed that N-WASP binds Cdc42 with 1:1 stoichiometry and that therefore, N-WASP density is determined by Cdc42 density on the membrane. Previous studies have shown that vesicles containing a high density of PIP2 (> 10 mol%) (Papayannopoulos et al. 2005) (Padrick et al. 2008) could recruit and activate N-WASP at the membrane in the absence of Cdc42. Nevertheless, in the context of a membrane with physiological levels of PIP2 (2 mol% in our experiments) (James et al. 2008), even when N-WASP is present in the micromolar range in solution, its membrane recruitment is minimal in the absence of Cdc42.

My results indicate that in order to reach an adequate N-WASP membrane density for actin assembly, a high concentration of Cdc42 (over 1 µM based on the binding data) is required. Based on reported measurements of Cdc42 concentrations in cellular extracts made from a defined number of neutrophils (Zigmond et al. 1997), and using the known cytoplasmic volume of neutrophils (~0.2 pL) (Schmid-Schonbein et al. 1980) (Ting-Beall et al. 1993), I estimate the cellular Cdc42 concentration to be ~150 nM. According to the N-WASP binding data (Figure 2), the maximum N-WASP density could be achieved with this concentration of Cdc42/RhoGDI would be well below the threshold required for actin network assembly. Moreover, in cells, the membrane surface area to cytoplasmic volume ratio is significantly higher than in my lipid-bead system. Therefore, even if every molecule of Cdc42 resides at the membrane and is bound to a molecule of N-WASP, when distributed evenly across the entire cell surface (assuming a spherical cell with a diameter of 10 µm (Roos et al. 1983)), the maximum N-WASP surface density could only reach ~50 molecules/µm². This amount of N-WASP at the surface is more than 2 orders of magnitude lower than the threshold surface density required for actin assembly in vitro experiments (Wiesner et al. 2003).

Together, these observations strongly suggest that additional amplification mechanisms are necessary in order to have sufficient N-WASP density at the cell membrane. In my in vitro system, the ability to assemble polarized actin structures even with initial sub-threshold levels of Cdc42 and N-WASP in the motility reaction suggests that N-WASP surface density must increase during actin comet tail assembly. This led me to hypothesize that an actin-dependent feedback loop locally increases N-WASP surface density to generate actin structures. This model could potentially explain my observation that nascent actin structures are produced locally rather than uniformly across the entire membrane. In the next chapter, I will critically test the hypothesis that the actin network locally increases N-WASP density. I will define the precise molecular interactions required for the actin-based feedback loop.
Materials and methods

Materials

Phospholipids were purchased from Avanti Polar Lipids Inc. Silica beads were purchased from Bangs Labs. Alexa Fluor dyes were purchased from Invitrogen. Rabbit muscle tissue and bovine calf brain were purchased from Pel-Freez.

Proteins used in the reconstituted system

N-WASP (aa150 – 501; ΔEVH1, “Wildtype”), intersectin DH-PH (Zamanian et al. 2003), and capping protein were expressed and purified from E. coli, and labeled as described (Co et al. 2007). Cdc42 was expressed in SF21 cells and RhoGDI was expressed in E. coli, and the Cdc42/RhoGDI complex was purified as described (Hoffman et al. 2000). Briefly, lysates of SF21 and E. coli were combined, at which point RhoGDI extracts Cdc42 from the SF21 membranes to form a complex. The Cdc42/RhoGDI complex was then purified from the clarified lysates by Ni-NTA and GST affinity chromatography. Actin from rabbit skeletal muscle and the Arp2/3 complex from bovine brains were purified as described (Co et al. 2007) (Spudich et al. 1971) (Egile et al. 1999). Actin was labeled on lysine with Alexa488-NHS (Invitrogen) (Isambert et al. 1995). Cofilin and profilin were amplified from a human placenta cDNA library (kind gift from Wendell Lim) by PCR, cloned into a His6-tagged vector (pSH200) for expression in E. coli, and purified by Ni-NTA affinity chromatography.

Preparation of fluorescent phospholipid

All glassware was dried in a 120 °C oven overnight and cooled in a desiccator. The preparation of all stock solutions and the synthesis of the labeled phospholipid were performed under an atmosphere of argon. Alexa Fluor 488 NHS ester (1 mg, 1.55 µmol) was dissolved in 50 µL anhydrous DMF and transferred to a glass vial followed by the addition of 8 µL of a 0.547 M (4.65 µmol) diisopropylethylamine solution in anhydrous DMF. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (3 mg, 4.65 µmol) was transferred to the reaction as a chloroform stock solution (138 µL). The reaction was stirred under argon and monitored by TLC for 24 hr. After removing the solvent by rotary evaporation, the Alexa Fluor 488-labeled DOPE (Alexa488 PE) was purified by prep TLC using a mobile phase of 2:1:1:1 ethyl acetate:water:acetic acid:n-butanol. The labeled lipid was extracted from the silica gel using a 4:1 CH3CN:H2O mixture, filtered and then concentrated under reduced pressure. The amount of purified lipid was quantified by measuring the absorbance in chloroform at 495 nm (Alexa Fluor 488 extinction coefficient = 71,000 cm⁻¹ M⁻¹).

Preparation of lipid-beads

I first prepared vesicles containing the desired composition of phospholipids as described (Klopfenstein et al. 2002) with minor modifications. For our standard membrane composition, I mixed solutions of phosphatidyl choline (egg yolk), phosphatidyl serine (brain), phosphoinositol 4,5-bisphosphate (PI(4,5)P2 or PIP2)
(brain), and Alexa488 PE in a 77.5:20:2:0.5 mol ratio (total amount of lipid = 1 µmol) in a small glass vial. The mixed lipid solution was then dried under an argon stream for ~10 min and subsequently under vacuum overnight. The dried lipid film was resuspended in 200 µL vesicle buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 250 mM sucrose) to form vesicles. I then sonicated the vesicles in a bath sonicator for ~15 sec, followed by 5 cycles of freezing and thawing in liquid nitrogen and room temperature water bath respectively. The vesicle suspension was aliquoted and stored at −80 °C.

To prepare lipid-beads, 5 µl of vesicles (5 mM of total lipids), 3.5 µl of silica beads (Bangs laboratories, ∅ = 2.3 µm, 10% solids w/v), and 31.5 µl of vesicle buffer were mixed together, bath sonicated three times at 10 sec intervals (with 20 sec pauses in between), and rotated for > 15 min at room temperature. The vesicles spontaneously fuse with the silica beads to create a supported lipid bilayer (Galneder et al. 2001) (Baksh et al. 2004). Lipid-beads were pelleted by centrifugation for ~7 sec with a tabletop microfuge, washed 2X with 200 µl of vesicle buffer, and resuspended in vesicle buffer back to the original volume. Lipid-coated beads were kept rotating at room temperature and were used within 12 hours of preparation. When beads of different sizes were used, the total volume of beads was adjusted to achieve the same final surface area based on manufacturer’s specifications.

N-WASP membrane recruitment assay
Lipid-coated microspheres (“lipid-beads”, ∅ = 2.3 µm, final surface density = 2.5 m²/L, bead density = 1.5 × 10⁸ beads/mL) were incubated with varying amounts of Alexa594 N-WASP (33% labeled) and Cdc42-RhoGDI in the reaction buffer (10 mM imidazole [pH 7.0], 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mg/mL BSA), supplemented by 10 µM GTP and 250 nM DH-PH, at room temperature for 30 min. A 1-microliter sample of each condition was fixed with 1.5% glutaraldehyde and imaged under the microscope.

Motility assays
Unless otherwise stated, our standard motility assays were performed as described with minor modifications (Co et al. 2007). Briefly, 2.3 µm lipid-beads (2:77.5:20:0.5, PIP₂:PC:PS:Alexa488-PE; prepared as described above) were diluted into the motility medium (final lipid-bead surface density = 0.5 m²/L), which consisted of the motility buffer (10 mM imidazole [pH 7.0], 50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 10 mg/ml BSA, 0.2% methylcellulose, 5 mM TCEP, 2 mM ATP, 1.25 mM DABCO, 20 mM ascorbic acid [pH 7.0]) and actin machinery (75 nM Arp2/3, 50 nM capping protein, 2.5 µM profilin, and 3.5 µM cofilin, 6 µM actin), supplemented by signaling components (2 µM GTP, 50 nM DH-PH, 100 nM N-WASP [33% labeled], and 250 nM Cdc42-RhoGDI). For N-WASP pre-loading, lipid-beads were incubated with signaling components at 5X final concentrations (500 nM N-WASP [33% labeled], 1.25 µM Cdc42-RhoGDI, 250 nM DH-PH, and 10 µM GTP) for 30 min at room temperature prior to 5X dilution into the motility medium.
Microscopy

Epi-fluorescence and phase contrast micrographs were acquired with a 60X objective (PlanApo, PH3, N.A. 1.40) on an Olympus IX70 inverted microscope with a CoolSnap HQ CCD camera (Photometrics) controlled by Metavue (Molecular Devices). For time-lapse imaging, glass slides and cover glass were silanized (Akin et al. 2008) or coated with BSA to minimize non-specific protein absorption (see below). For images of set time points, typically a 1-microliter sample of the reaction was fixed with 1.5% glutaraldehyde on the glass slide for imaging. Slides were sealed with VALAP (vaseline, lanolin, paraffin 1:1:1) to prevent evaporation. For quantitative fluorescence intensity measurements, ratiometric imaging was performed with Alexa488-PE as a normalization factor. The exposure and gain settings for each channel were kept constant to minimize day-to-day variations and allow direct comparisons from different experiments.

Surface treatment of slides and cover glass

To prepare treated (silanized or BSA-coated) slides and cover glass for live imaging, first they need to be cleaned thoroughly prior to surface treatment. I used staining racks made of glass (Electron Microscopy Sciences) and ceramic (Thermo Scientific) for slides and cover slips, respectively. Upon placing the slides/cover glass in the racks, I transferred the racks into a beaker filled with 70% ethanol. The slides/cover glass were sonicated in ethanol for 30 min with a bath sonicator. Then, the ethanol was exchanged for 0.1 M KOH, and the slides/cover glass were sonicated again for 30 min. After that, they were sonicated in ddH$_2$O for 30 min. The water was then exchanged for a fresh bath, and the cover glass/slides were kept there and covered until the next coating step.

For silanization of glass slides and coverslips, we follow the protocol as described (Akin et al. 2008) with minor modifications. Briefly, cleaned slides and cover glass were sonicated for 20 min in a silanization solution (95% isopropanol, 4.6% ddH$_2$O, 0.4% glacial acetic acid). Then they were sonicated in a 2% diethyldichlorosilane solution made with the silanization solution for an hour. After that, they were sonicated again in the silanization solution (without the silane) for 20 min and dried in a 70 °C oven overnight. The slides/cover glass were further sonicated in 100% ethanol for 30 min. They were then rinsed in ddH$_2$O and dried in the 70 °C oven overnight.

To prepare BSA-coated glass slides and coverslips, I sonicated the cleaned slides and cover glass in 20 mg/mL bovine serum albumin (BSA) solution, made with Fraction V BSA (Fisher Scientific) in water and filtered (0.22 µm pore size), for 30 min with a bath sonicator. The slides/cover glass were then washed by sonication in ddH$_2$O for 10 min, and dried in a 70 °C oven overnight.

Treated glass slides and cover glass are kept in the staining racks and covered to minimize dust adsorption.

Image analysis

Image analysis was performed with ImageJ. Fluorescence intensity measurements were performed as follows: raw images were background subtracted.
by the average intensity of an area away from lipid-beads. To measure overall 
membrane recruitment of N-WASP, the average intensity within a circular region of 
interest (ROI) surrounding the lipid-bead was calculated. To plot the degree of N-
WASP asymmetry at the lipid-bead, a line ROI (width = 5 pixels) with its length 
aligned with the axis of the actin comet tail was used. Fluorescence intensities of 
Alexa594 N-WASP were normalized against the intensity of Alexa488-PE on the 
corresponding lipid-bead using the identical ROI.

**N-WASP surface density calibration**

Silica beads (Ø = 2.3 µm, final surface area = 1.4 x 10⁹ µm²) were 
incubated with varying amounts of Alexa594 N-WASP (33% labeled) in buffer (10 
mM imidazole [pH 7.0], 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA) in a 50 µL volume at 
room temperature. After 30 minutes of incubation, BSA (1 mg/mL) was added to 
block any remaining exposed surfaces for ~10 min. The beads were pelleted by a 
20-second centrifugation step using a bench-top microfuge. The beads were then 
washed once (500 µL) and resuspended to the original volume with the same buffer 
supplemented by 1 mg/mL BSA. A small sample (1 µL) was removed for 
fluorescence imaging. Analyses by fluorescence microscopy and SDS-PAGE – 
Coomassie staining and fluorescence scanning with Typhoon scanner (GE 
Healthcare) – showed that the amount of N-WASP bound to silica beads increased 
linearly with the total amount of N-WASP input until a saturation point was reached. 
The linear range was used to calculate N-WASP surface density by assuming 100% 
of the N-WASP molecules were bound to the surface. A fluorescence-to-surface 
density calibration curve was generated. In parallel, an N-WASP membrane 
recruitment assay with the labeled lipid-beads was performed using identical optical 
settings on the microscope. This allows us to correlate N-WASP:lipid fluorescence 
ratio to N-WASP absolute fluorescence, and in turn, N-WASP surface density by 
applying the calibration curve generated above.
Chapter 3 – Dissecting the molecular mechanisms of local N-WASP amplification by actin
Abstract

In the preceding chapter, I described a new mechanism for actin symmetry breaking. Under conditions where Cdc42/RhoGDI concentration is limiting and the initial N-WASP surface density is low, an actin-driven feedback loop appears to provide the mechanism for promoting localized actin assembly and N-WASP clustering. In this chapter, I further explore the molecular mechanisms that govern the actin-driven feedback mechanism and symmetry breaking. I define the precise molecular interactions between N-WASP, the actin cytoskeleton, and membrane-associated signaling components that are required for the feedback mechanism. Finally, I examine how these interactions affect the dynamics of the signaling components at the membrane.
Introduction

Actin polymerization occurs in discrete zones in the cell and is regulated by WASP/WAVE-family proteins at the membrane (Mullins 2000). In turn, actin-dependent feedback loops are thought to locally amplify and spatially regulate upstream signaling components, such as Rac and the WAVE complex at the leading edge of a motile cell, causing cellular symmetry breaking (Bourne et al. 2002) (Charest 2006). In the previous chapter, I observed that sub-threshold levels of Cdc42 and N-WASP in the motility reaction were capable of inducing spontaneous assembly of polarized actin structures at the lipid-beads, suggesting that N-WASP surface density was increased during actin comet tail assembly. These observations led me to hypothesize that, in my in vitro system, actin-dependent feedback is responsible for the spontaneous formation of localized actin protrusions at the membrane, even though the surface lacks any pre-existing asymmetric cues.

Previous work from our laboratory showed that the WH2 domains of N-WASP make direct contacts with the barbed ends of actin networks at the membrane (Co et al. 2007). Although the affinity of the interactions between N-WASP WH2 and actin barbed ends is low, by virtue of the highly cross-linked nature of the actin network, the effective local concentration of barbed ends near the membrane surface is in the millimolar range. The WH2-barbed end interaction allows N-WASP to be transiently tethered at the membrane-actin interface, and it is essential for the asymmetric N-WASP distribution and co-localization at the actin comet tail during steady-state vesicle motility in cell-free extract (Taunton et al. 2000) (Co et al. 2007). However, it is unclear how the observed asymmetry is kinetically achieved along a uniform surface, i.e. how symmetry breaking occurs.

In addition to spatial regulation, actin polymerization appears to play a role in controlling the turnover dynamics of membrane-associated signaling molecules. A recent study of vaccinia virus motility found that actin polymerization increases the rate of recovery for N-WASP, WIP, and Nck at the membrane after photobleaching (Weisswange et al. 2009). Nevertheless, the molecular mechanisms by which a growing dendritic actin network exerts spatial and temporal control on signaling molecules at the membrane are not well understood.

In this chapter, I use the lipid-bead motility system to explore the underlying molecular mechanisms of the actin-driven feedback loop, which enables N-WASP and actin to spontaneously polarize at the membrane. I map the domains on N-WASP that are critical for its participation in feedback amplification. Finally, I quantify the effect of actin polymerization on turnover dynamics of N-WASP at the membrane.

Results

Actin-dependent feedback locally amplifies N-WASP

To understand how N-WASP surface density is dynamically controlled during actin assembly, I monitored the kinetics of N-WASP recruitment to lipid-beads during the earliest moments of actin comet tail formation (Figure 1a). I quantified N-WASP fluorescence by plotting the line profile through the center of the lipid-bead
along the axis parallel to the actin comet tail (Figure 1b, inset, yellow line). First, I found that N-WASP surface density increased over time, concomitant with actin comet tail elongation. Second, the rate of N-WASP accumulation proximal to the actin tail (Figure 1b, red arrows) was significantly faster than on the opposite side of the lipid-bead (Figure 1b, green arrows).

To test whether the apparent amplification of N-WASP density was dependent on actin polymerization, I performed the same experiment in the presence of Latrunculin B (LatB), which inhibits actin polymerization by binding tightly to actin monomers (Figure 2a). Line-scan profiles of N-WASP fluorescence – in a randomly chosen axis due to the lack of an actin structure – revealed no significant increase as a function of time. In addition, N-WASP distribution at the membrane was completely symmetrical during the course of observation (Figure 2b). Peak N-WASP density remained at basal levels (~900 molecules/μm²) in the absence of actin polymerization compared to the 4-fold increase (~3,900 molecules/μm²) observed during actin assembly (Figure 3). Note that this value is well below the ~16,000 molecules/μm² threshold defined in an earlier study with polystyrene beads and is closer to the ~1,900 molecules/μm² defined by a more recent study using Ni-NTA-liposomes and His6-VCA (Delatour et al. 2008). These results demonstrate that actin polymerization is essential for the increased density and polarized localization of N-WASP at the surface. A potential mechanism for this is as follows: upon N-WASP activation of Arp2/3 and creation of new barbed ends, N-WASP is “trapped” (or, preferentially retained by dissociation and rebinding) at the membrane-actin interface via interactions between its WH2 domains and actin barbed ends (Co et al. 2007). This promotes further nucleation events that are spatially restricted to regions where a dendritic actin structure already exists. As the density of barbed ends increases at the membrane-actin interface, more N-WASP molecules are trapped. As this cycle repeats, the local density of N-WASP would increase at the membrane-actin interface, concomitant with an increase of barbed ends, thus creating an actin-N-WASP positive feedback loop.
Figure 1 – N-WASP recruitment kinetics during initial actin assembly and symmetry breaking. 

**a.** Time-lapse sequence of actin network formation along a lipid-bead. **b.** Time evolution of line scans of Alexa594 N-WASP fluorescence (normalized against Alexa488-labeled membranes) along the central axis of the lipid-bead parallel to the actin network (inset, yellow line). N-WASP signal increases significantly at the side of the membrane proximal to the actin network (red arrows) compared to the distal side (green arrows) during actin tail formation and elongation. Scale bar: **a.** 5 µm; **b.** 2 µm.
Figure 2 – N-WASP recruitment kinetics when actin polymerization is inhibited by Latrunculin B (LatB). a. Time-lapse sequence of lipid-bead in the motility reaction supplemented by Latrunculin B. b. Time evolution of line scans of Alexa594 N-WASP fluorescence (normalized against Alexa488-labeled membranes) along the central axis of the lipid-bead (inset, yellow line). N-WASP did not increase or polarize during the course of observation. Scale bar: a. 5 µm; b. 2 µm.

Figure 3 – Maximal N-WASP:lipid fluorescence ratios (corresponding to red arrows in Figures 1b and 2b) over time obtained from time-lapse sequences from Figures 1a and 2a. (n = 8 lipid-beads per condition; error bars = s.d.)

Molecular requirements of the N-WASP-actin feedback amplification

To investigate which regions of N-WASP mediate the actin-driven feedback amplification mechanism, I developed an assay to quantify the local density of labeled mutant N-WASP constructs (50 nM) in the presence of excess (500 nM) unlabeled wildtype (WT) N-WASP. The low concentration of labeled mutants used, well below the detection limit of WT N-WASP in the absence of ongoing actin nucleation, ensures that the recruitment levels observed at the membrane can only be due to the actin-dependent feedback amplification mechanism.

<table>
<thead>
<tr>
<th>N-WASP Wildtype (WT)</th>
<th>151</th>
<th>B</th>
<th>GBD</th>
<th>PRD</th>
<th>Wa</th>
<th>Wb</th>
<th>C</th>
<th>A</th>
<th>501</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (9K)</td>
<td>KEKKGKAKKKRLTK</td>
<td>183</td>
<td>H208D (HD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4K</td>
<td>KEKAGAGGKRLTK</td>
<td>197</td>
<td>R410A/R438A (RA/RA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14K</td>
<td>KKKAKKKEKKGKAKKKRLTK</td>
<td></td>
<td>ΔA</td>
<td></td>
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</table>
A prediction from an actin-driven N-WASP amplification mechanism is that disruption of N-WASP interactions with actin network components – i.e. actin or Arp2/3 – would adversely affect N-WASP membrane recruitment. I found that mutations in the WH2 domains, R410A/R438A (RA/RA), which disrupt N-WASP–actin barbed end interactions (Co et al. 2007), essentially abolished N-WASP recruitment (ten-fold reduction relative to WT). By contrast, deletion of the Arp2/3-binding Acidic Region (ΔA) had little effect on actin-dependent recruitment of N-WASP (Figure 5). These results suggest that actin-dependent N-WASP amplification is a result of direct interactions between N-WASP and actin barbed ends; Arp2/3 binding is not required for the amplification mechanism.

Figure 5 – Representative images of Alexa594 N-WASP constructs (50 nM), with mutations that disrupt interactions with actin components, recruited to the lipid-beads undergoing actin-based motility driven by dark WT N-WASP (500 nM).

Figure 6 – Representative images of Alexa594 N-WASP constructs (50 nM), with mutations that disrupt interactions with membrane components, recruited to the lipid-beads undergoing actin-based motility driven by dark WT N-WASP (500 nM).
I next determined the role of N-WASP interactions with membrane-associated activators, PIP$_2$ and Cdc42, in the amplification mechanism. A point mutation in the GTPase-Binding Domain (GBD), H208D, which abolishes N-WASP-Cdc42 interaction (Miki et al. 1998), completely abrogated N-WASP localization to the membrane under these conditions. Similarly, an N-WASP construct with mutations that reduce the number of positively charged residues in the Basic Region (4K) and thereby weaken interactions with PIP$_2$ (Papayannopoulos et al. 2005) also failed to localize to the membrane-actin interface (Figure 6). These results show that N-WASP amplification absolutely requires binding to both PIP$_2$ and Cdc42 at the membrane even in the presence of ongoing actin nucleation mediated by excess WT N-WASP. Therefore, the actin-based feedback amplification mechanism does not bypass the requirement for Cdc42 binding as an initial activation step.

Combined with the result from Figure 5, these results suggest that the ability for N-WASP to localize to the membrane-actin interface requires either sequential or simultaneous interactions with PIP$_2$, Cdc42, and actin. To test whether weakened interaction with actin barbed ends could be rescued in cis, by enhanced binding to acidic phospholipids, I constructed an N-WASP mutant containing 5 additional lysine residues in the Basic Region coupled to the WH2 mutations (14K-RA/RA). During WT-powered motility, this N-WASP mutant (at 10-fold lower concentration) showed significant recruitment to the membrane (Figure 6). This observation suggests that recruitment defects caused by weakened affinity for actin barbed ends could be compensated by the increased affinity for PIP$_2$. It supports a model in which N-WASP binding to PIP$_2$ and actin is highly cooperative; each interaction significantly contributes to the overall N-WASP affinity for the membrane-actin interface.
Figure 7 – Normalized fluorescence intensities of Alexa594 N-WASP mutants (50 nM) recruited to the lipid-beads undergoing actin-based motility driven by dark WT N-WASP (500 nM) at steady state (t = 20 min; n = 30 lipid-beads per condition; error bars = s.d.)

![Graph showing normalized fluorescence intensities of N-WASP mutants](image)

Figure 8 – Membrane recruitment of Alexa594-labeled N-WASP constructs (WT, RA/RA, 4K, 14K, and 14K-RA/RA) to lipid-beads containing 2% PIP2. N-WASP fluorescence measurements are normalized against intensity of Alexa488-labeled lipid bilayer. N-WASP constructs bind to the membrane with apparent dissociation constants of $K_D \approx 0.3 \mu M$ for WT, RA/RA, 14K, and 14K-RA/RA, and $K_D \approx 1.1 \mu M$ for 4K (n = 30 per condition; error bars = s.d.).

![Graph showing membrane recruitment of N-WASP constructs](image)

Strikingly, the 14K extension did not increase the affinity of N-WASP for the lipid-beads in the absence of actin polymerization, despite the potential for increased PIP$_2$ affinity (Papayannopoulos et al. 2005). In fact, titration of 14K N-WASP in the presence of active Cdc42 revealed significantly decreased affinity for lipid-beads, presumably due to increased autoinhibition conferred by stronger intramolecular interactions between the Basic Region and the Acid Region of N-WASP (Figure 8). This result strongly suggests that the most critical contribution of the Basic Region/PIP$_2$ interaction is not in the initial membrane recruitment step; rather this interaction is essential for actin-mediated feedback amplification.

Collectively, these results indicate that recruitment of low concentrations (50 nM) of fluorescent N-WASP in the presence of excess dark N-WASP requires the cooperative interactions of: (1) Basic Region with PIP$_2$; (2) GBD with Cdc42; and (3) the WH2 Domains with actin barbed ends (Figure 7).
**Functional significance of the actin-N-WASP positive feedback loop**

The preceding experiments allowed us to specifically probe the molecular interactions required for N-WASP to participate in the actin-driven feedback loop. However, they did not allow us to test whether these interactions (and by extension, the feedback amplification mechanism) are essential for assembling a force-producing actin network. I therefore tested the effect of N-WASP mutations on the assembly of actin comet tails this time in the absence of WT N-WASP. Here, I quantified the steady-state comet tail lengths produced by the N-WASP mutants under my standard motility conditions, in which the initial N-WASP (and Cdc42/RhoGDI) concentrations are far too low to achieve a threshold density in the absence of the feedback amplification mechanism. I then tested the same mutants under pre-loading conditions, which bypass the requirement for actin-based feedback amplification, in order to rule out intrinsic defects in actin comet tail assembly.

As expected, both H208D and ΔA mutations rendered N-WASP completely inactive (Figure 9). This result is consistent with the known biochemical defects of the mutants; recruitment/activation by Cdc42 and recruitment/activation of the Arp2/3 complex, respectively.
Figure 9 – Actin assembly at the lipid-beads driven by N-WASP mutants.  

**a.** Representative images of actin tail morphologies at steady state (t = 20 min) under standard motility conditions, in the presence of N-WASP mutant (100 nM), Cdc42/RhoGDI (250 nM), GEF (50 nM DH-PH) and 2 µM GTP.  

**b.** Average lengths of actin comet tails generated by the N-WASP mutants in part a (n = 30 lipid-beads per condition; error bars = s.d.).

Figure 10 – Time-lapse images showing the birth of actin structures powered by N-WASP RA/RA mutant. While the construction of nascent actin structures at the lipid-beads can be observed, they rapidly detach and do not mature into force-generating actin comet tails. Scale bar = 5 µm.
Figure 11 – Representative images of actin tail morphologies (a) and corresponding tail length measurements (b) at steady state (t = 20 min). In this experiment, lipid-beads were pre-loaded with Cdc42 and N-WASP mutants prior to dilution into the motility mix. (n = 30 per condition; error bars = s.d.).
a

**PIP<sub>2</sub> density**

<table>
<thead>
<tr>
<th>2%</th>
<th>6%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA/RA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
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<tr>
<td>HD</td>
<td></td>
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<tr>
<td>Phase</td>
<td></td>
<td></td>
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<tr>
<td>Actin</td>
<td></td>
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</tbody>
</table>

b

![Graph showing actin comet tail length vs PIP<sub>2</sub> density for RA/RA and HD conditions](image)

- **RA/RA**
- **HD**

**Actin comet tail length (μm)**

- 2%
- 6%
- 10%
Figure 12 – Rescue of RA/RA and HD N-WASP with increasing PIP$_2$ density at the membrane.  a. Representative images of actin comet tails generated by RA/RA and HD N-WASP mutants using lipid-beads with varying mol% of PIP$_2$.  b. Tail length measurements for the lipid-beads in part a.  RA/RA and HD defects were partially rescued by increasing PIP$_2$ density at the surface.  Note that comet tails for RA/RA with 2% PIP$_2$ appeared slightly longer in this experiment compared to Figure 9.  Overall activity is influenced by batch-to-batch variability of different actin and Arp2/3 preparations.  Comparisons of tail lengths are only valid when performed in parallel.

RA/RA N-WASP was also significantly impaired in creating comet tails (Figure 9, 10), consistent with a requirement for barbed end binding in the N-WASP amplification mechanism.  Similarly, 4K N-WASP exhibited severe defects in producing a mature actin come tail.  The comet tails produced by 4K and RA/RA N-WASP were significantly shorter compared to WT N-WASP (Figure 9).  These results are consistent with the conclusion that N-WASP recruitment to the membrane and interactions with actin barbed ends are critical for mediating actin nucleation and stabilizing the connection between the actin network and the membrane (Co et al. 2007).

My results indicate that N-WASP mutants that failed to localize to the membrane-actin interface in the presence of excess WT N-WASP were also incapable of generating actin structures under conditions that required actin-dependent amplification of N-WASP density.  This correlation strongly suggests that the actin-N-WASP feedback loop is essential for recruitment and activation of a sufficient density of N-WASP to initiate and sustain lipid-bead motility.  The inability to participate in the feedback mechanism prevents N-WASP mutants from achieving the critical density needed for persistent actin network attachment, especially in the presence of negative actin regulators, such as capping protein and coflin, which place further demands on the ability of N-WASP to maintain stable connections between the membrane and the actin network.  To further substantiate a specific defect in the feedback amplification mechanism, I tested whether comet tail assembly defects caused by these mutations could be rescued by increasing their surface density via pre-loading, thereby bypassing the requirement of the amplification step.  Increasing the initial surface density of mutant N-WASP via pre-loading resulted in a striking increase in the length of comet tails assembled by both 4K and RA/RA N-WASP, such that they were almost indistinguishable from WT (Figure 11).  Nevertheless, HD N-WASP remained completely inactive in these experiments, further supporting the idea that N-WASP recruitment and activation critically depends on Cdc42 binding.

Previous work revealed that the Basic Region of N-WASP showed cooperative binding to PIP$_2$, and the apparent affinity was substantially elevated by increasing PIP$_2$ density (Papayannopoulos et al. 2005).  To test whether actin assembly defects exhibited by the mutants could be rescued by increasing PIP$_2$ surface density, I monitored actin assembly with lipid-beads containing higher PIP$_2$ densities (6% and 10%) under standard motility conditions (requiring feedback amplification).  N-WASP mutant activity was significantly enhanced when the PIP$_2$ density was at 6% and 10%, compared to 2%.  At the highest PIP$_2$ density (10%),
even HD N-WASP showed some ability to assemble actin comet tails (Figure 12). This is consistent with results from previous studies (Papayannopoulos et al. 2005) (Padrick et al. 2008) showing that N-WASP can be activated by a high density of PIP₂ (> 10 mol%), even in the absence of Cdc42. Moreover, the actin binding-defective RA/RA mutation was rescued in cis by the addition of 5 extra lysines in the Basic Region (14K-RA/RA), or in trans in the presence of lipid-beads that contained cholesterol (25%). Cholesterol presumably promotes microdomain formation, thereby increases the local density of PIP₂ (Figure 13). Time-lapse imaging revealed that relative to RA/RA, symmetry breaking and lipid-bead motility occurred sooner with 14K-RA/RA or with RA/RA in the presence of cholesterol. These results corroborate those in Figure 9 and demonstrate that an actin-dependent amplification mechanism is necessary for achieving sufficient density of N-WASP to initiate and sustain lipid-bead motility.

In summary, N-WASP amplification requires interactions with PIP₂, Cdc42, and actin barbed ends. M-WASP mutants with reduced affinity for any of these components fail to generate force-producing actin structures that are capable of propelling lipid-beads. However, defects in barbed end binding can be rescued by increasing N-WASP surface density or by increasing the density of PIP₂ and therefore the avidity of N-WASP for the membrane. Together, these results strongly support the notion that an actin-driven N-WASP amplification mechanism is essential for achieving sufficient N-WASP density at the membrane to initiate and sustain actin comet tail assembly, force generation, and lipid-bead motility.
Figure 13 – Time-lapse images showing initiation of actin assembly by N-WASP mutants with the RA/RA mutations. RA/RA N-WASP is severely defective in producing a force-generating actin comet tail, but the RA/RA defect could be rescued in cis by the 14K extension in the Basic Region or in trans by using lipid-beads that contained cholesterol (25 mol %), which likely promotes the formation of microdomains with increased PIP$_2$ density. Red arrows indicate the first moment of nascent actin structure assembly.

**The role of Cdc42 in the actin-N-WASP feedback loop**

The results shown in Figures 5 to 11 indicate that local formation of densely clustered N-WASP requires two elements: (1) Cdc42-dependent N-WASP localization to the membrane, and (2), local amplification of N-WASP density by actin barbed ends. Because Cdc42-binding is required for the initial N-WASP recruitment step as well as the subsequent amplification step (shown by HD N-WASP mutant, Figure 6), it would seem that Cdc42 density would also have to increase in an actin polymerization-dependent manner, even though Cdc42 is not known to directly interact with the actin network. Strikingly, this expectation was contradicted by careful quantitation of GTP-bound Cdc42 density in the presence and absence of actin assembly. Whereas N-WASP membrane density was augmented with increasing concentrations of N-WASP in a manner that required actin polymerization (Figure 15), Cdc42 density, as probed with fluorescent GTP (Figure 14), remained invariant under identical conditions (Figure 16). Furthermore, fluorescence images revealed that N-WASP distribution was highly polarized at the membrane during actin comet tail assembly (Figure 15), while Cdc42 was uniformly distributed with or without actin polymerization (Figure 16).
Figure 14 – Fluorescent GTP as a probe for Cdc42. Bodipy-Texas Red GTP was recruited to the membrane only in the presence of Cdc42 and the GEF, DHPH, in the reaction.
Figure 15 – N-WASP localization to the membrane in the presence of varying amounts of N-WASP, with or without actin polymerization.  

a. Representative images of Alexa594 N-WASP recruited to the membrane during actin polymerization with various concentrations of N-WASP in the reaction. N-WASP appeared polarized next to the actin comet tails. 

b. N-WASP fluorescence at the membrane, with or without actin polymerization. N-WASP signal is normalized to the condition with 100 nM N-WASP and no actin to indicate the fold increase in N-WASP surface density caused by actin polymerization and increasing N-WASP concentrations.
Figure 16 – Cdc42 localization to the membrane in the presence of varying amounts of N-WASP, with or without actin polymerization.  

a. Representative images of Cdc42 (probed by Bodipy-Texas Red GTP) recruited to the membrane during actin polymerization with various concentrations of N-WASP in the reaction. Unlike N-WASP, Cdc42/GTP was uniformly distributed along the membrane.

b. GTP fluorescence at the membrane, with or without actin polymerization. GTP signal is normalized to the condition with 100 nM N-WASP and no actin to indicate the fold increase in Cdc42 surface density caused by actin polymerization and increasing N-WASP concentrations.

The fact that Cdc42 density (unlike N-WASP density) is independent of N-WASP concentration and actin polymerization proves that the majority of N-WASP molecules at the actin-membrane interface are not bound to Cdc42. Thus, Cdc42 acts catalytically, rather than stoichiometrically, with respect to the number of N-WASP molecules at the membrane; although Cdc42-dependent N-WASP recruitment is a critical first step, N-WASP must be released from Cdc42 and maintained at the membrane. As my N-WASP structure-function analysis has shown, maintenance of N-WASP at the membrane requires interactions with PIP$_2$ and actin barbed ends.
Effects of actin polymerization on N-WASP dynamics at the membrane

An important and expected conclusion from the experiments presented above is that nucleation promoting factors, such as N-WASP, need not remain tightly bound to their upstream activators (e.g., Cdc42) as a 1:1 complex during steady-state actin-based motility. This model is consistent with the recent observation that the turnover rate of N-WASP at the membrane differs significantly from that of its upstream activator, Nck, during vaccinia virus motility in mammalian cells (Weisswange et al. 2009). In addition, the localization profile of active Rac was found to be significantly more diffuse than that of the WAVE complex at the leading edge of crawling neutrophils (Weiner et al. 2007). Similarly, Cdc42 distribution at the membrane is significantly more diffuse than N-WASP in our in vitro system (Figure 16).

A prediction of our model is that, depending on the interactions that maintain N-WASP molecules at the membrane – whether by actin, PIP2, and/or Cdc42 – their dynamics at the surface should be different. Using a pulse-chase experiment, I compared the turnover rates of labeled N-WASP recruited to the membrane via two different mechanisms – either by actin-driven feedback amplification or direct stoichiometric Cdc42 binding under pre-loading conditions.
Figure 17 – Pulse-chase experiment comparing the turnover dynamics of N-WASP recruited to the membrane by two different mechanisms – actin-driven amplification or Cdc42-dependent recruitment under pre-loading conditions. 

a. Representative images of labeled N-WASP dissociation from lipid-beads upon addition of a vast excess of dark N-WASP. 

b. N-WASP localized to the actin-membrane interface under amplification conditions exhibited significantly faster turnover rate (black curve, t$_{1/2}$ ~ 0.52 min) compared to N-WASP recruited by Cdc42 under pre-loading conditions (red curve, t$_{1/2}$ ~ 2.2 min).
Upon chasing with a ten-fold excess of unlabeled N-WASP, labeled N-WASP that has been recruited to the membrane during lipid-bead motility exhibited faster dissociation kinetics ($t_{1/2} \sim 0.52$ min) compared to N-WASP that had been pre-loaded onto lipid-beads via Cdc42 alone in the absence of actin polymerization ($t_{1/2} \sim 2.2$ min) (Figure 17). These results provide further evidence that N-WASP amplified by actin has dissociated from Cdc42 and is held at the membrane-actin interface via PIP$_2$ and actin barbed ends only. N-WASP residence at the membrane is more transient under conditions of actin-based feedback amplification conditions and lipid-bead motility.

**Discussion**

In this chapter, I have explored the molecular requirements for actin-driven N-WASP amplification at the membrane. Even though its initial recruitment and activation absolutely depends on Cdc42, N-WASP surface density is increased several fold during actin comet tail assembly and actin-dependent bead motility. To participate in the amplification step, N-WASP interactions with PIP$_2$, Cdc42, and actin barbed ends are essential. However, Cdc42 density is not increased concomitant with N-WASP, suggesting that Cdc42 activates N-WASP catalytically at the surface. N-WASP is released from Cdc42 upon activation, and at steady state, N-WASP is maintained at the membrane primarily by its interactions with PIP$_2$ and actin barbed ends. Consistent with this model, actin amplified N-WASP exhibits significantly faster turnover rates compared to N-WASP that is maintained at the membrane by Cdc42 and PIP$_2$ interactions. Even though the interaction between N-WASP and Cdc42 is significantly stronger (affinity of N-WASP B-GBD and Cdc42: $K_D \sim 70$ nM (Hemsath et al. 2005)) compared to that of N-WASP and actin barbed ends (affinity of N-WASP WH2 and G-actin: $K_D \sim 0.9$ µM, but $K_D$ is probably much higher for actin barbed ends (Co et al. 2007)), the sheer number of actin barbed ends in the dense actin network is significantly higher, and therefore acts as a thermodynamic sink which facilitates N-WASP release from Cdc42 while transiently trapping N-WASP at the membrane-actin interface.

**Functional significance of the actin-dependent feedback loop**

A key question that arose in the previous chapter is how actin assembly could spontaneously occur in a localized manner at the membrane. N-WASP surface density must be sufficiently high to facilitate actin assembly, yet when N-WASP is pre-loaded onto the membrane at high density, an isotropic actin structure appears uniformly across the entire surface rather than spontaneously appearing at a discrete sub-region. Here I have shown that, under conditions where Cdc42 and N-WASP concentrations are limiting (such that N-WASP density is below the threshold required to initiate lipid-bead motility), N-WASP is asymmetrically amplified at the surface by actin, leading to spontaneous symmetry breaking. The primary function of the actin-N-WASP feedback amplification mechanism is to generate a sufficiently high local N-WASP surface density to facilitate the assembly of a localized, force-producing actin structure. This model is supported by my observations that RA/RA N-WASP, which fails to participate in the amplification...
mechanism, is defective in assembling an actin comet tail under our standard motility conditions, even though it is perfectly capable of promoting Arp2/3-dependent actin nucleation in pyrene assays (Co et al. 2007). Consistent with our mechanistic model of feedback amplification, lipid-bead motility powered by RA/RA N-WASP was rescued under the following conditions: (1) pre-loading lipid-beads with high concentrations of Cdc42 and N-WASP (to achieve high initial densities) prior to adding motility components, (2) adding cholesterol to increase the local density of PIP$_2$, and thereby increasing N-WASP avidity, (3) increasing N-WASP avidity for PIP$_2$ by adding 5 lysines to the Basic Regions, or (4) directly increasing PIP$_2$ density, thereby increasing the avidity of RA/RA N-WASP for the actin-membrane interface, as well as potentially increasing the local density of N-WASP via PIP$_2$-mediated clustering (Padrick et al. 2008).

In addition to N-WASP surface density as a critical parameter for bead motility (Wiesner et al. 2003), recent mechanistic studies suggest that Arp2/3 activation is significantly enhanced when two N-WASP molecules bind to a single Arp2/3 complex, as compared to a 1:1 N-WASP/Arp2/3 stoichiometry (Padrick et al. 2008). This is corroborated by several studies showing that when multiple N-WASP molecules are in close proximity to one another, either via scaffolding by EspFU (Campellone et al. 2008) (Sallee et al. 2008) or oligomerization by SH3-containing adaptor proteins (Carlier et al. 2000) (Rohatgi et al. 2001) (Takenawa et al. 2007), actin assembly is significantly enhanced. Similar enhancement was achieved by artificial dimerization of GST-fused N-WASP VCA constructs (Padrick et al. 2008).

Nevertheless, despite the biochemical evidence suggesting that N-WASP needs to be densely arrayed at the membrane for optimal function, essentially nothing is known about the physiological mechanisms that lead to N-WASP recruitment at such a high local surface density, especially in the context of limiting Cdc42 concentrations in cells. My results strongly support the notion that, even in the absence of any forced dimerization mechanism (e.g. by poly-SH3 domain adaptors or GST), the actin-driven feedback loop could bring together neighboring molecules of N-WASP, potentially promoting the formation of 2:1 N-WASP/Arp2/3 complexes for efficient actin nucleation. The actin feedback loop could also promote further N-WASP clustering and enhanced Arp2/3 activation by facilitating the binding of the aforementioned adaptor and scaffolding proteins. Similar actin-dependent amplification mechanisms are likely to be important for membrane recruitment and clustering of cytosolic signaling factors that bind directly or indirectly to N-WASP (or WAVE), amplifying signal transduction cascades even when those factors are present at low concentrations in the cytoplasm and have low affinities for their downstream effectors or substrates.

**Implications of N-WASP activation by sub-stoichiometric levels of Cdc42**

A surprising observation from my studies is that Cdc42 activation of N-WASP appears to be catalytic. The idea that Cdc42 and N-WASP do not remain in a 1:1 stoichiometric complex is unexpected based on our current understanding of how Rho GTPases bind and activate WASP/WAVE-family proteins. However, my observations are consistent with recent studies showing that Rac and WAVE do not
perfectly co-localize at the leading edge, even though they are both polarized to the front of the motile cell (Weiner et al. 2007). In addition, turnover rates of N-WASP at the vaccinia actin comet tail are significantly slower than its upstream activators, WASP-interacting protein (WIP), Grb2, and Nck. These results further support the notion that N-WASP does not necessarily remain tightly associated with its activators, provided that a sufficient number of interactions exist to maintain N-WASP at the actin/membrane interface (i.e. sufficient density of barbed ends and PIP2). PIP2 and high density of actin barbed ends, while not sufficient to activate soluble auto-inhibited N-WASP, are clearly sufficient to maintain N-WASP in its active conformation at the membrane/actin interface once it has been activated by Cdc42.

As discussed in the last chapter, given the low cellular concentrations of Cdc42, the maximum density of N-WASP that could be recruited to the membrane surface is significantly below what is needed for actin assembly. When the actin-driven feedback loop is coupled to catalytic activation by Cdc42, N-WASP density can be amplified substantially without requiring the corresponding increase in Cdc42 levels at the membrane. There appear to be several potential advantages to such an amplification mechanism. First, when N-WASP is released from Cdc42 after being activated, it allows each molecule of Cdc42 to undergo multiple rounds of N-WASP recruitment and activation. A weak diffuse “signal” from a low density of Cdc42 can thus be converted into a strong highly localized signal of activated N-WASP. Perhaps even more important is the increase in N-WASP dynamics at the membrane. My in vitro data clearly indicate that if N-WASP remained bound to Cdc42 at the surface, it would have significantly slower dissociation rates (and therefore, slower flux rates) compared to actin-amplified N-WASP. These characteristics may contribute to the dynamics of signal transduction in cells, where signaling cascades that control cell motility have to be rapidly activated and deactivated as needed in response to environmental changes. My studies provide mechanistic insights into how the actin network could regulate the spatial organization and dynamics of signaling complexes at the membrane.

Materials and methods
Materials
Bodipy-Texas Red GTP was obtained from Invitrogen.

N-WASP mutant cloning, expression, and labeling
N-WASP mutants were generated by quick change or SOEing PCR using our ΔEVH1 N-WASP wildtype construct as a template. 14K N-WASP was amplified from a plasmid for mammalian transfection (Papayannopoulos et al. 2005) and cloned into a His6-tagged vector for expression in E coli. 14K RA/RA N-WASP was made with the technique of gene splicing by overlap extension (SOEing PCR) (Horton 1995) using the 14K construct and RA/RA as templates. All N-WASP mutant constructs were verified by sequencing. N-WASP mutants were expressed and purified from E coli following our standard procedure for N-WASP purification (Co et al. 2007). Mutants were labeled with Alexa594 maleimide using our standard
protocol (Co et al. 2007). Degree of labeling and concentration measurements were performed with a Nanodrop instrument (Thermo Scientific) and verified by Coomassie staining and fluorescence scanning with a Typhoon scanner (GE Healthcare) of SDS-PAGE gels.

**Time-lapse movies of lipid-bead propulsion**

Lipid-beads were added to the motility reaction with components as indicated in the text. Time was set to zero at the moment lipid-beads were introduced into the motility medium. A 2.3 µL sample was spotted onto silanized or BSA-coated glass slide and covered with 18 mm x 18 mm cover glass, giving a chamber height of 4.5 µm. The sample was then brought to the microscope, and imaging was started as quickly as possible to capture the initial moments of actin comet tail formation. Typical frame rates were set at 20 s intervals.

**Actin comet tail length measurement**

To measure steady-state lengths of actin comet tails, the motility reaction was supplemented with actin labeled with Alexa568 (1 µM). At t = 20 min all components had been combined, a 1 µL sample was spotted onto the glass slide and fixed with 1 µL of 3% glutaraldehyde (final conc = 1.5%). Microscopy images were acquired and analyzed with ImageJ. The tail lengths were measured using the segmented line ROI selection tool along the comet tail. Lipid-beads that did not have any comet tails attached were counted as having a tail length of zero. The measured lengths in pixels were converted to microns according to the calibration of our microscope system (at 60X: 1 pixel = 0.1066 µm).

**Pulse-chase experiments**

The turnover rates of N-WASP recruited to the membrane via actin-driven feedback and under pre-loading conditions were compared. Under the pre-loading condition, lipid-beads were incubated for 30 min at room temperature with 1.25 µM Cdc42-RhoGDI, 250 nM DH-PH, 10 µM GTP, and 3 µM N-WASP (5% labeled). After that the pre-loaded lipid-beads were diluted 5X into the motility, except without actin. For the amplification condition, lipid-beads were added to the standard motility reaction, except containing 600 nM N-WASP (5% Alexa594-labeled). Ten minutes after the lipid-beads were added to the reaction tube, an excess of dark N-WASP (6 µM) was added to the reactions, at which point the time was set to zero. A 1 µL sample of the reaction was fixed with 1.5% glutaraldehyde at each indicated time point and imaged under the microscope. N-WASP dissociation kinetics was estimated from single-exponential decay curve-fitting using Prism (MathPad).
Chapter 4 – Discussion
Summary

Actin polymerization provides the driving force for membrane protrusion and forward movement (Pollard et al. 2009). In addition, actin-dependent feedback loops are essential for compartmentalizing and amplifying signaling events at the membrane, as well as for cellular symmetry breaking (Charest et al. 2006) (Xu et al. 2003). How molecular interactions between actin and signaling components contribute to feedback amplification and spatial regulation at the membrane is not well understood. In this work, I have reconstituted a simple actin-N-WASP feedback circuit with purified components to elucidate the underlying molecular mechanisms. It should be noted that, prior to this work, the biochemical mechanisms underlying actin-mediated feedback loops were completely unknown. To my knowledge, this work is the first to reconstitute such a feedback loop from purified components.

First, I found that at physiological concentrations, Cdc42 could not recruit N-WASP at a sufficient membrane density to support actin comet tail assembly. Rather, local N-WASP amplification by actin-dependent feedback is absolutely required to promote the formation of force-generating actin structures. Second, I discovered that Cdc42 activation of N-WASP is catalytic; while Cdc42 is required for initial N-WASP recruitment and activation (Figure 1, step 1), activated N-WASP is released from Cdc42 (Figure 1, step 2) and is transiently associated with the membrane by cooperative interactions with PIP_2 and actin barbed ends (Figure 1, step 3). The fact that both are present at a high local density is likely the key thermodynamic and kinetic driving force for the feedback amplification mechanism. Third, the actin-N-WASP feedback loop results in a novel symmetry breaking mechanism, by which polarized actin networks and N-WASP recruitment are spontaneously localized to discrete sub-regions along the membrane.
Figure 1 – Model of the actin-N-WASP positive feedback loop in which N-WASP is catalytically activated by Cdc42 and amplified by the actin network. Initial N-WASP membrane recruitment requires interaction with Cdc42 (1), but N-WASP is then released (2) and remains transiently held at the membrane via its interactions with PIP$_2$ and the actin network barbed ends (3). This mechanism leads to the spontaneous accumulation and clustering of N-WASP and localized actin assembly. Arp2/3-dependent nucleation events and the presence of a highly dense network of barbed ends may increase the rate of N-WASP dissociation from Cdc42.

**Actin-N-WASP feedback in the context of existing models of positive feedback mechanisms**

At the heart of cellular symmetry breaking are signaling pathways that incorporate feedback amplification mechanisms (Drubin et al. 1996). For actin-driven processes, such as cell motility and polarization, it has long been thought that a local excitation/global inhibition (LEGI) mechanism, which involves generating regions of activated signals at the “front” via positive feedback loops (such as actin-dependent amplification of Rac and PIP$_3$) and global negative regulation that shuts off the activated signals elsewhere, is required for the asymmetry observed in cells (Van Haastert et al.) (Meinhardt et al. 2000). However, in my reconstituted system, it appears that a single actin-driven feedback loop is sufficient for producing localized N-WASP distribution and actin protrusions, although capping protein is present to prevent spontaneous actin polymerization away from the membrane, and therefore, could potentially act as a “global inhibitor”.

My experimental observations lend support to a recent theoretical model (Altschuler et al. 2008), in which spontaneous polarization and asymmetric membrane distribution of a single self-recruiting signaling species can be generated by a simple positive feedback loop. The key finding from the theoretical study is that to maintain polarized distribution of the activated signal, the total concentration of the signaling molecule must remain low. At regimes of high concentrations, recruitment of the signal is predominantly governed by its intrinsic kinetics for membrane association, and therefore, the signal is recruited everywhere along the membrane, and the system fails to polarize. By contrast, at low concentration regimes, membrane recruitment of the signaling molecule is primarily driven by the strength of the positive feedback, and thus a discrete zone enriched in the activated signal can be created and sustained at the membrane. Indeed, the two symmetry-breaking mechanisms in my *in vitro* assays capture the two extremes of this theoretical model. In my system, spontaneous polarization of the actin structure and asymmetric distribution of N-WASP at the surface is driven by the actin-N-WASP feedback loop only when the concentrations of Cdc42/N-WASP are “low” – i.e. the condition without N-WASP pre-loading. In the presence of high concentrations of Cdc42/N-WASP, such as when N-WASP is pre-loaded at a high density, actin is assembled everywhere along the membrane, and thus symmetry breaking requires the additional mechanism of fracture by mechanical stress.
Actin-driven feedback in the context of a motile cell

Actin-dependent feedback loops govern the overall directionality and dynamics of cellular movement. Neutrophils undergoing chemotaxis are capable of sensing minute amounts of chemotactic signals released by bacteria or a shallow gradient of ligands. In addition, neutrophils have the remarkable ability to rapidly adapt to changes in the direction of the signal gradient or bacterial movement (Xu et al. 2003). These observations suggest that the underlying actin-dependent feedback loops that promote actin protrusions at the leading edge must simultaneously confer sensitivity, adaptability, and spatial fidelity to the system. Numerous theoretical and in silico models have been developed to understand how these properties are propagated in feedback signaling networks (Welf et al. 2010) (Onsum et al. 2007), yet it remains unclear how molecular interactions could produce these behaviors during actin assembly. Could the mechanisms I have uncovered in the simple actin-N-WASP positive feedback loop explain some of these features?

Even though I have not directly addressed these properties in my in vitro reconstituted system, my experimental observations do provide a few mechanistic clues as to how sensitivity, adaptability, and spatial fidelity could be achieved in a simple actin-driven positive feedback loop. First, by virtue of the highly cross-linked actin network, and the fact that N-WASP amplification requires direct interactions with the actin barbed ends, actin-driven amplification of N-WASP is preferentially confined to sites where existing actin structures have already been built, thereby preserving the spatial fidelity and directionality of actin polymerization.

Second, since Cdc42 catalytically activates N-WASP during actin-driven amplification, each molecule of Cdc42 can potentially produce a large number of activated N-WASP molecules at the membrane, suggesting that N-WASP activation and actin assembly are “sensitive” to a small amount of Cdc42 signal at the surface. By contrast, if N-WASP were not released from Cdc42 upon activation, Cdc42 at the surface would quickly become saturated with activated N-WASP, preventing subsequent rounds of N-WASP activation and amplification. In addition, this system would require a much higher, stoichiometric number of Cdc42 molecules at the surface to achieve the same level of N-WASP recruitment for actin assembly.

As seen in the pulse-chase experiments, coupling actin-dependent N-WASP amplification to catalytic activation by Cdc42 provides the added benefit that amplified N-WASP has significantly faster turnover rates compared to N-WASP that remains bound to Cdc42. The transient nature of N-WASP localized to the membrane-actin interface could be the built-in instability that allows quick deactivation or changes in the direction of actin assembly. Just as Cdc42 is significantly more diffuse at the membrane compared to N-WASP, Rac appears to be more diffusely distributed compared to the WAVE complex at the leading edge of motile cells (Weiner et al. 2007). I hypothesize that upon activation by Rac, the WAVE complex is released from Rac and is transiently associated with the membrane by the actin network in a manner analogous to the Cdc42/N-WASP system. Furthermore, with the recent discovery that Arp2/3-dependent nucleation events and actin polymerization increase the flux of N-WASP at vaccinia actin
comet tails (Weisswange et al. 2009), the turnover rates of the WAVE complex at the membrane-actin interface would similarly be increased, and the actin structures produced could be more adaptable to changes.

**Impact of Cdc42 cycling on N-WASP signaling and amplification**

In this study, I have primarily focused on the kinetics and thermodynamics of N-WASP recruitment and amplification at the membrane. While it is clear that N-WASP membrane partitioning critically depends on Cdc42 levels at the surface, I have not explicitly considered how Cdc42 density and dynamics are controlled at the membrane. During actin-driven amplification, upon being activated at the membrane, N-WASP is released from Cdc42. What happens to the Cdc42 molecule that is freed up by the release of N-WASP? In my simple system, Cdc42 could become inactivated by hydrolyzing the GTP into GDP and be extracted by RhoGDI, or it could activate another N-WASP molecule at the surface. Physiologically, Rho-family GTPases are controlled by additional signaling factors, including GTase-activating proteins (GAP), which speed up the rate of GTP hydrolysis and GTase inactivation. Computer modeling shows that GEF and GAP work together to set the steady-state levels, as well as the turnover rates, of activated GTases (Goryachev et al. 2006). How these properties are affected in the presence of a membrane, N-WASP, and continuous actin polymerization remains unclear. I postulate that at the surface, N-WASP could compete with the GAP and protect Cdc42 from rapid GTP hydrolysis and RhoGDI extraction. Therefore, Cdc42 molecules that are proximal to existing actin structures, where a high density of amplified N-WASP is present, will likely remain active longer for multiple rounds of N-WASP activation. Conversely, the Cdc42 molecules that have diffused away from the membrane-actin interface will be quickly deactivated by GAP and extracted by RhoGDI. The net effect could be a strongly biased distribution of activated Cdc42 towards the actin comet tail, unlike the diffuse distribution I observe in my current experimental system. This could further strengthen the spatial fidelity of Cdc42/N-WASP signal transduction by sharply focusing Cdc42/N-WASP activation only at the sites of actin assembly.

**Feedback control of other signaling complexes**

Even though I have primarily focused on N-WASP activation and amplification in this study, actin-driven feedback loops could also amplify other signaling molecules at the membrane-actin interface by promoting their co-clustering with WASP/WAVE-family proteins. First, the proline-rich region of N-WASP is known to interact with a variety of SH3-containing proteins, such as Nck, Grb2, and Toca-1 (Rohatgi et al. 2001) (Carlier et al. 2000) (Ho et al. 2004). Many of these SH3-containing proteins have been shown to activate N-WASP in vitro, by dimerizing and oligomerizing N-WASP. The actin-dependent amplification step could further increase the rate, and perhaps the extent, of SH3-dependent N-WASP activation by bringing adjacent N-WASP molecules in close contact to one another and facilitating SH3-induced oligomerization.
Furthermore, actin-N-WASP feedback could also locally enhance phosphorylation events at the membrane by localizing Src-family kinases and their substrates together at the sites of actin assembly, effectively raising local concentrations of the reactants and speeding up the rate of reaction. N-WASP itself is a substrate of Src, which has been shown to increase the activity of WASP proteins by phosphorylation (Torres et al. 2003). In addition, many SH3-containing proteins implicated in actin polymerization, such as Tks5 and Cortactin, are also substrates of Src kinases. Their phosphorylation, as well as co-clustering with other SH3-containing proteins and WASP proteins, has been shown to enhance actin polymerization in vitro (Tehrani et al. 2007) and is essential for invadopodia/podosome assembly during cancer cell invasion in vivo (Stylli et al. 2009) (Oser et al. 2009). Together, the notion of an actin-dependent local enhancement of kinase activities, which in turn further promote actin assembly, would add another layer of feedback control and spatial regulation to signaling events at the membrane.

Aside from bringing signaling complexes together, the dendritic actin network assembled by N-WASP and Arp2/3 has been recently shown to be capable of modulating the biophysical properties of the lipid bilayer, including phase transition behaviors of microdomains (Liu et al. 2006). Therefore, an actin-driven feedback mechanism could further promote enrichment of membrane-associated and integral membrane protein complexes by inducing changes in the local lipid environment and stabilizing microdomains.

**Concluding remarks**

Traditional biochemical assays of actin polymerization have allowed us to probe only a small subset of components in isolation at a time. By integrating actin assembly and Cdc42/N-WASP signaling in a reconstituted system, I have gained invaluable insights about actin biology on a systems level and with finer molecular detail. I am now poised to further investigate how biochemical, biophysical, molecular, and mechanical mechanisms work in concert to create the complex behaviors of actin assembly and signaling at the membrane. With these new tools, future studies will, no doubt, “amplify” our understanding of how cells (and ultimately life) work.


