Supervillin Binding to Myosin II and Synergism with Anillin Are Required for Cytokinesis

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Abbreviations: AnilKD, anillin knockdown by specific dsRNA; bSV, bovine supervillin; ECT2, epithelial cell transforming sequence 2 oncogene; EPLIN/LIMA1, epithelial protein lost in neoplasm/LIM domain and actin binding 1; hSV, human supervillin; L-MLCK, long isoform of myosin light chain kinase; MHC, myosin II heavy chain; pMRLC, phosphorylated (activated) myosin regulatory light chain; PRC1, protein regulating cytokinesis 1; SVKD, supervillin knockdown by specific dsRNA.

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ABSTRACT

Cytokinesis, the process in which cytoplasm is apportioned between dividing daughter cells, requires coordination of myosin II function, membrane trafficking and central spindle organization. Most known regulators act during late cytokinesis; a few, including the myosin II-binding proteins anillin and supervillin, act earlier. Anillin’s role in scaffolding the membrane cortex with the central spindle is well established, but the mechanism of supervillin action is relatively uncharacterized. We show here that two regions within supervillin affect cell division: residues 831-1281, which bind central spindle proteins, and residues 1-170, which bind the myosin II heavy chain (MHC) and the long form of myosin light chain kinase (L-MLCK). MHC binding is required to rescue supervillin deficiency, and mutagenesis of this site creates a dominant-negative phenotype. Supervillin concentrates activated and total myosin II at the furrow, and simultaneous knockdown of supervillin and anillin additively increase cell division failure. Knockdown of either protein causes mislocalization of the other, and endogenous anillin increases upon supervillin knockdown. Proteomic identification of interaction partners recovered using a high-affinity GFP nanobody suggest that supervillin and anillin regulate the myosin II- and actin cortical cytoskeletons through separate pathways. We conclude that supervillin and anillin play complementary roles during vertebrate cytokinesis.

INTRODUCTION

Cytokinesis is a dynamic multi-step process in which the plasma membrane, the actin- and myosin II-associated membrane cortex, and components of the microtubule-rich central spindle coordinate the physical separation of a dividing cell into daughter cells [recently reviewed in (Green et al., 2012)]. As animal cells enter anaphase and the central spindle forms, it sends signals that recruit myosin II and actin to the cell equator (Werner et al., 2007; Salbreux et al., 2012). Ingression of the cleavage furrow requires continued myosin II activation at the cell equator and remodeling of the cortical actin cytoskeleton as the furrow narrows into an intracellular bridge with a midbody at its center by telophase (Wang, 2005; D'Avino, 2009). The cortex maintains a close association with the central spindle and midbody until abscission into daughter cells is complete at the end of cytokinesis (Frenette et al., 2012; Green et al., 2012). Successful cell division requires coordination of myosin activation, actin cytoskeletal proteins and central spindle assembly with membrane trafficking to and from the furrow (Poirier et al., 2012; Salbreux et al., 2012; Schiel and Prekeris, 2013).

Genetic deletion or RNAi-mediated knockdown of several membrane-associated, cytoskeletal proteins leads to division failure during early cytokinesis. These proteins include myosin II, citron kinase, ECT2, EPLIN/LIMA1, and two proteins that bind to both myosin II and F-actin: anillin and supervillin (Knecht and Loomis, 1987; Field and Alberts, 1995; Straight et al., 2005; Zhao and Fang, 2005; Chalamalasetty et al., 2006; Gruneberg et al., 2006; Piekny and Glotzer, 2008; Chircop et al., 2009; Smith et al., 2010; Watanabe et al., 2013). After RNAi-mediated depletion of either anillin or supervillin, furrows form and begin to ingress, but then the cells contract and bleb abnormally around their peripheries, with large movements of cytoplasm relative to the cleavage furrow (Straight et al., 2005; Zhao and Fang, 2005; Smith et al., 2010). Vertebrate supervillin and anillin each bind directly to the myosin II heavy chain (MHC) and can bind and bundle actin filaments (Field and Alberts, 1995; Chen et al., 2003; Straight et al., 2005). Anillin and supervillin also each interact with components of the central spindle (Piekny and Glotzer, 2008; Smith et al., 2010; Frenette et al., 2012). In addition, supervillin regulates cell proliferation through control of p53 levels (Fang and Luna, 2013) and contributes to cell motility, invasion, and rapid recycling of membrane vesicles (Crowley et al., 2009; Fang et al., 2010; Bhuwania et al., 2012). These reports suggest overlapping roles for these proteins in regulation of myosin II and actin assembly at the membrane.

Because vertebrate anillin and supervillin each contain binding sites for F-actin, myosin II, and central spindle proteins, we hypothesized that these proteins might play complementary roles in
regulating cortex organization relative to the central spindle during early cytokinesis. This hypothesis is consistent with the similarity in timing of cytokinesis failure after knockdown of either protein (Straight et al., 2005; Zhao and Fang, 2005; Smith et al., 2010). Supervillin homologues exist in worms and flies, but lack N-terminal sequences corresponding to the myosin II- and actin-binding sites in vertebrate supervillins (Pestonjamasp et al., 1997; Pope et al., 1998; Archer et al., 2005). Thus, the reports that loss of anillin from vertebrate cells results in a lower incidence of multinucleated cells than is observed after anillin depletion in C. elegans oocytes and Drosophila cells (Echard et al., 2004; Maddox et al., 2005; Straight et al., 2005; Zhao and Fang, 2005) may be due to the absence of functionalities specific to vertebrate supervillin.

We further hypothesized that, like anillin (Straight et al., 2005; Zhao and Fang, 2005; Hickson and O'Farrell, 2008; Pickny and Glotzer, 2008), supervillin might promote the localization of activated, phosphorylated myosin regulatory light chain (pMRLC) to the furrow, perhaps through its interactions with the long isoform of myosin light chain kinase (L-MLCK) (Takizawa et al., 2007; Bhuwania et al., 2012). Mice lacking MLCK survive to birth, indicating that this protein is dispensable for embryonic cell divisions (Somlyo et al., 2004), but inhibition of MLCK suggests synergy with other kinases during divisions leading to polyploid megakaryocytes (Avanzi et al., 2012). L-MLCK localizes to the cleavage furrow in vertebrates (Poperechnaya et al., 2000) and overexpression of the supervillin-binding L-MLCK N-terminus disrupts cytokinesis (Dulyaninova et al., 2004). We therefore have investigated whether supervillin and anillin cooperate during cell division and the role of supervillin and its ability to bind MHC and L-MLCK in this process. We have used HeLa cells, in which p53 levels are kept low by papilloma virus proteins in a supervillin-independent pathway (Li et al., 2004; Fang and Luna, 2013), to focus on the role of supervillin’s MHC-binding activity during early cytokinesis and how supervillin coordinates with anillin to maintain alignment of the cortical cytoskeleton with the central spindle.

RESULTS
Supervillin regulates myosin II in cell division

We showed previously that the knockdown of supervillin in HeLa cells leads to a significant increase in the number of bi- and multi-nucleated cells (Smith et al., 2010). We show here the rescue of that phenotype by expression of EGFP-tagged hSV (Figure 1A-D). This EGFP-hSV message is susceptible to a dsRNA targeting the coding region of hSV (SVKD-1) but not to a 3’-UTR-targeted dsRNA (SVKD-2). (Figure 1A, lanes 5-6). EGFP-hSV could not rescue the knockdown phenotype caused by SVKD-1, but reduced to control levels the numbers of bi/multinucleated cells generated by SVKD-2 (Figure 1B). Rescue also was observed with expression levels of EGFP-hSV only 2-3 fold higher than endogenous protein in a stable HeLa cell line (Figure 1C, D), but not by expression of EGFP-tagged bovine supervillin (EGFP-bSV, Figure 1E-F). Subsequent to this work, we became aware that the EGFP-hSV construct in the stable cell line contains a point mutation (K923E). Nevertheless, both higher levels of wild-type EGFP-hSV (Figure 1A, B) and near-endogenous levels of EGFP-hSV(K923E) (Figure 1C, D) rescued the bi/multinucleated phenotype of supervillin-knockdown cells.

Two regions of supervillin, including the myosin II regulatory sequence, are important for normal cell division, whereas actin-binding sequences target supervillin to the furrow. Although full-length EGFP-bSV did not rescue the knockdown phenotype, its overexpression was not detectably harmful (Figure 1G). We therefore used a panel of existing EGFP-bSV deletion mutants to determine whether their overexpression would result in dominant-negative increases in bi/multinucleated cells in the presence of endogenous hSV (Figure 1G; Supplemental Figures S1, S2). As compared to EGFP expression alone, cell division also was not detectably affected by the expression of bSV-1-171-EGFP, or EGFP-bSV-1010-1792. Expression of EGFP-bSV-1-830, which contains the myosin II regulatory sequence plus all three actin-binding sites, caused a small but significant decrease in the number of bi/multinucleated cells. By contrast, significant ≥ 2-fold increases in the percentages of bi/multinucleated
cells were seen upon expression of EGFP-bSV-171-1792, which lacks the myosin II/L-MLCK binding sequence, and after expression of EGFP-bSV-831-1281, which contains a coiled-coil sequence and binding sites for the central-spindle protein KIF14 and other cell cycle regulatory proteins (Wulfkuhle et al., 1999; Smith et al., 2010) (Figure 1G, red bars). Increases also were observed with other constructs but did not pass statistical testing. Consistent with dominant-negative effects on cell division, we also observed aberrant nuclear and cortical morphologies in cells expressing constructs that increased the numbers of bi/multinucleated cells (Supplemental Figure S2, panels q-t’, y-b”). All constructs that contained the three F-actin-binding sequences within bSV-171-830 (Chen et al., 2003) were concentrated at the furrow (Figure 1G; Supplemental Figure S2). These data suggest that while the myosin II/L-MLCK-interacting region within bSV-1-170 is not required to target supervillin to the furrow of dividing cells, its loss generates a dominant-negative phenotype (Figure 1G).

Supervillin is required for normal localization of total and activated myosin II at the equatorial cortex during cytokinesis (Figure 2). We previously reported that furrow ingression is disrupted after supervillin knockdown and that supervillin promotes myosin II activation at membranes in interphase cells by scaffolding MHC and L-MLCK (Takizawa et al., 2007; Bhuwania et al., 2012). We therefore explored whether supervillin knockdown would displace total or activated myosin II from the cleavage furrow during early cytokinesis (Figure 2), using antibodies against myosin IIA heavy chain (Figure 2A, MHC), the major myosin II isoform in HeLa cells (Maupin et al., 1994), and against phosphorylated Ser19 in myosin II regulatory light chain (pMRLC, Figure 2B) (Matsumura et al., 1998). MHC appearance was indistinguishable from controls through anaphase I (not shown). However, compared to controls (Figure 2Aa-d, i-l) myosin II concentrations at the polar cortices increased in supervillin-knockdown cells starting in anaphase II (Figure 2Ae-h, arrowhead) and remained mislocalized through bridge formation (Figure 2Am-p, arrowheads), resulting in significantly decreased ratios of equatorial-to-polar signals (Figure 2C, red vs. blue regions).

Interestingly, the effects of supervillin knockdown on pMRLC localization (Figure 2B, 2D) did not follow the same pattern as the effects on total myosin II. Compared to controls (Figure 2Ba-d, i-l), activated myosin II was mislocalized in supervillin-depleted cells in anaphase I (Figure 2Be-h), regained the expected localization to the invaginating furrow in anaphase II and telophase (Figure 2D), and then became mislocalized again during bridge formation (Figure 2Bm-p, 2D). These results suggest that supervillin is required to restrict both total and activated myosin II to the furrow during cell division, but it is not solely responsible for localizing activated myosin.

Identification of the separate Myosin II and L-MLCK binding sites

Separate sequences within the supervillin N-terminus interact with myosin II and the L-MLCK N-terminus (Figure 3; Supplemental Figures S3, S4, S5). Our laboratory showed previously that the N-terminal 174 amino acids of bSV (bSV-1-174) associate with myosin IIB in stress fibers in COS7 cells and cause myosin II to contract into stable punctae containing bSV-1-174-EGFP, myosin heavy chain (MHC) and L-MLCK (Takizawa et al., 2007). We used this system as a first assay for the importance of three well-conserved sequences within bSV1-174 (Figure 3A, black bars; Supplemental Figures S3, S4). As previously shown, cells expressing EGFP alone (Supplemental Figure S3, a-c) displayed normal stress fibers, whereas cells expressing bSV1-174 tagged with EGFP on either terminus contained punctae of MHC and EGFP (Supplemental Figure S3, d-i). These hypercontractile punctae were eliminated by deletion of either the N-terminal 11 residues or the C-terminal 47 amino acids of bSV-1-174 (Figure 3A; Supplemental Figure S3, j-x) and reduced in cells expressing bSV-93-174-EGFP (Supplemental Figure S3, y-a’). Point mutations made in the first two conserved regions had no detectable effect (Supplemental Figure S4, a-o), but charge-to-alanine replacements in the third conserved region (bSV-99-153) either reduced or eliminated the punctae (Supplemental Figure S4, p-x and y-a’, respectively). Taken together, these results implicate the first and third of the three highly conserved sequences within bSV1-174 in supervillin-induced myosin II hyper-contractionlity (Figure 3A).
Our second approach for identifying interaction sites within bSV-1-174 was to sediment EGFP-tagged mutated bSV-1-174 proteins with GST fusion proteins containing either the subfragment-2 domain of myosin IIA (GST-myo-S2) (Figure 3B-E) or the N-terminal 6 immunoglobulin-like domains of L-MLCK (GST-L-MLCK) (Supplemental Figure S5A-D). Our lab previously showed that GST-bSV-1-174, but not GST alone, binds directly to myosin IIA and IIB S2 domains and to the L-MLCK N-terminus (Chen et al., 2003; Takizawa et al., 2007). In this assay, EGFP-tagged bSV-1-174 bound to each GST construct, as expected (Figure 3A; Figure 3B, lane 2; Supplemental Figure S5A, lane 2). C-terminal deletion of bSV-128-174 (Figure 3A; Figure 3B, lanes 4, 5; Figure 3C, lane 3) eliminated binding to GST-myo-S2, but deletion of the first two conserved domains either maintained or enhanced binding (Figure 3A, 3B lanes 6, 7), suggesting the importance of the third conserved region (bSV99-153) for MHC binding. Point mutagenesis of R107/Y108, R112/R113, R140/K141, and K148/R149 within bSV99-153 also eliminated binding to MHC (Figure 3C lanes 5-8, 3D, lanes 6-7). Binding to GST-myo-S2 was also reduced for bSV-11-174 (Figure 3A, 3B lane 5, 3C lane 4), suggesting that the first conserved region contributes to the binding avidity. However, point mutations within the first two conserved domains did not detectably affect binding to GST-myo-S2 (Figure 3D, lanes 3-5; Figure 3E, lanes 4-5).

Results from the GST-L-MLCK pulldown assays showed that the MHC-binding sequence is not required for the interaction with L-MLCK (Figure 3A, Supplemental Figure S5). EGFP-bSV-1-101, which lacks the MHC-binding domain, interacts with GST-L-MLCK (Supplemental Figure S5A, lane 4), and mutations in SV99-153 had no effect on the GST-L-MLCK interaction (Supplemental Figure SSC, lane 5; Supplemental Figure SSD, lane 6). Interaction with GST-L-MLCK is lost by further deletion of the second domain (EGFP-bSV-1-23) (Supplemental Figure S5A, lane 3), but no mutations made in the first two conserved domains eliminated the interaction (Supplemental Figure SSB, lane 3; SSC, lanes 3-4; SSD, lane 4). We considered the possibility that myosin IIA might be acting as a bridge between GST-L-MLCK and bSV-1-174-EGFP in the pulldown assays, thereby masking the effectiveness of the point mutants. However, the GST-tagged L-MLCK bait lacks the MHC-binding site in the L-MLCK C-terminus, and endogenous MHC is absent from relevant bound fractions (Supplemental Figure S5E). These results show that our assay design precluded the recovery of significant amounts of the ternary complex of bSV-1-174 with MHC and L-MLCK. These results also show that the L-MLCK interaction site within the supervillin N-terminus is distinct from the binding site for MHC.

The sequences within supervillin investigated here are highly conserved among vertebrates (Figure 3F). The first conserved region within bSV1-174 contributes, but is not required, for binding to both MHC and L-MLCK. The second conserved region may be part of a larger site involved in L-MLCK binding. The first and third sequences are both required for supervillin-induced myosin II hypercontractility (Figure 3A, black bars; Supplemental Figure S3). The third conserved domain also is essential for binding to MHC, with numerous point mutations (Figure 3F, M) supporting the results of deletion analyses. Because the R140A,K141A mutation effectively abolished both MHC binding and the generation of hyper-contractile punctae in the COS7 experiments (Figure 3A, 3C; Supplemental Figure 4), we chose it for further experiments.

Myosin II binding is important for supervillin function during cell division
Supervillin proteins with point mutations at the MHC-binding site exhibit dominant-negative effects on cell division and are unable to rescue the supervillin-knockdown phenotype (Figure 4). To further test the requirement of the supervillin-myosin II interaction for cytokinesis, we transferred point mutations into full length EGFP-bSV and repeated the assay for dominant-negative effects on cell division in the presence of endogenous supervillin, using EGFP alone and bSV-171-1792 as controls (Figure 4A). Point mutations in the first two conserved domains did not significantly affect the percentages of bi/multinucleate cells (Figure 4A; K4A,R6A,R9A,R10A and E36A,E37A). By contrast,
the R140A,K141A mutation, which eliminated MHC binding in vitro and in vivo (Figure 3, Supplemental Figure S4), was as effective as bSV-171-1792 at inducing a dominant-negative defect in cytokinesis (Figure 4A). We then performed rescue experiments after mutating the same myosin II-affecting residues in the full-length EGFP-tagged human supervillin (Figure 4B). Wild-type supervillin (EGFP-hSV) again rescued the knockdown phenotype, but two mutants defective in binding to MHC (R140A,K141A; K148A,R149A) (Figure 3C, lanes 7, 8) failed to return the numbers of bi/multinucleate cells to control levels. While these experiments have not ruled out a role for the supervillin interaction with L-MLCK during cytokinesis, they show that the interaction with myosin II is critical for normal cell division.

Supervillin and anillin play non-redundant roles in cell division

Supervillin and anillin are both required for high fidelity production of daughter cells (Figure 5). The phenotypes observed upon supervillin knockdown are similar to that of another cortical cell division protein, anillin, which also is required for the localization of total and active myosin II at the cleavage furrow (Straight et al., 2005; Zhao and Fang, 2005). Although their amino acid sequences are very different, supervillin and anillin exhibit a similar organization of interaction domains: each binds directly to myosin II and F-actin, contains functional nuclear targeting sequences (Figure 5A, black T), and is predicted to have a central coiled-coil domain (Figure 5A, blue bars) (Pope et al., 1998; Wulfkuhle et al., 1999; Oegema et al., 2000; Chen et al., 2003; Straight et al., 2005; Piekny and Glotzer, 2008). Each also binds to proteins in the central spindle: anillin to citron kinase, RhoA, and the RhoA-GEF ECT2 (Piekny and Glotzer, 2008; Gai et al., 2011; Frenette et al., 2012); and supervillin to the kinase KIF14 and to protein regulator of cytokinesis 1 (PRC1) (Zhu et al., 2005; Carleton et al., 2006; Gruneberg et al., 2006; Smith et al., 2010; Hasegawa et al., 2013). In addition, the supervillin C-terminus binds to the furrow-regulatory protein, EPLIN (Chircop et al., 2009; Smith et al., 2010).

To determine the relationship between supervillin and anillin during cell division, we first quantified the effects on HeLa cell division after single and double knockdowns of these two proteins. The numbers of bi/multinucleated cells increased significantly (Figure 5B, white arrowheads; Figure 5C) after the knockdown of either supervillin alone (Figure 5Bb, SVKD) or anillin alone (Figure 5B, panels c, e; AnilKD-1, AnilKD-2), as compared to treatment with control dsRNA (Figure 5Ba; Figure 5C). Knockdown efficiencies (Figure 5D) and the percentage increases in the numbers of binucleated cells for the single knockdowns were similar to those reported previously (Straight et al., 2005; Zhao and Fang, 2005; Smith et al., 2010). Supervillin levels were unaffected by anillin knockdown, but surprisingly the amount of endogenous anillin increased an average of 1.9 ± 0.3 fold (both dsRNAs, n = 11; P < 0.0001, t-test) after supervillin knockdown (Figure 5D, lane 2). Simultaneous knockdown of supervillin and anillin approximately doubled the percentages of bi/multinucleated cells, as compared to either single knockdown, with up to ~80% of cells failing cytokinesis (Figure 5B, panels d, f; Figure 5C). These effects were more than additive, consistent with effects in parallel pathways.

We next asked whether overexpression of EGFP-hSV could rescue the binucleate phenotype caused by knockdown of anillin (Figure 5E, 5F). We simultaneously transfected HeLa cells with either control or anillin-specific dsRNAs and a plasmid encoding either EGFP alone or EGFP-hSV (Figure 5E, 5F); we also used the HeLa cell line that stably expresses EGFP-hSV(K923E) (not shown). In both cases, the percentages of binucleate cells upon anillin knockdown were unaffected by the overexpression of supervillin (Figure 5E). Knockdown of anillin and expression of both EGFP and EGFP-hSV were confirmed by western blot (Figure 5F). We also made several attempts at the reciprocal experiment, to rescue supervillin knockdown by overexpression of EGFP-anillin, but were unsuccessful due to an apparent loss of substrate adhesion by cells expressing EGFP-anillin for more than 24 hours. In this context, we note that the nearly 2-fold increase of endogenous anillin that occurs in supervillin-knockdown cells (Figure 5D) is insufficient to fully block the effects of supervillin knockdown (Figure 5C). However, partial compensation by increased anillin may explain why the bi/multinucleate phenotype caused by knockdown of supervillin alone is less penetrant than that seen in anillin-depleted cells (Figure 5C) and raises the possibility of intersecting pathways.
Supervillin is mislocalized in dividing cells depleted of anillin. To determine where and when supervillin and anillin pathways might cross-talk during cell division, we first used the HeLa stable cell line from Figure 1D to quantify supervillin localization in cells depleted of anillin (Figure 6). Consistent with our previous observation with bovine supervillin (Smith et al., 2010), human supervillin concentrated at the cleavage furrow in cells treated with control dsRNA (Figure 6Aa-h'). The equatorial-to-polar ratio of supervillin in these control cells increased during anaphase II and telophase, dropping somewhat at bridge formation (Figure 6B, black bars). By contrast, anillin knockdown mislocalized supervillin away from the cleavage furrow by anaphase II (Figure 6Ai-p', q-x', white arrowheads), as represented by a relatively constant equatorial-to-polar ratio of supervillin signals throughout cell division (Figure 6B gray and white bars). We note that anillin-depleted cells generally do not progress past anaphase II before furrowing fails (Straight et al., 2005; Zhao and Fang, 2005), making it impossible to determine whether supervillin can be recruited to the membrane around the midbody in late cytokinesis (Smith et al., 2010) after anillin knockdown.

Anillin localization is independent of supervillin from anaphase through telophase (not shown) but becomes mislocalized in supervillin-knockdown cells away from the midbody and into the cortices of the bridge and nascent daughter cells (Figure 7). Consistent with previous reports (Field and Alberts, 1995; Oegema et al., 2000), anillin concentrates at the center of the midbody within the cytokinetic bridge in dividing HeLa cells treated with control dsRNA (Figure 7Aa-d', white arrowheads, Figure 7B, red box). However, in supervillin-knockdown cells, anillin became redistributed away from the midbody into the cortices of the daughter cells at the proximal ends of the cytokinetic bridge (Figure 7Ae-h', 7Ai-l', white arrowheads; Figure 7B, blue region). This aberrant anillin signal is reminiscent of both the supervillin (Smith et al., 2010) and myosin II (Figure 2Ai) localizations in control cells at this stage of cell division. This localization also resembles that observed for anillin after knockdown of the central spindle component citron kinase (Gai et al., 2011). Taken together with the mislocalization of supervillin upon loss of anillin (Figure 6), these results suggest compensatory pathways that may crosstalk to regulate myosin II activity during cell division.

**Overlapping cytoskeletal interactors, but distinct signaling proteins**

Protein affinity isolations suggest overlapping cytoskeletal partners and distinct signaling pathways for supervillin and anillin (Table 1). We used a high-affinity anti-GFP nanobody on magnetic beads (Domanski et al., 2012) to collect complexes from extracts of HeLa cells that were transfected for 24 hours with either EGFP alone, EGFP-tagged hSV, or EGFP-tagged anillin. Cultures were enriched for dividing cells by treating with a Cdk2 inhibitor to synchronize them at the G2/M boundary; unsynchronized cells were used for comparison. Bead-associated proteins were eluted and identified by mass spectrometry using stringent criteria for protein assignments. The most abundant interacting proteins specific for supervillin and anillin are shown in Table 1. Nonspecific contaminants were defined as proteins that were represented by total spectral peptide counts in the EGFP-only samples that were ≥ 50% of those observed in the EGFP-supervillin or EGFP-anillin samples (Supplemental Table S2). The majority of these contaminants were skin epithelial proteins, but included abundant proteins from many intracellular compartments. All proteins specifically recovered with GFP nanobody beads from M-phase enriched and unsynchronized cells are listed in Supplemental Tables 3 and 4, respectively. Although supervillin and anillin did not appreciably co-isolate with each other, myosin II was the most abundant specific interactor for each, with both the myosin IIA heavy chain (MYH9) and light chains well represented (Table 1). Actin and actin-binding proteins, such as filamin A, also are abundant in the pulldowns for both proteins.

Additional known interactors for each protein are well represented in our affinity isolations (Table 1). These include the supervillin interactors, EPLIN, KIF14, and 14-3-3 proteins (Jin et al., 2004; Smith et al., 2010). All three of these proteins participate in signaling pathways that, like supervillin, regulate both cell motility and cytokinesis (Carleton et al., 2006; Gruneberg et al., 2006; Han et al., 2007;
Anillin-selective interacting proteins include CD2AP, SH3KBP1/CIN85/Cindr and citron Rho-interacting kinase (Table 1) (Monzo et al., 2005; Haglund et al., 2010; Gai et al., 2011). CD2AP and SH3KBP1 are SH3 domain-containing proteins that participate in membrane trafficking and actin re-modeling, as well as cytokinesis (Monzo et al., 2005; Havrylov et al., 2008; Johnson et al., 2008; Samoylenko et al., 2012), and citron kinase regulates cytokinesis through its interactions with Rho and KIF14 (Gruneberg et al., 2006; Watanabe et al., 2013). The presence of citron kinase in only the isolates from synchronized EGFP-anillin cells suggests that the enrichment for M-phase cells was effective. By contrast, supervillin-interacting proteins appear to be largely the same in interphase vs. M-phase and include many proteins (alpha-actinin, capping protein, tropomyosin, tropomodulin) associated with stress fibers and other unbranched actin filaments.

Many new candidate interactors for supervillin and anillin also were revealed (Table 1, Supplemental Tables 3 and 4). Novel direct or indirect interactors for supervillin include four membrane-associated myosins (myosins 1B, 1C, 6 and 18A), suggesting additional loci for cross-regulation of membrane dynamics and furrow structure. Supervillin family members flightless-1 and gelsolin also emerged as supervillin-selective interactors (Table 1). Flightless-1 promotes Rho-induced activation of linear actin assembly by formins (Higashi et al., 2010), and gelsolin may directly regulate myosin II activity at cell-substrate adhesions through calcium-dependent binding to MHC (Arora et al., 2013). Other supervillin-selective interactors are the signaling proteins IQGAP1, PRKDC, and MPRIP/p116Rip. IQGAP1 is a large multidomain protein that regulates numerous motile processes, including cytokinesis (Shannon, 2012). PRKDC is a large member of the phosphoinoside-3/phosphoinoside-4 kinase family that has been primarily studied for its role in DNA repair and p53-mediated apoptosis after exposure to ionizing radiation, but also has been implicated in mitotic progression (Stephan et al., 2009; Hill and Lee, 2010). Although it is not known to regulate cytokinesis, MPRIP recruits myosin phosphatase to unbranched actin filaments and thus regulates pMRLC activation (Koga and Ikebe, 2005; Surks et al., 2005). Conversely, serine/threonine protein phosphatase 2A emerged as a novel candidate interactor for anillin (Table 1); homologues of this protein are known to regulate myosin II assembly or cytokinesis in yeast and soil amoeba (Rai and Egelhoff, 2011; Goyal and Simanis, 2012). Thus, all of the newly identified abundant interactors are known or plausible participants in cytokinetic signaling pathways. The reproducible differences in interaction partners between supervillin and anillin suggest that these proteins participate in distinguishable pathways that overlap to regulate myosin II function at the furrow.

DISCUSSION

We show here that the MHC-binding site within supervillin lies within residues 99-153 and that the loss of myosin II binding leads to a dominant-negative disruption of cell division and the loss of ability to rescue supervillin depletion. Point mutagenesis suggests that at least 5 regions of local charge are involved in the supervillin-MHC interaction. Supervillin residues 93-125 were previously implicated in binding to myosin II by proteolytic mapping (Chen et al., 2003) and are unstructured although predicted to be capable of ligand-dependent folding (Meszaros et al., 2009; Fedechkin et al., 2012). Supervillin amino acids 1-10 also contribute to binding avidities for both MHC and L-MLCK although they are not sufficient for binding to either protein. These residues may be part of secondary, lower-avidity interactions for each protein, or they may be important for ligand-dependent structural rearrangements that facilitate tight binding.

We also show here that supervillin binding to myosin II is required for efficient cytokinesis; that this phenotype is similar to, but occurs somewhat later than, cell division defects caused by loss of anillin from human cells (Straight et al., 2005; Zhao and Fang, 2005); and that supervillin synergizes with anillin to regulate myosin II at the furrow. In supervillin-knockdown cells, some cortical MHC redistributes away from the furrow, beginning in late anaphase, whereas pMRLC distribution is aberrant only in early anaphase and at the bridge stage. Interestingly, Rab21-associated integrin trafficking, a process facilitated by supervillin (Fang
et al., 2010), is similarly required for both initial myosin activation signaling and membrane recruitment to the ends of the invaginating furrow (Pellinen et al., 2008). Because anillin localizes normally in supervillin-knockdown cells until bridge formation, it is apparently sufficient for myosin II activation at the furrow during late anaphase and telophase. In supervillin-knockdown cells, the overexpression and re-distribution of anillin to the bridge ends and the apical cortices of nascent daughter cells, which are characteristic localizations for supervillin (Smith et al., 2010), may allow anillin to partially compensate for supervillin depletion from cytokinetic bridges. Functional cross-talk between supervillin and anillin is further supported by the mislocalization of supervillin in anillin-knockdown cells and by the more-than-additive effects observed after double knockdowns of both proteins. Taken together, these results suggest that anillin and supervillin coordinate during the initial activation of myosin II in the anaphase cortex, that anillin plays the primary role in myosin activation during later anaphase and telophase, and that supervillin is required primarily for cortical constriction or membrane trafficking during bridge elongation or closure.

Proteomic identifications of supervillin and anillin interactors support the presence of separate functional pathways that coordinately regulate cytokinesis. Supervillin and anillin apparently do not interact directly, but each binds to both MHC and F-actin. Not all reported binding partners were identified in the affinity isolates, possibly due to insolubility of very large complexes, or the loss of proteins with lower avidities. Nonetheless, many interactors with signaling capabilities show specificity for one protein or the other. These results suggest overlap of function between supervillin and anillin at the level of myosin II recruitment and activation, with each protein playing a key role at the cortex at different stages of cell division. The greater association of actin-binding proteins with supervillin, especially the abundance of stress fiber-associated proteins, is reminiscent of observations in Drosophila cells, in which unbranched actin filaments are required for continued myosin II localization at the furrow (Dean et al., 2005). The number of supervillin-associated unconventional myosins raises questions about whether their motor or actin-binding activities contribute to membrane trafficking or cortical tension during the later stages of bridge narrowing.

The interactions of supervillin and anillin with different central spindle and signaling proteins suggest that each can promote scaffolding or signaling between the furrow cortex and the central spindle (Figure 5A). Furthermore, the second region of supervillin important for normal cell division (SV-831-1281) contains the KIF14-interaction site (Smith et al., 2010) and potentially a binding site for PRC1 (Hasegawa et al., 2013), both of which are required for central spindle assembly (Carleton et al., 2006; Neef et al., 2007; Shrestha et al., 2012). The interaction of KIF14 with citron kinase (Gruneberg et al., 2006; Watanabe et al., 2013), which co-isolates with anillin (Table 1) (Gai et al., 2011), suggests that supervillin and anillin could each link the cortex with separate-but-interacting components of the central spindle.

While this work was in revision, another report also proposed supervillin as a molecular link between myosin II activation and central spindle function (Hasegawa et al., 2013). These researchers showed that supervillin epitopes are found at the central spindle under fixation conditions that reduce the cortical signal and that supervillin residues 676-1009 bind directly to PRC1. In addition, cleavage furrows became elongated in the absence of the MHC/L-MLCK-interacting supervillin N-terminus or after mutation to alanine of Ser-238, a residue that is phosphorylated by polo-like kinase 1 (PLK1), a PRC1-binding partner and cytokinesis regulator (Hu et al., 2012). These supervillin mutations reduced pMRLC localization at the furrow during ingression (Hasegawa et al., 2013). These results are consistent with our observations that the loss of supervillin decreases the proportion of total MHC at the furrow during anaphase II and telophase and affects the proportion of furrow pMRLC during early anaphase and bridge formation. The results of Hasegawa et al. also may explain the failure of bSV, which naturally has an alanine at position 238, to substitute for human supervillin in rescue experiments and imply that not all functional regulatory sites in the supervillin N-terminus are conserved across species.

Supervillin is likely to be differentially important for cytokinesis in vertebrate cells, as opposed to other organisms. First, although a sequence 70% identical (85% similar) to the MHC-binding site in human
supervillin is found in sea urchin supervillin (XP_784024.3), no sequences similar to those implicated in L-MLCK binding (residues 1-10 and 23-101) are present. This suggests a different regulatory mechanism in sea urchin, the only organism in which MLCK is known to play a major role in activating myosin II during cell division (Lucero et al., 2006; Uehara et al., 2008). Second, the MHC- and L-MLCK-binding N-terminal sequences identified here are absent from fly and worm supervillin homologs (CG33232; viln-1/C10H11.1), potentially explaining why anillin knockdown in Drosophila Kc167 cells displays more penetrance than is observed in HeLa cells (Straight et al., 2005). Taken together, these observations suggest that supervillin may act as a genetic buffer for anillin function to ensure the fidelity of cytokinetic bridge formation and midbody formation in vertebrates.

We conclude that the myosin II-binding capability of supervillin is required for high-fidelity cell division and that supervillin helps to recruit and activate myosin II in the furrow cortex, especially during the bridge stage of cytokinesis. The identities of supervillin and anillin interactors suggest that each may scaffold the central spindle with the furrow cortex, with supervillin associating with myosin II, unbranched actin filaments, and signaling proteins, many of which play important roles in interphase processes, including cell migration and membrane trafficking. Thus, during both interphase and cytokinesis, supervillin integrates dynamic processes involving membrane signaling cascades, vesicle trafficking, microtubule-rich structures and the actin- and myosin II-associated membrane cortex.

MATERIALS AND METHODS

Cell Culture

Adherent HeLa S3 Tet-Off (Clontech, Mountain View, CA) and COS7 cells were grown as described previously (Smith et al., 2010). HeLa S3 Tet-Off cells stably expressing a GFP fusion to human supervillin, nonmuscle isoform 1 (Fang and Luna, 2013), latently discovered to bear a point mutation (K923E), were cultured in the HeLa medium. All experiments were performed in 6-well culture dishes containing 22 mm² coverslips, except for production of extracts used in GST-pulldowns and GFP nanobody affinity isolations (see below). All cultures were grown at 37°C in 5% CO₂.

Antibodies

For immunoblotting, rabbit-anti-supervillin (H340) (Nebl et al., 2002; Oh et al., 2003) was used at 1:1000. Rabbit antibody against amino acids 417-687 in human anillin was a kind gift from Dr. Michael Glotzer (Piekny and Glotzer, 2008) and used at 1:5000. Mouse monoclonal anti-actin (C4, Millipore, Temecula, CA) was used at 1:3000. Rabbit monoclonal anti-GFP (D5.1, Cell Signaling, Beverly, MA) was used at 1:1000. Rabbit polyclonal anti-myosin IIA (Covance, Princeton NJ) was used at 1:10,000. HRP-conjugated donkey anti-mouse, anti-rabbit, and anti-goat antibodies (Jackson ImmunoResearch, West Grove, PA) were used at 1:20,000.

For immunofluorescence, HeLa cells were stained with mouse monoclonal anti-pMRLC (Ser19) (1:200, Cell Signaling), rabbit anti-anillin (1:1500), or rabbit polyclonal anti-myosin IIA (1:200, Covance). COS7 cells were stained with rabbit polyclonal anti-myosin IIB (1:100; Covance). Rabbit polyclonal anti-EGFP (1:1000, Abcam, Cambridge, MA) was used to enhance the EGFP-supervillin signal in the stable HeLa cell line (Smith et al., 2010). Secondary antibodies were goat anti-rabbit or anti-mouse AlexaFluor 488 conjugates (HeLa) and goat anti-rabbit AlexaFluor 568 (COS7) (Life Technologies, Grand Island, NY). Actin and DNA were visualized using AlexaFluor 568 phalloidin (Life Technologies) and Hoechst, respectively.

RNA interference

Stealth (Life Technologies) duplex sequence for SV-6016-6040 (SVKD-2) was described previously (Smith et al., 2010); this dsRNA targets a 3'-UTR sequence absent from EGFP-supervillin. The following Stealth dsRNA sequences also were used: 5’-GACUAUGAAGGACCA CCAGAGAU (scrambled control) and 5’- GAAGAUAUCGAAGCCAGAGAU against a coding sequence present in both endogenous and
EGFP-tagged human supervillin (SVKD-1; SV-2473-2497).  5’-ACGAAAGGGUUUGUGCCAAUAUUCA (AnilKD-1; ANLN-3473-3497) and 5’ – CGUGAUAUGACUUGUACUAGGGUA (AnilKD-2; ANLN-3800-3824) were used to knock down anillin.

Plasmids

The nonmuscle myosin IIA-S2 region was sub-cloned from 6xHis-NMIIAS2 vector, a kind gift from Dr. Mitsuo Ikebe, and transferred into pGEX-6P-3 (GE Healthcare Life Sciences, Piscataway, NJ) by restriction digest using Eco RI and Xho I (New England Biolabs, Beverly, MA).  A vector encoding a GST fusion with the 6 Ig-domains of the L-MLCK N-terminus, amino acids 2 – 867 (L-MLCK-6Ig), was a kind gift from Dr. Anne Bresnick (Dulyaninova et al., 2004).  EGFP-tagged human supervillin (EGFP-hSV) was generated by PCR (Pope et al., 1998) and ligated into pEGFP-C2 between EcoRI and XbaI in two steps by Dr. Zhiyou Fang (Fang and Luna, 2013).  First, a 3’ EcoRV/XbaI fragment was ligated into pEGFP-C2 digested with Smal and XbaI.  This vector was then digested with EcoRI and ligated with an EcoRI fragment containing the hSV 5’ sequence.  EGFP (Clontech), EGFP-bSV, bSV-1-171-EGFP, EGFP-bSV-171-1792, EGFP-bSV-1-830, EGFP-bSV-831 - 1281, EGFP-bSV-1-1009, EGFP-bSV-1010-1792, EGFP-bSV-831-1792, EGFP-bSV-1-342 and bSV-1-174-EGFP were described previously (Wulfkuhle et al., 1999; Chen et al., 2003; Takizawa et al., 2007).  EGFP-bSV-1-174 was created by converting residue G175 in EGFP-bSV1-342 (Wulfkuhle et al., 1999) to a stop codon using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA).  Two C-terminal deletion constructs were made by restriction digestion of EGFP-bSV-1-342.  Digestion with BamHI I (New England Biolabs) and re-ligation resulted in EGFP-bSV-1-127; digestion with Xho I (New England Biolabs) and re-ligation produced EGFP-bSV-1-101.  EGFP-bSV-1-23 was created by converting residue L24 to a stop codon.  EGFP-bSV-11-174 and bSV-11-174-EGFP were created by PCR using EGFP-bSV-1-830 as template.  The resulting fragments were then ligated in-frame into either the pEGFP -C1 or –N3 vector (Clontech).  EGFP-bSV-72-174 and bSV-93-174-EGFP were created by converting residues R72 and S73 in bSV-1-174 to a Bgl II restriction site, followed by digestion with Bgl II (New England Biolabs) and re-ligation.  All primers for point mutagenesis and PCR are listed in Supplemental Table S1.

Targeted alanine mutations were introduced into bSV-1-174 and hSV-1-330 using the QuikChange kit as above, and primers listed in Supplemental Table S1.  To mutate a total of four residues in bSV-1-11 and bSV-33-51, a second set of primers was used to introduce additional mutations into bSV-1-174-R9A,R10A and bSV-1-174-M43A,R44A.  EGFP-bSV-1-174 mutants were restriction digested with Nhe I and Pvu I (New England Biolabs) and cloned into the corresponding restriction sites in EGFP-bSV-1-1792.  EGFP-hSV-1-330 mutants were restriction digested with Nhe I-HF and Bst EII-HF (New England Biolabs) and ligated into the corresponding sites in EGFP-hSV-1-1788.  All constructs and mutations were verified by sequencing.

Transfections and Protein Extractions

For protein knockdowns, HeLa cells were reverse-transfected with 20 nM total dsRNA (20 nM control, or 10 nM each of control and either supervillin or anillin dsRNAs) and 5 µl of Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer’s instructions.  In rescue experiments, 4 µl of Lipofectamine 2000 (Life Technologies) were used to reverse transfact dsRNAs (20 nM total concentration) and plasmid DNA (1.5 - 4.0 µg) together, and cells were grown on coverslips coated with bovine fibronectin (Sigma-Aldrich, St. Louis, MO) (Smith et al., 2010).  After transfection, cells were incubated for 48 hrs before fixation with 4% paraformaldehyde or extraction with RIPA buffer containing protease inhibitors (Smith et al., 2010).  To produce extracts for GST-pulldown experiments, HeLa cells were plated in 6-cm dishes and incubated overnight before transfection using Lipofectamine 2000 and either EGFP or EGFP-tagged BSV-1-174 deletion and mutation constructs.  After 24 hours of expression, cells were extracted with RIPA buffer lacking SDS and clarified by centrifugation at 16,000 x g for 15 min at 4°C (Smith et al., 2010).  Each 6-cm dish extract was divided for use in pulldowns with both GST-myo -S2 and GST- L-MLCK-6Ig.
For visualization of proteins during cell division, cells were synchronized at the G2/M transition by incubation for 20 h with 10 µM RO-3306 (Enzo Life Sciences, Farmingdale, NY), a reversible Cdk1 inhibitor (Vassilev, 2006). Cells were released by rinsing three times with D-PBS (Life Technologies) and then incubated at 37°C with fresh medium for 90-105 minutes before fixation in 4% paraformaldehyde and staining. To screen for dominant-negative effects of EGFP-bSV proteins on cell division, cells were reverse transfected as above, incubated overnight, synchronized with RO-3306, and then released for 4-18 hours before fixation and staining.

COS7 cells were seeded on coverslips for 24 hours before being transfected with 1-1.5 µg of EGFP-tagged constructs and 10 µl Effectene transfection reagent (Qiagen, Valencia, CA). Cells were then incubated for 24 hours before fixation with 4% paraformaldehyde and staining.

**Imaging and Equatorial:Polar Ratio Determination**

Cells were imaged using an Axioskop fluorescence microscope with Plan-NeoFluor objectives (Carl Zeiss Microscopy, LLC, Thornwood, NY), as described previously (Smith et al., 2010). Protein localizations in dividing HeLa cells and myosin II punctae in COS7 cells were imaged using a 100X (NA 1.3) oil immersion objective. Numbers of bi/multinucleated HeLa cells were assayed using a 40X (NA 0.9) objective lens, and representative overviews for Figure 5B were obtained with a 25x (NA 0.8) objective. Images were uniformly adjusted for brightness and contrast and merged images were assembled with Adobe Photoshop (San Jose, CA).

Average signal intensities at the equatorial and polar cortices were determined using a modification of the method of Robinson et al. (Robinson et al., 2002). The polygon tool in ImageJ (Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) was used to select areas with widths of ~0.4 µm that covered the cortex signal along the regions of the cell indicated in Figures 2 and 7. Mean signal intensities for these boxes were obtained by using the Measure function under the Analyze menu with area, mean signal intensity, and standard deviation selected for output. Measurements were made for each pole and each side of the furrow (Figure 2), which were then averaged using Excel (Microsoft Corporation, Redmond, WA) before calculating the ratio of Equatorial to Polar signal. For anillin localization at the midbody (Figure 7), signal intensities along the daughter cell cortices proximal to the midbody were analyzed and averaged as above, but the midbody signal itself was taken as one measurement. GraphPad InStat software version 3 (GraphPad Software, Inc., San Diego, CA) was used to calculate means, standard deviations, and to perform statistical tests.

**GST pull down and SDS-PAGE**

Fusion proteins were made using Rosetta™ DE3 pLysS competent E. coli (EMD4Biosciences, La Jolla, CA), as previously described (Swaffield and Johnston, 2001; Chen et al., 2003; Smith et al., 2010). GST pulldown assays were performed as previously described (Smith et al., 2010). HeLa cell lysates were separated on 5-15% gradient SDS-PAGE gels and transferred to Protran nitrocellulose (Whatman GmBH, Dassel, Germany) to confirm knockdown of supervillin and anillin proteins and expression of EGFP-SV. Extracts containing bSV-1-174 proteins were run on 10% SDS-PAGE gels. Blots were visualized using either Supersignal WestPico or WestFemto ECL reagents (Thermo Scientific, Rockford, IL), on a Kodak Image Station (Eastman Kodak, Rochester, NY). Protein band densities were obtained using Kodak Molecular Imaging Software version 4.0 (Eastman Kodak), and ratios calculated using Microsoft Excel.

**GFP Nanobody Affinity Isolations**

Protein affinity isolations were performed using a GFP nanobody covalently bound to magnetic beads. Isolations were performed in triplicate, with replicates carried out on separate days. HeLa cells from a sub-confluent T-75 flask were split into three 10 cm dishes and incubated overnight. Cells were then transfected using Lipofectamine 2000 and 13 µg of EGFP, EGFP-anillin, or EGFP-hSV, and incubated for 24 hours before extraction. To enrich for dividing cells, plates were incubated for 3 hours after transfection, and then treated
with 10 µM RO-3306 (Enzo Life Sciences), as above. Cells were released from synchronization for 100 minutes before extraction. Cells were extracted with 1 ml of RIPA buffer with protease inhibitors, but lacking SDS (Smith et al., 2010). Ten µl (per extract) of anti-GFP nanobody-conjugated beads (Domanski et al., 2012) were washed three times in cold RIPA buffer lacking SDS, before the addition of 900 µl of cleared lysate. Strong magnets were used at each step to separate the beads from the lysate and washed. After incubation for 1 hour at 4°C with rotation, the beads were transferred to a fresh tube and washed three times with ice-cold RIPA buffer lacking SDS. Proteins were eluted from the magnetic beads with 2% SDS and run on either 5-15% acrylamide SDS-gels (Laemmli, 1970) for silver staining or on 10% SDS-PAGE for in-gel digestion and analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS).

**In-gel Digestion**

Samples for in-gel digestion were run ~1.5 cm into the resolving area of 10% mini-gels and stained with a ProteoSilver™ Silver Stain Kit (Sigma-Aldrich). Protein-containing regions were excised, destained and cut into 1 x 1 mm pieces. Gel pieces were placed in 1.5-ml microcentrifuge tubes with 1 ml of water for 1 hr. The water was removed and 125 µl/tube 250 mM ammonium bicarbonate was added. Disulfide bonds were reduced by adding 25 µl/tube 45 mM 1,4-dithio-D-threitol (DTT) and incubating for 30 min at 50°C. Samples were cooled to room temperature and alkylated for 30 min with 25 µl/tube 100 mM iodoacetamide. Gel slices were washed twice with 1-ml aliquots of water and incubated at room temperature for 1 h with 1 ml/tube of a 50:50 mixture of 50 mM ammonium bicarbonate:acetonitrile. This solution was replaced with 200 µl/tube of acetonitrile, at which point the gel slices turned opaque white. After removal of acetonitrile, the gel slices were further dried in a Thermo Scientific Savant SpeedVac (Asheville, NC). Gel slices were rehydrated in 75 µl/tube of 2 ng/µl trypsin (Sigma-Aldrich) in 0.01% ProteaseMAX Surfactant (Promega Corporation, Madison, WI), 50 mM ammonium bicarbonate. Additional aliquots of 50 mM ammonium bicarbonate were added, as necessary, to fully submerge the gel slices. Samples were incubated for 21 h at 37°C. Supernatants were removed to separate 1.5-ml microcentrifuge tubes. Gel slices were extracted further with 100 µl/tube of 80:20 acetonitrile:1% formic acid. Extracts for each sample were combined, and the tryptic peptides were dried in a SpeedVac.

**LC-MS/MS**

Dried tryptic peptides were dissolved in 25 µl 0.1% trifluoroacetic acid, 5% acetonitrile, and a 3-µl aliquot was directly injected onto a custom packed trap column (2 cm x 100 µm C18). Peptides were eluted and sprayed from a custom-packed emitter (25 cm x 75 µm C18) with a linear gradient from 100% solvent A (0.1% formic acid, 5% acetonitrile) to 35% solvent B (0.1% formic acid, acetonitrile) in 90 minutes at a flow rate of 300 nL/min on a Proxeon Easy nanoLC system directly coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Data-dependent scans were acquired according to a scheme, in which full MS scans from 350 -2000 m/z were acquired in the Orbitrap Velos at a resolution of 60000 followed by 10 MS/MS scans acquired in the LTQ ion trap instrument. The raw data files were processed with Extract_MSN (Thermo Scientific) into peak lists and then searched against the Human index of the SwissProt database using the Mascot Search engine v. 2.4 (Matrixsciences, Ltd., London, United Kingdom). Parent mass tolerances were set to 10 ppm and fragment mass tolerances were set to 0.5 Da. The variable modifications of acetyl (protein N-terminus), pyro glutamic for N-terminal glutamine, carbamidomethylation of cysteine, and oxidation of methionine were used. Mascot search results were loaded into Scaffold4 software (Proteome Software, Inc., Portland, OR) for comparisons of sample results. Co-sedimenting proteins were screened using conservative parameters, with a positive identification requiring a protein threshold of 99% and a minimum of 2 peptides per protein with a peptide threshold of 95%. The estimated Prophet False Discovery Rate at these settings is < 0.4%.

Proteins that were present in EGFP-only affinity isolates at spectral count levels ≥ 50% of the number of peptides identified in EGFP-hHSV or EGFP-anillin fractions were defined as contaminants and eliminated; these proteins are listed in Supplemental Table S2. The most abundant interactors are presented.
in Table 1. All identified proteins meeting the cut-off parameters and the spectral counts from each experiment, including background counts, are reported in Supplemental Tables S3 and S4.

ACKNOWLEDGMENTS

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REFERENCES


### TABLE LEGEND

**Table 1.** Most abundant specific interactors with GFP-tagged supervillin and anillin.

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Total specific spectral counts, minus any background counts observed in EGFP-only samples, for the 43 most abundant (out of 265 total) proteins that co-isolated in triplicate analyses with EGFP-tagged human supervillin (SVIL) or anillin (ANLN) and anti-GFP nanobody affinity beads from M-phase enriched (M phase) and unsynchronized HeLa cells (Unsynchronized). Only proteins represented by >20 specific peptides, e.g. an average of ~7 peptides/experiment, are shown. Citron Rho-interacting kinase, in italics, is included because of its documented interaction with anillin. References cite previously documented interactions.
FIGURE 1. Overexpressed EGFP-hSV, but not EGFP-bSV, rescues the increase in bi/multinucleated cells caused by knockdown of supervillin; key roles for residues 1-171 and 831 - 1281 are suggested by dominant-negative effects of EGFP-bSV deletion proteins. (A) Immunoblots showing knockdown of endogenous supervillin and over-expression of EGFP-hSV; actin used as loading control. Lanes 1-3: EGFP with control (1), SVKD-1 (2), or SVKD-2 (3) dsRNAs; lanes 4-6: EGFP-hSV with control (4), SVKD-1 (5), or SVKD-2 (6) dsRNAs. (B) Quantification of bi/multinucleated HeLa cells simultaneously
transfected with EGFP (black bars) or EGFP-hSV (gray bars) and dsRNAs, as indicated. EGFP-hSV is immune to the SVKD-2 dsRNA. Means ± s.d.; N = 3; P (paired two-tailed t-test) * = 0.0424. (C) Immunoblots and (D) quantification of bi/multinucleated HeLa cells stably expressing EGFP-hSV(K923E) after transfection with 20 nM dsRNAs, as indicated. Means ± s.d.; N = 4; P (two-tailed paired t-test) * = 0.0103, ** = 0.0025. (E) Immunoblots and (F) quantification of bi/multinucleated HeLa cells co-transfected with EGFP constructs and either control or SVKD-2 dsRNA, as indicated in panel E. Means ± s.d.; N = 4, > 50 cells counted per condition per experiment; P (ANOVA) * < 0.05, **, < 0.01; ***, < 0.001. (G) Fold increase (dominant-negative effect) of bi/multinucleated HeLa cells after expression of the indicated EGFP-tagged bSV deletion mutants for 48 h (representative images in Supplemental Figure S1) and the localizations of these proteins during cell division (Supplemental Figure S2). Columns show means ± s.d., relative to EGFP-transfected cells; total numbers of cells and experiments; and localizations of deletion proteins in dividing cells. Red boxes denote statistically significant effects on cell division (P ≤ 0.05, two-tailed t-test). Red brackets, regions implicated as important for cytokinesis, either when absent (bSV1-171) or overexpressed (bSV831-1281). Supervillin domains are shown as (white) the intrinsically disordered N-terminus (Fedechkin et al., 2012), (purple) the central region with a predicted coiled-coil domain (Wulfkuhle et al., 1999), (blue) 5 sequences with homology to gelsolin repeats (Pestonjamasp et al., 1997), and (gray) the C-terminus with structural similarity to the villin headpiece (Vardar et al., 2002).
FIGURE 2. Supervillin is required for localization of myosin II at the cytokinetic furrow. (A, B) Immunofluorescence localizations of (A) total myosin IIA heavy chain (MHC) and (B) regulatory myosin II light chain phosphorylated at Ser-19 (pMRLC, active myosin II) in synchronized HeLa cells transfected with control (a-d, i-l) or supervillin-specific (SVKD-2; e-h, m-p) dsRNA. MHC or pMRLC signal (a, e, i, m, green in merges); F-actin (b, f, j, n, red in merges), DNA (c, g, k, o, blue in merges) in dividing cells. White arrowheads, MHC and pMRLC localizations. Bars, 10 µm. (C, D) Mean ratios of cortex-specific equatorial (red) to polar (blue) (C) total and (D) activated myosin II signals in cells at different stages; P (two-tailed t-test) * = 0.0483; ** = 0.0058; *** < 0.0007. Control cells (black bars); SVKD-2 cells (white bars). Numbers of cells analyzed were (C) anaphase I (N = 8 control, 13 SVKD), anaphase II (N = 12 control, 11 SVKD), telophase (N = 13 control, 23 SVKD), and cytokinesis (N = 29 control, 30 SVKD), N = 2 experiments; and (D) anaphase I (N = 15 control, 13 SVKD), anaphase II (N = 19 control, 24 SVKD), telophase (N = 13 control, 30 SVKD), and cytokinesis (N = 38 control, 44 SVKD), N = 5 experiments.
FIGURE 3. Deletion and point mutagenesis of the myosin II-binding site. (A) Summation of results with EGFP-tagged deletion and point mutants of bovine SV1-174 obtained from in vivo overexpression experiments in COS7 cells (hyper-contractile myosin punctae; Supplemental Figures S3, S4) and from pulldown experiments with GST-tagged myosin II S2 (GST-myo-S2) or GST-tagged L-MLCK N-terminus (GST-L-MLCK; Supplemental Figure S5). Highly conserved regions in SV1-174 are indicated in black.
White asterisks show approximate locations of point mutations. (B, C, D, E) Representative anti-GFP immunoblots of bound and unbound GFP-tagged SV1-174 mutant proteins, as indicated, after pulldown with GST-myo-S2. Ratios show the bound-to-unbound GFP signals as a fraction of the SV1-174-EGFP signal (positive control). N ≥ 2. Ratios ≤ 0.5 were considered binding reductions (‘reduced’ in panel A) and ratios ≤ 0.1 as no binding to myosin II (‘no’ in panel A). Ponceau-stained blots of bound proteins show levels of GST-myo-S2. (F) Sequences of highly conserved regions within supervillin amino acids 1 – 174. Amino acid sequences from human (Homo sapiens, NP_003165), cow (Bos taurus, NP_776615), mouse (Mus musculus, ADP02396.1), chicken (Gallus gallus, XP_418577.3), and frog [Xenopus (Silurana) tropicalis, NP_001090765.1] supervillins were aligned using CLUSTALW 2.1 (www.genome.jp/tools-bin/clustalw). Brackets, residues targeted for alanine or phenylalanine replacement, as described in Materials and Methods. The 4-residue mutation that reduced the L-MLCK interaction is indicated by “L”; mutations that reduced or eliminated myosin II interaction are shown by “M”. Point mutation (***) used in subsequent assays.
FIGURE 4. Point mutations that disrupt binding to myosin II mimic the dominant-negative phenotype of bSV171-1792 and eliminate rescue of the bi/multinucleate phenotype by full-length human EGFP-SV. (A) Dominant-negative overexpression of EGFP-bSV constructs. Cells were assayed as in Figure 1G. Means ± s.d.; N ≥ 3 experiments; > 30 - 151 cells per condition per experiment. P (paired two-tailed t-test) * = 0.0228, ** = 0.0055. (B) Lack of rescue of the bi/multinucleate phenotype in supervillin-knockdown (SVKD) cells by EGFP-hSV proteins containing point mutations that eliminate myosin II binding (hSV-R140A,K141A; hSV-K148A,R149A). Mean percentages of bi/multinucleate cells ± s.d.; N ≥ 3 experiments; ≥ 60 cells counted per condition per experiment. P (ANOVA) ** < 0.01, *** < 0.001.
FIGURE 5. Supervillin (SV) and anillin synergistically regulate cell division, but supervillin overexpression does not rescue anillin knockdown. (A) Similar organization of binding sites in supervillin and anillin. Both proteins bind directly to myosin II (pink), bind and bundle F-actin (orange), and contain nuclear localization signals (black Ts) and coiled-coil domains (dark blue). Both bind central spindle proteins: supervillin to PRC1 and KIF14, which also binds citron kinase; and anillin to citron kinase, RhoA and the Rho GEF ECT2. The supervillin N-terminus also binds to L-MLCK and its C-terminus interacts with EPLIN/LIMA1, another cytokinetic regulator. Gelsolin homology repeats (yellow); villin-like headpiece (black bar); anillin homology region (AH, green); PH domain (cyan). (B) Representative micrographs of each knockdown condition showing bi/multinucleated HeLa cells (white arrowheads). (a) Control, (b) control + SV knockdown (SVKD-2), (c) control + anillin knockdown 1 (AnilKD-1), (d) SVKD-2 + AnilKD-1, (e) control + anillin knockdown 2.
(AnilKD-2), (f) SVKD-2 + AnilKD-2. Actin shown in red, DNA in blue. Bar, 20 µm. (C) Percentages of cells with 2 or more nuclei 48 h after transfection with control, supervillin-specific, or anillin-specific dsRNAs. Means ± s.d., n > 250 cells counted per condition per experiment; N = 3 experiments; P (paired two-tailed t-test) * < 0.05. (D) Immunoblots of HeLa lysates showing supervillin and anillin knockdowns (20 nM total dsRNA), with actin as loading control. Numbers indicate the mean residual percentages of supervillin and anillin after 48 h of knockdown, N = 4. Endogenous anillin levels consistently increased at 48 hr in supervillin-knockdown cells. Lane 1: control dsRNA; lane 2: control + SVKD-2; lane 3: control + AnilKD-1; lane 4: control + AnilKD-2; lane 5: SVKD-2 + AnilKD-1; lane 6: SVKD-2 + AnilKD-2. (E) Quantification of bi/multinucleated HeLa cells simultaneously transfected with EGFP (black bars) or EGFP-hSV (gray bars) and anillin dsRNAs, as indicated. Means ± s.d.; N = 3 experiments, > 46 - 143 cells counted per condition per experiment. (F) Immunoblots showing overexpression of EGFP-hSV and knockdown of anillin; actin used as loading control. Anti-GFP confirms expression of EGFP and EGFP-hSV. Lanes 1-3: EGFP with control (1), Anil KD-1 (2), or Anil KD-2 (3) dsRNAs; lanes 4-6: EGFP-hSV with control (4), Anil KD-1 (5), or Anil KD-2 (6) dsRNAs.
FIGURE 6. Supervillin remains at the furrow during initial stages of division in anillin-knockdown cells, but becomes mislocalized in late anaphase. (A) HeLa cells stably expressing EGFP-hSV(K923E) were transfected
with either control (a-h’) or one of two anillin-specific dsRNAs (i-x’), and stained with anti-EGFP (a, c, i, m, q, u; green in merge), actin (b, f, j, n, r, v; red in merge), and DNA (c, g, k, o, s, w; blue in merge). Phase images shown in d’, h’, l’, p’, t’, x’. Bar, 10 µm. Supervillin is initially present at the invaginating furrow in dividing cells, but becomes mislocalized around the cortex beginning in anaphase II in anillin-knockdown cells (white arrowheads in e, i, m, q, u). (B) Ratio of cortex-specific equatorial-to-polar supervillin (SV) signal in cells at indicated stages of cell division, calculated as in figure 2; P (ANOVA) ** < 0.01, *** < 0.001. Control cells (black bars); AnilKD-1 cells (gray bars), AnilKD-2 cells (white bars). Numbers of cells analyzed at each stage, combined from 2 different experiments: anaphase I (N = 7 control, 13 Anil1, 6 Anil2), anaphase II (N = 8 control, 16 Anil1, 15 Anil2), telophase (N = 5 control, 12 Anil1, 15 Anil2), and cytokinesis (N = 23 control, 22 Anil1, 11 Anil2).
FIGURE 7. Anillin is mislocalized away from the midbody into the cortex during cytokinesis in supervillin-knockdown cells. (A) HeLa cells transfected with control (a-d') or supervillin-specific (SVKD-1, e-h'; SVKD-2, i-l') dsRNAs and stained for endogenous anillin (a, e, i; green in merge), actin (b, f, j; red in merge), and DNA (c, g, k; blue in merge). Phase images shown in d', h', l'. Bar, 10 µm. Anillin localization at the midbody and cytokinetic furrow shown by white arrowheads (a, e, i). (B) Ratio of anillin signal in the midbody (red box) vs. the cortices of the daughter cells proximal to the furrow (blue); Mean ratios ± s.d.; N = 3 experiments; P (two-tailed t-test) ** = 0.0019. Total number of cells assayed: 34 control (black bar) and 77 SVKD (white bar).