Defining the molecular interactions between the formin Bnr1 and its regulators Smy1 and Bud14

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Abstract

The polarization of eukaryotic cells often relies upon the dynamic assembly and turnover of filamentous actin (F-actin). In the budding yeast *S. cerevisiae*, this is accomplished through the well-regulated activities of the formins Bnr1 and Bni1. These proteins polymerize F-actin from actin monomers and incorporate the resulting filaments into actin cables used for polarized secretory traffic. The formin homology (FH2) domain of formins directly nucleates actin filament “seeds” and then elongates filaments that can be organized into cables with specific architectures. Several regulators of Bnr1 are used by cells to control the length and shape of their actin cables, including the negative regulators Bud14 and Smy1. However, the complete mechanism by which these factors mediate the inhibition of Bnr1 and serve to govern cable growth and architecture has yet to be illuminated. In this thesis, I describe my work done to map the surfaces of Bnr1 necessary for interactions with its regulators Bud14 and Smy1 through mutational analysis of the Bnr1 FH2 domain. I first tested the ability of Bnr1 FH2 mutants to assemble actin filaments in the presence of the inhibitors Smy1 or Bud14, and found several refractive alleles of Bnr1. I then integrated one such allele at the genomic *BNR1* locus to study the effects of the mutation on *in vivo* actin cable dynamics, thus shedding light on the importance of Bnr1 regulation by Smy1 and Bud14 in cells.

Introduction

Most eukaryotic cells maintain the ability to develop asymmetrically and to redistribute cellular contents unequally. This polarization is triggered by a variety of
intracellular or extracellular signals, and is frequently established by mechanisms involving the dynamic assembly and turnover of cytoskeletal polymer networks. In the budding yeast *S. cerevisiae*, actin structures known as ‘cables’ are indispensable for polarized cell growth because they facilitate myosin-based transport of secretory vesicles, mRNAs, and organelles into the growing daughter cell, or bud (Li et al., 1995).

The assembly of these actin cables in yeast relies upon formin proteins (Evangelista et al., 2002). The Formin family is highly conserved among all eukaryotes, including yeast and mammals. Formins are large (120-220 kDa) multidomain proteins that can assemble actin structures from monomeric G-actin by promoting its polymerization into linear filaments. Specifically, formins have both a robust ability to nucleate new actin filaments and to then accelerate polymer elongation, even in the presence of capping proteins. Interestingly, some formins also have the ability to bundle actin filaments (Chesarone et al., 2010). The assembly of yeast actin cables is mediated by two different formin proteins, Bni1 and Bnr1. Bni1 is dynamically recruited from the cytoplasm to the bud tip, whereas Bnr1 is more stably anchored to the bud neck throughout the cell cycle (Buttery et al, 2007). Neither Bni1 nor Bnr1 are essential for viability, but loss of both formins is lethal, presumably because cells cannot survive in the total absence of actin cables (Evangelista et al., 2002).

The N-terminal regulatory half of most formins contains a Rho-binding domain (RBD), and the C-terminal region contains two formin homology (FH) domains. FH1 is a proline-rich domain that binds profilin, as well as SH3 and WW domains. While the FH1 domain does not directly nucleate or elongate actin filaments, it is critical for the ability of formins to use profilin-bound actin monomers to accelerate filament elongation. Each
FH1 domain has multiple binding sites for Profilin, a protein that binds G-actin, and the FH1 profilin interactions allow the recruitment of profilin-actin complexes and the delivery of actin monomers to the site of elongation (the FH2 domain) (Chesarone et al., 2010).

The FH2 domain is the most conserved domain in the formin family and is responsible for directly nucleating actin filaments. Mostly alpha-helical, it forms a donut-shaped homodimer that sits atop the barbed end of the actin filament (Figure 1). The crystal structure of the FH2 domain of Bni1 has been solved (Xu et al., 2004), as has the FH2 domain of the mammalian formin Daam1, and it is predicted that all FH2 domains have a highly similar structure. The FH2 domain has five subdomains: an N-terminal lasso, a linker, a knob, a coiled coil, and a post. The lasso of one formin molecule facilitates dimerization by wrapping around the post of a second FH2 molecule. The linker produces a defined spacing and flexibility while lashing together the two halves (hemidimers) of the FH2, yielding a tethered dimer architecture (Xu et al., 2004). The functional FH2 dimer architecture includes two elongated actin-binding regions (on the knob and coiled-coil), connected at either end by the flexible linker segments. These two F-actin binding sites, relying on highly conserved isoleucine and lysine residues, are located on the inside surface of the FH2 “donut”.

By binding two actin subunits, the dimeric FH2 domain can selectively stabilize actin filament “seeds”, promoting the nucleation of new filaments, and bind tightly to the filament end. Following nucleation, the FH2 domain moves processively with the growing barbed end of the filament, while permitting further actin subunit addition. This allows the formin to provide protection from barbed end capping proteins and to
simultaneously mediate G-actin subunit addition (Otomo et al., 2010). The elongation activity of FH2 is greatly enhanced by FH1-profilin interactions, which allow delivery of new actin monomers to the growing barbed end of the filament. In addition, in some formins, sequences C-terminal to the FH2 can interact with nucleation factors in order to positively or negatively regulate formin nucleation activity (Chesarone et al., 2010; Gould et al. 2011). Nevertheless, the FH2 remains the focal point of a formin’s actin assembly activity and this domain is subject to tight regulation for optimal control of actin polymerization in cells.

Several forms of regulation of the FH2 domain specifically have been discovered in the case of the yeast formin Bnr1. They include: a nucleation-promoting factor (Bud6), a filament elongation damper (Smy1), and a filament elongation terminator, or formin displacer (Bud14). Inhibitors of Bnr1 are probably required in order to prevent unregulated activity in the form of “runaway” actin polymerization. Various in vivo studies were able to highlight this concept by showing that expression of dominant active FH1-FH2 fragments stimulates the assembly of excessive, disorganized actin arrays (Evangelista et al., 2002 and Sagot et al., 2001).

Negative regulation of formins is required in vivo in order for cells to control the length of actin filaments. Furthermore, in vitro tests have shown that formins persist on barbed ends even in the presence of capping proteins (Kovar and Pollard, 2004). To combat this, Bud14 terminates Bnr1-mediated actin filament elongation by displacing the FH2 domain from growing barbed ends (Chesarone et al., 2009). Consistent with its role as an inhibitor of Bnr1 function, loss of BUD14 in cells results in the formation of abnormally long and buckled actin cables, which leads to defects in secretory vesicle
transport and cell morphogenesis. These hyperelongated and kinked cables seem to be comprised of abnormally long actin filaments, as evidenced by resistance to the monomer-sequestering drug latrunculin A (LatA), suggesting that \textit{bud14}\textdelta\ yeast cells have a reduced ability to regulate the filament elongation activity of Bnr1. Finally, actin cable architecture defects observed in yeast with a hyperactivated \textit{bnr1} allele (\textit{bnr1}\textdelta\textit{DAD}) were suppressed only after also deleting \textit{BUD14}, demonstrating the rebalancing of actin filament length from many short filaments to fewer, longer ones (Chesarone et al., 2009).

Smy1 is another negative regulator of Bnr1 that binds directly to the FH2 domain. Smy1 shares homology with the kinesin-1 subfamily of motor proteins, but lacks detectable microtubule motor activity in vitro (Hodges et al., 2009). Smy1 lacks the formin displacement activity of Bud14, but instead dampens the elongation rate of Bnr1-capped actin filaments (Chesarone-Cataldo et al., 2011). Deletion of \textit{SMY1} from cells results in abnormally long and wavy cables that frequently change in thickness and direction, and fail to properly support efficient secretory traffic. \textit{bud14}\textdelta\textit{smy1}\textdelta\ double knockout cells display compounded defects, suggesting that Smy1 and Bud14 make different contributions to the cellular regulation of Bnr1 activity (Chesarone-Cataldo et al., 2011). Interestingly, Smy1 is trafficked along actin cables with secretory vesicles by myosin V, and thus, may be part of a negative feedback loop that senses cable length and prevents cables from growing too long, as longer cables deliver more Smy1 (Chesarone-Cataldo et al., 2011). The complete and combined mechanism by which Bud14 and Smy1 control formin activity to govern the length and shape of actin cables remains to be fully understood.
I sought to identify the specific surfaces on Bnr1 FH2 domain that bind each regulator and permit their effects on Bnr1. To this end, I used a previously designed panel of 32 Bnr1 FH2 mutants targeting residues that differ between the Bnr1 and Bni1 FH2 domains, since Smy1 and Bud14 specifically bind Bnr1 and not Bni1. After purifying Bud14 and Smy1 proteins, I used pyrene-actin assembly assays to identify three FH2 mutants that exhibited normal actin assembly activity yet were refractive to inhibition by the negative regulators. Next, I integrated the sequence for the most refractory mutant, Bnr1-14, at the genomic BNR1 locus in order to study its effects on in vivo actin cable assembly. I observed aberrant cable architecture in strains containing the BNR1-14 mutation. In addition, this mutation in a bni1Δ background results in a greater number of cables per cell. Future studies will dissect in greater depth the precise mechanism by which Bnr1 is regulated in yeast in order to maintain normal cable length and architecture.

Methods

Protein Purification

Bud14 (179-707) was expressed as a GST-TEV fusion protein in the E. coli strain BL21 (DE3). Cell cultures were grown to log phase at 37°C, then induced for 3 hr with 0.4 mM IPTG, and pelleted. For purification, pellets were resuspended in PBS + 0.5 mM DTT + standard protease inhibitors (Quintero-Monzon et al., 2005). Cells were lysed by sonication, centrifuged for 15 min at 12,500 RPM in a SA600 rotor (DuPont Instruments-Sorvall, DuPont Co.). The supernatant was incubated for 2 hr with glutathione agarose resin (Qiagen, Valencia, CA) at 4°C, and the beads were washed twice with PBS, twice
with PBS + 0.4 M NaCl, and twice with HEKG₅ buffer (20 mM HEPES, 1 mM EDTA, 50 mM KCl, 5% glycerol). Beads were then incubated for 30 min at room temperature with 10 µg TEV protease, then another 10 µg TEV protease were added and the beads incubated for a further 2 hr. The beads were then pelleted, and the supernatant containing TEV-released Bud14 was harvested. Finally, the protein was concentrated in a Cen-30 Microcon device (Milipore, Bedford, MA), aliquoted, snap-frozen in liquid N₂, and stored at -80°C.

Recombinant Smy1 (421-577) was expressed in E.coli as above, and then purified as a 6-Histidine fusion by nickel-affinity chromatography followed by desalting on a Profinia Protein Purification system (Bio-Rad). Purified Smy1 was eluted in HEKG₅ buffer (as above) and snap-frozen for storage at -80°C.

Bnr1 FH2 proteins were purified from E.coli as a fusion to the yeast SUMO protein Smt3. The 6-Histidine-tagged fusion protein was first isolated by nickel affinity chromatography. After cleavage with the SUMO-specific protease Ulp1, the Bnr1 FH2 protein was separated from Smt3 and other contaminants by gel filtration. Bnr1 was stored in HEK350 buffer (20 mM HEPES pH 7.5, 1 mM EDTA, 350 mM KCl).

**Actin Assembly Kinetics**

For actin assembly assays, monomeric rabbit muscle actin (RMA, 2 µM, 5% pyrene-labeled) in G-buffer (10 mM Tris pH 8.0, 0.2 mM ATP, 0.2 mM CaCl₂, and 0.2 mM DTT) was converted to Mg-ATP-actin in buffer containing 10 µM MgCl₂ and 0.4 mM EGTA for 2 min immediately prior to use in reactions. A total of 45 µl Mg-ATP-actin was then mixed with 12 µl of the indicated proteins or control buffer and 3 µl of
20x initiation mix (40 mM MgCl₂, 10 mM ATP, 1 M KCl) to produce each 60 µl reaction. Pyrene fluorescence signal was monitored at 25°C in a fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ) or in an Infinite M200 plate reader (Tecan) at excitation and emission wavelengths of 365 nm and 407 nm, respectively. For experiments in the Tecan plate reader, each reaction was performed in duplicate and the data was averaged, with the exception of rare occasions where one of the replicate reactions failed.

**Integration Vector**

A bnr1-14 integration vector was generated by using pBG1026, a yeast integration vector based on pRS305 (Sikorski & Hieter 1989) containing the terminal 1800 base pairs of BNR1 (including the FH2 domain). I used site-directed mutagenesis to generate the bnr1-14 mutation in this vector, and further had to use site-directed mutagenesis to correct a point mutation that I found in the BNR1 ORF of the “wild type” pBG1026 plasmid.

**Strain Construction**

All strains were in the W303 genetic background. The bnr1-14 integration vector (above) was linearized and transformed into a bni1Δ::TRP1 strain (BRY31) to produce a bni1Δ::TRP1, bnr1-14::LEU2 double mutant strain, and I unsuccessfullly attempted to integrate the allele into a wild type strain background as well. Instead, I obtained the bnr1-14 single mutant strain by crossing bni1Δbnr1-14 to CRN1-GFP::HIS3 (BGY6),
selecting for diploid cells on triple dropout media, then sporulating the diploid cells, and dissecting tetrads to isolate the \textit{bnr1}-14 allele.

**Cell Imaging**

To visualize F-actin, cells were grown in YPD media to OD\textsubscript{600} 0.1 or 1.0 depending on the experiment, fixed with 4% formaldehyde for 45 minutes, washed 3 times with PBS, stained with Alexa-488 phalloidin (Molecular Probes, Eugene, Oregon) overnight at 4°C and washed with PBS three further times. Images were captured on a Zeiss Axioskop-2 mot plus microscope (Carl Zeiss, Thornwood, NY) using a Hamamatsu ORCA-ER digital CCD camera (Hamamatsu Photonics, Bridgewater, NJ) running OpenLab software (Improvision, Lexington, MA).

**Growth Assay**

Yeast strains were grown to saturation in YPD at 25°C, serial diluted, and transferred to YPD plates. Growth was compared after 2-3 days at 25°C, 30°C, 34°C, and 37°C.

**Results**

To identify the surfaces on Bnr1 that are critical for mediating interactions with its regulators, we designed a panel of 32 Bnr1 mutants. The residues targeted for mutation are all surface-exposed on the FH2 domain of Bnr1, since Bud14 and Smy1 bind specifically that domain. The residues were chosen using a combined structure- and sequence-based approach based on two main criteria: they are well conserved in other
formins, yet significantly dissimilar between Bnr1 and Bni1 since the latter is not regulated by Bud14 or Smy1. All the sections of the FH2 domain (Lasso, Linker, Post, Coiled Coil, and Knob) are covered in this panel, and each chosen residue has been mutated to either alanine or the corresponding Bni1 residue. So far, 16 of the mutant Bnr1 proteins (FH2 fragment only, a.a. 861-1270) have been purified from E.coli. Using these, I performed actin assembly assays to identify any Bnr1 mutants that are comparable to the wild type protein in their actin assembly activity yet refractory to inhibition by either Smy1 or Bud14.

I began by testing the Bnr1 FH2 mutants for inhibition by Bud14 (179-707) using pyrene-actin assembly assays in a fluorescence spectrophotometer (PTI). I generated actin assembly curves using 5 nM of each formin protein in the presence and absence of 230 nM Bud14 (Supplementary Figure 1), then measured the amount of time required for each curve to reach 50% actin assembly (Figure 2B). Among the 16 mutants tested, I found that 13 assembled pyrene-actin at a rate within 2-fold of the wild type protein. Interestingly, one mutant appeared to be more active than wild type (Bnr1-14), while two mutants were significantly less active than wild type Bnr1 (Bnr1-21 and Bnr1-30). I then calculated the percent inhibition for each Bnr1 protein by Bud14 (Figure 2C), and found that the mutants Bnr1-14 (V995E), Bnr1-25 (K1209A, K1211A), and Bnr1-28 (K1106A, K1110A) were all defective in their ability to be inhibited by Bud14, where Bnr1-14 was the most defective. The residues mutated in Bnr1-14 and Bnr1-25 are located in the knob region of the Bnr1 FH2 domain, while those mutated in Bnr1-28 are located in the post region (Figure 3B).
I next tested the Bnr1 FH2 mutants for inhibition by Smy1 (421-577) using pyrene-actin assembly assays on a Tecan microplate-based fluorimeter. This being a more high-throughput experimental setup, I used multiple concentrations of each Bnr1 mutant, and a single dose of Smy1, to allow for the possibility of differences in the intrinsic activities of the mutant formin proteins (Supplementary Figure 2). In every case I calculated the % inhibition by 100 nM Smy1 (Figure 4B), which inhibits 5 nM wild type Bnr1 FH2 activity by 95%. From these experiments, two alleles emerged as possibly refractory to inhibition by Smy1. Just as in the Bud14 experiments above, Bnr1-14 and Bnr1-25 appeared to be less affected by Smy1, where Bnr1-14 was the most defective. However, Bnr1-28 did not exhibit refractivity to Smy1.

Next, I repeated the pyrene-actin assembly assays using the three mutants of interest (Bnr1-14, Bnr1-25, and Bnr1-28) with a broad range of Bud14 and Smy1 concentrations to define the half-maximal inhibitory concentration (IC_{50}) for each (Figure 5 and Supplemental Figure 3). Here, I confirmed the refractivity of all three mutants to Bud14 inhibition (Figure 5A), while only Bnr1-14 was confirmed as refractive to Smy1 (Figure 5B) with an IC_{50} above the highest Smy1 concentration tested (>1.6 µM). Two separate tests of Bnr1-28 function in the presence of Smy1 indicate that the residues mutated in this allele (a.a. 1106, 1110) do not contribute to interactions with Smy1. However, both Smy1 and Bud14 similarly fail to inhibit the mutants Bnr1-14 and Bnr1-25 to their respective extents, suggesting that these multiple regulators mediate their effect on the FH2 domain through mechanisms that involve at least some of the same Bnr1 surfaces.
Since the pyrene-actin assembly assays identified Bnr1-14 as the mutant most refractory to inhibition by both Smy1 and Bud14, I next asked what effects the BNR1-14 mutation might have on formin activity in vivo. Since deletion of the genes for either BUD14 or SMY1 in cells results in overgrown cables that have irregular, but distinct architectures, we reasoned that a bnrl-14 strain might display cables similar in appearance to those of the smylΔ or bud14Δ strains. This result would be a good confirmation of our model that BUD14 and SMY1 null phenotypes are in fact caused by improperly regulated Bnr1 activity. Since this mutation is refractive to both Bnr1 regulators in vitro, it was even conceivable that the bnrl-14 strain would show compounded defects similar to smylΔbud14Δ double mutant cells. Therefore, I generated a BNR1-14 integration vector and replaced the wild type genomic locus to express the mutant protein at endogenous levels.

I then used Alexa-488 phalloidin to stain the actin structures in fixed bnrl-14 cells and imaged them by epifluorescence microscopy in order to compare cables to those in wild type, smylΔ, and bud14Δ cells (Figure 6A). As expected, cables in bnrl-14 cells appeared abnormally long, wavy, and frequently changed in direction. Therefore, the phenotypes observed upon loss of BUD14 or SMY1 are quite likely to be the result of diminished negative regulation of Bnr1. In order to better understand the effects of my mutation on Bnr1 function, I turned to a bnl1Δ strain background, where all actin cables are necessarily produced by Bnr1. I generated a bnl1Δbnrl-14 double mutant strain and performed actin cable staining comparing it to bnl1Δ, bnl1Δbud14Δ, and the wild type strain (Figure 6C). As previously observed (Chesarone 2008), the bnl1Δbud14Δ double mutant control strain displayed cables that were long and kinked compared to wild type.
As expected, $bni1\Delta bnr1-14$ cells also had cables with markedly altered architectures looking elongated and curled up at the far end of the cell (distal to the bud neck), suggesting that the bnr1-14 mutation has indeed produced formin molecules that are not subject to their normal regulatory mechanisms.

In addition, $bni1\Delta bnr1-14$ cells also appeared to have more numerous cables than any other strain in this experiment. To quantify this observation I measured the number of cables per cell in each strain (Figure 6D). I determined that the $bni1\Delta bnr1-14$ strain had significantly more cables per cell than the other strains (including $bni1\Delta bud14\Delta$). This suggests that this mutation not only allows Bnr1 to assemble cables faster, but may also result in enhanced nucleation of actin filaments, which would then be incorporated into a large number of cables. Because of the above results and the previous observation that $bni1\Delta bud14\Delta$ shows a synthetic growth defect (Chesarone 2008), I tested the $bni1\Delta bnr1-14$ strain for similar synthetic defects. I spotted a dilution series of various strains on YPD plates and incubated them at both room (25°C) and elevated (37°C) temperature (Figure 6B). $bni1\Delta bnr1-14$ was able to grow similar to the $bni1\Delta$ strain at both temperatures, while the $bni1\Delta bud14\Delta$ control displayed the most severe growth defects at the elevated temperature.

**Discussion**

I set out to gain structural insights into the mechanism of Bnr1 regulation by Smy1 and Bud14, with the goal of determining whether the two regulators interact with the formin using distinct or overlapping surfaces. Since previous work had showed that both Smy1 and Bud14 target the FH2 domain of Bnr1, I reasoned that I should be able
to identify the surfaces required by mutagenic analysis of specifically that domain. Using a panel of purified Bnr1 FH2 mutants in pyrene-actin assembly assays, I identified three alleles (Bnr1-14, Bnr1-25, and Bnr1-28) that were able to normally polymerize G-actin into filaments, yet lacked the ability to be inhibited by Smy1 and Bud14 like the wild type formin (Figure 3B). Specifically, all three mutants displayed reduced inhibition by Bud14 while only Bnr1-14 was refractive to Smy1 function. The three residues targeted in Bnr1-14 and Bnr1-25 are all quite nearby each other on the “knob” region of the FH2 domain, and therefore may be parts of a combined “Bnr1-14/25” surface used for Bnr1 regulation by Bud14.

These results suggest that Smy1 and Bud14 target partially overlapping sites on the FH2 domain of Bnr1. This is not an unexpected outcome, and suggests a model by which they may function on the formin. The shared surface (V995, Bnr1-14) may correspond to the binding site of both regulators, which is consistent with the fact that both Smy1 and Bud14 themselves share a sequence motif that is important for each of their inhibitory functions (Julian Eskin and Sal Alioto; data not shown). I now hypothesize that this shared sequence motif in the two inhibitors is used for binding the FH2 domain at or near the Bnr1-14 site (V995). To test whether this Bnr1 surface is in fact a binding site as opposed to playing some other role in the regulatory mechanism, I now plan to test binding of Bnr1 (WT and mutant) to both Smy1 and Bud14.
Interestingly, if the two regulators indeed share a binding site on Bnr1, they would be expected to compete for binding in cells. Further experiments will be necessary to determine exactly how they interact, and whether Smy1 binding can protect the formin from the displacement effect of Bud14.
If the two Bnr1 regulators share a primary binding site on the FH2 domain, the differences in their inhibitory activities may stem from interactions with other Bnr1 surfaces. Alternatively, the differences may arise due to the distinct shapes of the inhibitory molecules, which could sterically interfere with formin function in different ways even if bound at the same site. One clue towards distinguishing between these two models comes from Bnr1-25 (K1209A, K1211A) and Bnr1-28 (K1106A, K1110A), a mutant that is refractive only to inhibition by Bud14 but not Smy1. This suggests that the lysine residues targeted in Bnr1-25 and Bnr1-28, respectively located on the knob and post regions of the FH2 (Figure 3B), are involved in the interaction with Bud14 but not Smy1. It is not immediately apparent how Bud14 might simultaneously make contact with both the Bnr1-14, 25 and the 28 sites, since these are on quite distinct areas of the FH2 domain and we have no structural information about Bud14. To address this, we may attempt to visualize the complex by electron microscopy, which could show the “donut”-shaped FH2 domain with further densities corresponding to Bud14 attached. Further sites that allow us to distinguish between the target sites of the two regulators might be defined through further mutational analysis. Alternatively, we could use mass spectrometry with samples of chemically crosslinked Bnr1-regulator pairs to better define the sequences where they interact (Sinz, A; 2003).

An electrostatic potential map of the FH2 domain (Figure 7) reveals that the two lysine residues mutated in Bnr1-25 (K1209A, K1211A) appear to be part of a larger positively charged patch, all of which might be involved in binding Bud14. To test this, I may attempt to create a more refractive mutation by targeting more of the basic residues in that region. In addition, a combined Bnr1-14/25 mutant allele could be used
to more completely knock out Bnr1 regulation by Bud14. Even though the Bnr1-14 (V995E) site is in the same general (knob) area of the FH2 as Bnr1-25, the hydrophobic valine is not a part of this basic patch.

The results above allow us to update our models for how Bnr1 FH2 activities are regulated by these two regulators. In the case of Bud14, it is known that its main effect is to displace the formin from the barbed ends of growing actin filaments (Chesarone 2008). One possible mechanism for this displacement effect would be if Bud14 were to compete with the actin filament end for binding to the formin. Since the residue targeted in Bnr1-14 is located quite near the actin-binding isoleucine residue of Bnr1, this model for competition between actin and Bud14 is quite plausible. Alternatively, the dimerization of the FH2 domain is believed to be essential for the persistence of the formin on the growing end. Therefore, disruption of the FH2 dimer by Bud14 would also cause displacement of the formin from barbed ends. Since the Bnr1-28 (K1106A, K1110A) mutation is located on the post (an FH2 subdomain that assists in dimerization) of each hemidimer, it is possible that the residues involved in this mutation represent a target site on Bnr1 normally used by Bud14 to destabilize the FH2 dimer.

We can also propose a new model for how Smy1 might function to dampen the elongation rate of Bnr1-mediated actin filament assembly. The processivity of the FH2 domain on elongating filaments is contingent upon its flexible tethered dimer architecture (Xu et al, 2004), since it “stair-steps” from one actin monomer to the next as they are inserted to the filament barbed end. This mechanism requires that the relative orientation of the two Bnr1 hemidimers be quite flexible. The Bnr1-14 mutation, being located on each hemidimer, could be simultaneously bound by one Smy1 molecule
(itself probably a dimer). This might result in greater rigidity in the dimer architecture, and lead to the dampening activity of Smy1. Alternatively, while bound to the FH2 domain, Smy1 could simply provide a steric hindrance to actin monomer insertion at barbed ends.

To better understand the mechanisms of these two Bnr1 regulators, I plan to generate the Bnr1-14 mutation (or a combination allele) in the context of the larger FH1-FH2-COOH fragment of Bnr1. This could be used for pyrene-actin assembly assays in the presence of the actin monomer-binding protein profilin, to test if the mutant is still refractive to inhibition in a slightly more physiological molecular context. If the FH1-FH2-COOH mutant Bnr1-14 protein were to lose its refractivity to Smy1 or Bud14, then they may act in a mechanism that also involves the FH1 domain or C-terminal portion of Bnr1. In addition, the FH1-FH2-COOH Bnr1-14 construct could be fluorescently labeled as a SNAP fusion and visualized in TIRF microscopy experiments to better quantify its processivity and understand the mechanistic differences between it and the wild type formin.

Because Bnr1-14 was by far the most refractive mutant to inhibition by both Smy1 and Bud14, I integrated its sequence at the genomic BNR1 locus in wild type cells and in a bni1Δ background. In both the single and double mutant strains, I observed marked defects in cable architecture, suggesting that the bnr1-14 mutation disrupts the regulation of Bnr1 by Smy1 and/or Bud14 in vivo. Also, the bni1Δbnr1-14 strain had on average more cables per cell than the wild type strain. This observation supports the idea that reduced inhibition of Bnr1 leads to the production of a larger pool of actin filaments ready to be added to growing cables. The bni1Δbnr1-14 strain did not
show temperature sensitive growth defects at elevated temperature compared to \textit{bni1}\textsuperscript{Δ}, lending little support to the argument that when Bnr1 is the only formin remaining, the interaction of the residues mutated in Bnr1-14 with negative regulators is critical for formation of actin cables with normal architecture and function. Additionally, the \textit{bnr1-14} strain was not sensitive to incubation at elevated temperatures. These in vivo findings may be the result of interactions of Bud14 or Smy1 with other binding partners that could deliver each inhibitor to the FH2 domain even if the formin is mutated for binding it. In fact, such a mechanism may explain the role of the Bud14/Kel1/Kel2 complex. Alternatively, a lack of temperature-dependent growth defects in this strain may be due to differences between the \textit{in vitro} and \textit{in vivo} situations. Inhibitor concentrations may be very different in cells compared to my pyrene-actin assembly assays, leading to different regulatory outcomes on Bnr1. One way to confirm my \textit{in vitro} data and test for concentration-dependence \textit{in vivo} would be to overexpress Bud14 or Smy1 in \textit{bni1Δbnr1-14} cells. I would expect the cells to exhibit some resistance to the overexpression defects (Chesarone 2008, Chesarone 2011), since the mutant formin would not be as susceptible to the overabundant regulators.

In the future, to better define the severity of the cable defects of \textit{bnr1-14}, I may also observe the distribution and movements of GFP-Sec4, a marker of secretory vesicles that can serve as a readout of cable function rather than their form. In addition, an interesting experiment would be to determine whether crossing \textit{bnr1-14} with \textit{aip1Δ} also causes compounded defects. Loss of the filament destabilizer Aip1 results in hyperstabilized cables that are relatively thick yet cause no obvious cell morphology defects (Okada et al., 2006, Rodal et al., 1999). \textit{bud14Δaip1Δ} double mutants showed
compounded defects in cell morphology, likely because of the overgrowth of cables due to loss of Bud14 activity. I would expect a \textit{bnr1-14; aip1Δ} double mutant strain to behave similarly.

Related modes of regulation may be used to control formins in other species. For example, cytoplasmic linker protein 170 (CLIP170) and Dia-interacting protein (DIP) bind to the FH2 domains of the mouse formin mDia1 (Chesarone et al., 2010), and could have activities and mechanisms similar to yeast Bud14 and Smy1. Therefore, I will do in-depth sequence analysis of mDia1 and other formins to determine whether the residues I have targeted in Bnr1 are conserved. If they are, this could indicate that those surfaces are universal formin-regulatory target sites. Formin activities are critical for many cellular processes, and understanding how they are regulated will provide valuable insights to the coordination of cytoskeletal networks.

References


Figure 1  Cartoon of a Diaphanous-related formin such as Bnr1 and its domain structure during actin assembly. The FH2 is the contact point with the barbed end of a growing actin filament.
Figure 2  (A) Monomeric actin (2 μM, 5% pyrene labeled) was polymerized in the presence of 5 nM Bnr1 (FH2) and 230 nM Bud14 (179-707) where noted. Shown here is data for wild type Bnr1; the data for all mutant FH2 domains is in Supplemental Figure 1 (B) Curves were normalized and their slopes were determined. Zero corresponds to the rate of actin assembly in the absence of a formin, and 1 corresponds to the rate of wild type Bnr1 (FH2) in the absence of Bud14. (C) Percent inhibition of each Bnr1 mutant by 230 nM Bud14.
**Figure 3**  (A) SDS-PAGE gels of three volumes each of purified wild type Bnr1 FH2, Bnr1-14, Bnr1-25, and Bnr1-28 proteins. (B) View of the Bnr1 FH2 domain from two angles with mutagenized residues in Bnr1-14, Bnr1-25, and Bnr1-28 indicated. Crystal structure is that of Bni1, expected to be highly similar.
Figure 4  (A) Pyrene-actin assembly assays performed as above with 2.5, 5, or 10 nM Bnr1 (FH2) and 100 nM Smy1 (421-577) as noted. Shown here is data for wild type Bnr1; the data for all mutant FH2 domains is in Supplemental Figure 2 (B) Curves were normalized and their slopes were determined as with Bud14 above. From the slopes, the percent inhibition of each Bnr1 mutant by 100 nM Smy1.
Figure 5  Pyrene-actin assembly assays were performed as above, using Bnr1 FH2, Bnr1-14, Bnr1-25, and Bnr1-28 proteins and a wide concentration range of either Bud14 or Smy1. The raw curves are shown in Supplemental Figure 3. (A) Concentration-dependence of Bud14 inhibitory effects on the indicated Bnr1 (FH2) mutants, determined from the slopes of the pyrene-actin assembly curves. Hyperbolic curves were fit to the data. (B) Concentration-dependence of Smy1 inhibition, determined as above. (C) Half-maximal inhibitory concentration ($IC_{50}$) of Bud14 and Smy1 on the indicated Bnr1 proteins, calculated from curve fits in (A).
**Figure 6** (A) Cells of the indicated strains were grown to \( \text{OD}_{600} = 1.0 \), fixed and stained with Alexa-488 Phalloidin. (B) Growth assays performed on the indicated strains by serial dilution followed by plating and incubation at 25 or 37°C. (C) Cells of the indicated strains were grown to \( \text{OD}_{600} = 0.1 \), fixed and stained with Alexa-488 Phalloidin. (D) Number of cables per cell of the indicated strains. N=22 cells of each strain. Error bars represent standard error of the mean. Significance was calculated by paired T-tests (p<0.01).
Figure 7  Electrostatic potential map showing the residues mutated in Bnr1-25 (K1209A, K1211A)
Supplemental Figure 1  Raw data for reactions of 5 nM formin and 230 nM Bud14. The blue curve is actin alone, red is formin with no inhibitor, and green is formin plus Bud14. Note maximum is different for Bnr1-14.
Supplemental Figure 2  Raw data for reactions of 2.5, 5, and 10 nM formin with 100 nM Smy1. Note maximum is different for Bnr1-14.
Supplemental Figure 3  (A) Raw data for reactions using a broad range of Bud14 concentrations with wild type Bnr1, Bnr1-14, Bnr1-25, and Bnr1-28. Note different range of concentrations used on Bnr1-14. (B) Raw data for reactions using a broad range of Smy1 concentrations with wild type Bnr1, Bnr1-14, Bnr1-25, and Bnr1-28. For Bnr1-14 and Bnr1-25 there are two charts each, with a higher range of Smy1 concentration depicted in the top chart and a lower range of Smy1 in the bottom.