DISCOVERY AND CHARACTERIZATION OF WISH/DIP/SPIN90 PROTEINS AS A CLASS OF ARP2/3 COMPLEX ACTIVATORS THAT FUNCTION TO SEED BRANCHED ACTIN NETWORKS

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Assembly of branched actin filaments produces dynamic structures required during membrane associated processes including cell motility and endocytosis. The Actin Related Protein 2/3 (Arp2/3) complex is the only known regulator capable of nucleating actin branches. To specify the sub cellular localization and timing of actin assembly the complex is tightly regulated. Canonical activation of the Arp2/3 complex by Wiskott-Aldrich Syndrome proteins (WASP), requires preformed actin filaments, ensuring the complex nucleates new actin filaments off the sides of preformed filaments. WASP proteins can therefore propagate branch formation but cannot initiate a Y-branch without performed filaments. A key question, then, is what is the source of preformed filaments that seed branched actin network formation in cells? It is unclear how activation of Arp2/3 by multiple regulators is balanced to specify actin filament architectures that are productive *in vivo*. In this dissertation, we identified WISH/DIP1/SPIN90 (WDS) family proteins as activators of the Arp2/3 complex that do not require preformed filaments, and evaluated whether WDS proteins seed branching nucleation.

In chapter II, we dissected the biochemical properties of WDS proteins and found they activate the Arp2/3 complex using a non-WASP like mechanism. Importantly, we discovered WDS-mediated Arp2/3 activation produces linear, unbranched filaments, and

this activity is conversed from yeast to mammals. These observations highlight that WDS proteins have the biochemical capacity to seed actin branches.

In chapter III, we observed WDS-generated linear filaments can seed WASP-mediated branching directly using single molecule microscopy with fluorescently labeled Dip1. We find that WDS-mediated nucleation co-opts features of branching nucleation.

In chapter IV, we investigated how WDS activity is balanced with WASP. We discovered WDS proteins use a single turnover mechanism to activate Arp2/3 and this is conserved during endocytosis. In contrast, WASP-mediated activation is multi-turnover, highlighting a crucial difference between WDS proteins and WASP. Our observations explain how Arp2/3 may limit linear filament production to initiate networks and favor branches during network propagation. Finally, we use fission yeast to show that increasing Dip1 is sufficient to cause defects in actin assembly and the timing of actin patches at sites of endocytosis.

This dissertation contains previously published and unpublished co-authored material.

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I dedicate this work to my brother Greg

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CHAPTER I

INTRODUCTION

THE CYTOSKELETON IS THE SCAFFOLD OF A CELL

Cells use a scaffolding system to organize molecules, adopt shape, and control the dynamic processes that give rise to life. The scaffold, called the cytoskeleton, is comprised of three filamentous protein networks; actin, microtubules, and intermediate filaments. Assembly of actin filaments allows cells to rapidly remodel in response to signals. Cell survival is actin dependent. The last common ancestor to bacteria and eukaryotes is thought to have an actin-like protein and all eukaryotic organisms from single celled fungi to multicellular mammals have well conserved actin genes (Welch and Mullins 2002). Actin is one of the most abundant proteins on earth because of high intracellular concentrations (Pollard and Cooper 2009).

The actin cytoskeleton is dynamic. Dense arrays of actin filaments are important for a diverse range of cellular activities including cell motility, phagocytosis, cell division, and protrusive structures like filopodia and invadapodia. In addition, pathogens hijack control of host-cells actin machinery to propel themselves and infect neighboring cells (Pollard and Borisy 2003). During these processes, actin provides mechanical forces to push, move, and pull on membranes (Blanchoin et al. 2014). Assembly of complex actin filament architectures requires the coordinated action of an assortment of actin binding proteins. To understand how the spatiotemporal undercurrents of actin networks are controlled, a complete list of the interacting proteins, their connections, and associated biochemical properties is needed. Determining how the actin cytoskeleton is regulated is an active topic of investigation with key questions left unanswered, how are these networks initiated? And what is the relationship between regulation and function? This dissertation will focus on attaining a mechanistic understanding of how actin systems are initiated by characterizing the proteins involved in the growth of these networks.

BUILDING A NETWORK FROM ACTIN MONOMERS TO FILAMENTS

A slow nucleation step is the key regulatory control point in actin polymerization. Before a filament is formed a critical nucleus of three actin monomers needs to exist (Cooper, Walker,

and Pollard 1983; Sept and McCammon 2001). Actin monomers stochastically collide within the cell to form dimers and trimers. These dimers of actin rapidly fall apart, which creates a barrier to the nucleation of new filaments. Moreover, in cells there are monomeric actin binding proteins, tymosin β_4 and profilin, that prevent the spontaneous nucleation of new actin filaments by sequestering and preventing monomers from sticking together (Pollard, Blanchoin, and Mullins 2000). As a result, specialized proteins have evolved to control the nucleation of actin within cells.

A variety of actin nucleators exist to overcome the barrier to making filaments from scratch. *De novo* actin nucleators fall into three distinct classes based on the mechanism used to bring actin monomers together and the type of filament generated. Two classes of nucleators produce linear filaments; formin family proteins and WH2 domain containing proteins like JMY, spire, and COBL (Campellone and Welch 2010). The third class has only one member, the Arp2/3 complex, with the unique in the ability to produce Y-branched filaments (Goley and Welch 2006). Nucleation is hallmark step in the creation of an actin network because it defines the subcellular localization where the first filaments are made. Additionally, the architecture of actin filaments is foremost the result of how actin filaments are formed.

How can a nanometer sized protein cause micron sized changes in cell shape? The ability of actin monomers to assemble into filaments turns nanometer physical dimensions into dense arrays with a micron sized influence (Blanchoin et al. 2014). The cellular pool of actin exists in equilibrium between its monomeric, or globular, and filamentous form. Monomers assemble into a two stranded helical polymer that elongates at a rate proportional to the available pool of globular actin (Cooper, Walker, and Pollard 1983). Actin filaments are polarized. The fast growing end, called the barbed end, is the physiologically relevant end of growth compared to the pointed end which elongates much more slowly (Pollard and Berro 2009). Actin binds and hydrolyzes ATP during polymerization, which has been hypothesized to form a timing mechanism that marks the age of actin filaments based on the presence of ATP or ADP (Iwasa and Mullins 2007). Over one hundred proteins interact directly with both monomers and filaments of actin to directly regulate its function. Monomeric actin binding proteins can influence how monomers are incorporated into filaments. Filament binding proteins modulate the arrangement of filaments and form crucial connections between filaments and other structures in

the cell, like the plasma membrane. Additionally, actin filaments are bundled, arranged in circular or linear arrays, and branched by these proteins to create diverse network geometries.

The organization of filaments is thought to be directly linked to the function of the network (Svitkina and Borisy 1999). In general, dense networks of F-actin form viscoelastic gels that can push or deform the plasma membrane (Walani, Torres, and Agrawal 2015). The amount of cross-linking and branching is directly proportional to the stiffness of the network (Pujol et al. 2012). Similarly, the F-actin binding proteins α-actinin and fascin both bundle filaments but have distinctive spacing between each individual filament. The separation between filaments is governed by the physical size of α-actinin and fascin. As a result, holes or pores with different sizes act as a filter to sort and segregate diffusing proteins when the filament network is encountered (Winkelman et al. 2016). The actin network architecture is defined as the overall arrangement and spacing of branches, cross-linked filaments, and bundled filaments. How filaments arrange into complex architectures and how architecture is related to cellular function are important unanswered questions.

Branched actin is necessary to achieve the architecture of actin networks observed *in vivo* and branching nucleation activity is controlled by a combination of regulatory proteins. Assembly of branched actin filaments by the Arp2/3 complex initiates a positive feedback loop where branches created by the complex form templates for additional branching. The kinetics of branched actin assembly are non-linear and proceed through an initial lag phase representing the delay before substrate mother filaments are nucleated (Marchand et al. 2001). Once the branching process begins the rate of filament formation increases exponentially. This mechanism is efficient and can generate dense arrays of filaments over a short period of time. At the leading edge of the cell, rapid branched actin assembly is coupled with capping of the newly formed ends and cross-linking filaments. While new filaments are generated at the front edge of the network, filaments at the tail are severed and disassemble. Monomers diffuse back to the front to create a tread milling network. A comprehensive model of the retrograde flow of a branched actin network has been named the dendritic nucleation model (Pollard, Blanchoin, and Mullins 2000).

ACTIN NUCLEATORS ARE REGULATED

Since spontaneous nucleation is actively inhibited within the cell, nucleator activity is also regulated to help define when and where filaments are generated. Surprisingly, nucleators are sufficient to define the composition of actin structures (Michelot and Drubin 2011; Michelot et al. 2010). Formins make linear actin filaments by using a Formin Homology domain, FH2, that is adequate to drive nucleation (Sagot et al. 2002; Pruyne et al. 2002). Formins are unique because the FH2 domain remains bound to the barbed end following nucleation and moves along with the newly growing end, acting as a cap. The processive movement of formins along the filament end increases the rate of elongation as the second formin homology domain, FH1, delivers profilin bound monomers to the newly growing end. Profilin regulates this activity by favoring formin nucleation and elongation over other filament nucleators, like the Arp2/3 complex (Suarez et al. 2015). All other linear filament nucleators fall into a group based on the presence of repeating actin monomer domains, namely WH2 domains. WH2 domains bind actin using an amphipathic helix that slides into the barbed end groove of the actin monomer. WH2 domains are arranged in tandem with a range of repeats. SPIRE, for example, has four repeats organized linearly whereas JMY has two adjacent repeats and a third separated by an intermediate poly proline region. WH2-domain containing nulceator activity is partially governed by the ratio of the protein:actin, with low ratios favoring nucleation and high ratios favoring sequestration (Campellone and Welch 2010). Since actin networks are assembled from a common pool of monomeric actin the regulation of nucleation activity helps distribute distinct actin networks within the cytoplasm.

The Arp2/3 complex is the only known actin regulator capable of nucleating Y-branched filaments. Branched actin filaments are important for cellular processes like endocytosis, cell motility, and neuritogenesis (Kaksonen, Toret, and Drubin 2006; Korobova and Svitkina 2008; Pollard and Borisy 2003). The Arp2/3 complex is a large 226 KDa stable assembly of seven proteins named for the largest two subunits, actin related proteins 2 (Arp2) and 3 (Arp3), which share a structural homology to actin itself (Robinson et al. 2001; Nolen and Pollard 2008). New daughter filaments are created off the sides of pre-existing mother filaments at a characteristic ~70 degree angle (Amann and Pollard 2001). To prevent ectopic branch formation, the complex is intrinsically inactive (Rodnick-Smith et al. 2016). Regulatory proteins bind and stimulate an

activating conformational change through a multi-step mechanism (Higgs, Blanchoin, and Pollard 1999). At this stage the complex is still not active; the regulator bound complex must bind to the side of a pre-existing mother filament before a new filament grows (Achard et al. 2010; Blanchoin et al. 2000). While inactive, Arp2 and Arp3 are oriented away from each other and split open in a splayed conformation. Upon activation the two subunits slide together to adopt an actin filament-like conformation that forms a template for daughter filament growth where Arp2 and Arp3 are the first two subunits of the filament (Hetrick et al. 2014). Once created, the daughter filament is free to grow from the barbed end while the pointed end remains firmly bound to the Arp2/3 complex at the branch point (Rouiller et al. 2008). The biochemical requirement for this assembly to bind to a preformed filament ensures that the complex creates exclusive Y-branched actin filaments.

Multiple types of regulatory proteins, called nucleation promoting factors (NPFs), tightly regulate Arp2/3 complex activity using partially redundant activation mechanisms. Almost all activating proteins known to date share a conserved region to bind the complex consisting of central (C), and acidic (A) sequences (Higgs, Blanchoin, and Pollard 1999). Wiskott-Aldrich Syndrome proteins (WASp) and SCAR/WAVE proteins form a family of archetypal activators that are the most well characterized (Goley and Welch 2006). Within this family there is a wide range of activation potency. Neural WASp (NWASp) is the strongest and ubiquitously expressed whereas WASp is weaker and only expressed in hematopoietic cells (Jonathan Zalevsky et al. 2001; Bompard and Caron 2004). The discovery of additional mammalian NPFs, including WASH and WHAMM, expanded the repertoire of Arp2/3 complex activity to include endosomal trafficking and ER-Golgi transport (Rottner, Hänisch, and Campellone 2010). The variety of Arp2/3 complex regulators implies that the function of the complex can be tuned for specific activities by utilizing different regulators. NPFs with a range of potency may function to fine tune Arp2/3 complex activity.

Balancing regulation of the Arp2/3 complex by multiple NPFs influences the kinetics and efficiency of branching nucleation. Most actin rich structures contain multiple NPFs. Invadapodia and sites of endocytosis, for example, contain the weak activator cortactin and type I myosins (a M. Weaver et al. 2001; Sirotkin et al. 2005; Ayala et al. 2008). How distinct Arp2/3 complex regulators function in concert is emerging as an important question. When presented with multiple NPFs, the Arp2/3 complex must integrate inputs from multiple signaling pathways.

A few models shed light on how the Arp2/3 complex balances input from multiple regulatory proteins, in the first the complex is coordinated regulated by NPFs and the second NPFs compete for activation of the complex. Response to regulatory input is crucial for Arp2/3 function because Arp2/3 complex has little to no NPF-independent activity, but even in the presence of an NPF the complex is an inefficient enzyme. The total number of barbed ends generated in Arp2/3 stimulated actin networks is far less than the total concentration of Arp2/3 (Marchand et al. 2001). Amazingly, only 1% of Arp2/3 complex binding events lead to branch formation (B. a. Smith et al. 2013). The majority of filament binding events might be unproductive because the CA region of WASp family proteins is bound tightly and has to be released before a new filament can grow (Benjamin a Smith et al. 2013; Marchand et al. 2001). Addition of other NPFS can speed up this process. For example, when cortactin is combined with NWasp-VCA, the two proteins synergistically activate the Arp2/3 complex (A. M. Weaver et al. 2002; Uruno et al. 2001). Single molecule studies dissected the NPF interplay and found that cortactin functions to displace WASp from Arp2/3 at a nascent branch junction (L. a. Helgeson and Nolen 2013; Luke a Helgeson et al. 2014). This mechanism speeds up a slower nucleation process through coordinated regulation of Arp2/3 complex. Importantly, NPFs could also compete with one another for activation of the complex.

THE SEARCH FOR A SEED FILAMENT

The requirement for Arp2/3 complex to bind to the side of a preformed filament before branching creates a paradox. New filaments cannot be created until an initial filament exists, but where does this initial filament come from? The search for the seed filament generator in cells has been the focus of many recent studies (Goode, Eskin, and Wendland 2015). Several candidate mechanisms and proteins have been proposed but the field has yet to reach a consensus. Linear nulceators, like formin family proteins, present the first obvious candidates based on the ability to make filaments *de novo*. However, Linear filament structures , like actin cables and contractile rings, are generally spatially separated from Arp2/3 generated networks (Kovar, Sirotkin, and Lord 2011). Moreover, formin nucleated filaments rapidly associate with the filament binding and stabilizing protein tropomyosin (Wawro et al. 2007; Ujfalusi et al. 2012). Tropomyosin decorated filaments are poor substrates for Arp2/3 mediated branching and are excluded from branched actin structures in fission yeast (DesMarais 2002; Skau, Neidt, and

Kovar 2009; Christensen et al. 2017). This data suggests that it is unlikely that formins are the primary seed generators in cells, but there may be special scenarios where this activity can contribute. For example, one study suggests that during lammelipodia initiation and ruffling the formin mDia1 cooperates with Arp2/3 (Isogai et al. 2015).

Some Arp2/3 complex activators like the budding yeast WASp family protein Las17 and mammalian JMY, contain actin monomer binding repeats and have been shown to nucleate short linear filaments (Zuchero et al. 2009; Allwood et al. 2016; Urbanek et al. 2012). It has been proposed that these short filaments could then template the branching reaction but the relevance of this activity still needs to be tested in cells. Moreover, not all mammalian WASp family proteins have this biochemical activity.

Another study postulated a cut and diffuse mechanism where short filament fragments generated by cofilin severing activity could then form substrates for Arp2/3 mediated branching in adjacent polymerizing structures (Pfaendtner, De La Cruz, and Voth 2010; Chen and Pollard 2013). During fission yeast endocytosis, the actin network internalizes with the nascent vesicle and could come in close proximity to an immature endocytic site while disassembling. In this model, the scaffolding protein Pan1 is proposed to capture the short filament seeds to localize them to the new sites of polymerization. While this activity is backed by evidence in cells, it does not provide a mechanism to create a seed *de novo* but could function to amplify the influence of another seeder. Moreover, aged filaments, i.e. ones that have hydrolyzed ATP, are thought to be poor substrates for the branching reaction (Ichetovkin et al. 2002).

Finally, WISH/DIP/SPIN90 proteins are conserved actin regulators that have been shown recently to play a role in initiating actin networks during endocytosis in fission yeast (Basu and Chang 2011). Given the range of possibilities, it will be an important advancement to elucidate the relative contribution of each seeding mechanism and whether a single pathway dominates or multiple pathways collaborate to robustly initiate branched actin assembly.

WISH/DIP/SPIN90 PROTEINS ARE ACTIN REGULATORS

WISH/DIP/SPIN90 (WDS) family proteins have been implicated as key actin regulators, but their precise function has been elusive. WISH, DIP, and SPIN90 were discovered simultaneously by three independent groups who each described an interaction between the mammalian protein and signaling molecules (Lim et al. 2001; Satoh and Tominaga 2001;

Fukuoka et al. 2001). WDS proteins are important during lammelipodia formation, endocytosis, synaptic remodeling, and membrane ruffling (Kim et al. 2006; Teodorof et al. 2009; Lee et al. 2006). WDS proteins have a conserved domain of unknown function defined by leucine rich repeats called the leucine rich domain (LRD). Mammalian orthologues have an N-terminal poly-proline region and Src homology domain III (SH3). The interactions at the N-terminus connect information from signaling pathways to reorganization of the actin cytoskeleton. Along those lines, knockdown of the mammalian orthologue, SPIN90, prevented PDGF-stimulated formation of lamellipodia (Kim et al., 2006) and reduced PSD-95 rich dendritic spine density (Lee et al. 2006). Despite a clear connection between WDS proteins and actin remodeling, the biochemical influence of WDS proteins on actin has yet to be well described, but some studies imply there is a connection with the Arp2/3 complex. SPIN90 has been shown to directly interact and active Arp2/3 complex, and based on sequence, it was hypothesized to be a WASp-like activator (Kim et al., 2006). In direct contrast, another study showed that SPIN90 could not directly activate Arp2/3 complex but could bind and relieve auto-inhibition of N-WASp to induce activation of Arp2/3 complex (Fukuoka et al., 2001). The role of WISH/DIP/SPIN90 proteins in regulating Arp2/3 has been ambiguous. One clue to the influence of WDS proteins on actin filaments was described when overexpression of DIP in HeLa cells led to membrane blebbing, a morphology consistent with a loose and disconnected actin array (Eisenmann et al. 2007). It will be an important focus to dissect these conflicting observations and shed light on the role WDS proteins play in regulating the actin cytoskeleton.

The fission yeast orthologue of WDS, Dip1, regulates the initiation of actin assembly during endocytosis (Basu and Chang, 2011). Sites of endocytosis, called actin patches, are nucleated by Arp2/3 complex. Endocytosis is a highly regulated portal of entry into the cell. An orchestrated series of events facilitate transport of solutes, bound receptors, and other macromolecules across the plasma membrane. Branched filaments in these networks are thought to drive endocytosis by providing a pushing force that opposes a cells internal turgor pressure and allows membrane ingression (Boulant et al. 2011; Basu, Munteanu, and Chang 2014). Endocytosis involves the coordinated action of approximately 60 proteins with remarkable precision in the timing of protein recruitment (Kaksonen 2008). The final stage of the process occurs with a well-timed burst of actin polymerization that coincides with the inward movement of a nascent vesicle. Normally, the *S. pombe* WASP protein, Wsp1, arrives at endocytic sites 8-

10 seconds before internalization (Sirotkin et al. 2010). In *dip1Δ* cells, the timing of this process is stochastic, with actin assembly and internalization sometimes delayed by hundreds of seconds. This delay was hypothesized to result from a failure in the actin initiation mechanism. Once actin started to accumulate in these cells the process occurred with the wildtype timing (Basu and Chang, 2011). These observations led us to ask how Dip1 and WDS proteins could regulate the initiation of branched actin networks and whether this regulation could occur directly through the Arp2/3 complex.

Initiation of actin polymerization is directly tied to the architecture of the resultant filament networks. Actin polymerization during endocytosis provides a good cellular model of branched actin network formation because the protein composition at an actin patch is similar to actin – rich motile structures (Pollard, Blanchoin, and Mullins 2000). Electron microscopy studies in yeast revealed that actin patches contain branched filaments that are relatively short, on the order of 50 nm, with a filament to branch ratio of 3:1 (Young, Cooper, and Bridgman 2004). Challenges in preparing samples, however, prevented a dissection of the contribution of actin patch components to the architecture of networks. Moreover, the relationship between the efficiency of endocytosis and the architecture of the actin networks is unknown. In addition to opposing internal turgor pressure, actin may function in moving the vesicle after it has internalized. Comet tails of actin propel the movement of intracellular pathogens and could propel newly formed vesicles within the cell (J Zalevsky, Grigorova, and Mullins 2001). One study suggested internalized vesicles travel along actin cables in yeast (Jr and Chang 2001) while another provided evidence that dense actin networks may function simply to limit the diffusion of vesicles as the network disassembles (Berro and Pollard 2014). These studies illustrate a need to dissect the relationship between the functions of actin network architectures as well as the exact arrangement of filaments. There are at least 5 resident endocytic actin patch proteins (Abp1, Pan1, Myo1, Crn1, and WASp) that have been shown *in vitro* to activate the Arp2/3 complex (Sirotkin et al. 2010; Mooren, Galletta, and Cooper 2012; Kaksonen, Toret, and Drubin 2006; Goode, Eskin, and Wendland 2015). Multiple Arp2/3 complex regulator proteins localized within the patch implies each activator may play non-redundant roles. Dissecting the activation mechanism of each regulator will shed light on how each activator contributes to distinct elements of endocytic actin polymerization.

Dissecting the biochemical properties of WISH/DIP/SPIN90 proteins will be important to defining how this protein family regulates actin polymerization. Given the disparate evidence on whether these proteins activate the Arp2/3 complex, studying the fission yeast orthologue may help clarify whether this protein has the capacity to activate the Arp2/3 complex. The robust knockout phenotype in yeast provides a good system to probe the relevance of *in vitro* findings *in vivo*.

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CHAPTER II

DIP1 DEFINES A CLASS OF ARP2/3 COMPLEX ACTIVATORS THAT FUNCTION WITHOUT PREFORMED ACTIN FILAMENTS

INTRODUCTION

The dynamic meshworks of filaments that make up the actin cytoskeleton are tightly regulated to orchestrate complex cellular process like endocytosis and cellular motility. Polymerization of actin filaments is limited by a slow nucleation step, in which the first few actin monomers assemble to form a template for elongation of a new filament (Cooper 1983). Cells contain multiple actin filament nucleators to regulate assembly (Chesarone and Goode 2009), but Arp2/3 complex is the only one known to nucleate branched actin networks (Goley and Welch 2006). Its activity is tightly regulated, and there are currently about a dozen known Arp2/3 complex activators, called nucleation promoting factors (NPFs) (Goley and Welch 2006, Rotty, Wu, and Bear 2013). WASP/Scar family proteins, the best-studied NPFs, recruit actin monomers to Arp2/3 complex to stimulate an activating conformational change (Chereau et al. 2005, Hetrick et al. 2013) However, nucleation occurs only when the complex is bound to the side of a pre-existing filament (Achard et al. 2010), ensuring that the complex creates exclusively branched actin networks. Once branching is initiated, Arp2/3 complex-nucleated filaments can serve as substrates to drive the reaction, but the source of the very first substrate actin filaments remains an open question. Cellular concentrations of actin filaments are high, but distinct pools of filaments are coated with characteristic actin binding proteins that may influence their suitability as substrates for the complex (Iwasa and Mullins 2007, Skau et al. 2011). For example, tropomyosin and coronin both bind to the sides of actin filaments and prevent them from activating Arp2/3 complex (Liu et al. 2011, Blanchoin, Pollard, and Hitchcock-DeGregori 2001).

WISH/DIP/SPIN90 proteins are poorly understood actin regulators that interact with Arp2/3 complex (Kim et al. 2006). SPIN90, the mammalian orthologue, was previously shown to activate Arp2/3 complex, and based on sequence alignments it was hypothesized to be a WASP-like activator (Kim et al. 2006). Consistent with this, knockdown of SPIN90 prevented PDGF-stimulated formation of lamelipodia and caused defects in actin organization (Kim et al. 2006). In contrast, another study showed that mammalian WISH/DIP/SPIN90 could bind N-WASP to relieve its auto-inhibition and induce activation of the Arp2/3 complex, but could not directly activate Arp2/3 complex (Fukuoka et al. 2001). Therefore, the role of WISH/DIP/SPIN90 proteins in regulating Arp2/3 complex is uncertain.

In *S. pombe,* Dip1 regulates the timing of actin assembly in endocytic actin patches (Basu and Chang 2011). Actin patches are nucleated by Arp2/3 complex, and branched filaments in these networks are thought to drive endocytosis by providing the pushing forces to invaginate membranes and internalize endocytic vesicles (Kaksonen, Toret, and Drubin 2006). Normally, the *S. pombe* WASP protein, Wsp1, arrives at endocytic sites 8-10 seconds before internalization and initiates a tightly controlled sequence of actin polymerization and recruitment of actin binding proteins (Basu and Chang 2011, Sirotkin et al. 2010). In *dip1Δ* cells, the timing of this process is random, with actin assembly and internalization sometimes delayed by hundreds of seconds. This delay was hypothesized to result from the absence of suitable substrate actin filaments for Wsp1-activated branching nucleation (Basu and Chang 2011). These observations led us to ask how Dip1 can regulate the initiation of branched actin networks, and how it might provide the initial substrate filaments for Arp2/3 complex.

Here we show that Dip1 directly activates Arp2/3 complex, but with a mechanism distinct from other NPFs. Dip1 does not interact with actin filaments or monomers like other NPFs, but instead uses a non-WASP-like interaction to bind to Arp2/3 complex and initiate an activating conformational change. Importantly, we show that Dip-mediated activation does not require preformed filaments, providing the biochemical mechanism by which Dip1 can control the timing of endocytic actin assembly. The biochemical properties of Dip1 are conserved in SPIN90, suggesting WISH/DIP/SPIN90 proteins may have a general role in providing seed filaments to initiate branching nucleation.

RESULTS

S. pombe Dip1 Is a Potent Activator of Arp2/3 complex

The *S. pombe* WISH/DIP/SPIN90 orthologue Dip1 has a conserved leucine rich domain (LRD), but has neither a poly-proline region nor Src homology domain III (SH3) present in other orthologues (Figure 1A, S1). To determine if Dip1 can influence the activity of Arp2/3 complex, we tested its activity in pyrene actin polymerization assays. Purified Dip1 dramatically increased polymerization rates in reactions containing Arp2/3 complex, but had no effect on reactions containing only actin, demonstrating that Dip1 activates Arp2/3 complex (Figure 1B). Comparing the activation potency of Dip1 to the canonical type I NPF from *S. pombe*, Wsp1, revealed striking differences between these two activators. First, Dip1 is a more potent activator of Arp2/3 complex than Wsp1. At saturation, the minimal construct of Wsp1 sufficient to activate Arp2/3 complex, Wsp1-VCA (Sirotkin et al. 2005), increased the maximum polymerization rate to a level nine fold higher than Arp2/3 complex alone. In contrast, near saturating concentrations of Dip1 increased the maximum polymerization rate forty-fold over Arp2/3 complex alone (Figure 1C). At each concentration tested, Dip1 produced faster polymerization rates than the equivalent concentration of Wsp1-VCA. With 50 nM Arp2/3 complex, saturating Wsp1-VCA produced 0.7 nM barbed ends while near saturating Dip1 (20 μM) produced 1.3 nM ends (Figure 1D). A second critical difference between the two NPFs is that at high concentrations, Wsp1-VCA potently inhibits actin polymerization, whereas Dip1 did not slow accumulation of polymer even at concentrations up to 20 μM (Figure 1C). VCA inhibits polymerization because its V-region binds to actin monomers, preventing them from spontaneously associating into nuclei (Higgs, Blanchoin, and Pollard 1999). That Dip1 does not slow polymerization suggests that it does not bind actin monomers like VCA, and that it may use a different mechanism to activate Arp2/3 complex. Finally, adding a GST tag to Dip1 did not increase its potency (Figure S1). This is in contrast to WASP family proteins, which show increased activity upon induced dimerization (Padrick et al. 2008).

Wsp1 and Dip1 colocalize to endocytic actin patches, and both contribute to the assembly of actin filament networks in patches (Basu and Chang 2011). To determine if these NPFs can cooperate to assemble actin filaments *in vitro*, we added Dip1 to a pyrene actin polymerization assay containing Wsp1 and Arp2/3 complex. Dip1 increased the

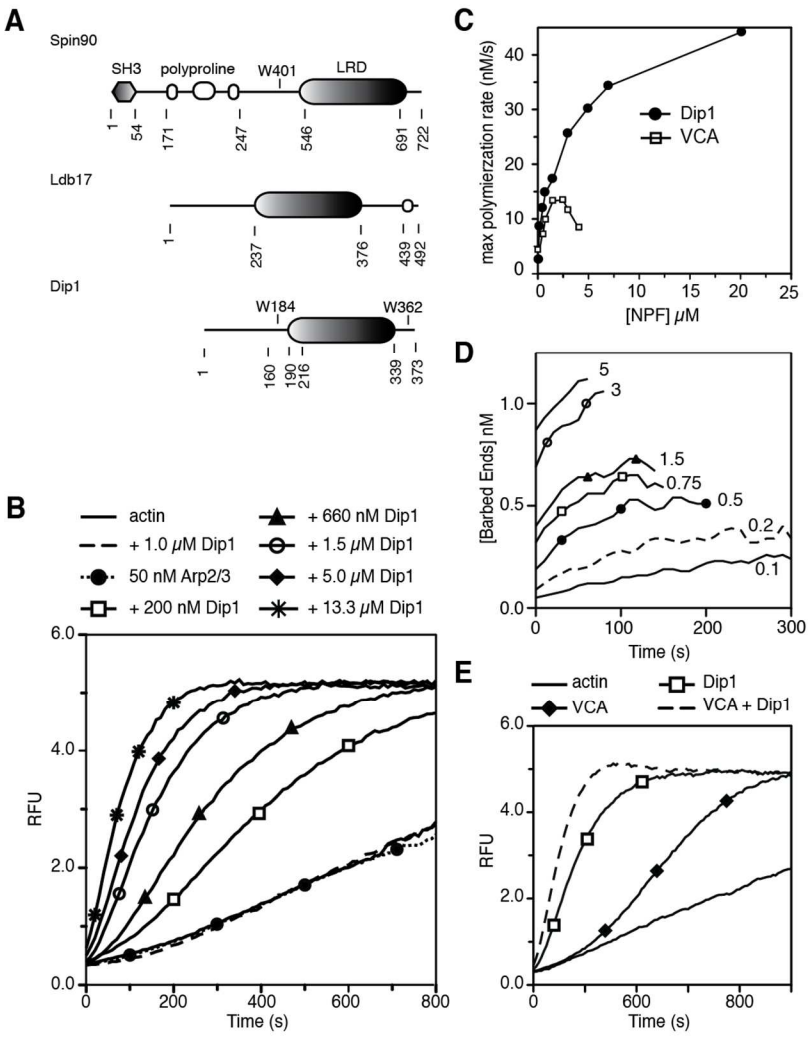

Figure 1: Dip1 is a potent activator of Arp2/3 complex. (A) Domain organization of human SPIN90, budding yeast Ldb17 and fission yeast Dip1. The leucine rich domain, LRD, is conserved in all species. The LRD not have sequence homology to leucine rich repeat domains. (B) Time course of polymerization of 3 μM 15 % pyrene-actin with or without 50 nM *S. pombe* Arp2/3 complex and a range of concentrations of Dip1. (C) Plot of maximum polymerization rate versus Dip1 or Wsp1-VCA concentration for pyrene-actin polymerization assays described in B. (D) Plot of calculated number of barbed ends versus time for reactions described in B. Micromolar concentration of Dip1 in each reaction is indicated. (E) Pyrene actin polymerization assays with Arp2/3 complex and pyrene actin as in (B), plus 1μM Dip1 and 200 nM Wsp1-VCA as indicated. See also Figure S1.

maximum polymerization rate 2.5-fold over reactions containing Arp2/3 complex activated by Wsp1 alone, suggesting that Dip1 and Wsp1 can act either additively or synergistically (Figure 1E). To explore further, we pre-incubated Arp2/3 complex with saturating Dip1 and a range of concentrations of GST-Wsp1-VCA before initiating polymerization assays. GST-Wsp1-VCA did not increase the maximum polymerization rate, and at high concentrations it slowed

polymerization (Figure S1). These data indicate that Dip1 and Wsp1 are unlikely to synergistically activate the complex by simultaneously engaging it. The decreased polymerization rates at high concentrations of GST-Wsp1-VCA could be due to a number of factors, including competition of the two NPFs for binding to the complex, or slowed spontaneous or induced nucleation from Wsp1-bound actin monomers.

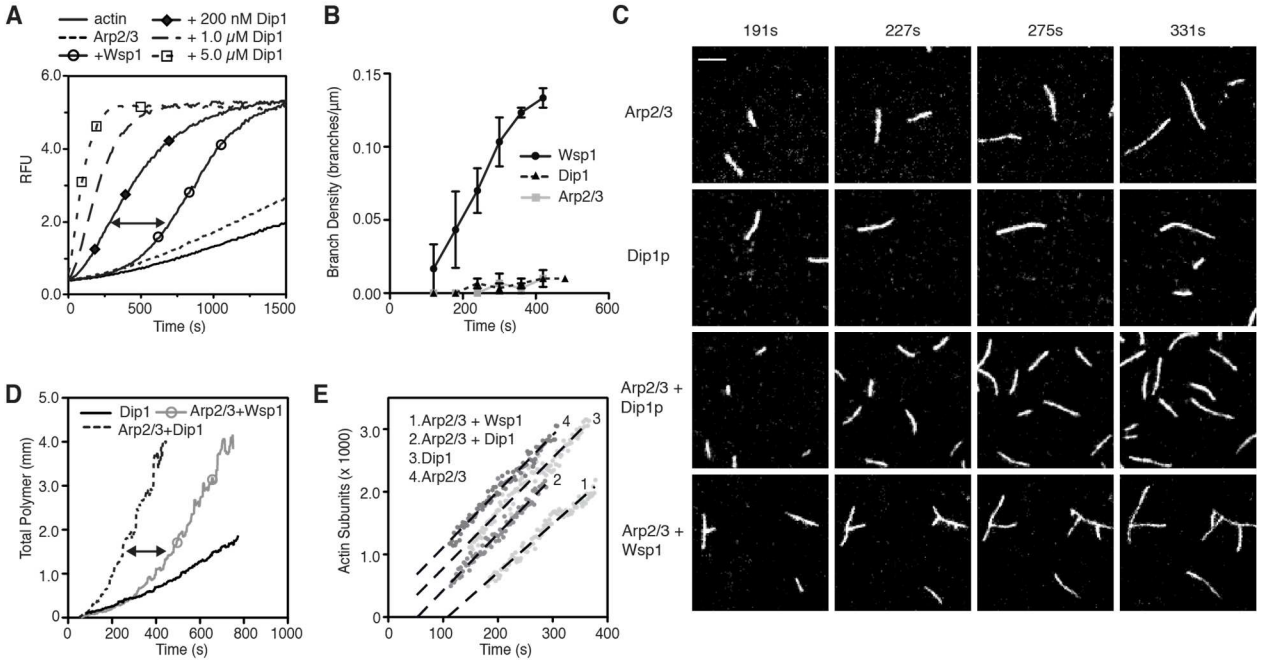

Figure 2: Dip1-mediated activation of Arp2/3 complex does not require preformed actin filaments. (A) Time course of polymerization of 3 μM 15% pyrene actin with 50 nM *S. pombe* Arp2/3 complex and 200 nM Wsp1-VCA or the indicated concentrations of Dip1. Arrow highlights lag in activation of Arp2/3 complex by Wsp1-VCA. (B) Plot of branch density versus time for TIRF data in panel C. Data are represented as mean +/- SEM. (C) Total internal reflection microscopy (TIRF) images of 33% Oregon Green-488 actin polymerizing with 50 nM *S. pombe* Arp2/3 complex, 150 nM Dip1 and 75 nM GST-Wsp1-VCA as indicated. Scale Bar = 2.2 μm. (D) Plot of total polymer length verses time for TIRF data in panel C. (E) Plot of filament lengths expressed in subunits of actin versus time for select single filaments in TIRF data in panel B. Dashed lines are linear fits of each filament growth. Global analysis of at least 7 filaments/reaction showed that the average growth rate in reactions with Arp2/3 alone was 9.0 ± 0.1 s^{-1} (n=541); Dip1 alone was 9.7 ± 0.3 s^{-1} (n=816); Arp2/3 + GST-Wsp1-VCA was 9.2 ± 0.2 s^{-1} (n=641); and Arp2/3 + dip1 was 9.5 ± 0.2 s^{-1} (n=775).

Dip1 Activates Arp2/3 Complex Without Preformed Actin Filaments

Because WASP-mediated activation of Arp2/3 complex requires binding of the complex to a preformed filament, accumulation of branched filaments proceeds through a lag phase caused by slow spontaneous nucleation (Higgs, Blanchoin, and Pollard 1999). However,

examination of the polymerization time courses revealed that the lag phase of Dip1-activated Arp2/3 complex was insignificant compared to the lag we observed with GST-Wsp1-VCA activated Arp2/3 complex (Figure 2A). An important consequence of the requirement for preformed filaments is that WASP-activated Arp2/3 complex specifically nucleates branched actin filaments. To determine if Dip1 activates Arp2/3 complex without requiring preformed filaments, we used TIRF microscopy to visualize actin polymerization in the presence or absence of Arp2/3 complex, with or without Dip1 or Wsp1-VCA (Movie S1-2). As expected, Arp2/3 complex activated by Wsp1-VCA produced branched filaments (Figure 2B, C, Movie S1). In contrast, Dip1-activated Arp2/3 complex produced many short, linear filaments that did not branch, indicating that preformed filaments are not required for activation of the complex (Figure 2B, C, Movie S1, S2). The accumulation of filamentous actin in TIRF assays was accelerated in reactions containing Dip1 versus Wsp1-VCA-activated Arp2/3 complex, consistent with the absence of a lag phase observed in bulk assays (Figure 2A, D). Filaments nucleated by Dip1 and Arp2/3 complex elongated from their barbed ends at the same rate as reactions containing Wsp1-VCA and Arp2/3 complex or actin alone, consistent with our conclusion that Dip1 acts directly on the complex rather than actin filament barbed ends (Figure 2E).

To provide additional evidence that actin filaments are not required for Dip1-Arp2/3 complex-mediated nucleation, we tested the influence of the Arp2/3 complex inhibitor protein coronin on Dip1 activity. Coronin binds to actin filament sides and blocks Arp2/3 complex from associating, thereby inhibiting nucleation (Liu et al. 2011). We reasoned that if Dip1-mediated activation of Arp2/3 complex does not require actin filament side binding, coronin will not antagonize Dip1. Indeed, we found that coronin had no effect on Dip1-mediated activation of the complex in pyrene actin polymerization assays, but blocked Wsp1-VCA-mediated activation (Figure 3A). As an additional test, we asked if increased concentrations of actin filament side binding sites stimulate Dip1 activity. Preformed actin filaments did not significantly affect the polymerization rate in a reaction containing Dip1 and Arp2/3 complex, but eliminated the lag phase in a reaction containing Wsp1-VCA and Arp2/3 complex (Figure 3B). These data demonstrate that Dip1 does not require preformed filaments to active Arp2/3 complex.

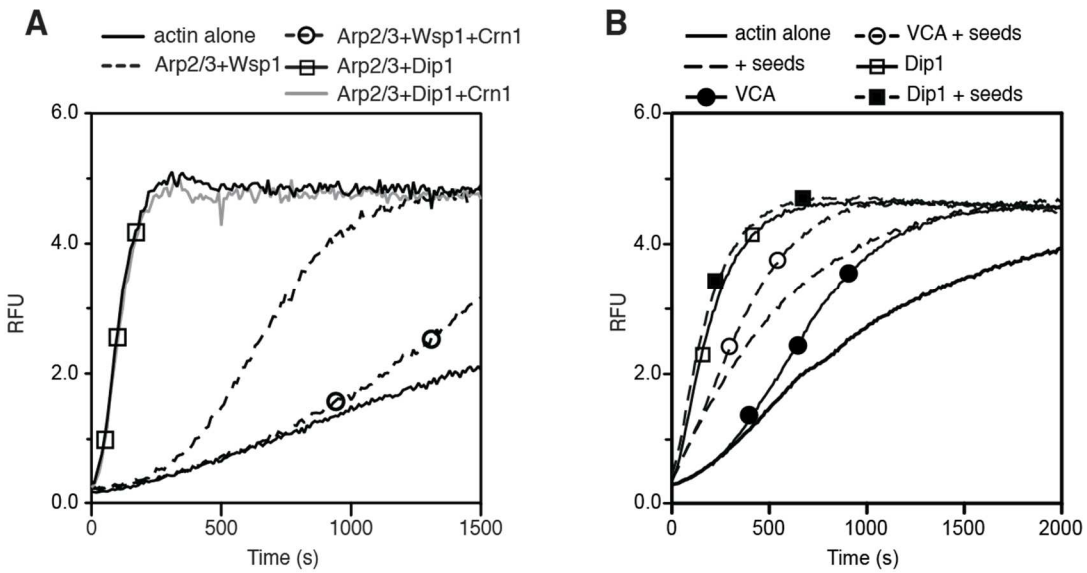

Figure 3: Bulk polymerization assays verify preformed filaments are not required for Dip1-mediated Arp2/3 complex activation. (A) Pyrene actin polymerization assay showing the influence of 1.5 μM Crn1 WD-CC construct (contains residues 1-410 and 594-651) on activation of 50 nM *S. pombe* Arp2/3 complex by 5 μM Dip1 or 200 nM GST-Wsp1-VCA. (B) Pyrene actin polymerization assay showing the influence of preformed actin filaments on Dip1- versus Wsp1-activated Arp2/3 complex. Reactions contained 50 nM *S. pombe* Arp2/3 complex, 1 μM Dip1, 200nM GST-Wsp1-VCA and 300 nM actin filament seeds as indicated.

Dip1 Uses a Mechanism Distinct from Known Type I or Type II NPFs to Activate Arp2/3 complex

NPFs have been separated into two categories: type I, which bind to Arp2/3 complex and actin monomers, and type II NPFs, which bind Arp2/3 complex and filamentous actin (Goley and Welch 2006). Importantly, the actin binding specificity affects the mechanism by which Arp2/3 is activated. Recruitment of actin monomers to the complex by WASP-VCA, the canonical type I NPF, stimulates formation of the actin "short-pitch" conformation of the Arp2-Arp3 subunits, thereby stimulating nucleation activity (Chereau et al. 2005, Hetrick et al. 2013). Less is known about how Type II NPFs regulate the complex, but their filament binding domains are important for activation (Goley and Welch 2006) (Helgeson and Nolen 2013). To determine how Dip1 activates the complex, we tested its interactions with actin. Dip1 did not interact with actin monomers in a pull down assay or a native gel shift assay, suggesting that it does not recruit actin monomers to Arp2/3 complex (Figure 4A, B). We next tested the ability of Dip1 to interact with actin filaments. Dip1 did not copellet with actin filaments at any concentration we tested, up to 4.9 μM, whereas ~99% of the prototypical type II NPF, cortactin, copelleted with

4.9 μM actin (Figure 4C). That Dip1 does not interact with either actin monomers or filaments demonstrates that it uses a different mechanism than known type I or type II NPFs.

Because Dip1 activates Arp2/3 complex but does not interact with filaments or monomers, we hypothesized that it might interact directly with Arp2/3 complex to influence its activity. To test this, we used GST-tagged Dip1 to pull down Arp2/3 complex. Dip1 at 8 μM pulled down 20% of a 1.14 μM solution of Arp2/3 complex, showing the two proteins interact directly (Figure 4D). Dip1 bound weakly to the complex compared to GST-Wsp1-VCA, and we did not saturate binding. This observation is consistent with our actin polymerization assays, which show the concentration at which Dip1 reaches half maximal activation is approximately 6-fold greater than Wsp1-VCA (Figure 1C).

Proflin binds actin monomers to catalyze nucleotide exchange and inhibit spontaneous nucleation of filaments (Mockrin and Korn 1980, Tseng and Pollard 1982). In addition, profilin has been shown to inhibit activation of Arp2/3 complex by some WASP/Scar family proteins (Machesky et al. 1999, Rodal et al. 2003). To determine if profilin affects Dip1-mediated activation of Arp2/3 complex, we added excess profilin to pyrene actin polymerization assays. Profilin at 7.5 μM decreased the number of barbed ends created by 5 μM Dip1 and 50 nM Arp2/3 complex from 0.38 to 0.12 nM (Figure 4E). This is more than the profilin-induced decrease in spontaneously nucleated ends (0.082 nM to 0.02 nM), suggesting that while Dip1 can activate Arp2/3 complex even in excess profilin, profilin may have a direct effect on Dip1-mediated activation of the complex that slows down nucleation.

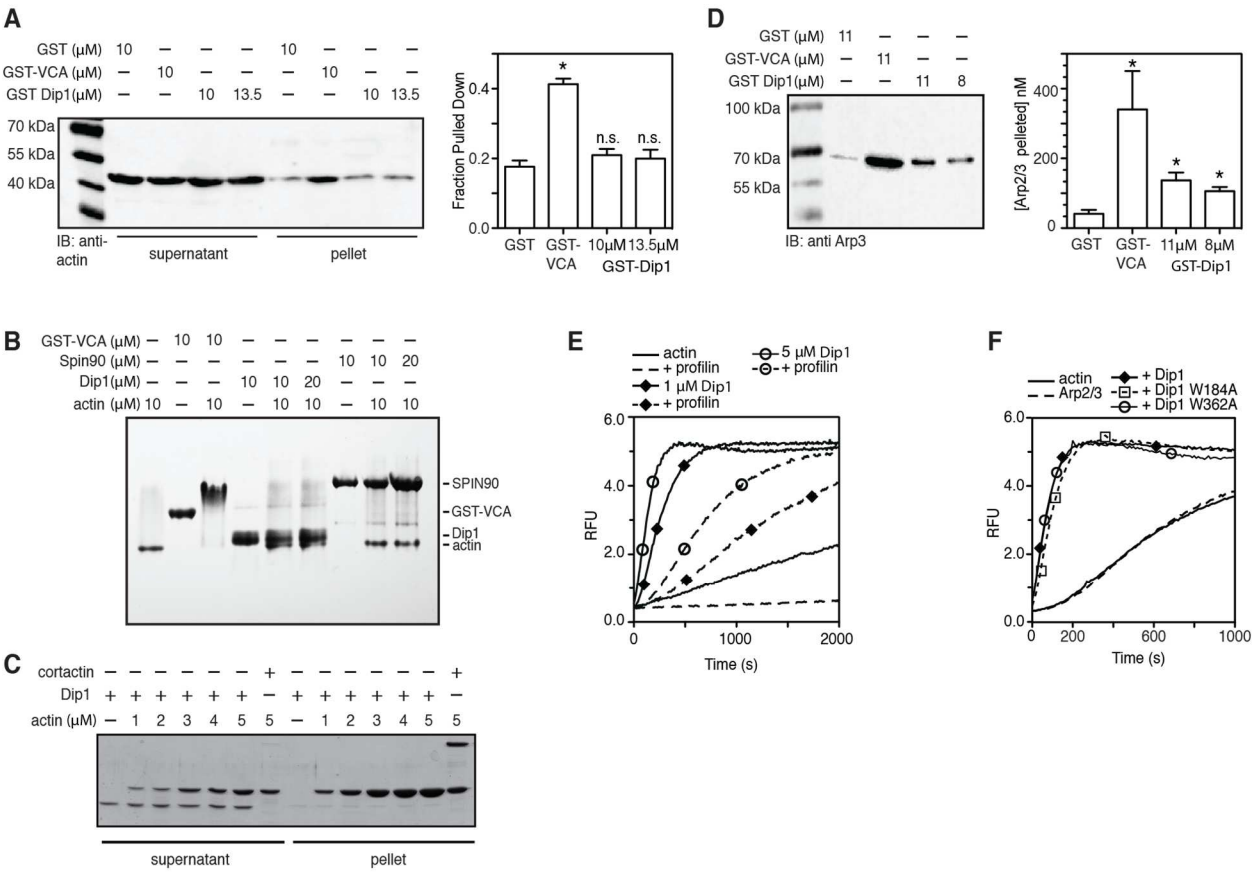

Figure 4: Dip1 uses a non-WASP-like mechanism to activate Arp2/3 complex. (A) Western blot of supernatant and pelleted fractions in actin monomer pull-down assay. Actin at 1.0 μM was pulled down with 10 μM GST-Wsp1-VCA, 10 or 13.5 μM GST-Dip1 or 10 μM GST control protein. Quantified data are represented as the mean +/- SEM (n=3), asterisk represents significant difference compared to GST control $p < 0.0001$ (parametric two-tailed T-test) (B) Coomassie-stained native gel shift binding assay. Reactions contained indicated concentrations of each protein plus 40 μM Latrunculin B to prevent actin polymerization. (C) Coomassie-stained SDS-PAGE gel of actin filament copelleting assay. Dip1 (750 nM) or cortactin (750 nM) were copelleted with a range of concentrations of polymerized actin (total actin concentration is indicated). (D) Anti-Arp3 western blot of pull-down assay containing GST-Dip1 and 1.14 μM *S. pombe* Arp2/3 complex. Control assays contained 11 μM GST or 11 μM GST-Wsp1-VCA. Quantified data are represented as the mean +/- SEM (n=3), asterisk represents significant difference compared to GST control $p < 0.05$ (parametric two-tailed T-test) (E, F) Time courses of polymerization of 3 μM 15% pyrene actin with 50 nM *S. pombe* Arp2/3 complex, 7.5 μM profilin and 5 μM mutant or wild-type Dip1, as indicated. Concentration of Dip1 in panel F is 5 μM.

Dip1 does not have a WASp-like CA region

Sequence analysis suggested that SPIN90, the mammalian orthologue of Dip1, has a CA-like region that might interact with and activate Arp2/3 complex using the same mechanism as WASP VCA (Kim et al. 2006) (Figure S2). The A sequence in WASP/Scar proteins consists of

a conserved tryptophan surrounded by variable numbers of acidic residues (Zalevsky et al. 2001). Previous data have shown that the trypotophan in the A region of WASP forms a contact with Arp2/3 complex important for binding and activation (Blanchoin et al. 2000, Ti et al. 2011) (Campellone et al. 2008). Analysis of the Dip1 sequence revealed two tryptophans, W184 and W382 (Figure 1A, Figure S2). To ask if either tryptophan marks a potential A region that could allow Dip1 to make a WASP-like interaction with the complex, we singly mutated each tryptophan to alanine. Neither tryptophan mutant affected Dip1 activity (Figure 4F). Therefore, we conclude that Dip1 does not interact with Arp2/3 complex using a WASP-like binding mode.

Dip1 Stimulates Formation of the Short Pitch Arp2-Arp3 dimer

Our data show that Dip1 activates the complex but does bind with significant affinity to actin monomers, and does not interact with the complex using a WASP-like binding mode. Therefore, we asked if Dip1 could stimulate formation of the short pitch conformation using a mechanism distinct from WASP. To test this, we used a previously described double cysteine mutant of budding yeast Arp2/3 complex (Hetrick et al. 2013). This mutant harbors engineered cysteine residues on Arp2 and Arp3 that can be cross-linked upon activation of the complex, when Arp2 and Arp3 adopt a short pitch filament-like conformation. Dip1 activates budding yeast Arp2/3 complex, so this assay can be used to determine the influence of Dip1 on formation of the short pitch conformation (Figure S3). We added the 8 Å crosslinker bismaleimideoethane (BMOE) to the engineered complex in the presence of Dip1 or N-WASP-VCA. Dip1 at 30 μM increased the formation of the short pitch crosslink 3.5 fold over Arp2/3 complex alone (Figure 5). Previous data showed that N-WASP-VCA alone weakly shifts the population toward the active state, but that actin monomers and VCA cooperate to strongly induce the short pitch conformation (Hetrick et al. 2013). In contrast, we found that actin monomers did not increase population of the short pitch conformation stimulated by Dip1 (Figure 5). Together, our data indicate that Dip1 activates Arp2/3 complex by stimulating formation of the short-pitch conformation, using a mechanism distinct from WASP.

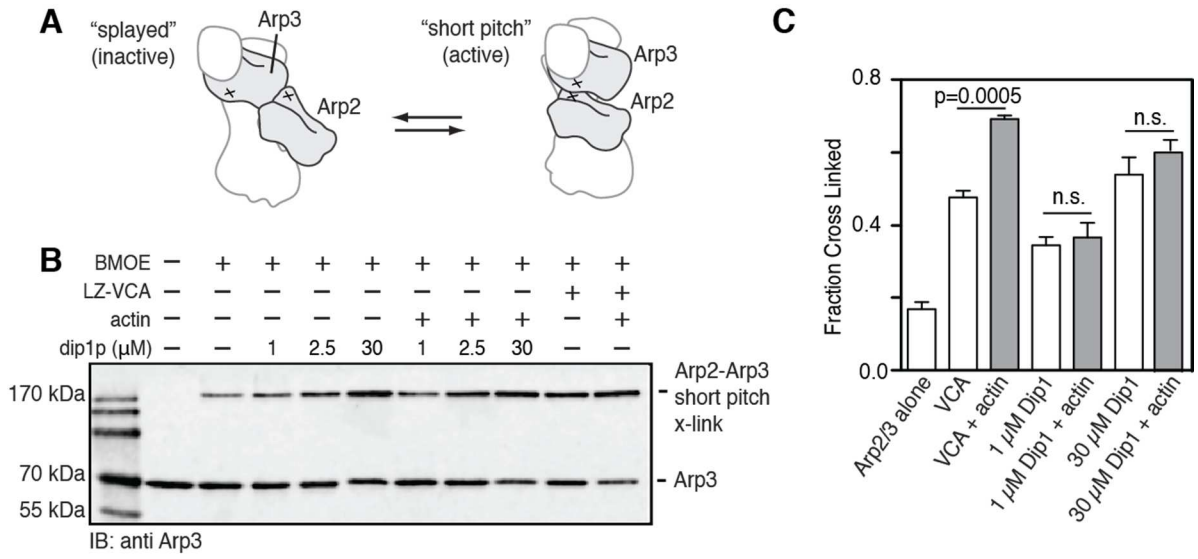

Figure 5: Dip1 stimulates formation of the short pitch conformation. (A) Cartoon schematic showing relative positions of engineered cysteine residues on Arp2 and Arp3 in active or inactive conformation. (B) Anti-Arp3 western blot of crosslinking assays containing 1.0 μM *S. cerevisiae* Arp2/3 complex (Arp3L155C/Arp2R198C) and 25 μM BMOE, 10 μM leucine-zipper (LZ) N-WASp-VCA, 10 μM latrunculin B-bound actin, and Dip1 as indicated. Reactions were allowed to proceed for 60 s before quenching with 1.25 mM dithiolthreitol and separating by SDS-PAGE. (C) Quantification of short-pitch Arp2-Arp3 crosslinking assays as described in panel. Data are represented as mean +/- SEM. P-value calculated from parametric two-tailed t-test

The Mechanism of Activation of Arp2/3 complex is Conserved Among WISH/DIP/SPIN90 Proteins

To determine which regions of Dip1 are required for activation, we tested the ability of Dip1 truncations to activate Arp2/3 complex. An N-terminal deletion construct starting at residue 160, Dip1(160-374), retained nearly full activity (Figure 1A, 6A, S1). This result is consistent with the lack of conservation of the N-terminal sequences among WISH/DIP/SPIN90 family proteins. Unexpectedly, the region C-terminal to the LRD domain, which is also poorly conserved, was required for activity (Figure 6A). However, truncation of the C-terminal sequence resulted in a poorly behaved protein with low solubility and a propensity to aggregate (data not shown). Therefore, this region could either be important for protein stability or may be directly involved in activating Arp2/3 complex, or both. A construct lacking the N-terminal 19 residues of the LRD domain, Dip1(216-374), was also inactive, consistent with the importance of the LRD in activation of Arp2/3 complex.

Because the LRD domain is conserved in all WISH/DIP/SPIN90 proteins, we hypothesized that the mechanism of Dip1-mediated activation of the complex might be conserved. As previously reported, human SPIN90 activated the complex ((Kim et al. 2006), Figure 6B). Activation required relatively high concentrations, but like Dip1, SPIN90-mediated activation lacked a lag phase. SPIN90-mediated activation of Arp2/3 complex was not inhibited by coronin, and addition of preformed filaments did not increase activation of the complex by SPIN90 (Figure 6C,D). SPIN90-activated Arp2/3 complex produced linear instead of branched actin filaments in a TIRF microscopy assay, in contrast to a previous report (Figure 6E, Movie S3,S4) (Kim et al. 2007). These data demonstrate that like Dip1, SPIN90 does not require preformed actin filaments to activate Arp2/3 complex. It was previously reported that SPIN90 harbors a V region similar to WASP proteins that allows it to interact with actin monomers, suggesting it may use a WASP-like activation mechanism (Kim et al. 2006). However, our sequence analysis indicates that the proposed V region in SPIN90 has significant differences from other V-region-containing proteins (Figure S4). We also found substantial biochemical differences between WASP-V and the proposed SPIN90 V-region. For instance, SPIN90 did not interact with actin monomers in a native gel shift assay, unlike N-WASP-VCA (Figure 4B). In addition, while high concentrations of WASP-VCA inhibited actin monomers from spontaneously nucleating (Figure 1C), SPIN90 did not inhibit spontaneous nucleation at the highest concentrations we tested, up to 27.4 μM (Figure 6F). Together our data suggest that WISH/DIP/SPIN90 proteins use a conserved mechanism, distinct from other NPFs, that allows them to activate Arp2/3 complex without requiring preformed filaments.

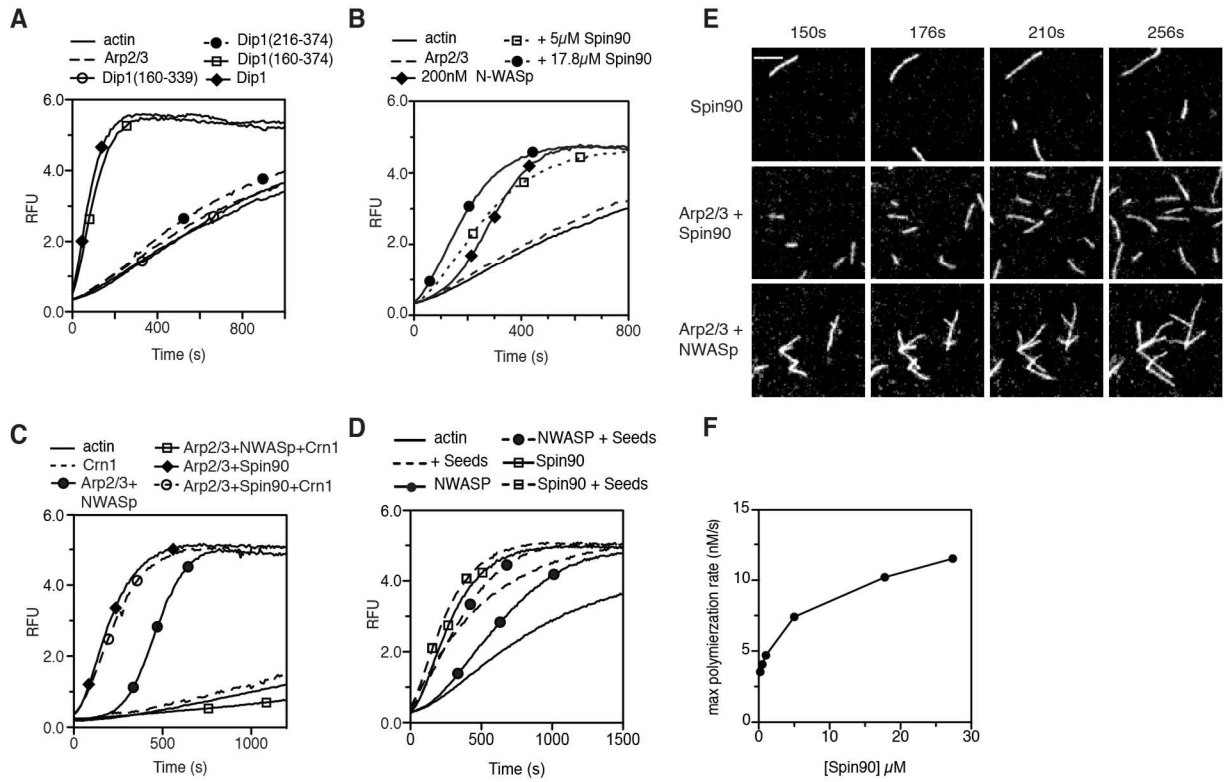

Figure 6: SPIN90 and Dip1 may use the same mechanism to activate Arp2/3 complex. (A) Time course of 3 μM 15% pyrene actin polymerization showing influence of wild type and 2.2 μM GST-Dip1 truncations on GST-Dip1-mediated activation of 50 nM *S. pombe* Arp2/3 complex. (B) Time course of 3 μM 15% pyrene actin polymerization containing SPIN90 (residues 269-722) or GST-N-WASP-VCA and 50 nM *B. taurus* (*Bt*) Arp2/3 complex. (C) Pyrene actin polymerization assays containing 50 nM *Bt*Arp2/3 complex, 2 μM Crn1 WD-CC construct, 17.8 μM SPIN90, and 200 nM GST-N-WASP-VCA as indicated. (D) Pyrene actin polymerization assay showing the influence of preformed actin filaments on SPIN90 versus GST-N-WASP-VCA activated bovine Arp2/3 complex. Reactions contained 50 nM bovine Arp2/3 complex, 10.6 μM SPIN90, 200 nM GST-N-WASP-VCA and 300 nM actin filament seeds as indicated. (E) Total internal reflection microscopy (TIRF) images of 33% Oregon Green-488 actin polymerizing with *Bt*Arp2/3 complex and 1.5 μM SPIN90 or 100 nM GST-N-WASP-VCA as indicated. Reaction with 1.5 μM SPIN90 contains 25 nM *Bt*Arp2/3 and reaction with N-WASP-VCA contains 20 nM *Bt*Arp2/3 complex. Scale Bar = 2.2 μm. (F) Plot of maximum polymerization rate versus SPIN90 concentration for pyrene-actin polymerization assays described in B.

DISCUSSION

Here we show that Dip1 defines a distinct class of NPFs that directly bind and activate Arp2/3 complex. Dip1 is distinct from other NPFs in that it does not bind actin monomers or filaments, and does not contain an Arp2/3 complex-interacting CA (or A) region. The biochemical properties of Dip1 explain its ability to serve as the master timer in initiating the

assembly of branched actin filaments during endocytosis. Based on our data, and on the work of Basu *et. al.* and others, we propose the following model for Dip1 function in actin patch assembly (Figure 7). Dip1 and Wsp1 are recruited to endocytic sites 8 to 10 seconds before internalization (Basu and Chang 2011, Sirotkin et al. 2010). Actin cables are nucleated by the formin For3 at the poles where they could potentially seed branching nucleation (Martin and Chang 2006) (Pelham and Chang 2001), but are coated with tropomyosin (Skoumpla et al. 2007, Skau and Kovar 2010, Arai, Nakano, and Mabuchi 1998), so likely cannot serve as substrates for Wsp1-mediated Arp/3 complex nucleation (Blanchoin, Pollard, and Hitchcock-DeGregori 2001). Dip1 binds Arp2/3 complex, stimulating nucleation of unbranched filaments that provide seeds for Wsp1-mediated Arp2/3 complex activation. This model predicts that tropomyosin does not block Dip1-nucleated filaments from activating Wsp1-bound Arp2/3 complex, though we cannot currently explain why. Wsp1-activated branching creates more substrates for nucleation, resulting in a positive feedback loop that causes rapid assembly of the actin network. Deletion of Dip1 destroys the timing mechanism and initiation of the network becomes dependent on the stochastic encounter of Wsp1-Arp2/3 complex with rare suitable actin filament substrates (Basu and Chang 2011).

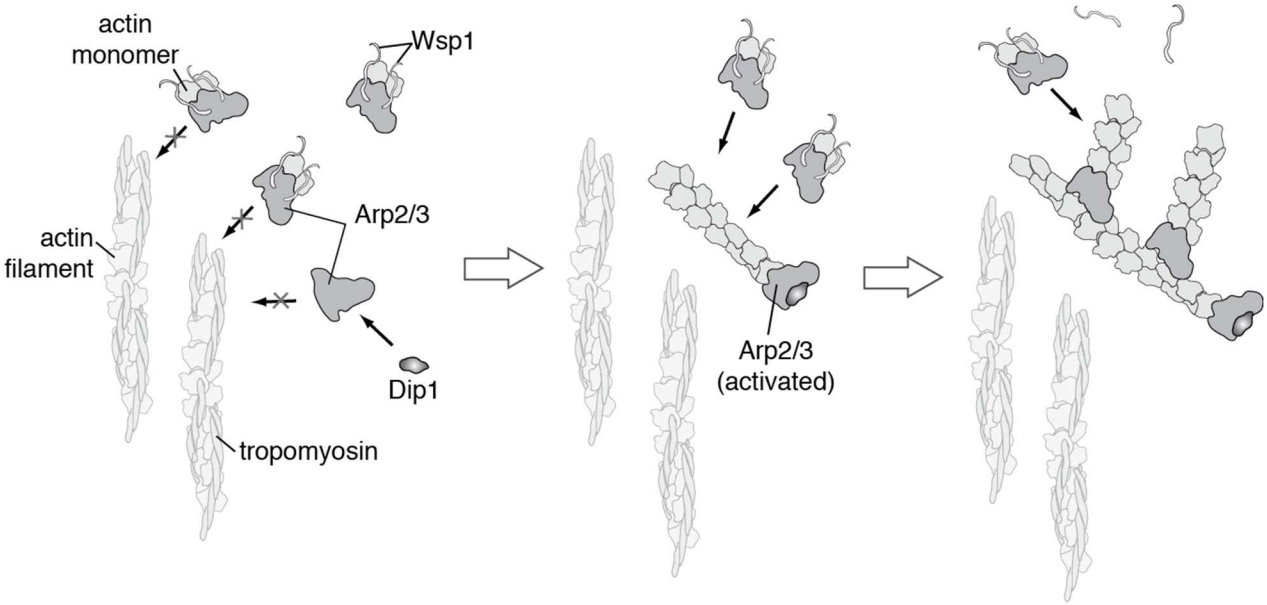

Figure 7: Cartoon model of initiation and propagation of Arp2/3-mediated branching nucleation by Dip1 and Wsp1. See text for details.

Our data show that Dip1 binds directly to Arp2/3 complex to stimulate formation of the short pitch conformation. Dip1 does not contain a CA region to bind Arp2/3 complex, and instead uses a non-WASP-like interaction to initiate this conformational change. We hypothesize that once the Dip1-Arp2/3 complex is in the short pitch conformation, actin monomers can associate with the barbed ends of both Arp2 and Arp3, creating a filament with a blocked pointed end and a barbed end that elongates at the same rate as spontaneously nucleated filaments. This mechanism is consistent with our elongation rate measurements and with structural and biochemical data indicating that Arp2/3 complex binds to the pointed end of the filaments it nucleates (Mullins, Heuser, and Pollard 1998, Rouiller et al. 2008). Importantly, we showed that like Dip1, SPIN90 activates Arp2/3 complex without preformed filaments. In addition, our data demonstrated that SPIN90 does not harbor a WASP-like V region, and binds actin monomers weakly or not at all. Together, these data indicate that SPIN90 and Dip1 use a common mechanism to activate Arp2/3 complex, which is likely conserved among WISH/DIP/SPIN90 proteins. One potentially important difference between SPIN90 and Dip1 is that SPIN90 contains a polyproline segment. While this segment is not required for SPIN90 activity (Figure 6B,C), it will be important to determine if it interacts with profilin-bound actin and how this interaction could influence Arp2/3 complex activation.

Multiple lines of evidence suggest that Wsp1 is the dominant NPF in controlling the architecture of endocytic actin patches. First, electron microscopy of the patches show Arp2/3 complex nucleates a densely branched network of short (19-38 subunit) filaments (Rodal et al. 2005, Young, Cooper, and Bridgman 2004), more similar to the Wsp1-initiated networks than the unbranched Dip1-activated networks we observed *in vitro*. Second, deletion of *dip1* influences the timing of patch initiation, but not patch assembly or internalization after initiation, whereas *wsp1* deletion strains have defective patches that fail to internalize (Basu and Chang 2011). An important question is how Wsp1 is maintained as the dominant NPF *in vivo*, despite the ability of Dip1 to strongly activate the complex. One possibility is that the concentration of Dip1 is relatively low in patches, allowing it to remain active during assembly without significantly decreasing branch density. Consistent with this hypothesis, there are approximately 20 molecules of Dip1 per patch compared to about 150 Wsp1 molecules (Basu and Chang 2011). It is also possible that Dip1 activity is down regulated after initiation of the actin network as Wsp1 becomes active.

A key finding of this work is that Dip1 does not require preformed actin filaments to activate Arp2/3 complex, unlike other NPFs tested (Achard et al. 2010, Higgs, Blanchoin, and Pollard 1999). This observation suggests that at least in some cases, cells use WISH/DIP/SPIN90, a specialized NPF, to create substrate filaments, instead of relying on nucleation machinery that functions independently of Arp2/3 complex. In actin patches, Dip1 may be better suited to initiate patch assembly than independently functioning nucleators. As previously mentioned, formin-nucleated filaments may not provide suitable filament substrates because they are coated with tropomyosin (Skau and Kovar 2010, Blanchoin, Pollard, and Hitchcock-DeGregori 2001). In addition, formins remain at elongating filament barbed ends, preventing capping protein from blocking ends to keep filaments short (Goode and Eck 2007). Dip1 likely acts at the pointed end of filaments, so it may not influence barbed end capping. In budding yeast, Las17, the WASP/Scar family protein, was recently reported to contain a poly-proline segment that nucleates actin filaments without requiring preformed branches or Arp2/3 complex (Urbanek et al. 2013). Mutation of these segments caused a phenotype distinct from the *dip1* knockout, extending the lifetime of the patches at the membrane and increasing the percentage of patches that fail to internalize. It will be important to determine if this segment works in concert with Ldb17, the budding yeast WISH/DIP/SPIN90 protein, or if the actin patch defects observed result from an inability of Las17 to deliver profilin-bound actin monomers to Arp2/3 complex. It will also be important to determine how short diffusing filaments severed by cofilin from disassembling patches might also play a role in initiating new patch assembly (Chen and Pollard 2013).

In endocytic actin patches, there are four known Arp2/3 complex activators (Pan1, Dip1, Myo1, Wsp1) in fission yeast and six in *S. cerevisiae* (Pan1, Myo3, Myo5, Las17, Crn1, and Abp1) (Kovar, Sirotkin, and Lord 2011, Weaver et al. 2003, Liu et al. 2011). While there are partial redundancies between some of these activators, mounting evidence suggests intricate but critical division of duties for these NPFs (Galletta, Chuang, and Cooper 2008, Sirotkin et al. 2005). Here we show that Wsp1 and Dip1 use different biochemical mechanisms to activate Arp2/3 complex, explaining how they carry out distinct functions in the assembly of branched actin networks during endocytosis. A similar division of duties may occur in other branched networks that contain multiple NPFs. Dissecting the biochemical underpinnings that allow multiple Arp2/3 complex regulators to coordinately regulate a single branched actin network will

be critical for our understanding of complex cellular processes like endocytosis and cellular motility.

EXPERIMENTAL PROCEDURES

Fission yeast Dip1, Arp2/3 complex, Wsp1-VCA, human SPIN90 (269-722), N-WASP-VCA, and budding yeast Crn1 (WD-CC) were purified as described in the supplemental material. The GST affinity tag of Dip was cleaved for all experiments, unless otherwise noted in the figure legend. Arp2/3 complex used in assays was from *S. pombe*, unless otherwise indicated. Rabbit skeletal muscle actin was purified and labeled with either pyrene iodoacetamide, or Oregon Green 488 maleimide as described previously (MacLean-Fletcher and Pollard 1980, Pollard 1984). Polymerization of pyrene actin was monitored as the increase in fluorescence at 407 nM as described previously (Liu et al. 2013). The polymerization of Oregon Green 488 actin was monitored using total internal reflection fluorescence microscopy (TIRF) essentially as previously described (Hetrick et al. 2013) with modifications detailed in the supplemental material. Details of other experimental procedures used can be found in the supplemental material.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein Expression and Purification

The coding region of *Schizosaccharomyces pombe* Dip1 was amplified from a cDNA library and cloned into pGV67 with flanking Not1 restriction sites, to generate a GST-TEV-Dip1 expression vector. The coding region of *Homo sapiens* SPIN90 (269-722) was amplified from a cDNA library and cloned into pGV67 expression vector with BamHI and XhoI restriction sites to generate a pGV67-TEV-SPIN90 (269-722) expression vector. For expression of full length and mutant protein, Bl21(DE3)RIL *E. coli* transformed with a pGV67 expression vector was grown to an O.D.595 of 0.6-0.7, induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, and grown overnight at 22 °C. Cells were lysed by sonication in lysis buffer; 20 mM Tris pH 8.0, 140 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor tablets (Roche). Lysate was clarified by centrifugation, and the soluble fraction was loaded on a glutathione sepharose column and eluted with 20 mM Tris pH 8.0, 140 mM NaCl and 50 mM glutathione. Peak fractions were pooled and a 25:1 ratio (by mass) of TEV

protease to recombinant proteins was added. The reaction mix was dialyzed overnight against 20 mM Tris pH 8.0, 50 mM NaCl and 1mM dithiothreitol. The sample was loaded onto a 6ml Resource Q column at pH 8.0 and eluted with a gradient of 50 mM to 500 mM NaCl. Protein was then concentrated in an Amicon-Ultra concentration device before loading on a Superdex 200 HiLoad 16/60 gel filtration column. Peak fractions were pooled, dialyzed against 20 mM Tris pH 8.0, 50 mM NaCl, and 1 mM dithiothreitol and concentrated before flash freezing in liquid nitrogen. Mutant Dip1 constructs were generated by amplifying pGv67-Dip1 using non-overlapping 5'-phosporylated primers, and expressed and purified as described above. Budding yeast Crn1 WD-CC construct (Liu et al. 2011), Fission yeast Arp2/3 complex (Liu et al. 2012), budding yeast Arp2/3 complexes (Hetrick 2013), and bovine Arp2/3 complex (Nolen and Pollard 2007) were purified as described previously.

Pyrene actin polymerization assays

Polymerization of actin was conducted essentially as described (Liu et al. 2011). Briefly, fluorescence measurements were made on a Tecan Safire2 plate reader using an excitation wavelength of 365 nm and an emission wavelength of 407 nm. Fluorescence values were normalized based on the maximum RFU value at equilibrium. The maximum rate of polymer formation was determined by plotting the slope of each polymerization curve at each time point and converting RFU/s to nM actin/s assuming that the total amount of polymer at equilibrium is equal to the total concentration of actin minus 0.1 μM, the critical concentration. Unless otherwise noted, the number of barbed ends was calculated by dividing the instantaneous rate of polymerization (when the reaction was 65 % complete) by the rate constant for actin monomer addition to the barbed end (11.6 $M^{-1}s^{-1}$, (Fujiwara, Vavylonis, and Pollard 2007)) and the instantaneous concentration of unpolymerized actin.

Total Internal Reflection Fluorescence Microscopy

TIRF chambers were constructed and reaction setup was carried out essentially as previously described with slight modifications (Kuhn and Pollard 2005). Images were collected using an EM-CCD camera (iXon3, Andor). TIRF chambers were created by sandwiching double-sided tape between a glass microscope slide and a 24 x 50 #1 coverslip to create a 12 μL, 0.5 cm wide

chamber. To initiate the reaction, 1 µL of 0.025 mM $MgCl_2$ and 1 mM EGTA was mixed with 5 µL of 9 µM 33% Oregon-green actin and incubated for 2 min before adding TIRF buffer (10 mM Imidazole pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 50 mM KCl, 100 mM DTT, 0.2 mM ATP, 25 mM Glucose, 0.5 % Metylcellulose (400 cP at 2%), 0.02 mg/mL Catalase (Sigma) and 0.1 mg/mL Glucose Oxidase (MP Biomedicals) to 1 x, *S. pombe* Arp2/3 complex to 50 nM, GST-Wsp1-VCA to 75 nM, or Dip1 to 300nM or 150 nM final concentration. The reaction was followed for 12 min with 50 ms exposure times at 2 second intervals.

TIRF Image Analysis

Images were prepared in Image J. Image sequences were subtracted for background signal with a 10-pixel rolling ball radius. The total actin polymer was calculated using a custom image processing script run in Matlab, described as follows. For each frame, pixels corresponding to filament fluorescence were identified using image segmentation followed by morphological area opening to remove non-filament small fluorescent objects. The final pixel number value was converted to micrometers (1px = 106.7 nm) to yield the total length of actin filaments in the image frame, and further converted to number of subunits using 370 subunits μm^{-1}. The filament growth rate was determined using an Image J plugin, courtesy of Jeff Kuhn.

Actin monomer binding assays

Actin (1.0 µM) in buffer G (2 mM Tris pH 8.0, 1 mM DTT, 0.2 mM ATP, 0.1 mM $CaCl_2$) was incubated with 10 µM GST-Dip1 or GST-Wsp1p-VCA bound to glutathione sepharose beads. Samples were pelleted and equal volumes of supernatant or re-suspended beads were analyzed by SDS-PAGE followed by immunoblotting with an anti-actin antibody (sc-1616 Santa Cruz Biotech).

Actin filament binding assays

Binding of Dip1 to filamentous actin was measured using copelleting assays. Actin was added to a solution containing 10 mM imadazole pH 7.0, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM dithiothreitol to polymerize actin and bring the final concentration of actin between 1-5 µM. The reaction was allowed to proceed for one hour. Reactions were then incubated with proteins of interest at 23 °C for 15 min before spinning in a TLA100 rotor at 80,000 rpm for 30 min.

Pellet and supernatant fractions were analyzed by SDS-PAGE and coomassie brilliant blue staining.

Arp2/3 Complex Binding Assays

Arp2/3 complex (1.14 μM) in 10 mM imadazole pH 7.0, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 0.2 mM ATP was incubated with 11 μM GST, 11 μM GST-Wsp1-VCA, or 8-11 μM GST-Dip1 bound to glutathione sepharose beads. Samples were pelleted and re-suspended beads were analyzed by SDS-PAGE followed by immunoblotting with an anti-*Sp*Arp3 antibody.

Short Pitch crosslinking assays

Six endogenous cysteine residues and one cysteine residue encoded by the Not1 restriction site were mutated to alanine in the context of the pGV67-Dip1 expression vector. Dip1-7cys-Ala was expressed and purified as described above. Cross linking assays were performed essentially as described [4]. Briefly,1 μM Arp2/3 complex, indicated concentration of activator, and 10 μM Latrunculin B and 10 μM actin (as indicated) were incubated in buffer (100mM imidazole pH 7.0, 500 mM KCl, 10mM EGTA, 10mM $MgCl_2$, 10 mM ATP, 10 mM $CaCl_2$). Latrunculin B was added to actin first in order to prevent spontaneous polymerization of the actin. BMOE was added to a final concentration of 25 μM at room temperature to initiate the crosslinking reaction. After 60 sec, dithiothreitol was added to a final concentration of 1.25 mM to quench the reaction. The presence of cross-linked Arp3-Arp2 was analyzed by separation by SDS-PAGE followed by immunoblotting. Immunoblots were probed for Arp3 antibody (sc-11973 Santa Cruz Biotech).

BRIDGE TO CHAPTER III

The work presented in this chapter established WISH/DIP/SPIN90 (WDS) proteins as potent activators of the Arp2/3 complex. We found that WDS proteins activate the complex using a non-WASP like mechanism that does not require preformed filaments. These data indicate that WDS proteins have the biochemical properties to seed WASP-mediated branch formation. In the next chapter, we will investigate whether WDS proteins can directly provide seeds to initiate branched actin formation.

CHAPTER III

DIP1 CREATES LINEAR ACTIN FILAMENTS THAT SEED BRANCHING NUCLEATION BY WASP-ACTIVATED ARP2/3 COMPLEX

This chapter contains unpublished co-authored material

Author contributions: Andrew Wagner, Luke Helgeson, and Brad Nolen designed research; Andrew Wagner and Luke Helgeson performed research and analyzed data; Andrew Wagner and Brad Nolen wrote the manuscript.

INTRODUCTION

Actin is one of the most highly expressed and highly conserved proteins in eukaryotes. It plays a central role in many cellular functions because monomers of actin can polymerize to form networks of interconnected actin filaments. Cells use these cytoskeletal networks as tracks for molecular motors or to provide pushing forces in processes like cell motility and endocytosis. To carry out its functions, the actin cytoskeleton must be tightly regulated by a suite of regulatory proteins that bind to actin monomers and/or filaments (Pollard and Cooper 1986, 2009). These regulatory proteins not only control when and where actin filament networks assemble and disassemble, but also architectures of actin networks. Arp2/3 complex is an important actin regulator because it nucleates new actin filaments in response to cellular signals, providing spatiotemporal control over actin network assembly (Rotty, Wu, and Bear 2013). Arp2/3 complex differs from other actin filament nucleators in that when activated by its canonical activators, WASP family proteins, Arp2/3 complex nucleates branched actin filaments (Blanchoin et al. 2000, Achard et al. 2010, Campellone and Welch 2010). Therefore, cells use WASP-activated Arp2/3 complex to assemble highly dendritic actin networks consisting of short, crosslinked filaments thought to be optimal for pushing against broad, flat membranes (Pollard and Borisy 2003). Accordingly, actin networks assembled by Arp2/3 complex and WASP family proteins drive processes like protrusion of the broad flat lamellipodial extensions at the front of motile cells and provide pushing forces to remodel membranes during endocytosis (Wu et al. 2012, Suraneni et al. 2012).

While the mechanism by which WASP proteins activate Arp2/3 complex is still being investigated, an important aspect of the mechanism is that WASP alone is insufficient to activate nucleation. WASP must directly tether actin monomers to the complex (Marchand et al. 2001,

Rohatgi et al. 1999), and the entire assembly must bind to the side of a preformed (mother) filament of actin before it can nucleate a new filament (Achard et al. 2010, Liu et al. 2011). The requirement for a preformed filament is important because it ensures that the complex creates exclusively branched actin filaments. This presents a paradox, however, because without a pre-existing filament, the WASP-bound Arp2/3 complex cannot generate branches. Therefore, assembly of branched actin networks by WASP and Arp2/3 complex requires an initial seed filament to prime network assembly. We recently identified a new potential seeding mechanism that can explain *de novo* seed generation. Specifically, we found that unlike Wsp1, the *S. pombe* WISH/DIP/SPIN90 family protein, Dip1, activates Arp2/3 complex without requiring a preformed filament. Therefore, Dip1 possesses the key biochemical property required for a seeder of branched actin network assembly (Wagner et al. 2013). In fission yeast, Dip1 controls the timing of actin assembly at endocytic sites (Basu and Chang 2011). Endocytic actin assembles cortical puncta called patches, which move inward and disassemble as the plasma membrane invaginates and internalizes (Weinberg and Drubin 2012, Goode, Eskin, and Wendland 2015). Deletion of Dip1 stalls Wsp1 at the cortex and decreases the rate at which new actin patches are initiated, leading to a significant decrease in the total number of patches in cells (Basu and Chang 2011). These observations, coupled with our previous biochemical data (Wagner et al. 2013), suggest actin filaments nucleated by Dip1-activated Arp2/3 complex can stimulate Wsp1-mediated activation of the complex to initiate branched network assembly. However, the evidence that filaments nucleated by Dip1 and Arp2/3 complex can activate WASP-bound Arp2/3 complex is indirect. In fact, while each of the potential seeding mechanisms is biochemically feasible, none of these mechanisms have been directly visualized, making it unclear if they occur. Directly testing the Dip1-mediated seeding mechanism is important considering several recent studies that show that the identity of an actin filament nucleator used to assemble an actin network influences the interactions of the nucleated filaments with binding partners. For instance, filaments nucleated by the fission yeast formin Cdc12 preferentially bind the fission yeast formin Cdc8 (Skau, Neidt, and Kovar 2009). Likewise, actin filaments nucleated by Arp2/3 complex are preferentially excluded from interactions with tropomyosin (Hsiao et al. 2015). These examples suggest long-range allosteric effects within actin filaments, in which the bound nucelator on the filament end influences the conformation of interior actin filament subunits (Papp et al. 2006). While the mechanism of

Dip1-mediated activation of the complex is still unclear, a similar allosteric mechanism could influence the suitability of Dip1-Arp2/3 complex nucleated filaments in WASP-mediated activation of the complex. Finally, our previous investigation of Dip1, we showed that the actin binding protein profilin slows actin polymerization in the presence of Arp2/3 complex and Dip1, suggesting it may inhibit directly inhibit Dip1. Because most actin monomers are bound to profilin in fission yeast(Suarez et al. 2014), it is unclear whether Dip1-Arp2/3 nucleated filaments could be an important source of seed filaments.

Several lines of evidence indicate that Dip1 and other WISH/DIP/SPIN90 (WDS) family proteins activate Arp2/3 complex using a different mechanism than WASP family proteins. First, WDS proteins lack the canonical Arp2/3 complex interacting region of WASP (CA), but instead use a C-terminal region containing a leucine rich domain of unknown fold to bind and activate the complex (Wagner et al. 2013). Second, unlike WASP, WDS proteins activate the complex without requiring pre-formed actin filaments (Wagner et al. 2013). Third, while WASP must use its conserved V region to recruit monomers directly to complex for activation(Marchand et al. 2001, Rohatgi et al. 1999), WDS proteins do not contain V regions nor bind with significant affinity to actin monomers(Wagner et al. 2013). Finally, while dimerizing WASP proteins can increase their potency at low concentrations, fusion of GST to Dip1 had no effect on its activity(Wagner et al. 2013). These observations demonstrate that WDS proteins represent a distinct class of NPFs. The sole biochemical property shared by WASP and Dip1 is that they both stimulate the short pitch conformational change, so despite their mechanistic differences, it is likely that both WASP and Dip1 use Arp2 and Arp3 to create a nucleus. Despite the potential importance of WDS family NPFs in initiating branched network assembly, little is known about how they activate Arp2/3 complex.

Here we use single molecule TIRF microscopy to investigate Dip1-mediated activation of Arp2/3 complex. We show that while Dip1 has a completely different activation mechanism that WASP proteins, it co-opts fundamental aspects of branching nucleation to activate the complex. Specifically, Dip1 activates the complex to create a linear filament that elongates from its barbed end, with its pointed end anchored to Arp2/3 complex. The linear filament nucleated by Dip1 is analogous to a daughter filament created by WASP-activated branching nucleation. This mechanism has implications for understanding how Dip1 might function with other NPFs and how branched networks are disassembled. In addition to these data, we directly demonstrate

seeding by Dip1 by showing that Dip1-Arp2/3 nucleated linear filaments can activate Wsp1-bound Arp2/3 complex. These data strongly support a model in which Dip1 activates Arp2/3 complex to create seed filaments that initiate actin patch assembly in cells.

RESULTS

Labeling and Characterization of Dip1 for Single Molecule Studies

To investigate the mechanism of Dip1-mediated nucleation and its role in seeding branched networks, we first fluorescently labeled Dip1 so that we could use TIRF microscopy to directly visualize its influence on the assembly of Oregon-green labeled actin. We mutated all six endogenous cysteine residues in Dip1 to alanine and appended an N-terminal cysteine residue. To determine if labeling influenced the activity of Dip1, we tested its activity in a pyrene actin polymerization assay (Fig. 1A). While Alexa 568-labeled Dip1 showed decreased activity compared to unlabeled Dip1, it significantly accelerated actin assembly in the presence of Arp2/3 complex, indicating the labeled protein retained activity. We set out to investigate whether this reagent could be used for single molecule studies. In addition, to determine if Dip1 functions as a monomer or oligomer, we added 2 nM Dip1-Alexa568 biotinylated on lysine residues to glass coverslips passivated with PEG and Avidin-Biotin-PEG to capture molecules for imaging and then monitored the Alexa 568 signal (Fig. 1B). Most Alexa 568 Dip1 puncta photobleached in a single step (92%, n = 183) (Fig 1C,D). Given that the labeling percentage of Dip1 was ~100%, this indicates that the majority of Dip1 molecules on the surface were monomers. However, a small fraction (8 %, n = 16) of events showed two photobleaching steps, consistent with measurements of the average intensities of the Dip1 puncta. Fitting this data to a sum of two Gaussians revealed a second minor peak indicative of a subset of brighter spots (Fig 1D, E). These data could either indicate that a small proportion of Dip1 forms dimers, or a few Dip1 monomers overlap on the imaging surface. These data validate the use of Dip1-Alexa568 in polymerization reactions and show Dip1-Alexa568 puncta correspond to single molecules of protein. We note that we showed previously that unlike WASP proteins, fusion of Dip1 to GST does not make it a more potent activator at any concentration, indicating that dimerization of Dip1 does not influence its activity.

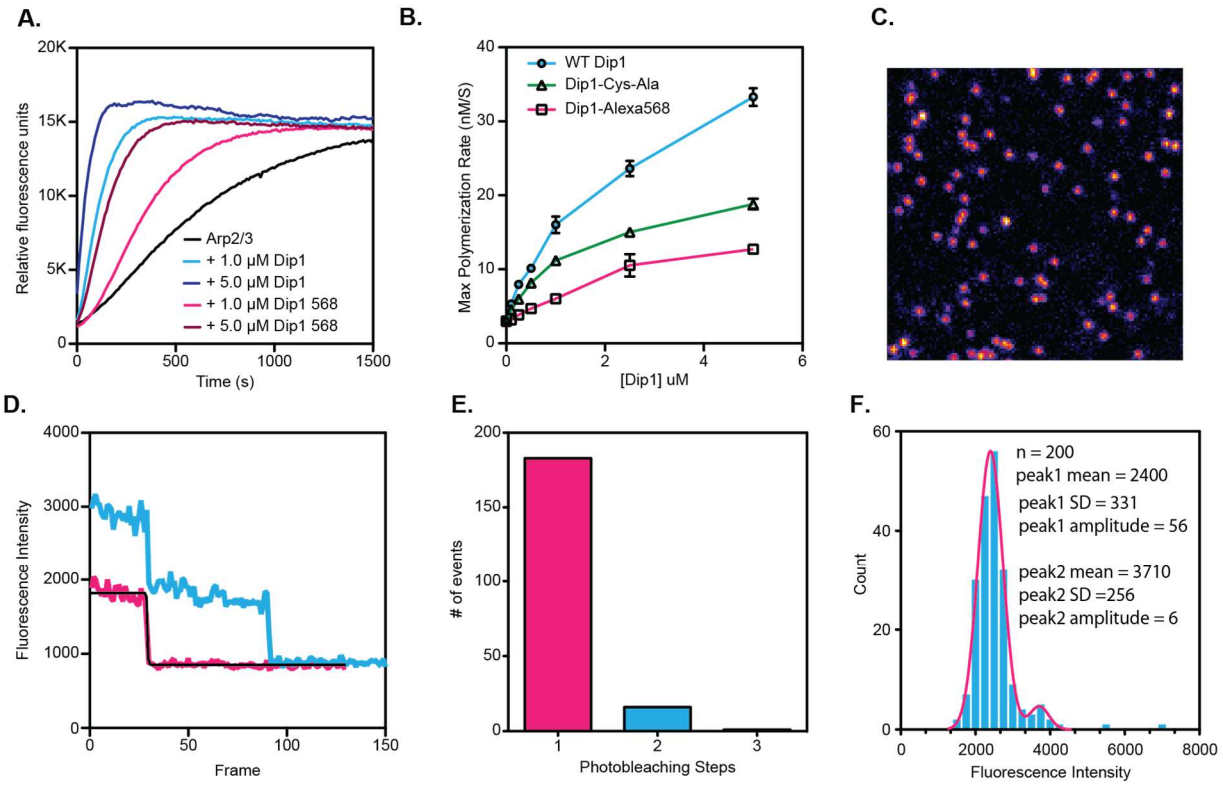

Figure 1: Characterization of Alexa-568 labeled Dip1. A. Time course of polymerization of 3 μM 15% pyrene-labeled actin in the presence of 50 nM SpArp2/3 complex and either unlabeled or Alexa568-labeled Dip1. **B.** Quantification of pyrene actin polymerization data in A comparing purified wildtype (WT) Dip1, the Dip1 labeling construct with 6 cysteines mutated to alanine (Dip1-Cys-Ala), and Dip1 labeled with the Alexa568 dye, Dip1-Alexa568. **C**. Representative image of Alexa568-Dip1 molecules bound to coverslip visualized by TIRF microscopy. **D.** Fluorescence of single Alexa-568 puncta over time. Solid line shows the non-linear fit from a one-phase decay function with a fluorescence intensity span of 968.9 **E.** Quantification showing the number of events in which Alexa568 Dip1 photo bleached in one versus multiple steps. **F.** Quantification of the number of observations versus the mean intensity of Alexa-568 Dip1 puncta. Magenta line shows the fit from a sum of two Gaussians. $R^2 = 0.993$.

Dip1 Co-opts Features of Branching Nucleation to Create Linear Filaments

Several biochemical differences between Dip1 and Wsp1 indicate that WISH/DIP/SPIN90 family proteins activate Arp2/3 complex using a different mechanism than WASP family proteins (Wagner et al. 2013). However, despite these differences, both WASP and WDS family proteins stimulate movement of Arp2 and Arp3 into a short pitch dimer arrangement, suggesting both NPFs use the actin-related subunits to construct a nucleus (Wagner et al. 2013). In the case of WASP-mediated activation, the nucleated filament (daughter filament)

elongates from the barbed ends of Arp2 and Arp3, while the pointed end of the Arps binds to the sides of a pre-existing (mother) filament (Fig. 2A). Therefore, we reasoned that like WASP, Dip1 could activate Arp2/3 complex to nucleate filaments that remain anchored to the complex on the pointed end, with their barbed ends free to elongate (Fig. 2A). In this mechanism, linear filaments generated by Dip1-Arp2/3 complex are analogous to branches generated by WASP-activated Arp2/3 complex. To test this model, we used single molecule TIRF to directly visualize the influence of Dip1-Alexa568 on actin polymerization in the presence of Arp2/3 complex.

In actin polymerization reactions containing Dip1, 1.5 μM 33% Oregon green 488 actin and unlabeled SpArp2/3 complex, we observed Dip1 molecules bound to one end of actin filaments that adhered to the imaging surface (Fig. 2B). Three distinct classes of events produced filaments with Dip1 bound at filament ends (Fig. 1B). In class I events (31 out of 141 observations), Dip1 non-specifically adsorbed to the surface and an actin filament appeared to nucleate from the Dip1 punctum (Fig. 2B). While we cannot eliminate the possibility that these events represent capture of a spontaneously nucleated actin filament by the surface-adsorbed Dip1, our observations argue against this interpretation. Therefore, we interpret these events as Dip1-Arp2/3 mediated nucleation of linear filaments. In a second, more frequent class of events (class II, 108 of 141), actin filaments bound to Dip1 were only observed after they landed on the coverslip surface (Fig. 2B, C). These events could represent surface capture of filaments nucleated by Dip1-Arp2/3 complex in the TIRF chamber. Alternatively, Dip1 might bind to the pointed end of a spontaneously nucleated filament within the reaction chamber, and later land on the imaging surface. However, our data indicate Dip1 pointed end binding events are infrequent, as we observed very few instances in which Dip1 bound to a pre-existing pointed end of a surface-captured filament (class III events, 2 out of 141 events). Therefore, most Dip1-filaments that land on the imaging surface (class II events) result from Dip1-Arp2/3 complex nucleating filaments that later land on the surface, rather than Dip1 and Arp2/3 binding to a pre-existing pointed end. When we repeated the reactions in the absence of Arp2/3 complex, we found that Dip1 did not bind to filament ends (Fig 2D, E.). Importantly, the free ends of Dip1-bound filaments from all three classes elongated at the same rate as free barbed ends, indicating Dip1 molecules bind the pointed end (Fig. 2F). Therefore, we conclude that Dip1 binds actin filaments indirectly through the Arp2/3 complex.

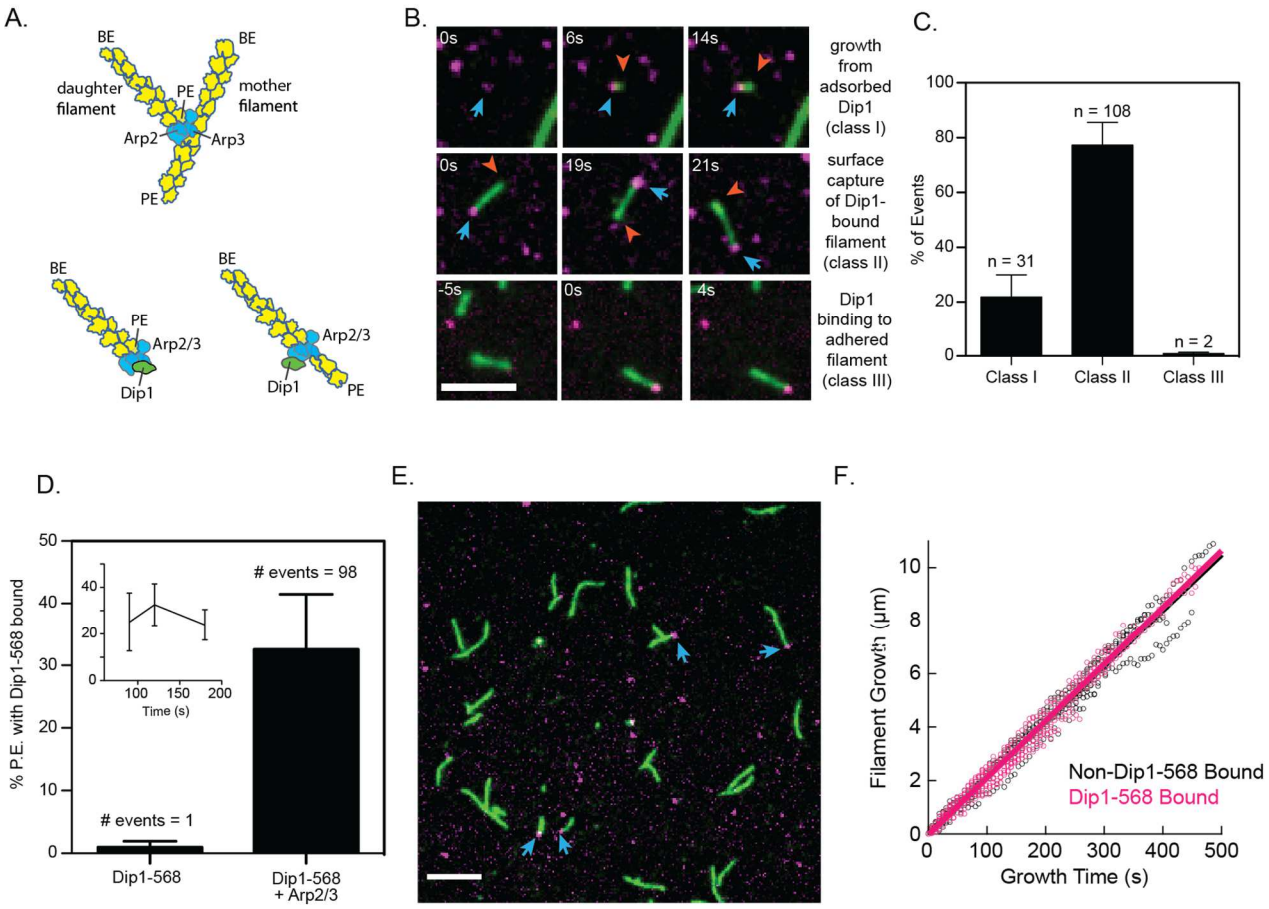

Figure 2: Dip1 co-opts features of branching nucleation to create linear filaments. **A.** Cartoon of branched filament nucleated by WASP-activated Arp2/3 complex (top) showing resulting filament polarity (barbed end, BE; pointed end, PE). Bottom half of panel shows a model of Dip1-mediated activation of Arp2/3 complex. In this model, the linear filament nucleated by Dip1-bound Arp2/3 complex is analogous to the daughter filament nucleated during branching nucleation. **B.** TIRF images of reactions containing 6nM Dip1-Alexa568, 1.5 µM 33% Oregon Green labeled actin and 500 nM SpArp2/3 complex. Scale bar 5 µm. **C.** Quantification of three classes of events in which Dip1 is observed on the ends of filaments by TIRF microscopy for the conditions described in B. Error bars show SEM. **D.** Quantification of the percentage of pointed ends with Dip1 bound in reactions containing 6nM Dip1-Alex568 and 1.5 µM 33% Oregon Green labeled actin with or without 500 nM SpArp2/3 complex. Error bars show SEM. **E.** TIRF image from conditions described in D with 500 nM SpArp2/3 complex Scale bar 5 µm. **F.** Plot of filament length versus time for filaments with or without Dip1-Alexa568 bound. Both conditions were fit to a linear regression.

Actin filaments nucleated by Dip1 and Arp2/3 complex activate Wsp1-bound Arp2/3 complex

Our data show that Dip1 co-opts features of branching nucleation to create linear filaments anchored at their pointed end by Arp2/3 complex. In the following chapter, we show that Dip1 stays bound to Arp2/3 complex on the pointed ends of filaments for more than 300 seconds on average (Chapter IV). Therefore, Alexa568 Dip1 molecules mark actin filaments nucleated by Dip1-Arp2/3 complex, allowing us to distinguish them from spontaneously nucleated filaments. Consequently, we next tested the activity of Dip1-Alexa568 in an assay containing 50 nM *S. pombe* Arp2/3 complex, 1.5 μM 33% Oregon green 488 actin and 250 nM of the Arp2/3 complex activating fragment of Wsp1, GST-Wsp1-VCA. This experiment was designed to determine if Dip1-Arp2/3 nucleated linear filaments can stimulate Wsp1-bound Arp2/3 complex. As in movies without Wsp1, we observed multiple events in which new actin filaments appeared to nucleate from Dip1 puncta non-specifically adsorbed to the surface (Fig. 3A). As these linear filaments elongated, we frequently observed branched filaments growing from their sides (Fig 3A). We also observed branches growing from Dip1 bound filaments that landed on the imaging surface after nucleation (Fig. 3B), which we demonstrated largely represent Dip1-Arp/23-nucelated filaments (Fig. 2B-E). Together, these experiments demonstrate that linear filaments nucleated by Dip1 and Arp2/3 complex can seed branching nucleation by Wsp1-bound Arp2/3 complex. Under the conditions of these reactions, we observed branching nucleation not only from Dip1-bound filaments, but also from free unbound filaments and from pre-existing branches.

DISCUSSION

Dip1 co-opts fundamental aspects of branching nucleation to activate linear filament nucleation

Our data demonstrate that Dip1 co-opts fundamental aspects of branching nucleation to activate linear filament nucleation. Specifically, an actin filament nucleated by Dip1 and Arp2/3 complex is analogous to the daughter filament of actin created by WASP-mediated activation of the complex. That Dip1 co-opts features of branching nucleation has implications for understanding multiple aspects of Arp2/3 complex function. For instance, while the pointed ends of the Arp2 and Arp3 subunits are not blocked by interactions with actin filaments during Dip1-

mediated activation, they do not create an elongating pointed end. Instead, Dip1-bound Arp2/3 complex caps pointed ends. Previous experiments demonstrated that Arp2/3 complex has intrinsic pointed end capping activity (Mullins, Heuser, and Pollard 1998). Our data demonstrates that Dip1 binding does not alter this intrinsic activity of the complex. While it is not clear why Arp2/3 complex does not nucleate filaments that elongate from their pointed ends, possible explanations include the large flexible inserts on the pointed ends of Arp2 and Arp3 that could block monomer association (Robinson et al. 2001), insufficient conservation of pointed end residues important for contacting actin monomers (Beltzner and Pollard 2004), or subunit arrangements during activation that block the pointed end.

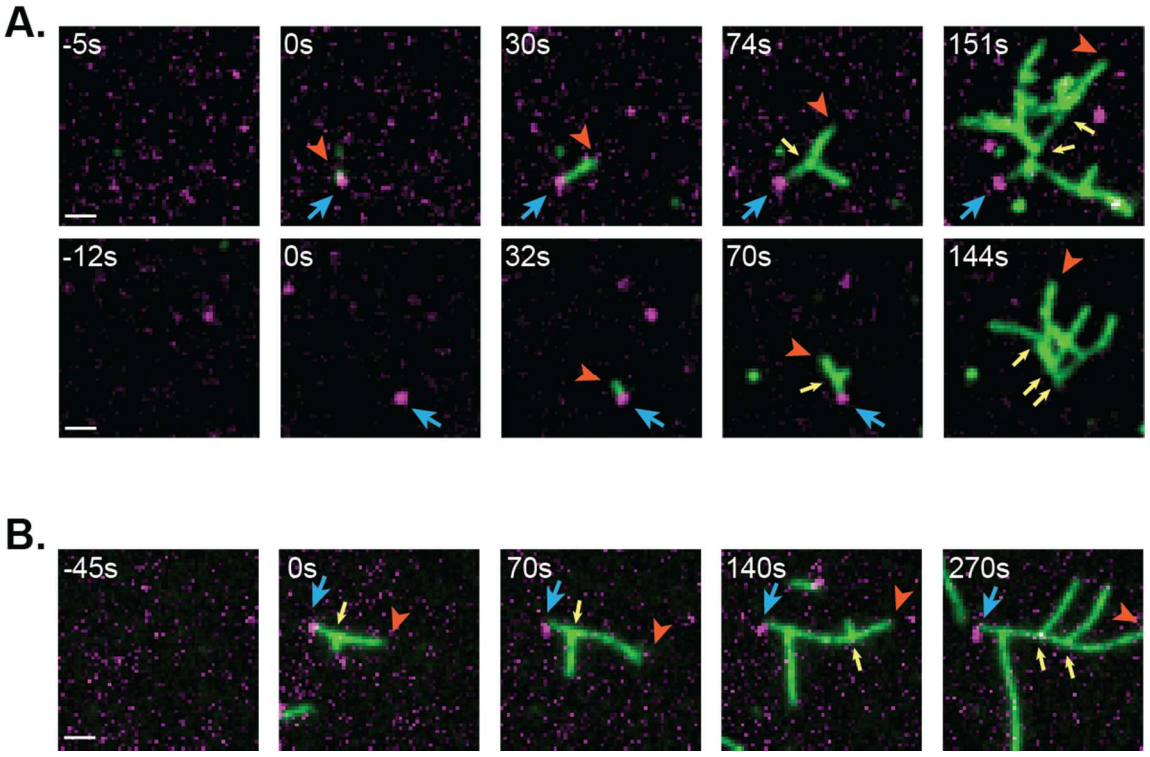

Figure 3: Actin filaments nucleated by Dip1 and Arp2/3 complex activate Wsp1-bound Arp2/3 complex. A, B. TIRF images of reactions containing 6nM Dip1-Alexa568, 1.5 µM 33% Oregon Green labeled actin, 500 nM SpArp2/3 complex, and 250 nM GST-Wsp1-VCA. C. TIRF image from conditions described in A showing two mother filament sources: branches (Wsp1-Arp2/3-mediated) and Dip1-Arp2/3 nucleated seed filaments.

Because Arp2/3 complex stays anchored on pointed ends during Dip1-mediated activation, the pointed ends of seed filaments nucleated by Dip1 are protected from depolymerization. To rapidly disassemble branched actin networks, cells cannot rely on the

relatively slow depolymerization of subunits from ADP-actin ends (Berro and Pollard 2014). Networks are actively disassembled by the severing activities of coflin and its accessory proteins, and the debranching activities of cofilin and the colfilin-like protein GMF (Lappalainen et al. 1997, Okreglak and Drubin 2007, Chan, Beltzner, and Pollard 2009, Gandhi et al. 2010) (Luan and Nolen 2013). Our data suggest that specific mechanisms may also be required to stimulate dissociation of Arp2/3 complex from the pointed ends of seed filaments. Because GMF binds directly to Arp2/3 complex to dissociate branches, it is tempting to speculate that GMF may dissociate Arp2/3 complex from the pointed ends of linear filaments.

Multiple NPFs are present at endocytic sites in *S. pombe*, including, Myo1, Wsp1 and Dip1, and potentially Pan1 (Sirotkin et al. 2005, Basu and Chang 2011, Kovar, Sirotkin, and Lord 2011). While few experiments address how NPFs coordinately regulate the complex, our results here show that Wsp1 and Dip1 use distinct mechanisms to activate a common mode of nucleation by Arp2/3 complex. If these mechanisms can work in concert, these two classes of NPFs could synergistically activate the complex, potentially influencing the rates of network seeding or propagation of branching, or both. Therefore, structural descriptions of the interactions of each NPF with Arp2/3 complex and quantitative biochemical studies of the influence of coordinated regulation of the complex by the two NPFs will be important to understand the kinetics of branched actin assembly in cells.

The seeding function of WDS family proteins is likely conserved

Here we directly demonstrate that actin filaments nucleated by Dip1-activated Arp2/3 complex can stimulate branching nucleation by WASP-bound Arp2/3 complex. Together with previous data showing that Dip1 deletion causes Wsp1 stalling at the cortex (Basu and Chang 2011), and our observation that Dip1 can activate Arp2/3 complex without preformed filaments (Wagner et al. 2013), these data strongly support a model in which Dip1 seeds assembly of branched actin networks at endocytic sites in cells. Several lines of evidence suggest the seeding function of WDS proteins is broadly conserved. Given that diverse WDS proteins activate the complex with similar biochemical properties and share a common Arp2/3 complex activating domain (Wagner et al. 2013), we anticipate that seeds generated by any WDS family protein will activate WASP-Arp2/3 complexes, as we observed here for Dip1. Second, previous studies provide preliminary support for roles for other WDS family proteins in network seeding. For

instance, deletion of the budding yeast homologue of Dip1, Ldb17, causes endocytosis defects, decreasing the number of endocytic actin patches and increasing their size (Burston et al. 2009). This phenotype is identical to the *dip1Δ* phenotype in yeast(Basu and Chang 2011), and is consistent with a model in which Ldb17 initiates new patches by providing preformed filaments to activate WASP-bound Arp2/3 complex. Less is known about the *in vivo* function of the mammalian WDS protein, SPIN90, but experiments suggest that it interacts with endocytic proteins, and it contributes to uptake of at least one endocytic cargo, EGFR(Oh et al. 2013). In addition to endocytosis, some studies suggest SPIN90 may play a role in assembly of actin in lamellipodia. Specifically, one group showed that knockdown of SPIN90 in Cos7 cells prevents PDGF-induced ruffling. It will be important to determine whether SPIN90 provides seed filaments to initiate branched actin assembly in lamelipodia.

WDS proteins are one of many potential sources of seed filaments.

We note that both lamelliopidal and endocytic branched actin networks contain multiple potential sources for seed filaments. For instance, several formins, which nucleate linear actin filaments, localize to lamelipodia and contribute to actin assembly (Isogai et al. 2015, Yang et al. 2007). It will be important to determine if formin-nucleated actin filaments seed Arp2/3 complex activation, or whether formins influence lamellipodial networks in other ways, for instance though elongation of filament barbed ends or by influencing the overall architecture of lamellipodial actin networks (e.g., the presence of lamellipodial actin bundles) (Yang et al. 2007). Another potential seeding mechanism relies on short sequences in certain NPFs that can nucleate actin filaments independently of Arp2/3 complex. Such sequences are found in JMY, an NPF that moonlights as a transcriptional regulator, and Las17, the WASP family protein in budding yeast (Zuchero et al. 2009, Urbanek et al. 2013). The Arp2/3-independent actin nucleation sequence in Las17 has been mapped the central poly-proline region, which contains both predicated and verified binding sites for many SH3 domain containing proteins, as well as the actin monomer binding protein profilin (Soulard et al. 2002, Feliciano and Di Pietro 2012, Tong et al. 2002, Allwood et al. 2016, Rodal et al. 2003, Spiess et al. 2013). While mutations that block nucleation by the Las17 nucleating segment show defects in early stages of endocytosis, it is not yet clear if this is due to a defect in seeding or failure to interact with one of its binding partners (Allwood et al. 2016).

Seeding of endocytic actin patches may also occur without *de novo* filament generation through the "sever and capture mechanism". In this mechanism, the actin filament severing protein cofilin severs short filaments from existing endocytic actin patches, and the filaments diffuse to and are captured by actin filament binding proteins at nascent endocytic sites (Chen and Pollard 2013). Mutations that selectively block cofilin-mediated severing cause delays in actin assembly, consistent with a role for severed filaments in seeding new actin patches (Chen and Pollard 2013). However, this seeding method relies on existing actin patches to generate seed filaments, so cannot explain the origin of the initial seed filament(s). Given the importance of the initiation step in assembly of branched actin networks, understanding the biochemical differences between each of these seeders, how they are regulated, and their relative contribution to different branched actin structures remain important open questions.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Fluorescent Labeling:

To generate a Dip1 construct for site specific labeling with a cysteine reactive fluorescent dye, the six endogenous cysteines were mutated to alanine by amplifying pGV67-Dip1 (described previously in Chapter III]) with non-overlapping 5'phosphoryated primers encoding the mutation. The N-terminal Not1 restriction site, used to generate the GST-TEV-Dip1 expression vector, codes for a cysteine that was exploited for labeling. For expression and labeling of mutant protein, BL21(DE3)RIL *E. coli* transformed with the pGV67 Dip1 expression vector was grown to an O.D.595 of 0.6-0.7, induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, and grown overnight at 22 °C. Cells were lysed by sonication in lysis buffer; 20 mM Tris pH 8.0, 140 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor tablets (Roche). Lysate was clarified by centrifugation, and the soluble fraction was loaded on a glutathione sepharose column and eluted with 20 mM Tris pH 8.0, 140 mM NaCl and 50 mM glutathione. Peak fractions were pooled and a 25:1 ratio (by mass) of TEV protease to recombinant proteins was added. The reaction mix was dialyzed overnight at 4 °C against 20 mM Tris pH 8.0, 50 mM NaCl and 1mM dithiothreitol. The sample was loaded onto a 6ml Resource Q column at pH 8.0 and eluted with a gradient of 50 mM to 500 mM NaCl. Protein was then concentrated in an Amicon-Ultra concentration device before loading on a Superdex 200 HiLoad 16/60 gel filtration column and eluted in 20 mM HEPES pH 7.0, 50 mM

NaCl. Peak fractions were pooled and concentrated to ~ 40 uM for labeling. A 10 mM solution of Alexa568 C5 Maleimide (Thermo Fisher) was prepared by dissolving in water according to manufacturer's protocol. Protein was labeled by the dropwise addition of a 10-40 molar ratio of dye:protein while stirring at 4°C. The reaction was quenched after 12-16 hrs by dialyzing against 20 mM Tris pH 8.0, 50 mM NaCl, and 1 mM dithiothreitol for 24 hours at 4 °C with buffer exchanges after 4hr and 8hrs. Labeled protein sample was loaded on a 5mL Hi-Trap desalting column and peak fractions were pooled and flash frozen in liquid nitrogen. *S. pombe* Wsp1-VCA and *S. pombe* (Sp) Arp2/3 complex (Liu et al. 2013) were purified as described previously (Liu et al. 2013).

TIRF microscopy of actin polymerization

TIRF flow chambers were constructed and reactions setup essentially as previously described with slight modifications (Kuhn and Pollard 2005). TIRF chambers were created by sandwiching double-sided tape between a glass microscope slide and a 24 x 50 #1.5 coverslip to create a 14 µL, 0.5 cm wide chamber. To initiate the reaction, 1 µL of 0.025 mM $MgCl_2$ and 1 mM EGTA was mixed with 5 µL of 9 µM 33% Oregon-green actin and incubated for 2 min before adding TIRF buffer (10 mM Imidazole pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 50 mM KCl, 100 mM DTT, 0.2 mM ATP, 25 mM Glucose, 0.5 % Metylcellulose (400 cP at 2%), 0.02 mg/mL Catalase (Sigma) and 0.1 mg/mL Glucose Oxidase (MP Biomedicals) to 1 x, *S. pombe* Arp2/3 complex to 50 nM, GST-Wsp1-VCA to 75 nM, or Dip1 to 300nM or 150 nM final concentration. For single color reactions, 50 ms exposures with the 488 nm laser were taken at 1000 ms intervals, and a typical polymerization reaction was imaged for 10 min. For two color reactions typical imaging conditions were 50 ms exposures with the 488 nm laser at 1000 ms intervals and 50 ms exposures with the 561 nm laser at a range of intervals. For low exposure imaging conditions, the interval between 561 frames was adjusted from 200 ms – 5000 ms to decrease the laser exposure by a factor of 25. The concentration of Dip1 was kept at 6 nM to minimize the background signal in the 561 channel.

TIRF Image Analysis:

Images were prepared in Image J. Image sequences were subtracted for background signal with a 10-pixel rolling ball radius. The total actin polymer was calculated using a custom image

processing script run in Matlab, described as follows. For each frame, pixels corresponding to filament fluorescence were identified using image segmentation followed by morphological area opening to remove non-filament small fluorescent objects. The final pixel number value was converted to micrometers (1px = 106.7 nm) to yield the total length of actin filaments in the image frame, and further converted to number of subunits using 370 subunits μm^{-1}. To calculate the ratio of branched to linear filaments, the number of branches and linear filaments were counted manually when the total polymer length in each movie was approximately 300 μm.

BRIDGE TO CHAPTER IV

In this chapter, we established that Dip1 co-opts features of branching nucleation to activate the Arp2/3 complex. We verified Dip1-Alexa568 as a tool for single molecule TIRF microscopy experiments. Importantly, we directly observed that filaments generated by Dip1-activated Arp2/3 complex can serve as seeds for WASP-mediated branching. In the next chapter, we investigate how Dip1 is regulated. We begin to investigate how the activity of Dip1 and WASP is balanced. Specifically, we investigate the influence of Dip1 generated linear filaments on actin network architectures.

CHAPTER IV

DIP1-MEDIATED ACTIVATION OF ARP2/3 COMPLEX USES A SINGLE TURNOVER MECHANISM THAT MAY PRESERVE THE DENDRITIC NATURE OF ACTIN NETWORKS IT SEEDS

This chapter contains unpublished co-authored material

Author contributions: Andrew Wagner and Brad Nolen designed research; Andrew Wagner performed research and analyzed data; Andrew Wagner and Brad Nolen wrote the manuscript.

INTRODUCTION

Actin filaments play essential roles in cells, forming interconnected networks that provide structural support, form tracks for transport of material or contraction by molecular motors, and generate pushing forces to remodel membranes (Blanchoin et al. 2014, Pollard and Cooper 2009). Cellular actin networks have diverse architectures, and whether actin filaments within a network are branched or linear, parallel or antiparallel, bundled or single, or long or short influences their ability to carry out specific biochemical functions (Hariadi, 2015; Reymann, 2012; Ennomani, 2016; Gressin, 2015; Blanchoin, 2014; Skau, 2015). Therefore, in addition to controlling the localization and dynamics of filamentous networks, actin regulatory proteins must exert tight control over actin network architectures. Actin filament nucleators are important actin regulators that not only control when and where filamentous actin networks assemble, but also influence network architectures by determining the topology of filaments they nucleate (Siton-Mendelson, 2017; Reymann, 2010). Two classes of actin filament nucleators, formins and tandem WH2 proteins, nucleate linear filaments, whereas Arp2/3 complex, a seven-subunit protein assembly, is the only nucleator that creates branched actin filaments (Campellone, 2010; Siton-Mendelson, 2017). The branching nucleation activity of Arp2/3 complex allows it to assemble dendritic networks thought to be optimal for pushing against broad surfaces, since short, crosslinked filaments resist buckling (Pollard, 2003; Pujol, 2012; Mogilner, 1996). Actin networks assembled by Arp2/3 complex are required for numerous cellular processes, including lamelipodial protrusion and membrane remodeling during endocytosis (Wu et al. 2012, Suraneni et al. 2012, Mooren, Galletta, and Cooper 2012, Kaksonen, Toret, and Drubin 2006, Goode,

Eskin, and Wendland 2015). Actin networks in lamellipodia are branched, consistent with the idea that branched networks are optimized to push against the broad leading edge of lamellipodial protrusions (Vinzenz, 2012; Urban, 2010; Small, 2011; Svitkina, 1997). Similarly, ultrastructural analysis demonstrates endocytic actin networks (patches) in yeast are also branched, though challenges in preparing yeast for EM imaging has precluded a precise understanding of actin architectures in these networks (Rodal et al. 2005, Young, Cooper, and Bridgman 2004).

Arp2/3 complex has little or no intrinsic nucleation activity, and its activation requires interaction with one of multiple classes of nucleation promoting factor (NPF) proteins (Goley and Welch 2006, Rotty, Wu, and Bear 2013). WASP family proteins, the best-studied NPFs, use a multi-step mechanism that ensures WASP-activated Arp2/3 complex always creates branches. Specifically, while WASP stimulates an activating conformational change in the complex, WASP alone is not sufficient for activation (Rodnick-Smith et al. 2016, Xu et al. 2011, Padrick et al. 2011, Ti et al. 2011). WASP-bound Arp2/3 complex nucleates filaments only once it is bound to the side of a preformed filament (Achard et al. 2010, Higgs, Blanchoin, and Pollard 1999). Importantly, once a branched filament has been nucleated, it can activate additional WASP-bound Arp2/3 complexes to stimulate additional rounds of branching, creating a feedback mechanism that allows WASP and Arp2/3 complex to propagate the assembly of highly dendritic networks.

While the biochemical mechanism of WASP ensures branching, it means that assembly of a WASP-Arp2/3 nucleated dendritic network must be primed with an initial seed filament. Multiple potential sources of seed filaments have been identified. For example, some WASP family proteins, including budding yeast Las17 and mammalian JMY, contain short actin monomer binding sequences that nucleate filaments in the absence of Arp2/3 complex (Zuchero et al. 2009, Urbanek et al. 2013). In fission yeast, two mechanisms contribute to seeding branched actin network assembly in endocytic actin patches. In one mechanism, the actin filament binding protein cofilin severs filaments from pre-exisiting actin patches (Chen and Pollard 2013). The severed filaments diffuse and are captured by actin binding proteins at new endocytic sites to prime branched actin network assembly. This mechanism cannot fully explain seeding, because it relies on pre-existing branched networks to create seeds. We recently discovered a second seeding mechanism that does not rely on pre-existing branched actin

networks (Wagner et al. 2013). Specifically, we identified a class of Arp2/3 complex activators called WISH/DIP/SPIN90 (WDS) proteins that unlike WASP, can activate Arp2/3 complex without preformed filaments. Importantly, we showed that filaments nucleated by Dip1, the WDS protein from fission yeast, and Arp2/3 complex activate Wsp1-bound Arp2/3 complex to create branches, directly demonstrating that Dip1 seeds branching (Chapter III). Like Wsp1, Dip1 localizes to cortical actin patches, and deletion of Dip1 causes actin patch assembly defects that support a role for Dip1 in seeding actin network assembly (Basu and Chang 2011). In wild type cells, endocytic actin patches begin to assemble within seconds of Wsp1 accumulation at endocytic sites (Sirotkin et al. 2010). Actin assembles for ~15 sec, before patches internalize into the cytoplasm and disassemble. In *dip1Δ* cells, Wsp1 stalls at the cortex, sometimes for hundreds of seconds, consistent with a model in which Dip1 and Arp2/3 complex provide seed filaments that activate WASP-bound Arp2/3 complex and initiate actin patch assembly (Basu and Chang 2011).

While Dip1 allows Arp2/3 complex to nucleate seed filaments, its activation mechanism presents a potential obstacle to assembling branched actin networks; Because Dip1 does not require preformed filaments, it activates Arp2/3 complex to create linear instead of branched actin filaments. How does an actin structure get the branch density required for proper function? It is not known how the coordinate action of Dip1 and Wsp1 influences the architectures of actin Arp2/3-assembled networks. Previous studies have shown that regulation of Arp2/3 complex by multiple distinct classes of NPFs can profoundly influence network topology, in some cases in a way distinct from the effect of individual NPFs. So while Dip1 on its own activates Arp2/3 complex to create linear filaments, the topology of actin filaments created by activation of Arp2/3 complex in the presence of both Dip1 and Wsp1 is unknown. It is possible that Wsp1 represses linear filament creation by Dip1, or that Dip1 synergizes with Wsp1 to create branches – either scenario could prevent Dip1 from significantly altering the branched architecture of actin networks assembled in the presence of both NPFs. Repressive or synergistic interactions between NPFs have been observed previously. For example, cortactin, a type II NPF, on its own activates Arp2/3 complex to create sparsely branched filament bundles (Helgeson et al. 2014), but synergizes with WASP to create highly dendritic actin networks. In addition, WASP strongly represses the bundling activity of cortactin. Therefore, understanding the influence of Dip1 on

actin architecture will requires biochemical studies of Dip1 function both alone and in the context of other NPFs.

Here, we investigate how the linear filament stimulating activity of Dip1 influences Arp2/3-mediated actin network assembly in the presence of branch-stimulating NPFs *in vitro* and *in vivo*. We show that Dip1 overexpression in *S. pombe* causes defects in endocytic actin patches, consistent with a model in which Dip1 activity must be limited to construct networks with the proper architecture. We use TIRF microscopy to show that *in vitro*, the linear filament nucleation activity of Dip1 potently antagonizes branching stimulated by Wsp1. Even in the presence of high concentrations of Wsp1, Dip1 causes disconnected linear arrays of actin filaments to assemble instead of highly branched networks, demonstrating that the presence of Wsp1 does not alter or limit the linear filament generating activity of Dip1. However, our data reveal a fundamental mechanistic difference between WASP proteins and Dip1 that could explain how their activities are balanced *in vivo*. Whereas WASP proteins are released from Arp2/3 complex upon nucleation, we use single molecule TIRF measurements to show that Dip1 stays bound to Arp2/3 complex for hundreds of seconds after nucleation, on average 10-fold longer than the lifetime of an average endocytic actin patch. Therefore, unlike WASP proteins, Dip1 is consumed in the linear filament nucleation reaction, and acts as a single turnover NPF. Dip1 is incorporated into treadmilling actin networks in *S. pombe*, whereas Wsp1 remains at the cortex, supporting a single turnover mechanism for Dip1 in vivo. This mechanistic difference between Dip1 and Wsp1 could explain how the activity of Dip1 is regulated to create enough linear filaments to seed network assembly, yet preserve the dendritic nature of the endocytic actin patches thought to be required for function.

RESULTS

Increased Dip1 concentrations at the endocytic sites cause defects in actin dynamics

As a first step in understanding how the level of Dip1 activity might influence actin structures *in vivo*, we asked if increasing the concentration of this linear filament nucleator causes defects in endocytic actin patches. To accomplish this, we first tagged *DIP1* at the endogenous locus with a C-terminal mEGFP tag. To confirm that the GFP tag did not influence function, we used Fim1-mCherry, an actin filament binding protein that marks endocytic actin networks (ref), to compare the number of endocytic actin patches in wild type versus Dip-GFP

strains. As previously reported, Dip1 knockout strains show an ~3 fold reduction in the number of endocytic patches, presumably due to a lack of seed filaments to initiate patches (Basu and Chang 2011).

Expression of GFP-tagged Dip1 under its native promoter rescued this defect, showing the same number of endocytic actin patches as the wild type strain (Fig S1). When observed using confocal microscopy, Dip1-GFP appeared diffuse within the cytoplasm, in linear structures within the cytoplasm, and in a few cases in cortical puncta previously identified as endocytic actin patches (Fig S2). Because the signal for Dip1-GFP was weak, we also imaged the strains using TIRF microscopy. TIRF images showed many more Dip1-GFP puncta, all of which overlapped with Fim1-mCherry, confirming that Dip1 localizes to actin patches (Fig 1A.) (Basu and Chang 2011). To carry out overexpression, we created an *S. pombe* strain with *dip1-GFP* under control of a strong thiamine-repressible promoter P3nmt1 (Forsburg 1993). When switched to a growth medium lacking thiamine, this strain showed an increase in total Dip1-GFP expression when compared to the wild type promoter (Fig 1A.) After 22 hours of expression, some cells appeared misshapen (Fig 1B.), and the P3ntm1 dip1-GFP strain was less viable compared to non-overexpressing cells (unpublished observations). We noticed abnormal actin patch dynamics in a subset of cells over expressing Dip1-GFP. Specifically, we saw rare events where a Dip1 puncta would originate at the cortex and Fim1-marked actin assembled similar to wild type actin patches. After building to a peak a large comet tail of actin, containing both Fim1 and Dip1, would streak into the cytoplasm and last for hundreds of seconds (Fig 1C.). We hypothesized these defects resulted from disproportionate accumulation of Dip1 at a single endocytic site. To determine if excess Dip1-GFP accumulates at endocytic sites in overexpressing strains, we measured the intensity of the GFP signal over the course of assembly and disassembly. Endogenous Dip1-GFP signal is weak, in order to quantify accumulation of signal in wildtype and over expressing we used oblique angle TIRF microscopy. To standardize the signal intensity, we imaged over expressing and wildtype cells on the same slide and asked how much Dip1-GFP accumulated in patches compared to wild type cells. The Dip1-GFP signal for an average endocytic patch in P3nmt1-Dip1-GFP overexpression stains was ~3-fold more intense than for wild type patches, demonstrating that excess Dip1-GFP is recruited to the patches. Some patches contained as much as 10 to 15 times as much Dip1-GFP signal. (Fig 1D, E.).

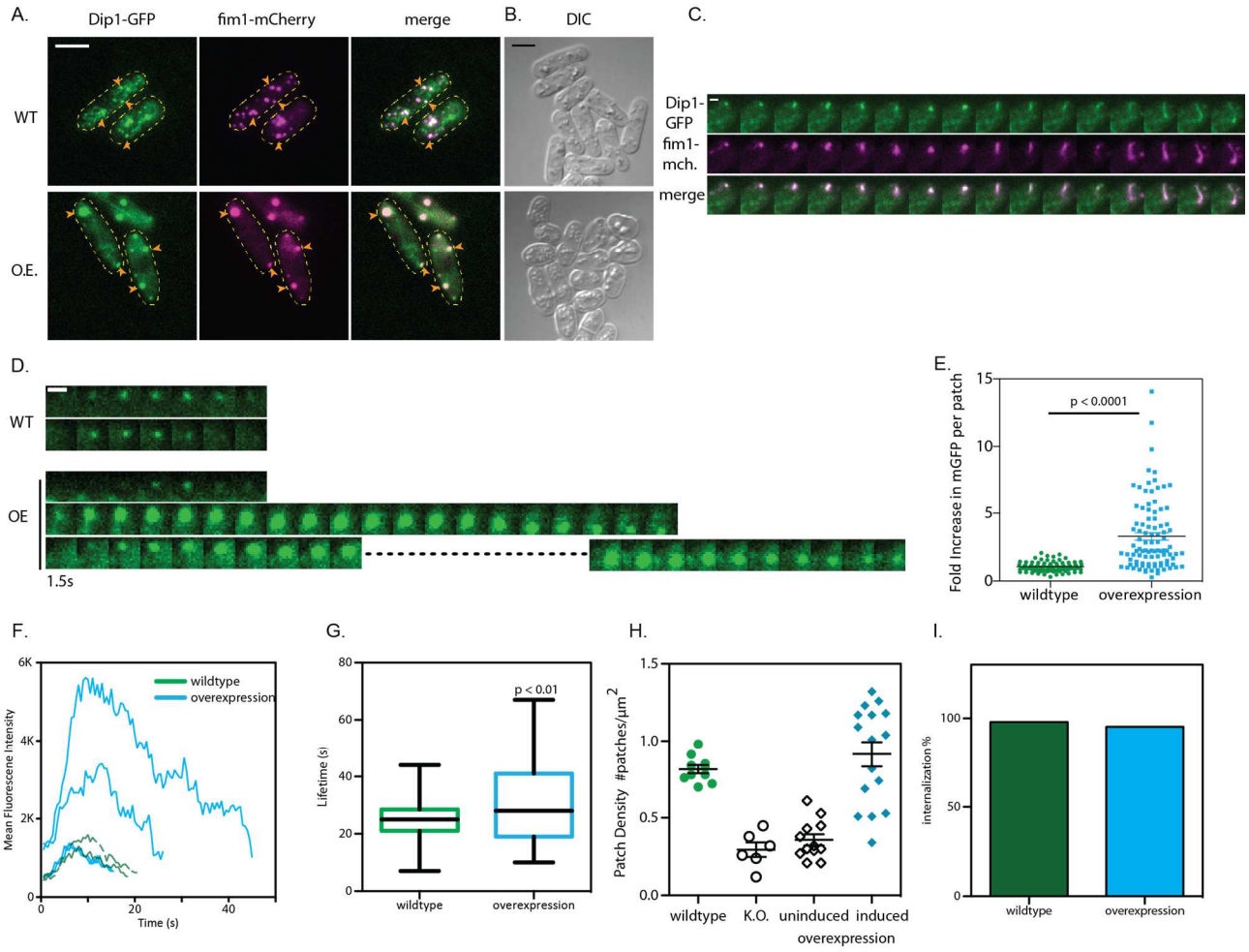

Figure 1: Increased Dip1 concentrations at the endocytic sites cause defects in actin dynamics. **A.** Oblique angle TIRF microscopy images of *S. pombe* cells expressing Fim1-mCherry and Dip1-GFP under its endogenous promotor or the thiamine repressible p3nmt1 promotor in inducing conditions lacking thiamine. Labeled O.E. throughout figure **B.** DIC images of cells expressing Dip1-GFP under and endogenous promotor or under the induced p3nmt1 promotor. **C.** TIRF microscopy images of cells under the induced p3nmt1 promoter showing a rare comet tail event. **D.** Montages of individual actin patches from wildtype or cells under the induced p3nmt1 promoter. **E.** Scatter plot of the fold increase in maximum total intensity of Dip1-GFP signal of individual patches relative to the average maximum intensity of Dip1-GFP signal in non-overexpressing strains. Plots are shown for individual patches in strains expressing Dip1-GFP under its endogenous promotor (n = 81) or an induced p3nmt1 promotor (n = 96). Intensities were quantified from TIRF images (see methods). P-value shown from a two tailed Mann-Whitney test using a Gaussian approximation. **F.** Plot of mean fluorescence intensity of Fim1-mCherry signal versus time for cells expressing Fim1-mCherry plus Dip1-GFP under its native promotor or induced P3nmt1-Dip1-GFP. **G.** Box and whisker plot showing lifetime of patches from wildtype patches (n=49) or induced p3nmt1-Dip1-GFP patches (n = 42). P-value shown from two tailed t-test with Welch's correction for unequal variance. **H.** Patch density in either wildtype cells or the genotype indicated. (wildtype: n = 10 cells, Dip1 knockout (K.O.) n = 6 cells, uninduced p3nmt1-Dip1-GFP n = 12 cells, induced p3nmt1-Dip1-GFP n = 16 cells). **I.** Percent of patches that internalize in wildtype cells or p3nmt1-Dip1-GFP expressing cells. (wildtype n = 49 patches in 7 cells, p3nmt-Dip1-GFP n=42 patches in 6 cells).

To determine how higher concentrations of Dip1 influence actin dynamics at endocytic sites, we analyzed the Fim1-mCherry signal in these cells. At endogenous Dip1 expression levels, Fim1-marked actin patches assemble at the cortex over ~10 s, then move into the cytoplasm as the actin patch disassembles (Fig 1F.) In contrast, in the overexpression strain, fim1 signal accumulation was more variable, in some cases assembling to a peak much greater than wildtype patches and assembling actin more quickly but lasting for longer than wild type patches. The longer lasting patches in the over expressing cells significantly increased the average lifetime from 20 to 30 seconds (Fig 1G.) Importantly, the average lifetime reflects the variability between patches in over expressing cells, with a subset of patches exhibiting wildtype lifetimes and a subset with much longer lifetimes, up to 60 seconds (Fig 1D.). The increase in fim1 signal, Dip1 signal, and average lifetime of patches in over expressing cells indicates Dip1 is sufficient to increase the amount of actin at sites of endocytosis. Along these lines, we quantified the patch density which is a reflection of patch initiation and lifetime. Consistent with previously published results, the uninduced cells reassembled the dip1 knockout. When induced, cells over expressing Dip1 rescued the wild type number of patches, albeit with some cells containing a higher density than wildtype and some with a lower density (Fig 1H.) Together, these observations indicate that increased Dip1-GFP at endocytic sites cases defects in endocytic actin patches, supporting the hypothesis that the activity of Dip1 must be limited to properly regulate endocytic actin. We note that despite the pronounced defects, some aspects of patch dynamics were relatively normal, including the percentage of patches that internalize. While we cannot currently explain this observation, it suggests patches containing excess Dip1 are not completely defective (Fig 1I).

Dip1 potently induces linear actin network architectures, even in the presence of Wsp1

We wondered if an imbalance of linear and branched filament nucleation activities could cause defective actin architectures at endocytic sites, explaining the overexpression phenotype. To address this, we used TIRF microscopy to visualize how actin network assembly is influenced by the combined influence of linear and branching NPFs. We first measured the influence of titrating Dip1 into reactions containing 150 nM Wsp1-VCA, 50 nM *S. pombe* (Sp) Arp2/3 complex and 1.5 μM 33% Oregon Green labeled actin. In reactions lacking Dip1, many branches grew from a few spontaneous nucleated filaments, generating networks with high

branch density and a high branching order, in which branches frequently grew off of other branches (Fig 2 A, B.).

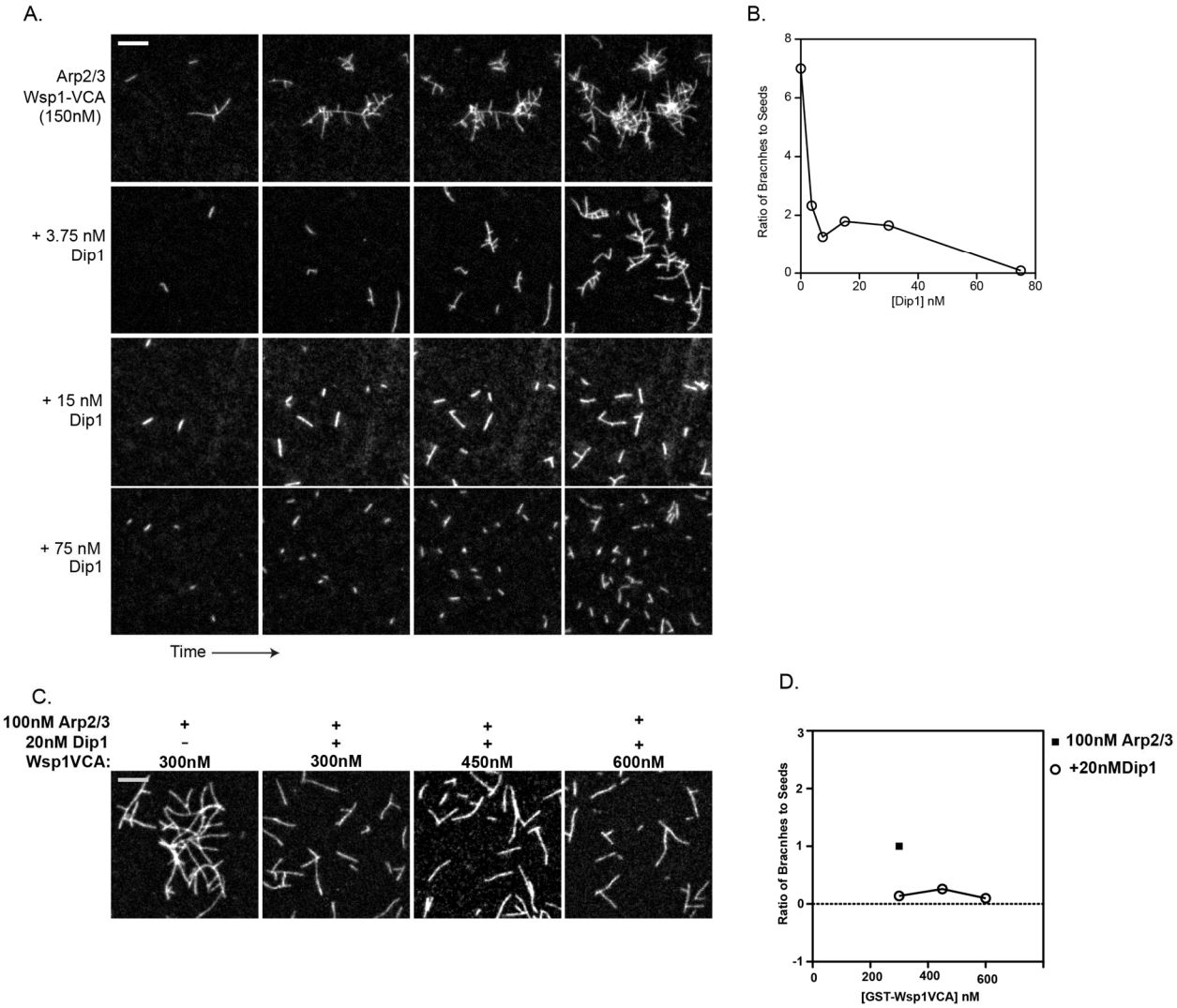

Figure 2: Dip1 potently induces linear actin network architectures, even in the presence of Wsp1. **A.** Frames from TIRF microscopy movies imaging polymerization of 1.5 µM 33% labeled Oregon actin in the presence of 50 nM *Sp*Arp2/3 complex, 150 nM GST-Wsp1-VCA, and the indicated concentration of Dip1. Columns show time points of reactions when the total actin polymer was roughly equivalent across conditions (quantification detailed in materials and methods). **B.** Ratio of branched to linear filaments from movies shown in A. Quantification was measured at time points when total polymer was roughly equivalent to 300 µm. **C.** TIRF panels showing influence of increasing GST-Wsp1-VCA on reactions imaging the polymerization 1.5 µM 33% labeled Oregon Green actin in the presence of 100 nM *Sp* Arp2/3 complex. Each panel shows reaction when total polymer was equivalent roughly 300 µm. **D.** Ratio of branched to linear filaments from movies shown in D. Quantification was measured at time points when total polymer was roughly equivalent to 300 µm.

Adding Dip1 to the reactions significantly influenced the actin filament architectures. Even at low concentrations of Dip1 (*e.g.,* 3.8 nM), both the ratio of branched to linear filaments and the

overall branching order decreased, causing network architectures to become less dendritic (Fig 2 A, B.). At 75 nM Dip1 the ratio of branches was decreased 70-fold compared to reactions without Dip1, and 300 nM Dip1 completely eliminated branching in the reactions, even though 150 nM Wsp1-VCA was present (unpublished observation). These data show that Dip1 potently induces actin networks assembled by Arp2/3 complex to form linear instead of branched filaments, even in the presence of Wsp1. Therefore, Wsp1 does not attenuate the linear filament creation activity of Dip1, nor does Dip1 increase branching by Wsp1-Arp2/3 complex.

We wondered if the combined influence of Dip1 and Wsp1 could be explained by the relative ratio of Dip1 to Wsp1, which would imply a simple competition between the two NPFs. To test this, we repeated the experiment with a fixed concentration of Dip1, and titrated increasing concentrations of Wsp1. Unexpectedly, addition of Wsp1 did not significantly change the ratio of branched to linear filaments (or the branch density) (Fig 2C, D.) Therefore, under these conditions, the absolute concentration of Dip1, not its relative concentration compared to Wsp1, controls the influence of Dip1 on the architecture of the assembled networks. We suggest the dominance of Dip1 in determining the network architecture may be caused by the rapid kinetics of Dip1-mediated activation of Arp2/3 complex compared to Wsp1-mediated activation (Wagner et al. 2013). Together, these observations indicate that the effect of Dip1 overexpression may be to change the architectures of the networks.

Dip1 binds to treadmilling actin networks in *S. pombe*

We showed that *in vitro*, Wsp1 fails to limit linear filament creation by Dip1, so reactions containing Dip1 and Wsp1 are dominated by the linear filament nucleation activity of Dip1. Given this dominance, we wondered whether Wsp1 and Dip1 might be spatially separated at endocytic sites to prevent Dip1 from antagonizing the activity of Wsp1 in highly branched regions of endocytic actin networks. Precedence for distinct localization of different NPFs at endocytic sites derives from gold antibody labeled electron micrographs of budding yeast, which showed that Las17 and Myo5p localize in distinct patterns along the length of the endocytic invagination (Idrissi et al. 2008). While Dip1 and Wsp1 both localize to actin patches in fluorescence micrographs (Basu and Chang 2011), actin patches are diffraction limited, so differences in the distribution of the two NPFs at endocytic sites cannot be resolved.

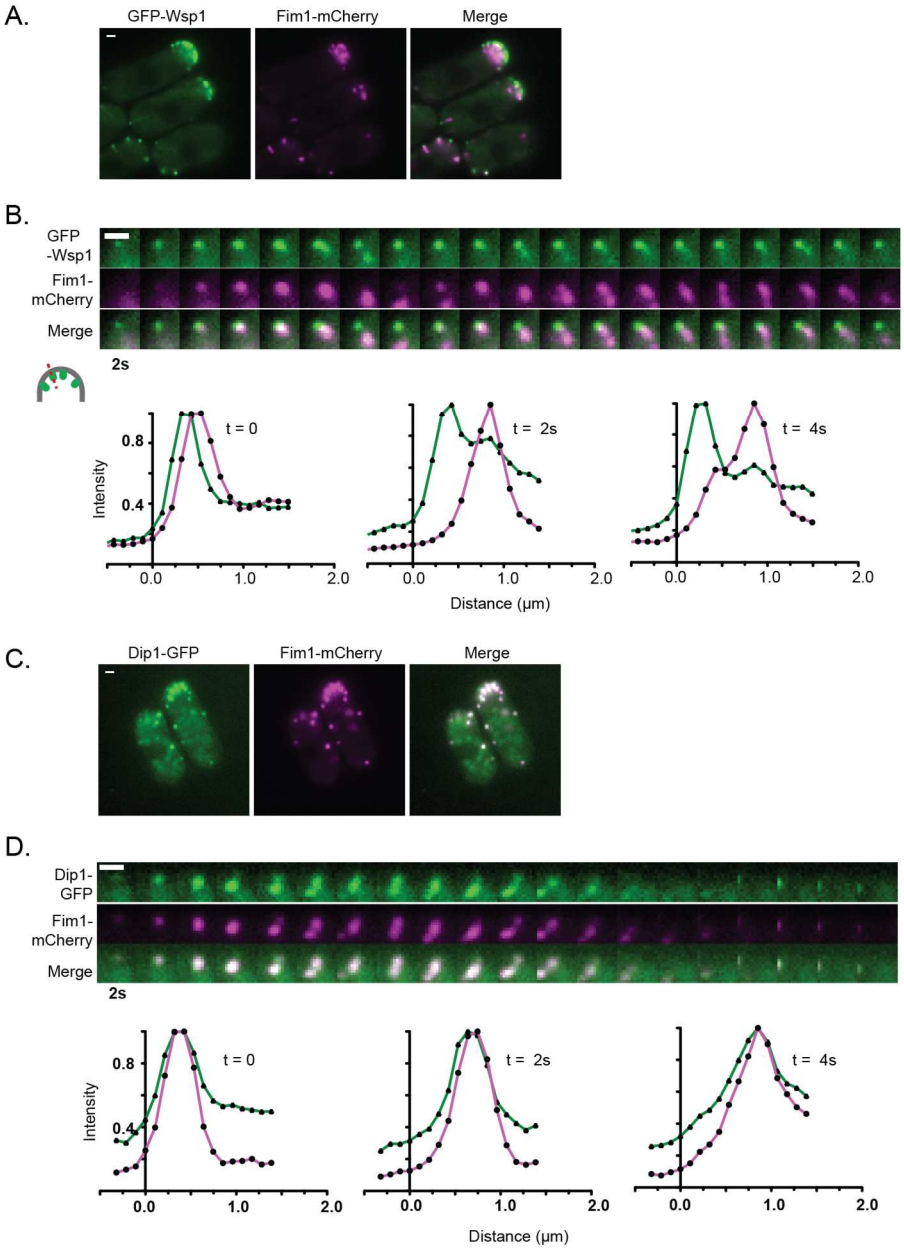

Figure 3: Dip1 binds to treadmilling actin networks in *S. pombe.* **A.** Images from TIRF microscopy experiments of *S. pombe* cells expressing Fim1-mCherry plus Wsp1-GFP in the *end4Δ* background. (scale bar = 1 μm) **B.** Montage of TIRF images showing two temporally separated comet tails treadmilling from a single Wsp1-GFP punctum in Fim1-mCherry-marked comet tails. Plots below montage show Wsp1-GFP and Fim1-mCherry intensity along axis perpendicular to the cortex for a single comet tail at three separate time points. (scale bar = 1 μm) **C.** TIRF microscopy images of *S. pombe* cells expressing Fim1-mCherry plus endogenously expressed Dip1-GFP in the *end4Δ* background. **D.** Montage of TIRF images showing Fim1-mCherry and Dip1-GFP signal in a single comet tail. Plots below montage show Dip1-GFP and Fim1-mCherry intensity along axis perpendicular to the cortex for a single comet tail at three separate time points. (scale bar = 1 μm)

To circumvent this issue, we labeled these NPFs and the actin patch marker Fim1 in the context of an *end4Δ* strain. *END4* deletion converts punctate and transient endocytic actin networks into continuously polymerizing actin comet tails (0.75 – 1.5 μm long) that treadmill away from endocytic adaptors on the cortex and into the cytoplasm (Newpher et al. 2005, Basu and Chang 2011, Kaksonen, Sun, and Drubin 2003) (Fig 3A).

In *S. pombe* strains marked with Wsp1-GFP, Fim1-mCherry, and *end4Δ* imaged by TIRF microscopy, most Wsp1-GFP localized at the junction between the cell cortex and the actin comet tail, as reported for the same mutation in budding yeast cells (Kaksonen, Sun, and Drubin 2003) (Fig 3B). A relatively small amount of Wsp1-GFP colocalized with the Fim1-marked treadmilling actin network. This is consistent with other studies showing most WASP remains bound at the cortex in treadmilling actin networks, and does not significantly incorporate into the network. Wsp1-GFP cortical puncta sometimes produced consecutive treadmilling comet tails (Fig 3B). In these instances, the first comet tail polymerized inward and eventually released from the cortex, but most Wsp1 remained cortical. Additional Fim1-mCherry marked tails then elongated from the same cortical Wsp1 punctum. These observations are consistent with biophysical studies that indicate WASP release is programmed into the nucleation mechanism. By releasing from branch junctions and remaining cortical, WASP can likely mediate multiple rounds of branching nucleation that allow continued comet tail polymerization from the cortex. Note that we cannot eliminate the possibility that all Wsp1 moves inward after nucleation with the pool of Wsp1 being constantly replenished at the cortex, but this is unlikely given that in other treadmilling systems, only a small fraction of cortical WASP molecules undergo retrograde flow (Mllius, 2012).

S. pombe strains with Dip1-GFP, Fim1-mCherry, and the *end4Δ* mutation cells showed that Dip1 colocalized strongly with Fim1 at the cortex when treadmilling actin structures initiated, indicating Wsp1 and Dip1 colocalize at the cortex (Fig 3C) (Basu and Chang 2011). However, in contrast to Wsp1, as the comet tails grew away from the cortex, Dip1 did not remain cortical, but instead localized along the length of the treadmilling actin network (Fig 3D). This suggests that unlike Wsp1, most or all Dip1 associates with the treadmilling actin.

When a comet tail was released from the cortex in the Dip1-GFP strain, the Dip1 signal did not remain at the cortex but instead moved into the cortex along with the Fim1-marked comet tail. By examining the localization of Dip1 and Wsp1 in the context of the End4 deletion we found remarkable differences in the dynamics of these two proteins. Dip1 becomes incorporated with the actin network during polymerization while Wsp1 remains cortical. These observations suggest a fundamental difference between biochemical mechanisms of NPFs.

Dip1 remains bound to actin filaments for hundreds of seconds after nucleation

Our *in vivo* data indicate that Dip1 is incorporated into treadmilling networks, while WASP remains at the cortex. We reasoned that this observation might hint at a critical difference in the mechanisms of Arp2/3 complex activation by the two NPFs. Specifically, WASP release is required for nucleation, and this may allow WASP to remain largely cortical, since (unreleased) WASP bound at newly formed branch junctions would treadmill inward and colocalize with the treadmilling network (Fig 4A). On the other hand, Dip1 colocalizes with the treadmilling actin network, and does not appear to be attached to the cortex. Dip1 does not bind directly to actin filaments (Wagner et al. 2013), but instead binds indirectly through Arp2/3 complex (chapter III). Therefore, we wondered whether unlike Wsp1, Dip1 might stay attached to Arp2/3 complex after nucleation, explaining why it remains bound to Arp2/3-assembled networks (Fig. 4A) To test this, we labeled Dip1 with Alexa568 on an appended N-terminal cysteine residue and directly visualized its action on Arp2/3 complex in reactions visualizing Oregon green-labeled actin in TIRF microcopy. We characterized our labeling strategy and verified that we could observe single molecules of Dip1 under these conditions in Chapter III. In these movies detailed here, we observed multiple events in which a new linear actin filament grew from an Dip1-Alexa568 molecule adsorbed to the surface (Fig. 4A). Given that Dip1 does not nucleate filaments on its own (Wagner et al. 2013), we interpret these events as Dip1-mediated Arp2/3 linear filament nucleation events (Chapter III). Importantly, Dip1 did not release from filament ends upon nucleation, and the Alexa 568 signal was visible at the filament end for many seconds as the filament elongated. Because Dip1 only binds filament ends indirectly through Arp2/3 complex (Chapter III), we conclude that Dip1 remains bound to Arp2/3 complex after nucleation.

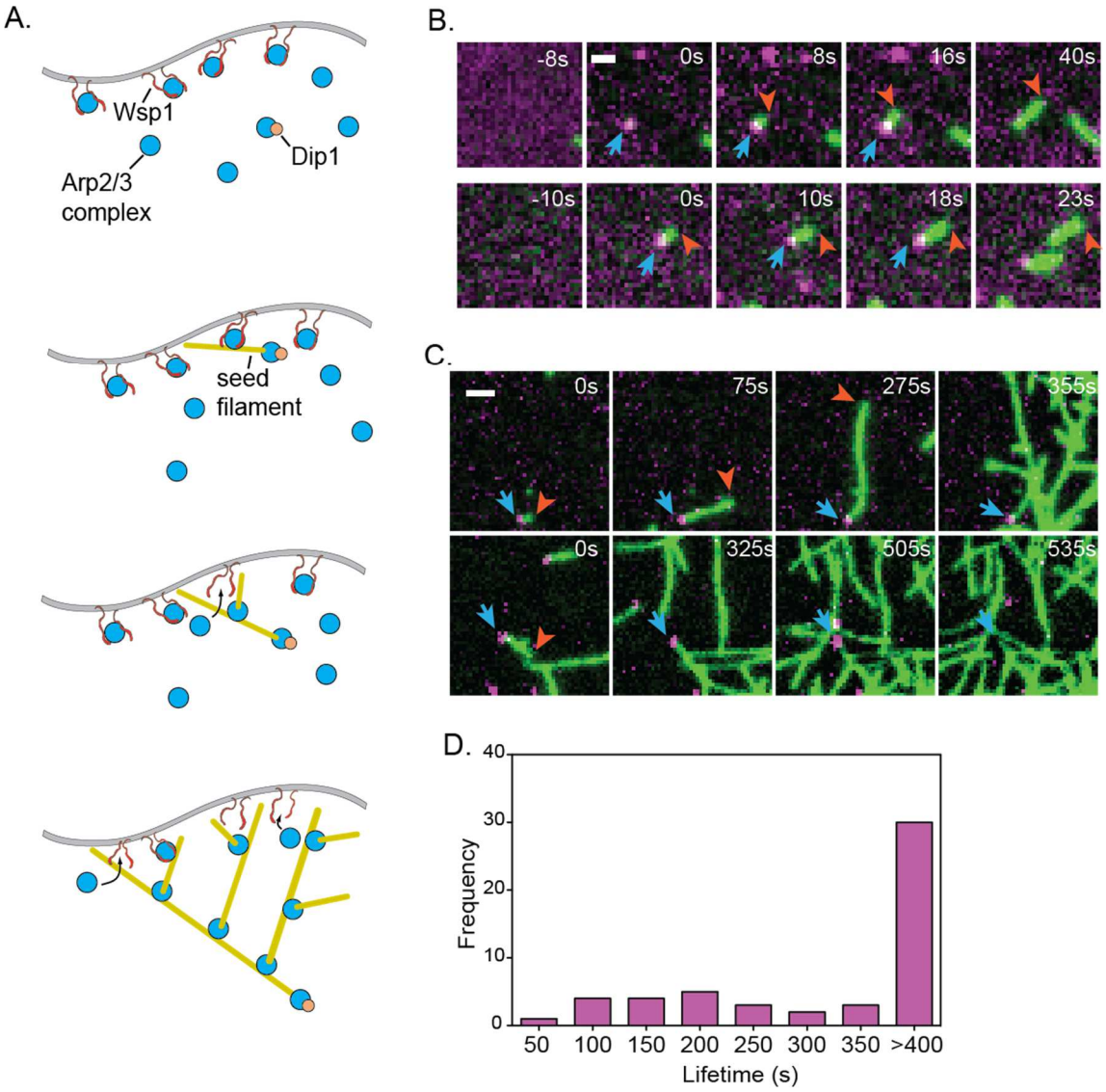

Figure 4: Dip1 Remains Bound to Actin Filaments After Nucleation. A. Cartoon model of assembly of a treadmilling branched actin network by Wsp1, Dip1 and Arp2/3 complex. Number of molecules of Dip1, Wsp1 and Arp2/3 complex are approximately proportional to their relative concentration in endocytic actin patches. **B.** Images from TIRF microscopy experiments imaging the polymerization of 1.5 µM 33% Oregon green labeled actin in the presence of 6 nM Dip1-Alexa568, and 500 nM SpArp2/3 complex under conditions with high relative laser exposure. Top row shows an apparent Dip1-mediated nucleation event. Bottom row shows an event in which a Dip1-bound actin filament bound to the imaging surface. Disappearance of Dip1-568 puncta in both of these cases could be from dissociation or photo-bleaching. **C.** Same as B, except that the frame rate for data collection was changed from 50 ms at 200ms intervals to 50 ms at 5s intervals. **D.** Histogram of single molecule lifetimes on actin filament ends under low laser exposure conditions.

In addition to the events noted above, many filaments landed on the imaging surface with Dip1 already bound to filament ends, and our previous experiments indicate that the majority of these events represent Dip1-Arp2/3 complex nucleated filaments, since Dip1 rarely binds to Arp2/3 complex preloaded on actin filament pointed ends (Chapter III, Fig 4B). To determine the lifetime of Dip1 on filament ends, we measured the average length of Dip1 binding events from both classes of events, extrapolating the birth time of the Dip1-Arp2/3 nucleated filaments in the second class based on the filament length and measured elongation rate of the filaments. Under our original imaging conditions, the average lifetime of Dip1 on the ends of filaments was ~185s (Fig S3). To determine if this lifetime was determined by photobleaching or dissociation, we repeated the imaging using decreased laser exposure (Fig 4C). At the lowest exposure levels we tested, the majority of Dip1 binding events lasted longer than the duration of the experiment (~ 7 min, Fig 4 C, D. TIRF panels and histogram), so we could not determine the rate constant for dissociation of Dip1 from Arp2/3 complex on filament ends. However, these data set a lower limit on the average Dip1 lifetime on the filament end at 185 seconds (Fig S3).
Together, these data indicate that Dip1-mediated activation of the complex does not require Dip1 release, and that Dip1 stays tightly bound to Arp2/3 complex after nucleation and throughout elongation of the filament. Importantly, this means that unlike Wsp1, Dip1 molecules are consumed in the nucleation reaction. Given that than the lifetime of Dip1 on filament ends is at least 10-fold longer than the lifetime of the average endocytic actin patch, we conclude that Dip1 functions as a single turnover NPF in actin patch assembly.

DISCUSSION

A fast single turnover mechanism of Dip1-mediated activation may to allow cells to rapidly initiate branched networks while preserving their dendritic nature. Here we showed that *in vitro* Dip1 activates Arp2/3 complex more rapidly that Wsp1 (Wagner et al. 2013), and that even in the presence of high concentrations of Wsp1, Dip1 activity dominates. Endocytic actin patches contain at peak ~20 Dip1 molecules, ~150-230 Wsp1 molecules and ~320 Arp2/3 complexes (Basu and Chang 2011, Sirotkin et al. 2010). Given the dominance of linear filament generation when Dip1 is present, most or all of the ~20 molecules of Dip1 at endocytic sites likely activate the complex to nucleate a linear seed filament. However, because Dip1 is consumed in the reaction, this number represents an upper limit on the number of Dip1-Arp2/3

nucleated linear filaments per patch. In other words, consumption of Dip1 during seed generation may preserve the dendritic nature of the patches.

Producing linear filaments to seed branched actin influences the architecture of those networks. Ultrastructural studies demonstrated that endocytic actin patches contain branches, but the precise architecture of these structures is still unknown. Measurements of the concentrations of actin and Arp2/3 complex in patches, coupled with the assumption that Arp2/3 complex always creates branches, has led to models in which patch filaments are ~100-200 subunits long and exclusively branched (Young, Cooper, and Bridgman 2004). However, we now know that Dip1 can activate the complex to create linear filaments, and based on kinetic arguments and concentration measurements, we presume that Dip1 probably creates around 20 linear filaments per actin patch. Wsp1 and Myo1, the other two potent NPFs at endocytic sites, active the complex to create branches, so the remaining ~300 Arp2/3-nucelated filaments are likely branched.

Our data show that overexpression of Dip1 caused accumulation of Dip1 in the patches and significant actin defects, suggesting that nucleating more than 20 linear filaments causes architectural changes that influence network function. However, we note that despite these defects, actin patches still internalized in Dip1 overexpression strains, suggesting the actin network remained at least partially functional. Experiments in cultured mammalian cell lines support some degree of tolerance of linear filaments in branched actin networks in lamellipodia. For instance, in cells expressing constitutively active mDia2, a formin normally present in lamellipodia, sheet-like lamellipodial structures form and protrude from the cell body, even though these structures contained an increased proportion of linear actin filaments (Yang et al. 2007). In fact, building evidence indicates that linear filaments nucleated by formins are not merely tolerated, but play important roles in assembling lamellipodial actin networks, either by seeding branches, elongating filaments, or otherwise influencing the network architecture (Isogai et al. 2015). Overexpression of mammalian DIP, a member of the WDS family, cause membrane blebbing, though reported to be due to its ability to inhibit mDia1, not through Arp2/3 complex (Eisenmann et al. 2007). Understanding the role of linear filament generators in influencing the architecture and function of Arp2/3-assembled cellular actin networks will be an important future direction.

While our data indicate that production of too many Dip1-mediated linear actin filaments causes defects in endocytic actin, endocytic networks, unlike lamelipodial structure, appear to tolerate the absence of linear filament generators. Specifically, while deletion of Dip1 causes a significant decrease in the rate of new actin patch initiation, once initiated, actin patches assemble at the same rate and internalize nearly identically to with and without Dip1. This suggests that linear filaments nucleated by Dip1 are not required to build a functional endocytic actin network architecture once assembly is initiated. Consistent with this observation, mathematical modeling showed that, given the autocatalytic nature of branching nucleation, one or two seed filaments can seed actin assembly in endocytic actin patches at the rates observed by live cell imaging (Berro, Sirotkin, and Pollard 2010). Why then, are 20 molecules of Dip1 recruited to actin patches? We speculate cellular concentrations of Dip1 are likely tuned to allow rapid seeding of new actin networks at endocytic sites, and that while only one or two seeds are sufficient to initiate the networks, excess Dip1, and the associated linear filaments created, are tolerated to ensure rapid initiation of new patches. Understanding the role of WDS proteins in initiating, propagating, and ultimately the function of actin networks will require a detailed understanding of how WDS proteins are regulated and distributed amongst distinct actin architectures.

How are WDS proteins regulated?

Arp2/3 complex activators are responsive to cellular signals and determine how WDS proteins are regulated will shed light on how linear filament production is limited in structures that require branched actin. Here we showed that the *S. pombe* WDS family protein, Dip1, utilizes a single turnover mechanism to activate Arp2/3 complex. Our experiments indicate that diverse WDS family proteins (eg. S. pombe Dip vs H. sapiens SPIN90) have identical biochemical properties as NPFs, suggesting the single turnover mechanism is likely conserved among WDS family proteins. However, we note that unlike Dip1, SPIN90 harbors regions flanking its Arp2/3 activating LRD domain known to interact with proteins that could regulate its activation of the complex, including Grb2 (Satoh and Tominaga 2001), Nck1 (Lim et al. 2001), PSD95 (Lee et al. 2006), IRSp53 (Teodorof et al. 2009) and N-WASP (Fukuoka et al. 2001). Several of these proteins contain SH3 domains, and could activate SPIN90 by binding to its proline-rich region, a mechanism SH3 domain-containing proteins use to activate WASP. Regulation of SPIN90 by trans factors could control when it is turned on to initiate new

networks, and modulate how many linear filaments are nucleated during assembly of branched structures. We note that it is possible that linear filament nucleation by Dip1 is controlled both through its single turnover mechanism, and by the action of trans regulatory factors. For instance, both Dip1 and Ldb17, the budding yeast homologue, localize to cortical sites independently of actin, suggesting they may interact with molecules at the endocytic site that could influence their seeding activity. Ldb17 has a C-terminal proline rich region that allows it to interact with Sla1 and other SH3 domain containing proteins that may influence localization or regulation. While Dip1 does not contain a proline rich sequence, its N-terminus is conserved among fungal species, and may be play a role in regulating the localization or activity of Dip1 in cells. Given the importance of seeding in assembly of branched actin networks, it will be important to understand both the molecular mechanisms underlying the regulation Dip1 and the other WDS family proteins.

WASP proteins make branches while WDS proteins make linear filaments. We have demonstrated here and elsewhere that WDS family proteins have distinct biochemical properties compared to WASP family proteins (Wagner et al. 2013). These mechanistic differences have important implications for understanding how WDS proteins function *in vivo*. For example, while we showed that Dip1 stays bound to Arp2/3 complex after nucleation, WASP release is programmed into its activation mechanism (Smith et al. 2013, Helgeson and Nolen 2013). WASP release is required to avoid excessive connectivity between the actin network (mediated by Arp2/3 complex) and membrane-tethered WASP, which can cause compression of actin networks and reduced force output. Given that Dip1 stays bound to the complex after nucleation, it's likely that a mechanism must exist to release Dip1 from its putative cortical binding partner(s). Further, the strong binding of Dip1 to filament ends and its incorporation into actin networks also necessitates a mechanism for recycling Dip1. One possibility is that ADF family protein GMF, which binds to Arp2/3 complex at branch junctions and stimulates branch dissociation, might also bind to Arp2/3 complex on Dip1-Arp/23 capped pointed ends to stimulate Dip1 or Dip1-Arp2/3 release from the end of the filament. Perhaps the most critical mechanistic difference between WDS and WASP family proteins is that WDS proteins activate the complex to produce linear instead of branched actin filaments. Linear filaments created by Arp2/3-Dip1 could theoretically anneal to the side of pre-existing filaments, converting the linear filament into a branch and preventing Dip1 activity from altering the architecture of the network.

However, we never observed end to side annealing events in any of our TIRF reactions. Because Dip1 stays bound to Arp2/3 complex on filament pointed ends, it is possible it blocks the annealing reaction by preventing Arp2/3-bound ends from interacting with the sides of actin filaments. Such a result would suggest that Dip1 might bind to the filament binding surface of Arp2/3 complex, providing an important clue as to how it might activate. It will be important to uncover both the binding interaction of Dip1-Arp2/3 complex and how Dip1 stimulates activating conformational changes in the complex. These mechanistic details are important to generate a model of how Arp2/3 complex balances the input of multiple NPFs during network generation that ultimately gives rise to a functional actin network.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Fluorescent Labeling:

To generate a Dip1 construct for site specific labeling with a cysteine reactive fluorescent dye, the six endogenous cysteines were mutated to alanine by amplifying pGV67-Dip1 (described previously in Chapter III]) with non-overlapping 5'phosphoryated primers encoding the mutation. The N-terminal Not1 restriction site, used to generate the GST-TEV-Dip1 expression vector, codes for a cysteine that was exploited for labeling. For expression and labeling of mutant protein, BL21(DE3)RIL *E. coli* transformed with the pGV67 Dip1 expression vector was grown to an O.D.595 of 0.6-0.7, induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, and grown overnight at 22 °C. Cells were lysed by sonication in lysis buffer; 20 mM Tris pH 8.0, 140 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor tablets (Roche). Lysate was clarified by centrifugation, and the soluble fraction was loaded on a glutathione sepharose column and eluted with 20 mM Tris pH 8.0, 140 mM NaCl and 50 mM glutathione. Peak fractions were pooled and a 25:1 ratio (by mass) of TEV protease to recombinant proteins was added. The reaction mix was dialyzed overnight at 4 °C against 20 mM Tris pH 8.0, 50 mM NaCl and 1mM dithiothreitol. The sample was loaded onto a 6ml Resource Q column at pH 8.0 and eluted with a gradient of 50 mM to 500 mM NaCl. Protein was then concentrated in an Amicon-Ultra concentration device before loading on a Superdex 200 HiLoad 16/60 gel filtration column and eluted in 20 mM HEPES pH 7.0, 50 mM NaCl. Peak fractions were pooled and concentrated to ~ 40 uM for labeling. A 10 mM solution of Alexa568 C5 Maleimide (Thermo Fisher) was prepared by dissolving in water according to

manufacturer's protocol. Protein was labeled by the dropwise addition of a 10-40 molar ratio of dye:protein while stirring at 4°C. The reaction was quenched after 12-16 hrs by dialyzing against 20 mM Tris pH 8.0, 50 mM NaCl, and 1 mM dithiothreitol for 24 hours at 4 °C with buffer exchanges after 4hr and 8hrs. Labeled protein sample was loaded on a 5mL Hi-Trap desalting column and peak fractions were pooled and flash frozen in liquid nitrogen. *S. pombe* Wsp1-VCA and *S. pombe* (Sp)Arp2/3 complex(Liu et al. 2013) were purified as described previously(Liu et al. 2013).

Fission Yeast Strains and Molecular Biology

Table S1 lists all *Schizosaccharomyces pombe* strains used in this study. Dip1 was knocked out from a strain carrying fim1-mCherry and replaced with the URA4+ cassette from the KS-ura4 plasmid using PCR based genomic integration to make the BNXXX strain harboring Dip1Δ::Ura4+ (Bähler et al. 1998). To generate a p3nmt1-Dip1-GFP strain, a plasmid with a p3nmt1-Dip1 was constructed first by amplifying the strongest no-message in thiamine promoter from pFA6a-KanMx6-P3nmt1 and annealing to the Dip1 sequence using overlap extension PCR. The resultant PCR product was sub cloned into the pFA6A backbone to generate a pFA6a-P3nmt1-Dip1. PCR based genomic integration was used to integrate the P3nmt1-Dip1 cassette into the *dip1*Δ::Ura4+ strain by counter selecting against ura4 using YNB+5'FOA. The P3nmt1-Dip1 strain was tagged at the C-terminus with mEGFP (Bähler et al. 1998). We used the strain VS1133 which expresses Dip1-mEGFP and fim1-mCherry both from their native loci. *End4Δ* strains were generated by genetic crosses with strains expressing either Dip1-mEGFP and fim1-mCherry or mEGFP-pWsp1-Wsp1 and fim1-mCherry. *End4*Δ::Ura4+ strains were crossed the fluorescent fusion carrying strains by mixing cells in nitrogen starving conditions. 15 uL of sterile H_20 was dropped on an SPA5S plate and a pinhead of each strain to be crossed was mixed well with a wooden stick. The mixture was incubated for three days at 25°C. Tetrad dissection was used to isolate spores that germinated on YE5S plates at 25°C for 3-5 days. Triple mutants were identified using replica plating and fluorescence microscopy.

Preparation of *S. pombe* for imaging

Cells were grown in EMM5S at 25°C for 2 days while maintaining exponential growth to a final OD595 – 0.2-0.8 before imaging. Cells were collected by centrifuging at 900x*g* for 3 minutes,

washed once with EMM5S, and finally resuspended in 20-100μL EMM5S + 1:100 ProLong Live antifade reagent (Thermo Fisher). Cells were mounted on 0.25% gelatin pads and sealed with a mix of 1:1:1 mix of petroleum jelly, paraffin wax, and lanolin. Cells were generally imaged within 1 hr after placing them on the gelatin pads. To induce expression from the P3nmt1 promoter, cells were grown in EMM5S + 5 μg/mL thiamine for 2 days then washed 3 x by pelleting cells at 900x*g* for 3 minutes and re-suspending in fresh EMM5S. Cells were grown for 22hr without thiamine before imaging.

Confocal microscopy imaging of fission yeast.

S. pombe cells on gelatin pads were imaged on a Nikon TE2000 inverted microscope equipped with 100x/1.49 numerical aperture objective with a 1.5x magnification slider and a Yokagawa Spinning Disk scanhead (?). Images were collected on an EMCCD camera (iXon3, Andor). For quantification of the Fim1-mCherry signal, images were taken in the 561 channel using 300 ms exposures at 500 ms intervals at a single focal plane taken at the center of the cell. For quantification of the actin patch density Fim1-mCherry signal, images were taken in the 561 channel using 300 ms exposures over 21 slices spaced 360 nm on the Z-axis to collect entire fluorescence from the cell. As described in the main text, the Dip1-GFP signal was typically too faint to reliably identify puncta of fluorescence in cells expressing Dip1-GFP at wild type levels.

TIRF imaging of fission yeast.

S. pombe cells on gelatin pads were imaged on a Nikon TE2000 inverted microscope equipped with 100x/ 1.49 numerical aperature TIRF objective with a 1.5x magnification slider. To adjust TIRF illumination to near TIRF, or oblique angle TIRF, the incident angle was first adjusted obtain a perpendicular laser beam. The focus was adjusted to the side facing the coverslip. The incident angle of the laser was adjusted until the signal disappeared, then the angle was slowly increased until the fluorescence at the cell surface reappeared. The Z-focus was adjusted once more to obtain optimal image focus. The TIRF angle was considered acceptable when no blurred edges were observed next to the cells (Spira et al. 2012). Images were collected using an EM-CCD camera (iXon3, Andor). Samples were exposed for 100 ms at 200 ms intervals for the 488 channel for 20 ms at 200 ms intervals for the 561 channel. These setting could be used to collect 2 min movies with minimal photobleaching over the course of the imaging.

Analysis of live cell images

Images were analyzed using the FIJI distribution of ImageJ (ref). To make direct comparisons between overexpressing and non-overexpressing strains using oblique angle TIRF microscopy imaged at a single focal plane, were performed an internal calibration for each slide used. We mixed cells expressing Dip1-GFP under its native promoter with cells expressing both Fim1-mCherry and P3nmt1-Dip1-GFP. The mCherry signal was used as a marker for the overexpressing cells. To track Dip1-GFP signal in TIRF images we used the following workflow. The image background around cells was subtracted using FIJI background correction with a rolling ball radius of 130 pixels, larger than any cell in the field of view, to preserve entire fluorescence signals within the cell. Dip1-GFP localizes to additional structures within the cytoplasm that contribute to actin patch fluorescence. To subtract cytoplasmic background, background subtracted images were duplicated and a median filter (radius = 10) was applied, the median filtered image was subtracted from the background corrected image to generate an image for actin patch tracking. The total fluorescence intensity of the GFP signal in patches was measured using the manual tracking with TrackMate plugin in FIJI. The radius was determined by measuring the largest size of each patch and tracking the entire fluorescent event. To track fim1-mCherry signal in TIRF images we used the following workflow. The image background around cells was subtracted using FIJI background correction with a rolling ball radius of 130 pixels, larger than any cell in the field of view, to preserve entire fluorescence signals within the cell. To correct for photobleaching, each image was scaled such that the average fluorescence intensity within the cell remains constant in successive frames (Picco and Kaksonen 2017). Patches were tracked that met the following criteria, objects whose fluorescence intensity increased with time, were born after the time series started, and did not collide with other fluorescent objects. The FIJI plugin Manual tracking with TrackMate was used with a circular ROI with a diameter of 8.0 pixels was used to measure the fluorescence over the entire patch lifetime.

TIRF microscopy of actin polymerization

TIRF flow chambers were constructed and reactions setup essentially as previously described with slight modifications (Kuhn and Pollard 2005). TIRF chambers were created by sandwiching double-sided tape between a glass microscope slide and a 24 x 50 #1.5 coverslip to create a 14

μL, 0.5 cm wide chamber. To initiate the reaction, 1 μL of 0.025 mM $MgCl_2$ and 1 mM EGTA was mixed with 5 μL of 9 μM 33% Oregon-green actin and incubated for 2 min before adding TIRF buffer (10 mM Imidazole pH 7.0, 1 mM $MgCl_2$, 1 mM EGTA, 50 mM KCl, 100 mM DTT, 0.2 mM ATP, 25 mM Glucose, 0.5 % Metylcellulose (400 cP at 2%), 0.02 mg/mL Catalase (Sigma) and 0.1 mg/mL Glucose Oxidase (MP Biomedicals) to 1 x, *S. pombe* Arp2/3 complex to 50 nM, GST-Wsp1-VCA to 75 nM, or Dip1 to 300nM or 150 nM final concentration. For single color reactions, 50 ms exposures with the 488 nm laser were taken at 1000 ms intervals, and a typical polymerization reaction was imaged for 10 min. For two color reactions typical imaging conditions were 50 ms exposures with the 488 nm laser at 1000 ms intervals and 50 ms exposures with the 561 nm laser at a range of intervals. For low exposure imaging conditions, the interval between 561 frames was adjusted from 200 ms – 5000 ms to decrease the laser exposure by a factor of 25. The concentration of Dip1 was kept at 6 nM to minimize the background signal in the 561 channel.

TIRF Image Analysis:

Images were prepared in Image J. Image sequences were subtracted for background signal with a 10-pixel rolling ball radius. The total actin polymer was calculated using a custom image processing script run in Matlab, described as follows. For each frame, pixels corresponding to filament fluorescence were identified using image segmentation followed by morphological area opening to remove non-filament small fluorescent objects. The final pixel number value was converted to micrometers (1px = 106.7 nm) to yield the total length of actin filaments in the image frame, and further converted to number of subunits using 370 subunits μm^{-1}. To calculate the ratio of branched to linear filaments, the number of branches and linear filaments were counted manually when the total polymer length in each movie was approximately 300 μm.

BRIDGE TO CHAPTER V

In this chapter, we established that Dip1 uses a single turnover mechanism to activate the Arp2/3 complex. We identify this as a potential regulatory mechanism that limits WDS protein activity. We discovered Dip1 generated linear filaments directly influence the architecture of WASP-mediated branched networks. In the next chapter, we will synthesize the implications of our findings from the previous three chapters. Specifically, we will discuss how the characterization of WDS proteins shows how biochemically distinct NPFs can both activate the Arp2/3 complex but with separate influence on branched actin networks.

CHAPTER V

DISCUSSION

Since the discovery of the Arp2/3 complex over 20 years ago, regulation of its activity and the consequences of improper regulation have been a major focus in cell and structural biological research. Around twenty proteins directly interact with the complex to regulate its function, most of which are NPFs that promote activity. Here we have characterized the actin regulators, WISH/DIP/SPIN90 (WDS) proteins, as a class of Arp2/3 complex activators that function to convert the complex from a branched to a linear filament nucleator. We were motivated to investigate WDS protein activity because accumulating evidence suggested Arp2/3 complex regulators may each play a unique and non-redundant role. In addition, our understanding of how distinct activators function in concert is building which first requires a description of how regulators function alone and then a study of them together. Some NPFs work in concert to synergistically activating the complex. Our findings implicate WDS proteins as branched actin network initiation factors that directly influence the architecture of the networks they initiate.

In chapter II, we show that WDS proteins do not require preformed filaments to activate the Arp2/3 complex. WDS proteins are distinct from other NPFs in that they do not bind monomers or filaments, and do not contain an Arp2/3 complex-interacting-CA (or A) region. Importantly, the activation mechanism does not require preformed filaments. The consequence is that the Arp2/3 complex makes linear filaments in response to stimulation by WDS proteins. Our data suggests that WDS protein monomers function to activate the complex. Forced dimerization of fission yeast Dip1 through a GST domain does not increase activity and our characterization of fluorescently labeled Dip1 with Alexa568 showed the protein behaves as a monomer in TIRF experiments. In contrast, other NPFs, including WASP family proteins, more potently activate the complex when dimerized by GST, probably because Arp2/3 contains two binding sites capable of interacting with CA (or A) regions (Padrick et al. 2008; Padrick and Rosen 2010; Ti et al. 2011). WDS proteins and other NPFs both stimulate a conformational change in the activation pathway in which the Arp2 and Arp3 subunits slide together to form an actin filament-like dimer (Hetrick et al. 2014; Rodnick-Smith et al. 2016). The conformational change is

sufficient to bypass the need for NPF activity (Rodnick-smith et al. 2016). Although the conformational change is sufficient to bypass the requirement for NPFs, the complex is still not fully active, and requires a performed filament. The biochemical properties of WDS proteins explain the ability to serve as a master timer in initiating the assembly of branched actin filaments during endocytosis.

Given the similarities and differences between WDS proteins and other NPFs it will be important to determine the structural requirements in WDS proteins necessary for activation. WDS proteins have a conserved leucine rich domain (LRD) flanked by conserved sequences in fungal species which together are sufficient for activity of fission yeast Dip1. Similarly, in mammalian Spin90 the N-terminal SH3 and poly-proline regions are dispensable for activity. Taken together the LRD is an excellent candidate to form the Arp2/3 interacting region of WDS proteins. Structural studies are needed to determine where on the Arp2/3 complex WDS proteins bind compared to other NPFs. Because WDS proteins function as monomers and WASP family proteins can engage the complex as dimers, it remains an open question if there a single binding site unique for WDS proteins, or if they use the same binding sites as other characterized NPFs? Additionally, it is unclear if multiple Arp2/3 complex regulators can simultaneously engage the complex. It is tempting to speculate that WDS proteins could mimic a preformed filament by binding the filament binding interface on the complex. If WDS proteins have a unique binding site it opens the possibility that other NPFs could synergistically activate Arp2/3 with WDS proteins, as has been shown with cortactin and WASP family proteins (L. a. Helgeson and Nolen 2013; Luke a Helgeson et al. 2014). Multiple Arp2/3 complex regulatory proteins exist in actin rich structures. Dissecting the structural details of activation mechanisms helps shed light on how the complex is coordinately regulated by multiple proteins.

In chapter III, we test our hypothesis from chapter II directly, do WDS proteins have the capacity to seed WASP-mediated actin-branches? We show a mechanism where Dip1 nucleates seed filaments that then serve as templates for WASP-activated Arp2/3 to land and form a daughter filament. This observation addresses a long-standing paradox, that new branched filaments cannot be created until an initial filament exists. Before the characterization of WDS proteins, existing data suggested Arp2/3 complex was incapable of making filaments *de novo*, because the complex must bind to the side of a pre-existing (mother) filament before a new (daughter) filament is made. The function of branched actin is dependent on two properties

during network development. The timing of new filament production and the overall architecture within the network. The Arp2/3 complex can satisfy both of these properties because it nucleates new filaments and nucleation is coupled to branching. Within the cell, the Arp2/3 complex is sufficient to start a new network and once started, dictate the arrangement of filaments because branches form off of branches in a dense array.

We show that while WDS proteins use a non-WASP like mechanism, they co-opt features of the branching nucleation mechanism. Importantly, WDS-mediated linear filaments are anchored by the Arp2/3 complex at the pointed end, just like at a branch junction. The barbed end of the actin filament is then free to elongate. Filaments nucleated by WDS-mediated activation elongate at the same rate as freely formed or branched filaments. These data show that WDS proteins have the biochemical capacity to serve as seed filament generators that function to initiate branched actin formation. Our observations show that the Arp2/3 activation mechanism is conserved from fission yeast Dip1 to mammalian Spin90. We predict that human Spin90 can also seed NPF-mediated branching, but note that we did not test this idea directly. For the first time, our experiments directly observe a seeding pathway and demonstrate that WDS proteins function to provide initial substrate filaments for WASP-mediated branching.

Actin filament nucleators are sufficient to define the composition of actin networks. Multiple seeding mechanisms have been proposed to overcome the need for initial mother filaments. We note that while we have now confirmed that WDS proteins function to seed branched actin networks *in vitro* whether this activity is sufficient to initiate branched actin networks *in vivo* remains an open question. In order for actin filaments to serve as seeds for Arp2/3-mediated branching they first must localize to sites of branched actin assembly and second be free of filament binding proteins that may block the binding of Arp2/3 to filament sides. A cut, diffuse, and capture mechanism was proposed to initiate actin patches at sites of endocytosis in yeast, in which filaments severed by cofilin diffused to immature patches and provided the first substrate filaments (Chen and Pollard 2013). While this activity is supported by evidence in cells, this mechanism cannot generate filaments *de novo* like WDS-mediated seeds. One possibility is that WDS proteins provide initial seeding activity that is then complemented by the cofilin severing mechanism. Actin patches at sites of endocytosis are dynamically distributed in yeast cells, localizing to sites of active membrane synthesis and remodeling, namely at the poles or septa (Kovar, Sirotkin, and Lord 2011; Berro and Pollard

2014). The mechanism by which actin patches are actively redistributed is currently unknown. It's possible that cofilin severing and Dip1-mediated seeding mechanisms function to initiate distinct sub-cellular hot spots of endocytosis in yeast. Formins have been implicated as potential seed filament generators because they specifically nucleate linear filaments. Evidence in mammalian cells supports a role for formins during initiation of lammelipodia and membrane ruffles (Isogai et al. 2015). In yeast, however, formins are excluded from actin patches at sites of endocytosis (Skau, Neidt, and Kovar 2009). In addition, formin nucleated filaments are characteristically decorated with tropomyosin which antagonizes Arp2/3 activity (Blanchoin, Pollard, and Hitchcock-degregori 2001). Moreover, mammalian Spin90 was shown to directly inhibit the activity of the formins mDia2 and mDia1 (Eisenmann et al. 2007). It's intriguing to consider the possibility that WDS proteins down regulate formin activity and upregulate Arp2/3 complex activity. This could function to exclude formin activity where branched filaments are needed. By using WDS-activated Arp2/3 complex to generate seeds, branched filament initiation is coupled to network propagation through a common enzyme. This could function to efficiently specify the localization of seed filaments exactly where branches are needed. Along those lines, Arp2/3 complex bound at the pointed end of a seed filament may allosterically influence the filament conformation many subunits away and better serve as templates for branching compared to unbound filaments. It will be an exciting future direction to determine the relative contribution of each seeding mechanism *in vivo* and address whether there are unique seeding mechanisms for specific sub-cellular actin structures.

In chapter IV, we shift our focus from how WDS proteins make filaments to how seeding activity influences network architectures and show a mechanism that may limit linear filament production *in vivo.* We show that the number of linear filaments produced by Dip1 has a profound effect on the branch density in a network. The architecture of actin filaments is tied to the physical properties and functionality of the network. The branch is the basic repeating unit in dendritic arrays and it is clear that filament length and the frequency of branching impacts the stiffness of the network (Pujol et al. 2012). Specifically, the branch density decreases markedly in response to a relatively small amount of Dip1compared to WASP. Our results indicate the branched density is directly proportional to the number of Dip1 generated seeds. The branching nucleation reaction is highly cooperative, as each new filament is made, the filament area for Arp2/3 complex binding grows exponentially. Adding a few Dip1 generated seeds effectively

distributes WASP-mediated branches demonstrating that this positive feedback loop is sensitive to the number of input filaments. Surprisingly, we found that increasing the amount of WASP in a reaction with Dip1 was incapable of recovering branch density. While we cannot fully explain this observation, one possibility is that Dip1 needs to be completely consumed before WASP-mediated branching can resume.

We show in chapter IV that excess Dip1 activity causes defects in actin assembly and the timing of endocytosis in fission yeast. Mathematical modeling of actin assembly at sites of endocytosis showed that 2-3 seed filaments were sufficient to stimulate both the rate and peak of actin assembly in patches (Berro, Sirotkin, and Pollard 2010). Previous studies measured the protein concentration in actin patches to contain roughly 20 molecules of Dip1, 150 molecules of WASP, and 230-320 molecules of Arp2/3 at peak (Basu and Chang 2011; Sirotkin et al. 2010; Wu and Pollard 2005). Our experiments show that increasing the concentration of Dip1 at sites of endocytosis is sufficient to increase the amount of actin, which suggests the nucleation activity of Dip1 is in part limited by Dip1 concentration at endocytic sites. Interestingly, the amount of seed filaments from modeling experiments that is sufficient to generate actin is far less than what we predict is generated during our experiments. In wildtype cells, 20 molecules of Dip1 could give rise to 20 linear seed filaments. In our experiments, as much as 15 times more Dip1 than wildtype is present at sites of endocytosis. Both our experiments and wildtype Dip1 levels that are capable of producing more than 2-3 linear filaments. If the branch is the important structural feature in dendritic actin arrays than why make more linear filaments than is necessary? It's possible that excess Dip1 functions to protect against initiation failure or functions to define a specific actin architecture at endocytic sites. From our comparison of NPF activity *in vitro,* Dip1 appears to activate Arp2/3 complex faster than WASP for activation of the Arp2/3 complex, which implies that Dip1 could out-compete WASP for activity of Arp2/3 early in endocytic actin assembly. It will be important to dissect how both WASP and Dip1 function together *in vivo*. In addition, defining the attributes of functional actin architectures and the specific arrangement of filaments will be important future directions in understanding actin driven processes.

In Chapter IV, we were also motivated to understand how Dip1 and WASP activity is balanced. We show that Dip1 uses a single turnover mechanism to activate the Arp2/3 complex and hypothesize that this could function to regulate Dip1 activity. We show that Dip1 stably

incorporates into the networks it seeds in fission yeast endocytosis, consistent with our observations *in vitro*. Dip1 makes linear filaments while WASP makes branches. Since branched filaments have been observed *in vivo* and our results show that Dip1 competes with WASP, WDS protein activity must be limited in cells (Young, Cooper, and Bridgman 2004; Svitkina and Borisy 1999; Korobova and Svitkina 2008). Consistent with our results in yeast, over expression of the LRD domain of mammalian DIP causes membrane blebbing in Hela cells which reflects a linear and disconnected actin array (Eisenmann et al. 2007) If our single turnover mechanism holds true, Dip1 activity would be limited to the number of molecules present. However, we currently don't understand how WDS proteins are localized to actin rich structures in cells. In addition, it's unclear whether WDS proteins generate seed filaments before or after localization to nascent sites of branched actin. It's possible that seeds generated by WDS proteins are freely diffusing within the cytoplasm and initiation of networks depends on a stochastic encounter with one of these filaments. Actin patches at sites of endocytosis use a single burst of actin polymerization that coincides with membrane ingression and vesicle internalization (Kaksonen, Toret, and Drubin 2006). In contrast, retrograde flow of actin in lamellipodia lasts for minutes to hours. Actin patches need a single initiation event to turn on actin polymerization but how many are needed in lamellipodia or other longer lasting structures? Are multiple pulses of seeding activity required to sustain a tread milling actin network?

The discovery and characterization of WDS proteins as activators of the Arp2/3 complex provide one explanation for how actin networks are initiated in cells. WDS proteins convert Arp2/3 complex from a branched filament nucleator into a linear filament nucleator. WDS proteins could be used as tools to interrogate the importance of branched actin filaments in cells. The effects of ablating Arp2/3 complex activity as a whole have been used to conclude that branched filaments are required for processes like endocytosis and lamellipodia formation. While EM studies have directly visualized branches at these structures, the function of the branch within these networks has remained elusive. It's exciting to consider the possibility of using WDS proteins as engineering tools. Could all Arp2/3 complex activity be converted to linear filament production? With an understanding of the WDS activation mechanism, Arp2/3 complex could be engineered to only respond to WDS proteins. This would be an invaluable tool in understanding the role of individual branched filaments in cells. Given the wide range of actin function in cells, from forming tracks for motors to pushing or deforming membranes, it will be

exciting to determine how actin filament architectures are tied to function. More studies are needed to understand how Arp2/3 integrates inputs from multiple regulatory pathways at distinct structures within cells.

APPENDIX A

SUPPLEMENTARY FIGURES AND VIDEOS FOR CHAPTER II

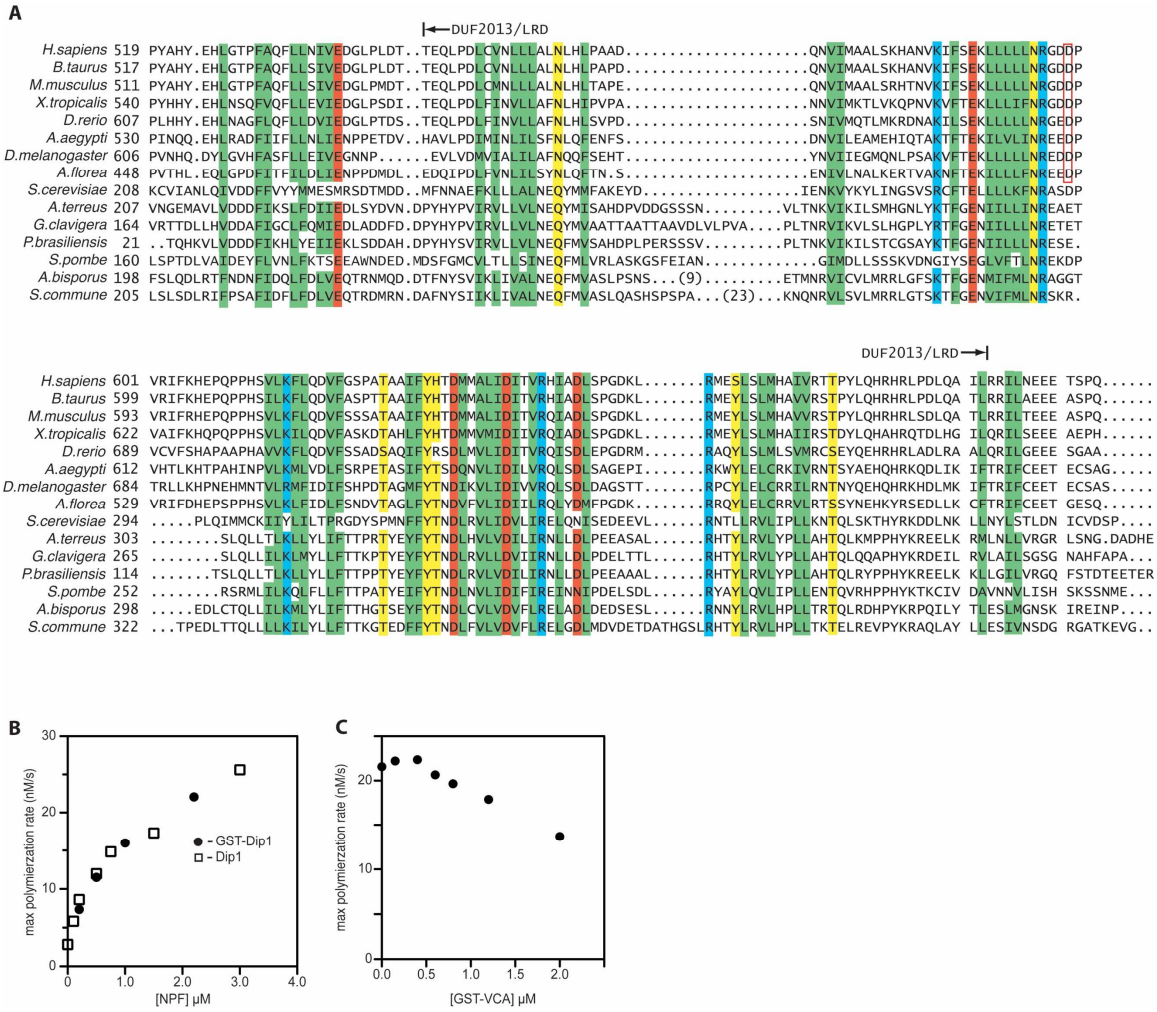

Figure S1, related to Figure 1: (A) Sequence alignment of LRD domain and flanking regions from WISH/DIP/SPIN90 sequences. Residues are boxed as followed: conserved hydrophobic, green; conserved acidic, red; conserved basic, blue; conserved polar, uncharged, yellow. Sequence identification numbers are as follows (from top to bottom): NP_057537.1, DAA17033.1, NP_109654.2, NP_001123414.1, XP_002666143.2, XP_001662980.1, NP_572439.1, XP_003691808.1, NP_010135.1, XP_001212459.1, EFX00826.1, EEH22318.1, NP_595964.1, EKM78508.1, XP_003031417.1. (B) Plot of maximum polymerization rate versus GST-Dip1 or untagged Dip1 concentration for reactions containing 3 µM 15% pyrene labeled actin and 50 nM *S. pombe* (*Sp*)Arp2/3 complex. (C) Plot of maximum polymerization rate versus GST-Wsp1-VCA concentration for reactions containing 3 µM 15% pyrene labeled actin, 50 nM *Sp*Arp2/3 complex, and 5 µM Dip1.

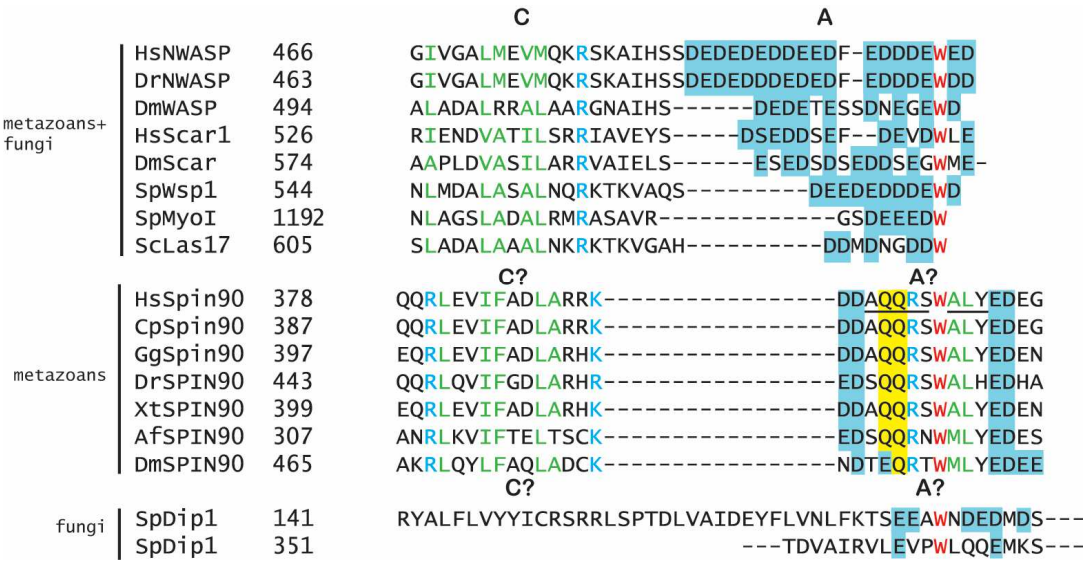

Figure S2, related to Figure 4: Sequences of putative WISH/DIP/SPIN90 CA sequences compared to CA regions from known type I NPFs. Top sequences show alignment of metazoan WASP/Scar family CA regions. Middle sequences show putative metazoan WISH/DIP/SPIN90 CA regions (Kim et al. 2006). Bottom two sequences show potential CA or A regions from *S. pombe* Dip1 investigated in this study. Conserved hydrophobic, basic, or polar uncharged resides are green, cyan or boxed in yellow, respectively. Underlined segments indicate residues from human SPIN90 that were omitted from a previously published sequence alignment (Kim et al. 2006). The conserved Trp residue in WASP/Scar proteins and the putative acidic region Trp residues in WISH/DIP/SPIN90 are colored red. Acidic resides within known or putative acidic regions are boxed in cyan. For the fungal Dip1 sequences, only the potential acidic residues and tryptophan are indicated. Species abbreviations are as follows: Hs, *Homo sapiens*; Dr, *Danio rerio*; Dm, *Drosophila melanogaster*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Cp, *Cavia porcellus*; Gg, *Gallus gallus*; Xt, *Xenopus tropicalis*; Af, *Apis florea*.

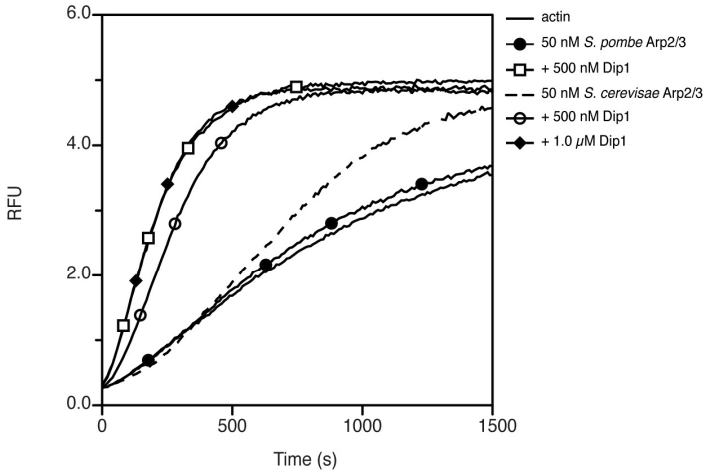

Figure S3, related to Figure 5: Time course of polymerization of 3 μM 15 % pyrene-actin with 50 nM *S. pombe* Arp2/3 complex or 50nM *S. cerevisiae* Arp2/3 complex and a range of concentrations of *S. pombe* Dip1.

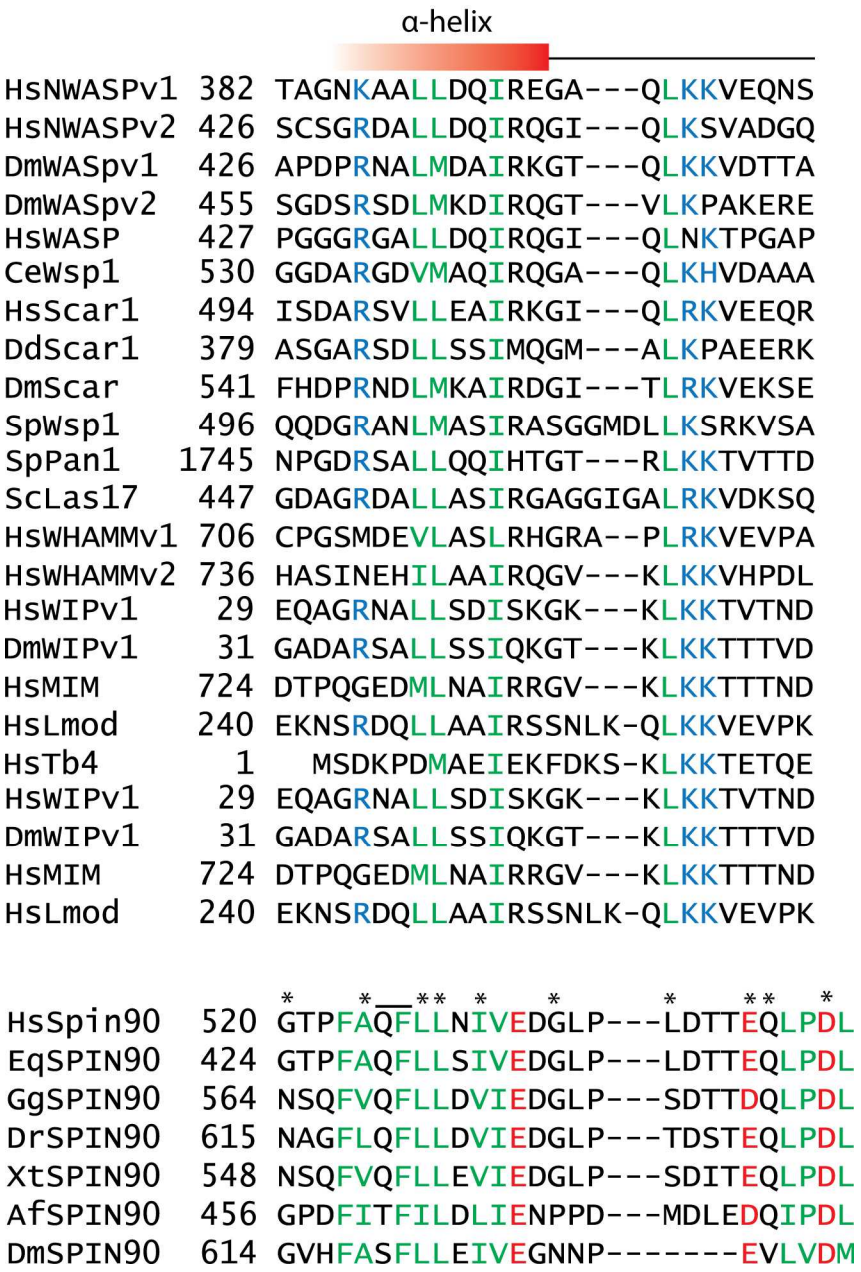

Figure S4, related to Figure 6: Sequence alignment of known V regions (top) with proposed V regions of metazoan SPIN90 sequences (bottom). Conserved residues are green (hydrophobic), blue (basic) or red (acidic). Species names are abbreviated as follows: Hs, *Homo sapiens*; Eq, *Equus caballus*; Gg, *Gallus gallus*; Dr, *Danio rerio*; Xt, *Xenopus tropicalis*; Af, *Apis florea*; Dm, *Drosophila melanogaster*. Black lines above SPIN90 sequences indicate residues omitted from V region alignments as published in Kim, *et. al*. (Kim et al. 2006). Asterisks indicate residues marked as key conserved V region residues in Kim, *et. al.* (Kim et al. 2006).

Movie S1, related to Figure 2: TIRF microscopy movie showing polymerization of 1.0 μM 33% Oregon Green actin polymerizing in the presence of 50 nM SpArp2/3 complex (left panel), 300 nM Dip1 (middle left panel), 50 nM SpArp2/3 complex and 75 nM GST-Wsp1-VCA (middle right panel), or 50 nM SpArp2/3 complex and 300 nM Dip1 (right panel). Movies are 20-fold faster than real time and show a 27 by 27 μm area of reaction chamber.

Movie S2, related to Figure 2: TIRF microscopy movie showing polymerization of 1.0 μM 33 % Oregon Green actin polymerizing in the presence of 50 nM SpArp2/3 complex and 150 nM Dip1. Movie is 20-fold faster than real time and shows a 27 by 27 μm area of reaction chamber.

Movie S3, related to Figure 6: TIRF microscopy movie showing polymerization of 1.0 μM 33% Oregon Green actin polymerizing in the presence of 1.1 μM SPIN90 (left panel), 20 nM BtArp2/3 and 100nM GST-N-WASP-VCA (middle panel), or 20 nM BtArp2/3 complex and 1.1 μM SPIN90 (right panel). Movies are 20-fold faster than real time and show a 27 by 27 μm area of reaction chamber.

Movie S4, related to Figure 6: TIRF microscopy movie showing polymerization of 1.0 μM 33% Oregon Green actin polymerizing in the presence of 25 nM BtArp2/3 complex and 1.5 μM SPIN90. Movie is 20-fold faster than real time and shows a 27 by 27 μm area of reaction chamber.

APPENDIX B

SUPPLEMENTAL FIGURES FOR CHAPTER IV

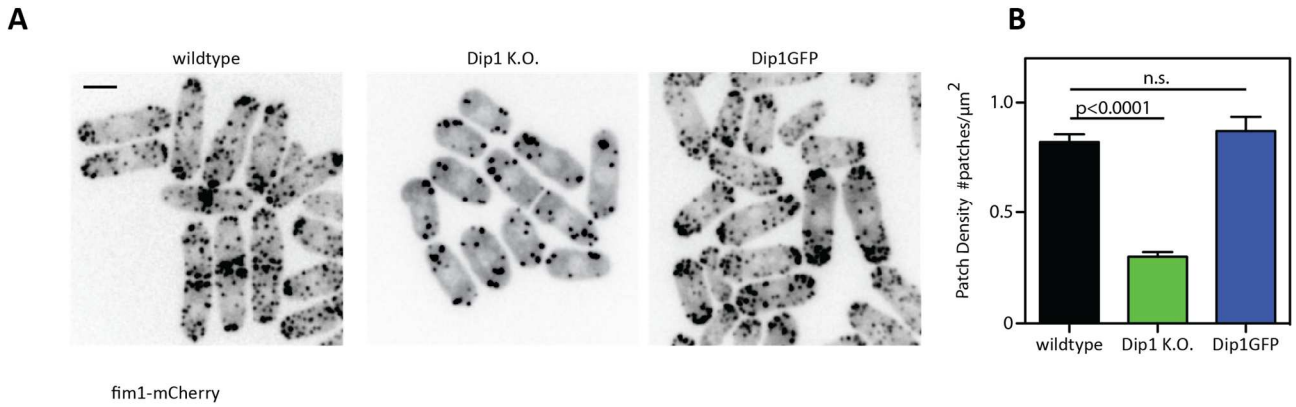

Figure S1, related to Figure 1: Dip1 is Functional with a C-terminal mGFP Tag
A. Maximum intensity projections of *S. pombe* cells expressing fim1-mcherry from spinning disc confocal Z-stacks spanning the entire cell. Scale bar is 5 μm. **B.** Quantification of patch density in cells comparing wildtype (n = 6 cells), Dip1 knockout (K.O.) (n = 6 cells), and Dip1-GFP (n = 6 cells) expressing cells. Error bars show SEM. Not significant (n.s.) or significant P-value shown calculated from an un-paired two-tailed t-test.

Table S1. List of Strains

Name	Genotype	Source
VS1133a	h+ ade6-M210 leu1-32 ura4-D18 Dip1-mGFP-kanMX6 fim1-mCherry-natMX6	V. Sirotkin
BN229	h+ ade6-M210 leu1-32 his3-D1 ura4-D18 KanMX6-Pwsp1-mGFP-wsp1 fim1-mCherry-natMX6 dip1Δ::ura4	This Study
BN206	h+ ade6-M210 leu1-32 his3-D1 ura4-D18 dip1Δ::p3nmt1-Dip1-mGFP-KanMx6 fim1-mCherry-cloNAT	This Study
VS1124a	h+ ade6-M210 leu1-32 his3-D1 ura4-D18 KanMX6-Pwsp1-mGFP-wsp1 fim1-mCherry-natMX6	V. Sirotkin
VS1220	h+ ade6-M210 leu1-32 ura4-D18 his3-D1 Dip1-3xmGFP-kanMX6	V. Sirotkin
BN157	leu1-32 his3-D1 ura4-D18 KanMX6-Pwsp1-mGFP-wsp1 fim1-mCherry-natMX6 end4::ura4+	This Study
BN165	leu1-32 his3-D1 ura4-D18 Fim1-mCherry-clonNAT Dip1-mGFP-kanMX6 end4::ura4+	This Study
VS982	h+ ade6-M210 leu1-32 ura4-D18 his3-D1 Dip1-mGFP-kanMX6	V. Sirotkin

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CHAPTER III

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CHAPTER IV

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