

Qualifying Exam Question

Mechanisms of Formin Activation

An early look into the cellular regulatory methods behind these actin cable nucleators.

Introduction

The Dynamic Actin Protein

All eukaryotic cells rely on actin in some way, shape or form. Single-celled yeast utilize actin networks in the processes of establishing cell polarity, cytokinesis, endocytosis, vesicle transport, cellular secretion, mitotic spindle orientation and mating. Amoeboid organisms base their movement on dynamically manipulating their actin cytoskeletons and higher order metazoans evolved extensive arrays of actin into coordinating muscle contraction. This roughly 42-kDa protein is versatile in many ways, but in its most basic form it exists in either a head-to-tail filamentous configuration (F-actin) or as a globular monomer solubilized in the cytosol (G-actin) [1]. The ability to control the polymerization of actin filaments is evidently very important considering the fact that so many cellular activities depend on the actin cytoskeleton to properly function.

As expected from the head-to-tail polymerization of actin monomers, the resulting filament exhibits a distinct polarity. The two ends of a filament are referred to as either 'pointed' or 'barbed' due to the stacked arrowhead morphology of F-actin when saturated with S1 subunits of myosin and visualized on an electron micrograph [1, 2]. It is important to note that monomer addition to the barbed end of an existing filament is generally faster than addition to the pointed end. In budding yeast (*Saccharomyces cerevisiae*) barbed end addition is approximately nine times faster than pointed end addition ($11.6 \text{ monomers } \mu\text{M}^{-1}\text{s}^{-1}$ versus $1.3 \text{ monomers } \mu\text{M}^{-1}\text{s}^{-1}$) [1]. The asymmetrical nature of F-actin allowed the evolution of various cellular systems to sense its polarity, localize F-actin nucleation sites, and regulate filament growth rate.

G-actin does have the ability to polymerize into F-actin on its own but the conditions in the intracellular environment discourage spontaneous assembly. Actin dimers and trimers are extremely unstable. However, once a tetramer of actin forms, it becomes a proper nucleation point for filament growth. Even then, filament severing proteins (such as cofilin and Aip1 [3]) and monomer capping proteins (such as profilin [4]) are regularly expressed as a method to control actin assembly. Spontaneous G-actin polymerization is suppressed at both the nucleation step and at the elongation step.

Three Classes of Actin Nucleators

Instead of relying on spontaneous polymerization of F-actin, eukaryotic cells express proteins that are able to bind G-actin in an active conformation. The stably bound actin monomer then functions as a nucleation site for actin filaments [5, 6]. There are three main F-actin nucleator classes: the Arp2/3 complex, the Spire protein, and the formins.

The Arp2/3 complex is the most extensively studied nucleator of the three classes. It requires attachment to a preexisting actin filament before it will nucleate actin [1, 5]. Upon activation, the Arp2 and Arp3 subunits come together to mimic the surface of a barbed end actin monomer [1, 5]. The mimicked barbed end attracts and binds G-actin, promoting barbed end growth away from the complex [1, 5, 7]. These nascent filaments branch off from the old actin filament at a 70° angle [5]. Since the Arp2/3 complex is not associated with the growing end of its nucleated filament, the nascent filament is vulnerable to capping proteins which terminate the addition of actin monomers [8]. Arp2/3 promotes the formation of branched actin filaments typically found in endosomes, cortical actin patches, and the leading edge of motile eukaryotic cells [5].

Spire is the newest addition to the F-actin nucleator class of proteins. Spire was first identified as an actin filament nucleator by Quinlan *et al.* in *Drosophila melanogaster* and published their results in early 2005 [9]. Spire promotes the assembly of F-actin by having four evenly spaced, inline WH2 repeats which bind to G-actin [9]. Once bound to four actin monomers, Spire holds them together in a stable tetramer to instigate nucleation of actin filaments [6]. Spire explicitly promotes barbed end elongation of unbranched filaments due to its C-terminal region which caps the pointed end of the bound tetramer, effectively capping the filament preventing both dissociation and association of actin subunits [9]. Similar to the Arp2/3 complex, the Spire protein does not associate with the growing, barbed end of its filament which is also vulnerable to capping proteins [8]. Due to its recent discovery as an actin nucleator, not much is known about the Spire family of proteins. However, orthologs of the Spire protein have been discovered throughout metazoan species [6].

The most distinguishing features of the formin class of actin nucleators are its FH2 domain and size. The FH2 domain is a highly conserved amino acid sequence between these proteins and was used to identify them well before their actin nucleating activity was discovered. The size of a formin protein is typically over 1000 amino acids in length and the difficulty in studying these proteins mainly stems from its insolubility when attempting to express full length protein or even individual domains in prokaryotic hosts [10, 11, 12, 13, 14]. The mechanism formins use to nucleate actin is not well understood. Formins promote the assembly of actin filaments by forming a processive cap over the barbed end with its FH2 domain. The FH2 domain protects the end of the filament from capping proteins while simultaneously adding G-actin onto its end. This mechanism results in the formation of long, unbranched filaments. Prior to the discovery of its actin polymerization ability,

formin proteins were believed to be merely scaffolds due to their size and high number of protein-protein interactions.[15] Research into formins is still in its relative infancy and remains an area of intense study.

The theme for the remainder of this paper is centered on proteins of the formin class of actin nucleators and the latest discoveries regarding the cellular mechanisms in place to regulate the activity of these potent filament assemblers. The paper will start off covering the formins in a general sense and continue onward to focus on the five yeast formins and the murine formin mDia1 since the bulk of the knowledgebase regarding formins stems from the research based on these proteins. The end of the paper will discuss methods to measure localized formin activation in living eukaryotic cells.

The Rho Family of Small G-Proteins

The Rho family of GTPase proteins is characterized by their small size (~21 kDa) and ability to be bound to either GTP or GDP. Dependent on its association with GTP or GDP, Rho GTPases exhibit conformational differences in the protein's tertiary structure, especially in areas of effector protein-protein interaction [16]. When bound to GTP, the protein is considered to be active and when bound to GDP, it is considered dormant. This property allows the Rho family to act as 'molecular switches' to signal intracellularly within eukaryotic cells [17]. Rho proteins undergo three post-translational modifications at their C-termini which end in a CAAX motif [17, 18]. First, the cysteine residue is isoprenylated by either a geranyl-geranyl or farnesyl moiety, second the three C-terminal amino acids are cleaved off the protein, and finally, the exposed C-terminal, modified cysteine receives a carboxyl methylation [17, 18].

Cellular control of Rho activity is directly orchestrated by at least three types of proteins. Guanine dissociation inhibitors (GDIs) stabilize the GDP bound form of Rho and keep the protein cytosolic by binding over the lipid moiety which localizes Rho to phospholipid membranes [18, 19]. GDI disassociation factors (GDFs) displace the GDI from the Rho protein, causing it to become membrane bound. GDFs do not necessarily have to be proteins since some phospholipids destabilize the interaction of certain GDIs and their bound Rho protein [19]. Guanine nucleotide exchange factors (GEFs) encourage the exchange of GDP to GTP, or the activation of Rho proteins. GTPase activating proteins (GAPs) deactivate the activated Rho protein by promoting GTP hydrolysis which ultimately results in the loss of the gamma inorganic phosphate and an inactive GDP bound Rho protein [18, 19]. Rho protein activity is regulated at two tiers: at the membrane where GEFs and GAPs interact and in the cytosol where GDIs would associate and dissociate.

A link between Rho activation and remodeling of the actin cytoskeleton has been known since 1992 CE when Ridley *et al.* exposed Swiss 3T3 fibroblasts to an extracellular Rho-activating ligand, lipophosphatidic acid, which led to the assembly of stress fibers and maturation of focal adhesions [20, 21]. This association was further probed in 1994 CE when Yamochi *et al.* localized N-terminal hemagglutinin (HA) tagged Rho1p in *S. cerevisiae* to emergent bud sites, the bud tip and at the mother-bud neck region at cytokinesis [18]. In the same study, they stained F-actin with rhodamine phalloidin and found localization of HA-Rho1 at sites of actin nucleation [18]. The connection between actin remodeling and Rho activity is apparent in both mammalian cells and budding yeast and the evidence suggests its mechanisms are conserved across all eukaryotic species.

The Formin Discovery Timeline

The term 'formin' first emerged in the year 1990 CE as a protein product that was identified by Woychik *et al.* in the 'limb deformity' locus in mice [22]. The locus was first characterized by a random transgene insertion into the mouse chromosome that deleted 1.5 kilobases of genomic DNA and the phenotype includes oligodactyly and syndactyly of the forelimb and hind limb digits and fusion of the tibia and fibula amongst other less obvious detrimental characteristics [23]. The transgene and deletion of chromosomal DNA was mapped to the promoter region of an open reading frame and subsequently named Formin-1 [22].

In 1994 CE, Castrillion *et al.* discovered the *diaphanous* gene product in *Drosophila melanogaster* is necessary for cytokinesis. It was originally identified in a genetic screen for recessive male-sterile mutants. BLASTP analysis of its amino acid sequence revealed two regions of homology between the mouse 'limb deformity' gene product and *S. cerevisiae* Bni1p. They designated these two regions of homology as formin homology domain 1 (FH1) and formin homology domain 2 (FH2) [24].

Three years later in 1997 CE, Evangelista *et al.* drew the connection between formin proteins and polymerization of actin filaments in *S. cerevisiae* through genetic experiments. They also truncated the N-terminus of Bni1p and discovered an abundance of F-actin in the form of cables and cortical patches, inadvertently generating a constitutively active form of the protein. In addition, they also discovered the direct

interaction between the N-terminus of Bni1p and GTP-bound Cdc42p (a Rho family protein) through a two-hybrid screen [25].

The FH3 domain was discovered and characterized by Petersen *et al.* in 1998 CE. They demonstrated the FH3 domain from Fus1 in *S. pombe* is necessary for proper localization of the formin to the projection tip during mating [26].

In 2001 CE, Arthur Alberts identified the C-terminal diaphanous autoregulatory domain (DAD) in mice and demonstrated its evolutionary conservation through the yeast formins. He also proposed an autoregulatory mechanism based on the binding of GTP-Rho. The GTPase binding domain (GBD) and the DAD binding domain are found in the same N-terminal region and the bound GTP-Rho would displace the DAD through either steric hindrance or destabilization of the GBD-DAD interaction [27].

Research into formins sharply escalated in 2002 CE when Pruyne *et al.* and Sagot *et al.* applied *in vitro* methods and demonstrated that the *S. cerevisiae* formin Bni1p functions as an active actin nucleator instead of a passive scaffold that recruits a separate nucleator. Both groups successfully expressed the C-terminal portion of Bni1 in *E. coli*, purified it, and then incubated it with purified actin. With this setup they were able to observe actin polymerization [7, 28]. These results led to numerous studies into deciphering the structures, functions and mechanisms behind formin proteins.

The years 2004 CE to 2006 CE produced several papers displaying the crystal structure of various diaphanous related formin (DRF) domains. Xu *et al.* solved the crystal structure of the FH2 domain of *S. cerevisiae* Bni1p (amino acids 1348-1766) which dimerizes in a head-to-tail donut fashion [29]. Shimada *et al.* were able to crystallize the FH2 domain of mDia1 and found a similar structure to yeast Bni1p FH2, although the fragment they crystallized lacked the dimerization domain [30]. Otomo *et al.* were also able to co-crystallize the FH2 domain of murine mDia1 with tetramethylrhodamine-actin. This crystal structure revealed a mechanism in which mDia1 is able to nucleate actin filaments by aligning two actin monomers in a stable dimer configuration [31]. In the same year Otomo *et al.*, Rose *et al.*, and Lammers *et al.* crystallized the N-terminal regulatory domain of murine mDia1 [32, 33, 34]. Nezami *et al.* published their solved N-terminal structure the mechanisms will be analyzed in greater detail later in this paper.

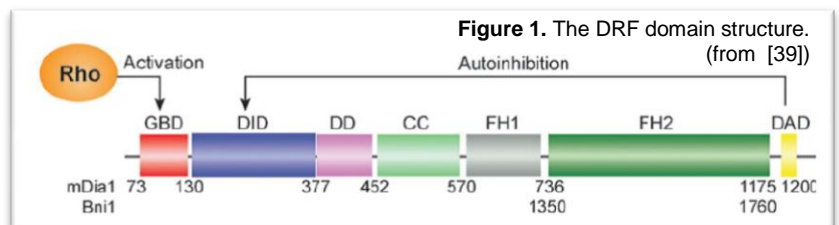
Database Mining for Formins

At the turn of the millennia, various genome sequencing projects completed their work and published entire genomic sequences onto database servers online. Enthused with the knowledge of the recently solved Bni1p FH2 crystal structure, Higgs *et al.* and Rivero *et al.* mined available genome databases to discover proteins harboring the distinctive FH2 domain. These studies resulted in the identification of 107 proteins containing formin FH2 domains in genomic databases of animals, plants and yeasts [36, 37]. Higgs and Peterson ran a phylogenetic analysis on their data using algorithms based on minimum evolution, maximum parsimony, and maximum likelihood and determined that mammalian formins fall into seven divergent groups: Diaphanous (Dia), Disheveled-Associated Activators of Morphogenesis (DAAM), Formin-Related proteins identified in Leukocytes (FRL), Formin Homology Domain-containing proteins (FHOD), the 'original' formins (FMN), the 'inverted' formins (INF) and Delphilin [36, 38]. Also, it is important to note that they found the yeast formin FH2 sequences to be phylogenetically segregated from all other eukaryotes [36]. The amino acid sequences of yeast formin FH2 domains do not align strongly with the metazoan FH2 domains. However in respect to domains flanking the FH2 region, the structure of *S. cerevisiae* yeast formins Bni1p and Bnr1p are classified as diaphanous related formins (DRFs) due to being ligands of Rho-GTPases as a part of their regulatory mechanism [15]. The mammalian groups Dia, DAAM and FRL also belong to the DRFs [39]. Of the seven identified formin groups, the DRFs are the best understood.

Domain Organization of the Diaphanous Related Formins

Domain Structure

The DRFs are structurally related and they share the same general domain organization [39, 40]. The basic structure consists of a GTPase binding domain (GBD), Diaphanous Inhibitory Domain (DID), Dimerization Domain (DD), Coiled Coil domain (CC), Formin Homology 1 (FH1) domain, Formin Homology 2 (FH2) domain, and Diaphanous Autoregulatory Domain (DAD) in order from the amino- to carboxy-terminus [39, Figure 1]. The Formin Homology 3 (FH3) domain is not depicted in Figure 1 and some may argue about its definition as a legit homology domain due to it being the



least conserved of the FH domains [15]. If included, the FH3 domain would span the DID and DD in the above illustration [26].

Domain Roles

DRFs can be divided into two halves, the N-terminus being the regulatory half and the C-terminus would house the actin nucleation and extension mechanism. Inactive DRFs are believed to be in an autoinhibited state. An intramolecular interaction between the DID and the DAD impedes actin nucleation activity of the FH1-FH2 domain [27, 41]. Upon GTP-Rho binding of the GBD, the DID releases the DAD and the formin is considered to be active [34]. It is from the crystal structure that we know the FH1 and FH2 domains dimerize in a head-to-tail donut fashion [29]. The FH1 domain contains repeating polyproline motifs which bind to profilin [24, 42]. Through an unknown mechanism, the FH1-FH2 domain separates profilin from actin, adds the freed actin to the growing filament and releases profilin to the cytoplasm. The DD and the CC regions (the FH3 domain) enable the N-terminus to dimerize and may be important for the proper localization of the protein [26, 43, 44].

Formins are powerful actin filament nucleators. Yeast and mammalian cells expressing N-terminal truncated DRFs display drastically altered gross morphologies and are unable to properly complete subsequent cell divisions [25, 41, 45]. The observation that these proteins constitutively nucleate and extend actin filaments as an N-terminal truncation cements the concept of DRFs structured into a regulatory half and catalytic half. The DRF regulatory methods discussed in this paper are still early in their understanding; however most of these mechanisms have been conserved across this formin group. As more of the basic factors of regulation become better characterized, the research will naturally progress into the smaller details behind DRF regulation.

Schizosaccharomyces pombe Formins

Currently, research into *S. pombe* formins is a little behind its single-celled cousin *S. cerevisiae*, but is rapidly catching up. Even though the regulatory mechanisms behind the activation and deactivation of these formins are not well understood, from what is known we can draw parallels to other DRFs, especially with those found in *S. cerevisiae*. *S. pombe* has three formins with distinct roles in cellular processes. For3p, cdc12p and Fus1p are responsible for longitudinal actin cables in interphase cells, the median actin ring in cell mitosis, and the cell projection tip during conjugation, respectively [26, 46, 47]. Unlike Bni1p and Bnr1p in *S. cerevisiae*, the *S. pombe* formins cannot rescue each other in the event of a knockout mutation [26, 46, 47]. *for3Δ* cells have a 'dumpy' or 'bent' phenotype and lack longitudinal F-actin cables during interphase [46]. *cdc12Δ* cells are inviable [47]. *fus1Δ* cannot form mating projections in response to pheromone [26]. Since these proteins basically perform the same function, yet cannot compensate each other is evidence of the tight control these proteins are regulated under.

For3p

For3p is the best understood formin out of the three *S. pombe* formins. Through yeast two-hybrid studies we know that the N-terminus of For3 (aa. 149-488) interacts with constitutively active Rho3(Q71L) and Cdc42(Q61L) but not Rho1(Q64L). It was not clarified in that manuscript whether the full-length For3p is activated by the direct binding of activated GTP-Rho [46]. Subsequent studies determined that the activity of For3p displayed no change in activity in *rho3Δ* cells, but cdc42-GTP is necessary to activate the protein [48, 49].

In an *in vitro* binding assay, truncated For3 (aa. 137-515) is able to directly co-precipitate tea4p, associating microtubule ends to actin assembly in *S. pombe*. Also, overexpression of tea4p causes the overproduction of actin cables from For3p, suggesting spatial activation once it is positioned in complex at the end of the microtubule [50]. Later studies determined the C-terminal region of For3p directly interacts with Bud6p [51]. *S. pombe* Bud6p is homologous to *S. cerevisiae* Bud6p, which are both actin binding proteins and cell polarity determinants [51, 52, 53]. Genetic studies between For3p and Bud6p further bolstered the theory of For3p spatial regulation. In *bud6Δ* cells, For3p-derived actin cables were thinner and the cable growth rate was significantly reduced. In addition, once Bud6p-RFP and For3p-3GFP signals lost co-localization, For3p-3GFP activity halted [48]. A proposed mechanism of activation and localization for For3p starts with a small fraction of the inactive protein binding to the Tea4 complex at the end of the microtubule. The formin rides to the polar end of the cell where it binds to Bud6p. Upon activation by Cdc42-GTP, For3p nucleates an actin cable towards the center of the cell where Myosin V is able to transport more For3p to the cell tip [49].

Species	
<i>S. pombe</i>	For1p cdc12p Fus1p
<i>S. cerevisiae</i>	Bni1p Bnr1p
<i>H. Sapien</i>	hDia2C delphilin
<i>M. musculus</i>	mDia1

Phylogenetic analysis of For3p failed to find an obvious DAD-like sequence and it was suggested that the protein was not regulated by autoinhibition, but through alternative mechanisms [36]. The recent discovery that For3p is actually a DRF emphasizes the complications behind domain analyses based on primary amino acid sequences and the importance of hand-testing outliers obtained through high-throughput data analyses.

cdc12p

The *cdc12* protein has not been studied to the same extent of For3p. Genetic analysis has revealed a synthetic-lethal interaction with *cdc3p* (profilin). The phenotype of haploid *cdc12Δ* cells is elongated and multinucleate, characteristic of defects in cytokinesis but not nuclear division [54]. An N-terminal truncation mutant, *cdc12(FH1FH2)p*, results in multinucleated cells with misshapen septa and an abundance of actin cables with no cortical patches or contractile rings [45]. This confirms the existence of an autoregulatory region in the N-terminus through intramolecular interactions. However, an interesting property of the formin was discovered in an *in vitro* study with the constitutively active mutant. In order for *cdc12* to assemble actin cables, it needs access to profilin-actin in a phenomenon referred to as 'profilin gating'. Without profilin, *cdc12(FH1FH2)p* would bind actin but will not add monomers to the barbed end. The rate of filament growth from *cdc12(FH1FH2)p* without profilin is comparable to the rate of filament elongation of pointed end growth (ie. zero formin activity) [45, 55]. This may be used as a method to regulate the activity of *cdc12p* by limiting the amount of profilin expressed in the cell. For this formin, it is not yet known whether or not small G-proteins are able to activate actin nucleation activity or how it is localized to the cell median for the production of the mitotic actin ring [2].

Fus1p

Very little is known about *Fus1p*. It is a formin that is involved in extending the projection tip during conjugation. Petersen *et al.* defined the FH3 domain from this protein as a targeting motif important for proper localization to sites of action [26]. They also demonstrated the existence a targeting motif from *cdc12p* in the same area with respect to the *Fus1p* FH3 domain [26]. However, due to amino acid alignments between formins in the FH3 area not being well conserved, some experts disagree on its function as a 'homology domain' [36, 38]. Considering the fact that different formins would require different binding partners to localize to different zones in the cell, it is not surprising that the area defined as the FH3 domain (now DD and CC) shares little sequence homology across the formins. Taking into account strong evidence that localization of certain DRFs contributes strongly to their activity, investigation into this region might uncover a general activation or regulatory mechanism that ties GTP-Rho binding and localization in a cooperative manner [13, 14, 51].

Schizosaccharomyces pombe as an example

The *S. pombe* formins are strictly regulated to their specific function in the cell. Since they cannot compensate for the activity entitled to another, the methods regulating individual formin activation must be strictly controlled and specific. The studies surrounding For3p have discovered a link between its localization and activation. Studies into *S. cerevisiae* Bni1p have also displayed that same association with its own Bud6p. The *S. cerevisiae* discovery proves spatially-controlled formin activation is not isolated to the *S. pombe* species [13, 14]. The domain of a localization motif discovered in *Fus1p* and *cdc12p*, could lead into future investigations regarding formin localization and activation. Unfortunately, Petersen *et al.* did not perform two-hybrid or co-immunoprecipitation assays with their FH3 peptides and it remains ambiguous as to which proteins are involved that properly localize *Fus1p* and *cdc12*. For3p activity re-localizes from the bud tips to the mid-section and the mechanism behind this switch is still not well understood, suggesting the list of For3p binding partners have yet to be fully fleshed out [2]. The discovery that *cdc12(FH1FH2)p* requires *cdc3p* (profilin) in order to elongate its attached actin filament leads to interesting ideas regarding mechanisms of formin regulation [45, 55]. It is possible that the cell could regulate *cdc12p* activity by controlling the transcription of *cdc3p*. It is yet to be determined whether the cell actually uses this method as a means to regulate *cdc12p* activity.

***Saccharomyces cerevisiae* Formins**

Of all the information on formin proteins, the most is known about the *S. cerevisiae* DRF Bni1p. Its second formin, Bnr1p is trailing closely behind due to most studies including both proteins in their experiments. Live imaging studies localize Bni1p to the bud site, then into the growing bud and then to the mother-bud neck just before cytokinesis [43]. These locations are also known as 'sites of growth'. The same technique was used to localize Bnr1p to the bud neck where it stayed for as long as the mother and daughter cell were attached [56]. Single knockouts of *bni1Δ* or *bnr1Δ* do not have much of an effect on growth compared to wild type cells [10, 57]. A double knockout, *bni1Δbnr1Δ*, is temperature sensitive, grows slowly, shows an

increased fraction of multinucleate cells, and loses cell polarity [10]. Its phenotype is similar to a *pfy1Δ* (profilin) knockout strain [58]. The drastic difference between a single knockout and the double knockout suggests that these two proteins are able to compensate for each other in one's absence. This is in stark contrast to the *S. pombe*, in which a single null mutant of one of its three formins results in an observable detrimental phenotype [26, 46, 47]. Most of the experiments performed on *S. pombe* to determine its formin regulatory mechanisms were based on research performed on *S. cerevisiae* and the following observations may sound very familiar.

The Formins Bni1p and Bnr1p as Rho-GTPase Ligands

Even though Bni1p and Bnr1p are able to rescue the other's null mutation, they still play specific and different roles within the cell [14]. Therefore, it is not surprising the two formins interact with different sets of charged Rho-GTPase proteins. The *S. cerevisiae* genome encodes five Rho family GTPases: Rho1p, Rho2p, Rho3p, Rho4p, Rho5p, and Cdc42p [1]. All six proteins have some sort of influence in the regulation of the actin cytoskeleton [59].

The *S. cerevisiae* Rho1p is the homolog to mammalian RhoA. They share about 70% identity between their amino acid sequences. The *rho1Δ* null mutant is inviable, but the mammalian RhoA can rescue the deletion with a temperature sensitive phenotype [18]. Two-hybrid and recombinant *in vitro* pulldown experiments have pinned a direct association between constitutively active Rho1(Q68L)p and a fragment of Bni1p containing the GBD (aa. 90-489). Interestingly, they also managed to score a hit between the GDP-bound form of Rho1p with the N-terminus of Bni1p (aa. 1-524) in a recombinant pulldown experiment, but not in the two-hybrid experiment [57]. Immunofluorescence studies have localized Rho1p to sites of growth, similar to the localization of Bni1p [18]. However, more recent studies have challenged the direct interaction between activated Rho1p and Bni1p. Dong *et al.* claim that, through genetic studies, GTP-Rho1p does not directly regulate Bni1p through GBD interaction but indirectly through an effector, protein kinase C (Pkc1p) [59]. A yeast strain expressing both constitutively active Pkc1p* and *rho1-2p* remained viable at a nonpermissive temperature (37°C) for *rho1-2p* yeast [59, 60]. Also, a *pkc1Δ* knockout strain loses actin cables and is also inviable at 37°C. Based on these criteria, they decided that Bni1p activation through the Pkc1p/Rho1p pathway is only necessary at elevated temperatures [59]. Unfortunately, it remains vague as to how activated Pkc1p leads to the activation of Bni1p. This is the only evidence of formin regulation in response to environmental temperature.

Cdc42p is another small G-protein found in budding yeast. During logarithmic growth, it is localized to the bud site, growing bud tip, or the mother-bud neck, similar to Bnip and Rho1p [61]. The first evidence of interaction between Bni1p and Cdc42p was examined by Evangelista *et al.* in a two-hybrid assay between the N-terminus of Bni1p (aa. 1-1214) and constitutively active Cdc42(G12V)p [25]. This interaction was also observed through live cell imaging techniques. A temperature sensitive *cdc42-1p* strain is mislocalized at a nonpermissive temperature [61]. A yeast strain expressing both *cdc42-1p* and GFP-Bni1p displays GFP mislocalization at nonpermissive temperatures and it was determined that Cdc42p is essential for the proper localization of Bni1p [43]. In the same study, it was also determined that Bni1 is not dependant on Rho1p for proper localization, which is consistent with (but not substantial to confirm) the theory that Rho1p does not directly interact with Bni1p [43, 59]. Interestingly, nonpolarized actin cables persisted in *cdc42-1* strains at nonpermissive temperatures suggesting that Cdc42p is essential for localization during logarithmic growth and is not the exclusive activator of Bni1p [59]. This leads to more theories about the regulation of Bni1p activity. In a situation where the cell would need to nucleate actin cables at a site other than where active Cdc42p localizes, the option to express a Bni1p binding protein that doesn't localize at growth sites and an alternate Bni1p activating factor is definitely feasible. The only recognized situation that would make the cell alter the direction of polarized growth is in the formation of mating projections in the direction towards a pheromone gradient. In that situation, Cdc42p is already known to be recruited to the tip of the mating projection [62]. Any other situation where budding yeast would need to alter its polarization has yet to be explored and the mechanism behind the change in polarity would be intriguing.

While Rho1p and Cdc42p are essential for cellular viability, Rho2p, Rho3p, Rho4p and Rho5p are non-essential genes [18, 59, 61]. Evangelista *et al.* observed the formation of complexes of Bni1p-Rho3p and Bni1p-Rho4p but did not publish their data [25]. Meanwhile, yeast two-hybrid experiments and recombinant *in vitro* pulldown assays suggested a direct interaction between an N-terminal fragment of Bnr1p (aa. 63-421) and constitutively active Rho4(Q70L)p [10]. The analysis of three strains harboring separate null mutations of *rho2Δ*, *rho4Δ* or *rho5Δ* did not result in any aberrant phenotypes. The *rho3Δ* strain had a slower growth rate than the other mutants and was the only strain unable to produce actin cables in the bud with a Bni1p overexpression plasmid [59]. It was concluded that Bni1p requires Rho3p to produce cables in the bud.

Overexpression of Rho4p can overcome the sick phenotype of a *rho3Δ* strain, indicating the functionality of the two GTPases is partially redundant [63]. The double knockout *rho3Δrho4Δ* strain is extremely sick with severe cytoskeletal defects [63, 64]. Expression of a N-terminal truncated form of Bni1p (aa. 452-1954, constitutively active) lacking its GBD or overexpression of the Bni1p DAD (aa. 1796 -1954) (which would compete with the full-length Bni1p's DAD to relieve autoinhibition) were both able to rescue the growth defects in the *rho3Δrho4Δ* strain [59]. It is suggested that the detrimental phenotype in the *rho3Δrho4Δ* strain is due to the inability of Bni1p to overcome autoinhibition [59].

Of all the Rho family proteins found in *S. cerevisiae*, there has not been any evidence that Rho5p is able to influence the activity of Bni1p or Bnr1p. Overexpressed Rho2p is able to rescue the lethal *rho1Δ* knockout strain, indicating some degree of functional redundancy with Rho1p [65]. Two-hybrid data suggests Bni1p directly interacts with activated Rho1p, Rho3p, Rho4p and Cdc42p even though the Bni1p-Rho1p interaction has been challenged [25, 57, 59]. Genetic experiments indicate that Bni1p is activated by Rho1p through Pkc1p, is localized but not activated by Cdc42p and is only truly activated by Rho3p [59]. The only positive Rho-GTPase two-hybrid hit for Bnr1p was with activated Rho4p [10]. It is still not certain whether Rho4p regulates actin filament nucleation and elongation activity of Bnr1p. It is also not clear if Bnr1p is unable to activate in a *Rho4pΔ* strain. In most studies featuring the two budding yeast formins, Bnr1p regularly ends up on the backburner.

Bni1p & Bnr1p Non-Rho Protein-Protein Interactions

If Bni1p and Bnr1p are anything similar to the *S. pombe* formins, then several non-Rho family proteins should be able to influence their activity. One protein that binds to every DRF is Pfy1 (profilin) [24, 42]. This interaction is accomplished through the polyproline motifs in the FH1 domain, adjacent to the FH2 domain [58, Figure 1]. The interaction between profilin and the FH1 domain does have potential to regulate formin activity as observed with *S. pombe* cdc12p through a process dubbed 'profilin gating' [45]. The interaction between the FH1 domains of both Bni1p (aa.1239-1397) and Bnr1p (aa. 756-807) with profilin have been confirmed by two-hybrid assays [10, 25]. Densitometric tracing of the two-hybrid blot suggested a molar ratio of 2:1 for profilin:Bni1(1239-1650)p [10]. The stoichiometric binding ratio of Bnr1p to profilin has yet to be determined. Elongation activity of N-terminal truncated Bni1p or Bnr1p *in vitro* was not arrested by the absence of profilin [7, 14, 66]. Therefore, the activity of the two budding yeast formins is not gated by profilin.

The protein Spa2p localizes to sites of growth and is considered to play a role in establishing cell polarity [67]. Two-hybrid experiments and recombinant *in vitro* pulldown assays have indicated a protein-protein interaction between Bni1(826-987)p and Spa2(1213-1466)p [11]. Bni1p interacts with Spa2 at its FH3 domain which some consider to house DRF targeting motifs [26]. A *spa2Δ* knockout strain displays a random budding pattern, akin to a *bud6Δ* strain [11, 52, 53]. Live cell imaging confirms the mislocalization of Bni1p in the absence of Spa2p [43, 56]. It is proven that Spa2p is necessary for the proper localization of Bni1p to the bud tip. However, it is still not certain whether or not Bni1p binding to Spa2 contributes to its activation.

A construct composed of a protein fragment derived from Bni1p that spans its FH1 and FH2 domains (aa.1229-1650) was used to probe for interactions from the yeast cytosolic fraction. Translational elongation factor EF-1 alpha (Tef1/2p) was isolated and identified as the major binding partner to that particular fragment [12]. Tef1/2p is an essential protein of the translational machinery that catalyses the elongation of the nascent polypeptide chain [68]. EF-1 alpha from *Dictyostelium* has been shown to bind and bundle F-actin and the same holds true for Tef1/2p [68, 69]. Co-incubation and sedimentation assays suggest that the Bni1p interaction with Tef1/2p disrupts Tef1/2p from properly bundling actin [68]. Also, there is no evidence of interaction between Bnr1p and Tef1/2p. It is still not clear what the importance is behind the association involving Bni1p and Tef1/2p. In an unrelated study, it was observed that Bnr1p is able to bundle actin filaments while Bni1p does not [14]. It is possible that the bundling of actin filaments is necessary for cable formation and proper formin function. The meaning behind this association remains a curious issue that warrants further study.

The protein Bud6p from *S. pombe* was found to interact with a formin, Fus3p, which is responsible for the generation of longitudinal cables. A two-hybrid assay in *S. cerevisiae* suggested a protein-protein interaction between the C-terminal end of Bni1p (aa. 1647-1953, that encompasses the DAD) and the C-terminal end of Bud6p (478-788) [25]. Bud6p, like Spa2p share similar roles in establishing polarity in budding cells since deletions in either gene confer similar gross phenotypes [52, 53, 67]. Data from live cell imaging of GFP-Bni1p in a *bud6Δ* knockout strain showed that Bni1p was properly localized until the cell cycle progressed through small- to middle-sized buds when the GFP-Bni1p signal would split into punctate dots on the bud cortex [43]. Continued bud growth resulted in rectangular-shaped buds [43]. It was also shown that Bud6p has an influence on formin activation, similar to its influence on *S. pombe* For3p. In a *bud6Δ* knockout strain,

overexpressed Bni1p is unable to generate actin cables. This suggests a role for Bud6 is to stabilize the active conformation of Bni1p through DAD binding [14, 70]. In addition, a Western Blot analysis of synchronized yeast cells displayed an upregulation of Bud6p during bud emergence and growth [14]. In the same blot, Bud6p ran as a doublet. Treatment of the extract with λ -protease abolished the doublet which indicates genuine phosphorylation of Bud6p [14]. The significance of Bud6p phosphorylation is not yet understood.

There is a known protein-protein interaction which Bnr1p does not share with Bni1p. The FH1 domain of Bnr1p (aa. 757-887) interacts with the SH3 domain of Hof1p (aa. 551-669) in a Rho4-dependent manner [71]. Immunofluorescence of Hof1p localized the protein to the bud neck in a similar pattern to Bnr1p localization [56, 71]. Unfortunately, it was never tested whether Bnr1p mislocalizes in a *Hof1* Δ knockout strain or if Bnr1p activity is regulated upon interaction with Hof1p.

The non-Rho protein-protein interactions of the yeast formins may contribute to their regulation. Only Bud6p thus far has been determined to contribute to the activation of Bni1. It is possible with continued research that other potential direct regulators of Bni1p and Bnr1p may be found.

Other hinted mechanisms of Bni1p regulation include phosphorylation. In two separate studies, phosphorylation of Bni1 was observed [1, 62]. Matheos *et al.* observed the phosphorylation of full length Bni1p specifically by Fus3 kinase *in vivo* and *in vitro* [62]. Moseley and Goode observed phosphorylation of their C-terminal construct Bni1(FH1-COOH)p (aa. 1227-1953), but did not identify the kinase [1]. Unrelated, two double-overexpression screens were performed to discover dosage rescue interactions against N-terminal truncation mutants for Bni1p or Bnr1p [25, 72]. There were no hits for proteins with direct interactions with these formins except for profilin. This indicates that there may be no proteins that bind directly to the C-terminal domain that would deactivate the catalytic apparatus. Phosphorylation on top of GTP-Rho activation coupled with non-Rho activators indicates the many dimensions of possible mechanisms that may regulate formin proteins.

Mammalian Formins

Possible Insights into Formin Activation

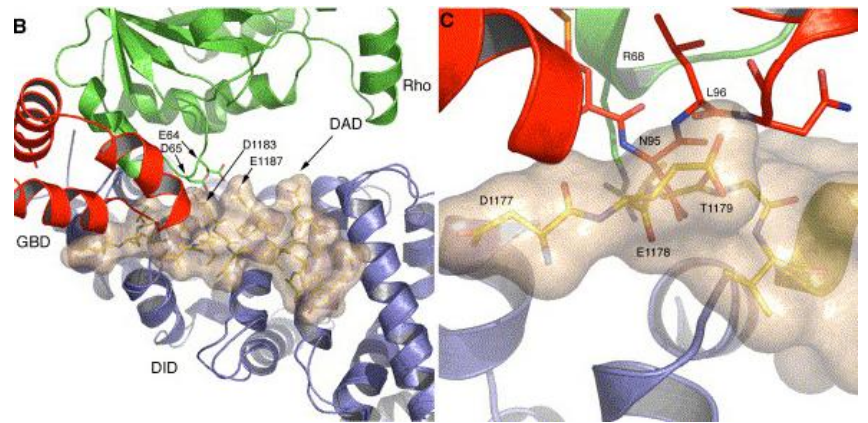
The mammalian DRFs share many parallels with the yeast formins [38, 39]. The details will be different but the mechanisms for the most part, remain very similar. However, there are a handful of regulatory mechanisms observed in mammalian formins that have yet to be found in yeast.

Gasman *et al.* discovered the human DRF hDia2C through a yeast two-hybrid assay probing for a constitutive RhoD^{G26V} binding partner in a HeLa cDNA library [73]. This formin is identical to hDia2B except for an 11 amino acid deletion and a 7 amino acid insertion within the GBD and this difference is coded on separate exons within the hDia2 locus, indicating these two proteins (hDia2B and hDia2C) are splice variants. The formin hDia2B was only identified and never characterized so it is impossible to draw parallels between the two proteins [74]. The only difference we do know is that hDia2B was not detected in the yeast two-hybrid assay as a protein that interacts with GTP-RhoD [73]. However, whether or not hDia2B is present in the HeLa cDNA library or if hDia2B-GBD protein fragments are soluble when expressed in yeast has not been addressed. The main point of this example is that mammalian formins have the potential to be regulated at the mRNA processing stage through alternative splicing. These variants may have differences in GTP-Rho binding partners, cellular localization, actin nucleating/filament extension rates, scaffold properties, protein half-lives, or cytosolic protein concentrations, just to list a few examples. Also, these splice variants will be difficult to hit in high throughput genomic alignments [36, 37]. Mammalian systems most likely express many more formin varieties than expected.

The activation of mDia1 was observed upon application of shear stress to human and mouse fibroblasts. The application of force to serum starved fibroblasts caused mDia1-dependant recruitment of actin, vinculin, and paxillin suggesting the maturation of focal adhesions [75]. The mechanosensor responsible for the instigation of this event has not been identified. These experiments were also conducted before recombinant formins were observed nucleating actin *in vitro* by Pruyne *et al.* and Sagot *et al.* [7, 28]. It may be possible that formins act as mechanosensors, considering that Bni1p is able to interact with multiple proteins that anchor it to the membrane (Cdc42p, Spa2p and Bud6p) and at sites at its N- (Cdc42p, Spa2p) and C-terminus (Bud6p) [see above]. Whether a reasonable amount of strain to both ends of the protein would overcome autoinhibition has yet to be tested.

The mammalian Delphilin is a unique formin that is selectively expressed in cerebellar Purkinje cells [76]. Its primary sequence is so different from the other known formins that it is classified in a group by itself [36]. Delphilin has recognizable FH1 and FH2 domains but the remaining 94% of the protein does not harbor another recognizable formin domain [36, 76]. There has been no physical evidence that Delphilin interacts with actin and its activity as a formin is assumed through its primary sequence. Its N-terminus however, houses a PDZ domain which allows it to complex with the glutamate receptor $\delta 2$ subunit [76]. This is a curious association between an unorthodox formin and a cerebellar neuroreceptor. The existence of a connection between neuronal signaling and Delphilin formin activation (if it does) remains an open question.

Figure 2. The crystal structure of Rho-GBD and DAD-DID superimposed. (from [35])



The Crystal Structure of mDia1

A powerful way to determine the molecular mechanisms behind a protein's functionality is to analyze its crystal structure. Unfortunately, the crystal structure of the N-terminal regulatory domain of Bni1p has not been solved thus far. This is most likely due to the difficulty of keeping recombinant yeast formins soluble in prokaryotic hosts. Surprisingly, the N-terminal fragment of murine mDia1 is expressed better in prokaryotes than the N-terminus of yeast Bni1p. To date, four manuscripts present solved crystal structures of mDia1 N-termini. The crystal structure of mDia1 in its entirety has not yet been solved.

Otomo *et al.* were the first to crystallize the N-terminus of mDia1. Their structure did not have Rho or DAD co-crystallized in complex to mDia1 [32]. Their data suggest the DID is composed of a superhelix of five armadillo repeats. The armadillo repeats form into a cupped structure with a hydrophobic interior [32]. This finding makes sense since the mDia1 DAD binding is mainly due to hydrophobic interactions [27]. Rose *et al.* co-crystallized the N-terminus of mDia1 with RhoC while Lammers *et al.* and Nezami *et al.* were able to solve the N-terminal structure of mDia1 in complex with DAD [33, 34, 35]. Their amalgamated findings shed some insight into the nature of GTP-Rho mediated release of the DAD peptide. The ejection of DAD from DID seems to transpire in a two-stage process. As GTP-Rho approaches the GBD of mDia1, long-range electrostatic repulsion between residues on RhoC (E64, D65, D67) and DAD (D1183, E1197) clash [33, 34, 35, Figure 2]. The RhoC-GBD interaction is thermodynamically favorable (K_d 9 nM) over the DAD-DID interaction (K_d 57 nM) and is able to compete off the C-terminal DAD residue [35]. The second stage commences once GTP-RhoC contacts the GBD. The GTP-RhoC footprint partially overlaps the binding footprint of DAD. Bound GTP-RhoC prevents the annealing of DAD to DID through mechanical obstruction [34, 35].

Keep in mind that the release of autoinhibition is just one factor in the activation of formins, as is the case for *S. cerevisiae* Bni1p, *S. pombe* For3p, and murine mDia1 [14, 49, 70, 77].

Live Cell Imaging of Formin Activation

The ability to image live cells definitely has its advantages. Collecting data as a function of time literally adds another dimension to the results. Several experiments have directly observed the formin-induced *in vitro* polymerization of actin but the method does have its limitations [40, 45]. The only way to properly measure formin activity in relation to the cell cycle is through live-cell imaging.

Several groups have already successfully imaged active formins in living cells and were able to quantify actin polymerization rates by observing filaments or measuring the translocation of individual formin proteins. A study by Higashida *et al.* involved the expression of various EGFP-fused recombinant mDia1 constructs in XTC *Xenopus* fibroblasts [78]. The N-terminal truncated construct was imaged using single-molecule speckle analysis techniques and they were able to track a single molecule of EGFP-mDia1(FH1FH2) as it moved about the cell. Average speckle velocity was measured to be 2.0 $\mu\text{m}/\text{sec}$ and occasionally traveled distances over 10 μm [78]. This experiment exhibits an available technique that can be used to track a single protein in a live cell. This technique could be used to measure average turnover rates of Bnr1p in accordance to the cell cycle since in its inaugural paper, it was described to be highly susceptible to proteolysis [10].

A study by Martin and Chang involved the construct For3p-3GFP in *S. pombe* expressed under its own promoter [48]. They observed punctate cortical localization during all cell cycle phases except before cytokinesis where the spots relocated to the cellular mid-region. They also noticed some spots exhibiting retrograde movement along actin cables, which allowed them to assess the dynamic turnover rate of For3p at the cell tip [48]. This observation was only possible through the live-cell formin imaging.

Detecting actin filaments *in vivo* is usually tricky. A popular way to visualize the polymerization of actin is to label cysteine 374 with pyrene. However, the labeling process can only be performed *in vitro*, limiting its usefulness for live-cell imaging. Also, an Actin-GFP fusion has been engineered but its functionality remains questionable since it is unable to complement null mutations [83]. As an alternative, tagging the actin binding protein Abp140p with a fluorescent protein has been proven to label filaments without altering actin kinetics [79].

A recent study by Buttery *et al.* utilized the Fluorescence Recovery After Photobleaching (FRAP) technique on 3CFP-tagged Abp140 to measure actin cable elongation rates in budding yeast [80]. They were able to observe cables extending directly from Bni1p-3GFP punctate cortical spots and were able to measure the rates of elongation. They also noticed retrograde movement of Bni1-3GFP speckles down actin cables, similar to what Martin and Chang observed with For3-GFP [48, 80]. On the other hand, Bnr1-GFP did not display retrograde speckle movement. In combination with FRAP, they were able to determine the half life

In order to activate a DRF, it needs to be liberated from autoinhibition, typically through interaction with its GTP-Rho protein. Fluorescent colocalization of a Rho protein and its DRF does not necessarily mean they are interacting. The laboratory of Althur Alberts has integrated the technique of Fluorescence Resonance Energy Transfer (FRET) into their studies into mammalian formins with convincing results [81, 82, Figure 3]. This technique is more useful than colocalization since the two fluorophores need to be in close proximity for efficient energy transfer and detection, signifying genuine and stable protein-protein interactions [83]. In addition, if the N- and C-terminus in active DRFs exceed the Förster's radius of an inefficient fluorescent protein pair, it might be lucrative to pursue labeling the N-terminus and C-terminus of a DRF to directly detect its release from autoinhibition. Unfortunately, since a crystal structure of a full length DRF has yet to be solved, we are unable to estimate the change in distance between the N-terminus and the C-terminus from autoinhibited and active states.

Fluorescence microscopy is a very versatile and useful tool for the lab. Many innovative techniques and tricks have been developed over the years. The FRET technique seems to be the best choice to image activated DRFs due to the activation requirements of a bound Rho-GTPase. The Bimolecular Fluorescence Complementation (BiFC) technique (which requires a time-consuming annealing step of two halves of a fluorescent protein) is another method utilized to image protein-protein interactions. However, that system takes too long to generate an active fluorophore, especially considering the dynamic interaction of Rho-GTPases and their ligands.

In Closing

Research on formins is still in its infancy, especially considering how the pique in interest within the greater scientific community started as recent as 2002 CE when formins became more than protein scaffolds. Many aspects have been studied, yet there is still a lot of work that needs to be done. There are many difficulties ahead, mainly due to its insolubility when expressed in prokaryotic hosts. Formins have been showed to be regulated through various means: Rho-GTPase binding, other regulating protein-protein interactions or splice variation. Other theoretical (yet hinted) methods of formin regulation include mechanical stress, elevated temperature, phosphorylation and neurotransmitter signaling. These methods have yet to be confirmed or denied.

Fluorescence microscopy had already established itself as a powerful tool in formin research. With the emergence of live-cell formin imagery allowing the observation of localization and half-life dynamics, fluorescence microscopy proves to be an even more versatile tool for those with the creativity to harness it. Considering the ground that has been covered thus far, it is interesting to imagine where the frontier will be in the not too distant future.

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