RhoGTPases and Their Regulators

An analysis of how GEFs and GAPs interact with and regulate Rho GTPases

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Table of Contents

Abstract

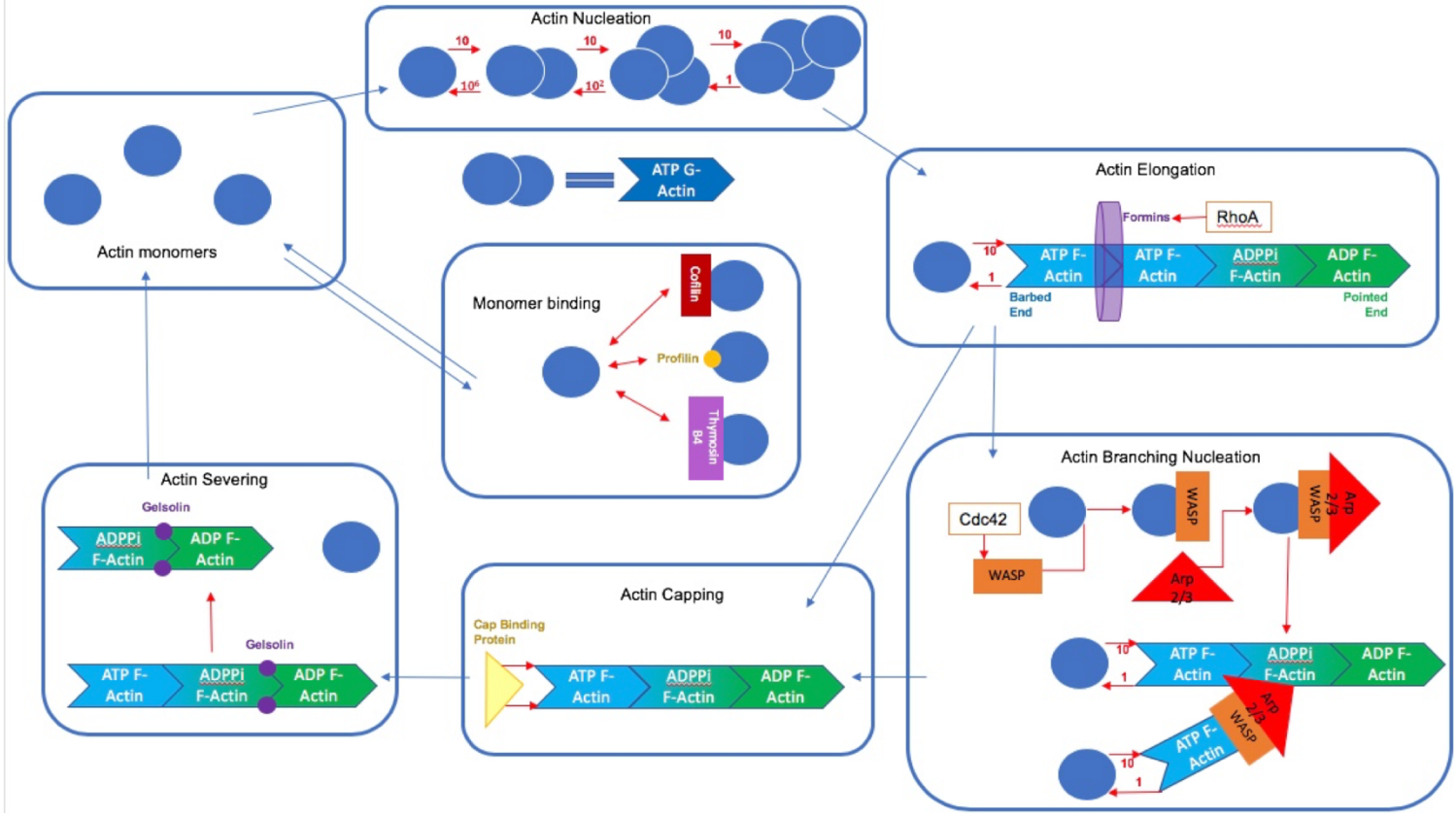
Regulation of actin assembly and disassembly are important for cellular functions such as cell division, cell locomotion/motility, and wound healing. Actin networks are regulated by Rho GTPases, of which the three best characterized are Rho, Cdc42, and Rac. Rho GTPases, in turn, are regulated by Guanine Nucleotide Exchange Factors (GEFs), which activate GTPases, and GTPase activating proteins (GAPs), that inactivate GTPases. GEFs and GAPs are regulated by numerous effector proteins that are currently being studied in great detail. This paper addresses the functions of selected GEFs and GAPs and how they are regulated by specific effector proteins. The proteins that will be described in detail are anillin, cortactin, septins, par6, and moesin. These proteins regulate and influence the localization of a wide variety of GEFs and GAPs. The goal of this review is to better understand the function of GEF and GAP effector proteins and their interactions with GEFs, GAPs, GTPases, and other proteins.

Overview of the Regulation of the Actin Network

Actin is a 42kDa protein that serves to provide cells with internal mechanical support, tracks for transport of intracellular materials, and forces to drive cell movement (Ladt et al., 2016; Pollard and Cooper, 2009). Actin is the most abundant protein in eukaryotic cells with a concentration from 20uM to 500uM (Pollard and Borisy, 2003). When polymerized, filamentous actin (F-actin) is an integral part of the cellular cytoskeleton involved in cell locomotion, wound healing, and cytokinesis (Pollard and Cooper, 2009). Among the regulators of actin dynamics, there are the Rho GTPases Rho, Cdc42, and Rac (Maeda et al., 2011), nucleators of actin formation such as Arp2/3 and Formins (Jaffe and Hall, 2005), capping proteins and sequestering proteins inhibiting actin polymerization (Symons, 1996), and a variety of proteins involved in keeping actin monomeric and creating new places for actin polymerization to form (Bamburg and Bernstein, 2010; Sanders et al., 1992). In this section, I will describe how actin filaments are formed and regulated through a host of protein interactions.

Actin is present in cells in two forms: globular actin (G-actin) which are present as monomers, and filamentous actin (F-actin) which are made of polymerized actin monomers in the cell (Welch and Mullins, 2002). Actin filaments are polar, with a fast-growing barbed end and slower growing pointed end (Welch and Mullins, 2002). Barbed ends undergo rapid actin polymerization, due in large to the protein profilin which binds G-actin and prevents it associating with pointed ends (Pollard et al., 2000). Barbed ends are usually capped in cells to prevent continual actin polymerization (Schafer, 1996). Free barbed ends only last ~0.2 seconds in a cell before something is added to continue polymerization or initiate depolymerization, compared to capped barbed ends which last ~28 minutes (Schafer, 1996). Actin cycles between monomeric and polymerized states (Pollard et al., 2000) Actin assembly factors can stimulate actin filament polymerization and help elongate actin polymers, while actin severing and capping proteins break down actin filaments either into smaller polymers or back into their monomer state (Pollard et al., 2000). This cycle is needed for two reasons: 1) to provide a pool of actin monomers so that actin filaments are always able to grow, and 2) to ensure there is an abundance of actin filaments to ensure cellular integrity. ATP G-actin is brought to the barbed ends of the filaments and is protected by cortactin which has the highest affinity to ATP-actin (Bamburg and Bernstein, 2010). ATP-actin transitions to ADP-actin once attached to the actin polymer and ADP F-actin preferentially wants to dissociate from the filament as more monomers are added. After the filament is depolymerized, the ADP actin turns into ATP G-actin, ready to be added to a new actin filament (Bamburg and Bernstein, 2010).

Figure 1: Cycle of Actin Polymerization and Depolymerization



Actin is in a constant cycle of monomers and polymers. This diagram shows the processes of actin polymerization and depolymerization through nucleation, elongation, branching, capping, severing, and monomer binding. Adapted from Pollard and Cooper, 2000.

Actin nucleation, which is the initial step for polymerization of actin filaments, can occur by three different processes: de novo filament nucleation, uncapping pre-existing filaments, or severing filaments (Condeelis, 2001; Welch and Mullins, 2002). De novo filament nucleation is the most unfavorable reaction to occur of the three mechanisms due to cofilin, profilin and thymosin-B4 which bind ATP actin monomers preventing polymerization (Pollard et al., 2000; Welch and Mullins, 2002). Arp2/3 is the main regulator of de novo nucleation, is activated by the GTPase Cdc42 via WASP, and is made up of seven polypeptides. Two of the seven Arp2/3 polypeptides, actin-related proteins Arp2 and Arp3, create a mock barbed end for polymerization, joining ATP actin monomers. G-actin is free to join this mock barbed end (Jaffe and Hall, 2005; Pollard et al., 2000). In order for de novo filament nucleation to occur, there needs to be a concentration of ~10 mM of actin monomers to drive successful dimer and trimer assembly (Pollard et al., 2000).Arp2/3 preferentially generates a branched network of actin from preformed actin filaments by binding to the side of an F-actin polymer (Jaffe and Hall, 2005; Pollard et al., 2000). As long as there is a concentration of at least ~1uM of G-actin in cells, polymerization on a pre-existing actin filament can occur (Condeelis, 2001; Welch and Mullins, 2002). The second way to nucleate actin is through uncapping actin filaments, largely done through phosphatidylinositol 4,5- bisphosphate (PIP2) and phosphatidylinositol 4-monophosphate (PIP) but can also happen spontaneously (Condeelis, 2001; Schafer, 1996). Actin filaments are capped by capping proteins (CP) to prevent further actin polymerization (Schafer, 1996). Spontaneous uncapping does occur on actin filaments, but proceeds slowly because the half-life on CP bound to barbed ends is ~28 minutes which means cells need a faster mechanism of uncapping to ensure further F-actin polymerization (Schafer, 1996). PIP2, and to a lesser extent PIP, function to fill this need by removing the capping protein from the barbed ends of actin

filaments (Schafer, 1996; Symons, 1996). PIP2's mechanism for uncapping seems to be by binding directly to CP and increase its rate constant for dissociation from F-actin (Schafer, 1996). This is seen in experiments maximizing PIP2 functionality in vitro where the half-life for a barbed end dropped from ~28 minutes to ~46 seconds with a calculated 500 uncapped barbed ends being produced every 19 seconds (Schafer, 1996). This implicates PIP2 and PIP as major contributors to uncapping the capping protein. The third mechanism to nucleate actin is severing actin filaments. The most well studied severing protein is ADF/Cofilin which, upon severing, generates a free barbed end allowing for actin polymerization (Condeelis, 2001).

After actin is nucleated, elongation of the filament occurs primarily via formins (Jaffe and Hall, 2005). mDia is part of the formin family and functions to add monomers to a growing actin filament and, once attached to the filament, it remains bound to the barbed end ready to help add the next monomer (Jaffe and Hall, 2005). Actin monomers are often bound to profilin and the FH1 domains of formins bind to profilin, creating a system of formins recruiting actin monomers to the barbed end (Vidali et al., 2009). To prevent excessive actin polymerization and ensure that there is always a pool of actin monomers to make actin for new processes, actin capping is essential (Condeelis, 2001). The most important capping proteins include CapZ and Gelsolin-related proteins (Schafer, 1996; Weeds and Maciver, 1993). Capping proteins bind the barbed ends of actin at a rate of $3uMs^{-1}$ and prevent dissociation or addition of actin monomers (Pollard et al., 2000). Caps are usually kept on the barbed ends until ADF/cofilin comes to sever the actin filament or PIP and PIP2 remove the cap for further actin polymerization (Pollard et al., 2000). The final step in the cycle is actin depolymerization, which is performed by cofilin's severing mechanism (Pollard et al., 2000). When there is less than ~1uM G-actin in cells, the freeing of a barbed end by cofilin is unable to polymerize new actin monomers to it. In these cases, cofilin promotes the dissociation of actin subunits from the ends of the filaments, which is enhanced by actin-interacting protein 1 (Aip1) (Condeelis, 2001; Pollard et al., 2000). Thymosin-B4 sequesters G-actin, aiding in the depolymerization of actin filaments by lowering the concentration of available G-actin (Sanders et al., 1992).

Functions of Rho GTPases

F-actin and its effector proteins are regulated by Rho GTPases (Maeda et al., 2011). Rho GTPases are molecular switches that regulate a variety of functions in with GTP activating GTPases and GDP inactivating them (Jaffe and Hall, 2005). Rho GTPases are a family of GTPases with more than 20 members identified in mammalian cells and constitute a branch of the Ras superfamily of small GTPases (Maeda et al., 2011; Miyamoto and Yamauchi, 2010).The Rho GTPase family works in cascades affecting each other, and the three most common are Rho, Cdc42, and Rac (Symons, 1996). Rho is shown to direct actomyosin assembly at the cell equator and precedes F-actin polymerization after anaphase (Bement et al., 2015). Rho proteins have lipid binding activity and help localize Guanine Nucleotide Exchange Factors (GEFs) and GTPase activating proteins (GAPs) (Sahai and Marshall, 2002). When Rac is activated it leads to the assembly of actin filaments at the cell periphery including lamellipodia and membrane ruffling (Hall, 1998; Symons, 1996). The most recently discovered of the three major GTPases is Cdc42 which controls polarized cell growth and induces actin rich surface protrusions called filopodia (Hall, 1998; Symons, 1996). Cdc42 proteins display ~40% similarity to other ras-like GTPases and contain the same C-terminal sequence Cys-Xaa-Xaa-Leu which is used for proper membrane anchorage (Johnson, 1999).

GEFs, GAPs, and GDIs

Guanine Nucleotide Exchange Factors (GEFs) and GTPase activating proteins (GAPs) bind directly to and regulate Rho GTPases (Kaibuchi et al., 1999). GEFs catalyze the exchange of GDP for GTP bound by the

GTPase, thereby activating them (Jaffe and Hall, 2005). GEFs stabilize the nucleotide free G protein and nucleotide binding sites which causes GDP dissociation (Johnson, 1999). GAPs stimulate intrinsic GTPase activity to inactivate the Rho GTPases (Jaffe and Hall, 2005). Guanine Nucleotide Dissociation Inhibitors (GDIs) are the least studied of the three Rho GTPase regulators, thus will not be discussed in depth here, however there is consensus on GDIs blocking spontaneous activation of Rho GTPases by sequestering the GTPases in the cytoplasm (Jaffe and Hall, 2005). GDIs are able to extract GTPases from the membrane and inhibit guanine nucleotide exchange, likely using the mechanism of sterically locking the GTPase in either a GDP or GTP bound state (Johnson, 1999). It is in the GTP bound state that GTPases interact with effector proteins like serine/threonine kinases and scaffolding proteins (Jaffe and Hall, 2005).

GEFs and GAPs share similar domain homologies which allows them to interact with GTPases and other proteins that regulate the GEFs and GAPs (Rossman et al., 2005). The biggest domain homology groups of GEFs are Dbl homology (DH), Lsc homology (LH), regulator of G protein signaling homology (RH), pleckstrin homology (PH), and DOCK homology (Crompton et al., 2000; Fukuhara et al., 1999; Goicoechea et al., 2014; Rossman et al., 2005; Schmidt, 2002; Sterne-Marr et al., 2004). Dbl homology (DH or seen elsewhere as dbl) mediates binding to inactive GTPases and catalyzes the exchange of GDP for GTP, thus making it necessary in all GEFs (Rossman et al., 2005; Schmidt, 2002). DH is a highly alpha-helical catalytically active domain but shares little conserved homology among DH domains with less than 20% sequence identity (Johnson, 1999; Schmidt, 2002). The DH domain needs a pleckstrin homology (PH) domain to target the DH to its proper subcellular location (Johnson, 1999). The interaction between DH and PH domains is termed the DH-PH module and is a signature of all DH homology GEFs (Hoffman and Cerione, 2002). The DH-PH module has been shown to directly interact with and activate Rho GTPases in *C. elegans* and can promote nucleotide exchange in vivo, while in vitro only the DH part of the module is needed (Johnson, 1999; Lenoir et al., 2015; Qadota et al., 2008). PH domains reside on the N-terminus of GEFS and are regulated by phosphoinositides, which are involved in membrane localization and regulate activity of an associated catalytic domain (Crompton et al., 2000; Lenoir et al., 2015; Viaud et al., 2012). Deletion of the PH domain has been shown to result in loss of activity of numerous GEFs including Dbs, Dbl, Lsc, Lfc, and Lbc (Coleman and Olson, 2002; Whitehead et al., 1995; Zheng et al., 1996). It has been suggested the reason there is loss of activity in these GEFs when the PH domain is removed is that the PH domain mediates the translocation of GEFs to membrane and cytoskeletal structures (Johnson, 1999). Lsc homology domain (LH) has been suggested to have a negative regulatory role as mutants without LH were more active (Fukuhara et al., 1999). The regulator of G-protein signaling homology (RH) domain is primarily located in the N-terminus and works with G alpha cell surface receptors that transmit signals through heterotrimeric G proteins which, in turn, activate Rho-dependent pathways (Sterne-Marr et al., 2004). Separate from the previous domains mentioned are DOCK homology domains which act only on Rac and Cdc42 GTPases (Goicoechea et al., 2014). DOCK domains are characterized by DOCK homology region 2 (DHR2) and a phospholipid binding domain (DHR1) that can target GEFs to the membrane using a novel mechanism than DH homology (Goicoechea et al., 2014; Rossman et al., 2005). RhoGTPase GAPs historically have been less studied than GEFs but it is known that there is a common RhoGAP domain consisting of 140 amino acids and has only 20-40% similarity between the GAPs (Lamarche and Hall, 1994).

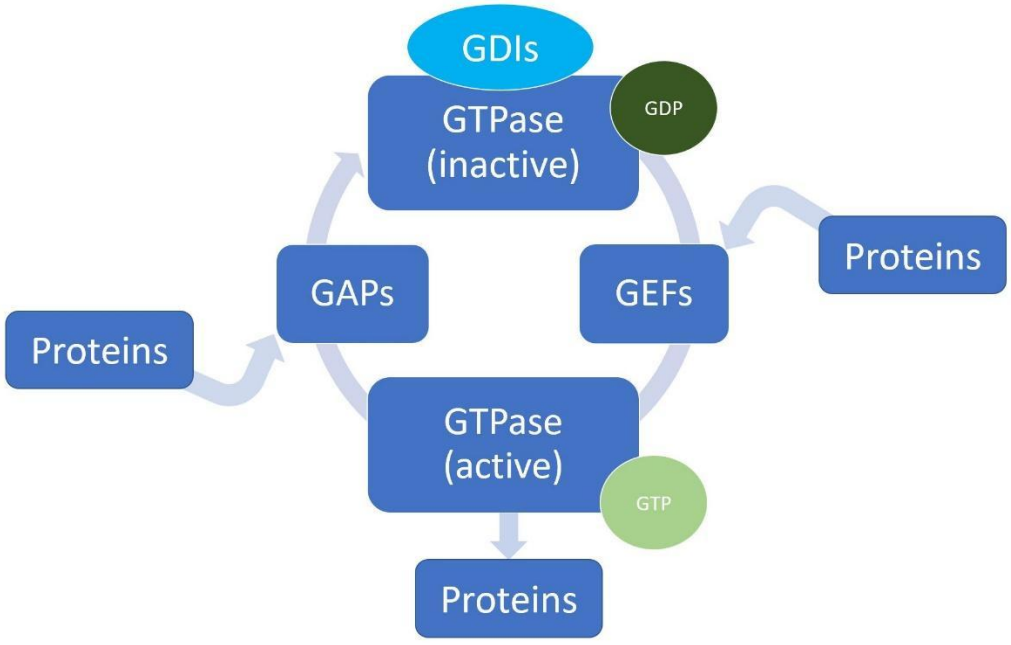
Table 1: Domain Homology of Selected Rho GTPase GEFs

	DH Domain	PH Domain	RH Domain	LH Domain	DOCK Domain
P115 Rho GEF	Yes	Yes	Yes	Yes	No
Lbc Rho GEF	Yes	Yes	Yes	Unknown	No
Lsc Rho GEF	Yes	Yes	Unknown	Yes	No
Ect2 Rho GEF	Yes	Yes	Unknown	Unknown	No
DOCK180	No	No	No	No	Yes
MOCA (DOCK3)	No	No	No	No	Yes
Smg GDS Rho GEF	Yes	Yes	Unknown	Unknown	No

Previously identified domain information about highly conserved domains in GEFs. The DH and PH domain are present in all GEFs to date while the RH and LH domains seem to be present in most proteins with PH and DH. The DOCK domain works independently of the DH domain and all GEFs to date only have one or the other.

There are over three times as many Rho GEFs as Rho GAPs (Viaud et al., 2012). Some of the Rho GEFs of interest are P115 (Chen et al., 2012), lbc (Dutt et al., 2004; Martin et al.; Medina et al., 2013), lsc (Dubash et al., 2007), ect2 (Mikawa et al., 2008; Su et al., 2011), and smg GDS(Hamel et al., 2011). Some Rho GAPs that will be discussed later include p190 (Mikawa et al., 2008; Ponik et al., 2013), p122 (Kawai et al., 2005), Myr (Müller et al., 1997), and ARHGAP18 (Maeda et al., 2011). Cdc42 and Rac GEFs and GAPs overlap considerably with each other and some of them are Tiam Cdc42 and Rac GEF (Lawson and Ridley, 2018; Narayanan et al., 2013), Trio Rac GEF (Steven et al., 2005), BCR Rac GEF and GAP(Cho et al., 2007; Oh et al., 2010), ABR Rac and Cdc42 GAP(Cho et al., 2007; Oh et al., 2010), Pix Rac and Cdc42 GEF (Koh et al., 2001), Beta-chimaerin Rac GAP (Griner et al., 2010), Alpha-chimaerin Rac GAP (Yagi et al., 2012), p190 Rac GAP (Bustos et al., 2008), racGAP50c (Gregory et al., 2008), RacGAP1 (Warga et al., 2016), Intersectin-1 Cdc42 GEF (Humphries et al., 2014), Intersectin-2 Cdc42 GEF (Rodriguez-Fraticelli et al., 2010), Fgd1 Cdc42 GEF (Estrada et al., 2001), and p21 Cdc42 and Rac GAP (Sahai and Marshall, 2002). These proteins will be talked about in more detail as they relate to proteins that effect GEFs and GAPs. (See appendix for more information.)

Figure 2: Flowchart of Rho GTPase Regulation Cycle



Proteins that Interact with GEFs and GAPs

GEFs and GAPs are regulated by effector proteins that modulate the activity and localization of GEFs and GAPs (Sahai and Marshall, 2002). Some of the proteins that have been shown to effect GEFs and GAPs include anillin (Reyes et al., 2014), septins (Caviston et al., 2003; Gladfelter et al., 2002), cortactin (Kirkbride et al., 2011; Lua and Low, 2004), ROCK, Kinectin, PAK1,2,3, MLK2,3, Par6, p67PHOX, POSH, IRSp53, N-WASP/WASP, MRCK1,2 (Sahai and Marshall, 2002), PRK/PKN1 (Schmidt et al., 2007), and Moesin (Sherrard and Fehon, 2015; Speck et al., 2003). (See appendix for more information.) The proteins that will be focused on for this paper are anillin, septins, cortactin, Par6, and moesin.

Table 2: GEFs and GAPs and What Proteins Affect Them

Protein that Affect GEFs/GAPs	GTPases Affected	GEFs/GAPs Affected	Function of Protein
Anillin	Rac, Rho	racGAP50c/MgcRacGAP, p190 Rho GAP, Ect2, IQGAP1	Scaffolding
Septins	Cdc42	Cdc42 GAPs: Bem3p, Rga1p, Raga2p.	Scaffolding
Cortactin	Rho	P190 Rho GAP, BPGAP1 Rho GAP	Cell migration
ROCK	RhoA,C	P190 Rho GAP	As a kinase: actomyosin contraction, transformation, transcription. As a Scaffold: actin polymerization, transcription, microtubules
PRK1/PKN	RhoA/C,RhoB	Ect2	Endocytosis, transcription, cytokinesis, motility, cell adhesion
Kinectin	RhoG	Unknown	Scaffolding
PAK1,2,3	Rac1,2,3, Cdc42	Cdc42p GEF	JNK activation, transformation
MLK2,3	Rac1,2,3, Cdc42	Unknown	JNK activation
Par6	Rac1,2,3, Cdc42	Tiam GEF Cdc42 and rac	Cell polarity, transformation
P67PHOX	Rac 1,2,3	Vav1	Transcription
POSH	Rac 1,2,3	Unknown	Scaffolding, JNK activation, transcription
IRSp53	Rac 1,2,3	Tiam1 GEF Cdc42 and rac	Actin polymerization
NWASP/WASP	Cdc42	Unknown	Scaffolding, actin polymerization
MRCK1,2	Cdc42	Unknown	Actin organization
Moesin	Rac, Cdc42, Rho	ARHGAP18	Cell polarity

This table provides a quick reference for what proteins affect which GEFs and GAPs and how they function. The proteins may affect more GEFs and GAPs than are listed, but to date these are what has been found. Adapted from Sahai and Marshall, 2002.

Anillin

Anillin is a scaffolding protein that interacts with cytoskeletal components and recruits structural and signaling constituents of the contractile ring (Piekny and Maddox, 2010; Schwayer et al., 2016). Anillin contains a conserved C-terminal domain that is required for its function and localization; the domain shares homology with the RhoA binding protein Rhotekin and it has been shown it directly interacts with RhoA (Piekny and

Glotzer, 2008). Anillin has been found in all stages of the cell cycle. In Drosophila during metaphase, it is present in both the cytoplasm and the cortex, while in anaphase and telophase it increases in abundance at the cleavage furrow (Field, 1995). This indicates that anillin is used for both structural integrity of cells and cell division (Field, 1995). One of the most highly studied interactions of anillin is its interaction with F-actin, but anillin does not bind all F-actin in a cell and instead seems to be specific to cell-division related cytoskeletal structures (Piekny and Maddox, 2010). Of note, anillin is not present during Drosophila dorsal closure or in stress fibers during interphase of mammalian cell culture (Piekny and Maddox, 2010).

Anillin primarily localizes to cell-cell junctions and the cytokinetic contractile ring (Reyes et al., 2014). At cell-cell junctions, anillin is required for integrity of the cell and is needed for proper adherens junctions and tight junctions (Reyes et al., 2014). Cells lacking anillin show intercellular spaces and reduced signaling of E-cadherin, an apical junction membrane protein (Reyes et al., 2014). Anillin is seen at cell-cell boundaries in mitosis and interphase and it is known anillin interacts with Rho, F-actin, and myosin which all are involved in cell-cell junction integrity (Reyes et al., 2014). In Drosophila early embryos, anillin is seen at the connections of the base of each cell junction shortly after contraction and without anillin the cells lose their rounded shape. This suggests that anillin is required for cell-cell junctions and cells' rounded shapes (Field, 1995; Reyes et al., 2014). Anillin's most important role is in cytokinesis where it scaffolds the contractile actomyosin ring and works with Rho at the cleavage furrow (Piekny and Glotzer, 2008). Anillin promotes Rho activity while Rho is needed for anillin to localize at the cleavage furrow, creating a positive feedback loop (Reyes et al., 2014). The protein is also needed to stabilize and distribute tension in the actomyosin belt while spatially regulating the contraction of myosin (Piekny and Glotzer, 2008; Straight et al., 2004). Further, anillin connects the actomyosin ring to spindle microtubules through RacGAP (D'Avino, 2009). Interestingly, anillin has also been found in Drosophila early syncytial blastoderm at the cap above the nucleus where actin is also found (Field, 1995).

As anillin relates to regulating GEFs and GAPs, anillin contains a conserved PH and Anillin Homology (AH) domain which allows it to interact with GEFs and GAPs that also contain the PH domain and that interact with the AH domain (Piekny and Maddox, 2010). Three GEFs and GAPs anillin is seen to regulate are Ect2, RacGAP50, and p190. The interaction between anillin and Ect2 is shown to require the AH domain of anillin and the PH domain of Ect2 (Piekny and Maddox, 2010). Anillin seems to be involved in junctional regulation of Rho GTPase because when a mutation of Ect2 in its PH domain occurred, not only did it decrease affinity for anillin but it also showed Ect2 did not as readily migrate to the plasma membrane and there was decreased Rho production (Piekny and Maddox, 2010). Ect2 also binds with RacGAP50 but at a different place than RacGAP50 binds with Anillin (Piekny and Maddox, 2010). Anillin's C-terminus binds RacGAP50's N-terminus and they are shown to be dependent on each other (Gregory et al., 2008; Piekny and Maddox, 2010; Reyes et al., 2014). RacGAP50 is the term for it in Drosophila, but this GAP is also known as MgcRacGAP in mammals or Cyk-4 in other model organisms (Piekny and Maddox, 2010). RacGAP50 specifies the site of cleavage but without anillin RacGAP loses association with the equatorial cortex and cytokinesis fails (Reyes et al., 2014). More recently, p190 is a Rho GAP which has been shown to directly bind to anillin and modulate RhoA-GTP levels at the cleavage furrow (Manukyan et al., 2015).

Septins

Septins are scaffolding ring components that link actin filaments into curved arrays and join the actomyosin ring to the plasma membrane either directly or through anillin (Schwayer et al., 2016). Septins don't localize to the actomyosin ring until after other components such as Rho have made a clear ring, but it has been shown that septins arrive to the vicinity of contractile ring formation several minutes before they begin to localize (Iwase et al., 2006). The protein assembles into heteromeric, rod shaped, nonpolar complexes which have been found to make hetero-octamers in *Saccharomyces cerevisiae* (Cannon et al.,

2019). They were first found in *Saccharomyces cerevisiae* in the plasma membrane of the mother-bud neck (Longtine et al., 1996). Their structure normally consists of a variable N-terminal and C-terminal region with a conserved core which has GTP-binding site elements (Caviston et al., 2003). Septin localization is dependent on anillin and without anillin the maintenance and organization of septins are disturbed (Piekny and Maddox, 2010). Septin binding activity is conferred by part of the C-terminus of anillin that encompasses the PH and AH domains (Piekny and Maddox, 2010) The ring of septins form at the neck of the actomyosin ring and seems to function as a scaffold to recruit other proteins for contraction (Caviston et al., 2003). Septins can sense the curvature of the membrane and ensure actin also curves around the membrane (Cannon et al., 2019). Septins are dependent on Cdc42 GTPase but independent of actin. Cdc42 GAPs Bem3p, Rga1p, and Rga2p work with septins to localize septins and actomyosin to the contractile ring (Caviston et al., 2003; Gladfelter et al., 2002). GTPases beyond Cdc42 help regulate septins functions and localization. Tem1 GTPase is seen to also control septins dynamics during cytokinesis (Lippincott). Rho GTPase disrupts septins filament structures in rat embryonic fibroblast REF52 cells but Rhotekin, a Rho binding protein, helps reorganize septins and seems to help regulate the link between septins and Rho (Ito et al., 2005).

Cortactin

Cortactin plays a role in actin polymerization as well as cellular migration and cancer cell-based processes such as invasion (Kirkbride et al., 2011). Cortactin contains an acidic N-terminal domain, tandem repeat domain, and a proline rich C-terminal domain which includes phosphorylation sites and a SH3 domain (Kirkbride et al., 2011). Cortactin is recruited upon Rac GTPase activation as seen in Swiss 3T3 fibroblasts where, upon Rac activation, platelet-derived growth factor (PDGF) was triggered to translocate cortactin (Daly, 2004; Kirkbride et al., 2011). After being recruited, cortactin is activated by phosphorylation on multiple serine and threonine residues (Daly, 2004). The most well studied role of cortactin is in actin polymerization, specifically the activation of the Arp2/3 complex which triggers branched actin polymerization (Kirkbride et al., 2011; Uruno et al., 2001). There are two mechanisms by which cortactin activates Arp2/3, either directly triggering the branch at the N-terminus of cortactin or indirectly by using WASP proteins (Kirkbride et al., 2011). The N-terminal domain of cortactin is necessary for it to be recruited to actin rich areas of the cell and any mutations in this region prevent its interaction with Arp2/3 (Uruno et al., 2001). Cortactin is also seen in membrane ruffles and lamellipodia and it is suggested it localizes here to enhance formation of branched actin (Daly, 2004; Kirkbride et al., 2011). This protein is also prominent in cell motility functions where increased cortactin increases cell motility (Kirkbride et al., 2011) and cortactin helps form adherens junctions by localizing E-cadherin, necessary for adherens junction formation (Daly, 2004).

Two GAPs are suggested to interact with cortactin: BPGAP1 and p190 RhoGAP. BPGAP1 is a Rho GAP that regulates cell migration through proline rich domains (Lua and Low, 2004). BPGAP1 interacts with cortactin and facilitates it movement to the periphery to enhance cell migration (Lua and Low, 2004). The other GAP is p190 RhoGAP which disrupts actin organization (Fincham et al., 1999). The protrusion localization sequence (PLS) helps p190 target the leading edges of a moving cell through negative regulation, and cortactin binds the PLS (Binamé et al., 2016).

Par6

Par6 is part of the Par3/Par6/aPKC complex which regulates cell polarity in all eukaryotes (Chen and Zhang, 2013). Originally, this complex was identified in C. elegans as necessary for anterior/posterior cell polarity, and its now been shown that the complex works together to regulate early stages of Drosophila and C. Elegans polarization (Chen and Zhang, 2013). Both Par3 and Par6 are scaffold proteins that are able to bind to each other as well as many other proteins. Using these interactions they are able to ensure the Par complex is correctly localized (Chen and Zhang, 2013). Par6 contains an N-terminal PB1 domain, a C-terminal PDZ

domain, and a semi-CRIB motif immediately preceding PDX domain (Chen and Zhang, 2013). The PB1 domain complexes with aPKC and the semi-crib domain binds to active Cdc42 GTPase (Nishimura et al., 2005). Upon Cdc42 binding to Par6 at the semi-crib domain, aPKC is activated which phosphorylates Par3 and causes Par3 to dissociate from the Par6/aPKC complex (Nishimura et al., 2005). Par6 effects both Rac 1,2,3 as well as Cdc42 GTPases (Sahai and Marshall, 2002). One of the mechanisms how Par6 effects cell polarity is once the Par6/aPKC complex is activated it promotes the localized association of adenomatous polyposis coli (APC) with microtubule plus ends (Etienne-Manneville et al., 2005). APC works with Dlg1 for the polarization of the microtubule cytoskeleton (Etienne-Manneville et al., 2005). Par6 has also been shown to negatively regulate the assembly of epithelial tight junctions (Gao et al., 2002).

Tiam1 GEF and p190 RhoGAP have both been shown to play roles in regulating Par6. P190 has been linked to the Par6/aPKC complex through RhoA activity (Zhang and Macara, 2008). Tiam1 is a GEF for Cdc42 and Rac and is necessary for cell migration (Lawson and Ridley, 2018). Tiam1 is recruited by the Par complex to promote local Rac1 activation and cytoskeletal remodeling for controlling cell polarity (Narayanan et al., 2013; Nishimura et al., 2005). The Par-Tiam interaction is critical for cell function, and any deregulation of this interaction is implicated in tumorigenesis (Mertens et al., 2006).

Moesin

Moesin is part of the exrin, radixin, and moesin (ERM) family that links the actin cytoskeleton to the plasma membrane and sends signals for cytoskeletal remodeling (Ivetic and Ridley, 2004; Pearson et al., 2000). ERM proteins are highly concentrated at the cleavage furrow (Sato et al., 1991) and on cytoplasmic actin stress filaments like stress fibers (Arpin et al., 2011). The family has a highly conserved N-terminal region with ~85% identity, and they are regulated by their FERM and C-terminal domains which mask their binding sites (Pearson et al., 2000; Tsukita and Yonemura, 1999). The C-terminus and N-terminus is able to bind to F-actin while the middle regions bind both G-actin and F-actin (Tsukita and Yonemura, 1999). There are two activation pathways for ERM proteins found in vitro, either phosphorylation of their C-terminal threonine residue or PIP2 binding to their N-terminal domains (Tsukita and Yonemura, 1999). PIP2 is also activated by Rho, which suggests a pathway of Rho activating PIP2 which in turn activates ERM proteins (Tsukita and Yonemura, 1999). Additionally, a hallmark of ERM proteins is their ability to interact both upstream and downstream of Rho GTPase indicating a positive feedback pathway between Rho and ERM proteins (Ivetic and Ridley, 2004).

Moesin is the only Drosophila member of the ERM family (Kunda et al., 2008). Moesin has been shown to aid cortical rigidity, cell rounding, and is implicated in microvilli structure as seen when moesin is removed microvilli grow longer than normal (Kunda et al., 2008; Sherrard and Fehon, 2015). Moesin has been shown to directly recruit the RhoGAP Conundrum to the cell cortex. This interaction leads to decreased RhoA activity at the cortex (Neisch et al., 2013). It is also proposed to interact with crumbs which is a transmembrane protein that functions in apical polarity, epithelial integrity, and stability of adherens junctions (Sherrard and Fehon, 2015). Moesin limits crumbs at the membrane by reducing its interaction with the Par complex, while moesin depletion allows for redistribution of crumbs moving from the membrane to the marginal zone and intracellular vesicles (Sherrard and Fehon, 2015).

Rho GAP ARHGAP18 has been found to regulate moesin and RhoA. ARHGAP18, or conundrum in Drosophila, is recruited to the cell cortex by moesin which negatively regulates RhoA activity (Neisch et al., 2013). This Rho GAP is dependent on Rac activity and is activated when moesin localizes to the correct location (Neisch et al., 2013). ARHGAP18 is shown to be necessary at the leading edge for correct cell spreading and migration as well as required for cell polarization (Maeda et al., 2011). Moesin helps

ARHGAP18 localize to the plasma membrane and the cell cortex where ARHGAP18 can act as a GAP to Rho1 (Neisch et al., 2013).

Conclusion/Future Directions

More than 20 members of the Rho GTPases have been identified in mammalian cells, each having their own regulators of GEFs, GAPs, GDIs, and other effectors (Jaffe and Hall, 2005). While there has been tremendous work in discovering the pathways and interactions among these proteins, there is much more work needed to understand the full picture of the Rho GTPase family interactions. GDIs are an emerging field with many chances for discovery understanding how GDIs are regulated and their mechanism of RhoGTPase extraction/sequestration. The Bement lab at the University of Wisconsin-Madison has efforts underway to characterize many of the pathways of Rho GTPases. Previous work in the lab has established that there are waves of Rho followed by waves of actin in pre-cytokinetic embryos (Bement et al., 2015). One of the biggest mysteries surrounding these Rho and actin waves is how Rho is turned off at the trailing edge of the waves. Cortactin is hypothesized to be turning off Rho at the trailing edge of the actomyosin contractile ring by bringing Rho GAPs to the trailing edge and current work does show that cortactin is part of an “enzymatic corral” that organizes F-actin assembly (unpublished data). Its also suggested that anillin could be turning off Rho at the trailing edge of the pre-cytokinetic waves of Rho and F-actin by recruiting a GAP or GAPs. (Bement et al., 2015; unpublished data). Understanding how GEFs and GAPs are regulated by effector proteins is a vital part to solving how Rho waves are turned off, but more work is still needed to better understand the interactions between GEFs, GAPs, and regulator proteins of Rho GTPases.

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Appendix

GEFs and GAPs

1. P115 Rho GEF: contains DH and RH homology similar with all other GEFs, transduces Galpha13 signals to Rho and inhibits Galpha12/13 signals through the LH domain. (Chen et al., 2012)
2. BPGAP1: RhoGAP that regulates cell migration, proline rich domains, work with cortactin. (Lua and Low, 2004)
3. DOCK180 (or DOCK1): a Rac GEF. (Miyamoto and Yamauchi, 2010)
4. DOCK3 (or MOCA): a Rac GEF that modifies cell adhesion. (Miyamoto and Yamauchi, 2010)
5. Lsc Rho GEF: LH binding domain, associates with microtubules, lfc binding domain AKAP121 phosphorylates Lsc. (Dubash et al., 2007)
6. Ect2 Rho GEF: helps with formation of cytokinetic furrow, requires PH domain, works with p190GAP to negatively coregulate each other. Help define Rho zone. (Bement et al., 2015; Mikawa et al., 2008; Su et al., 2011)
7. Smg GDS: promotes cell proliferation and migration, is upregulated in cancer, a Rho GEF (Hamel et al., 2011)
8. P190GAP: disrupts organized actin (Fincham et al., 1999). Rho GAP, works with Ect2 to negatively coregulate each other(Mikawa et al., 2008), localizes with p120 catenin at sites of cell-cell contact (Ponik et al., 2013), binds anillin which regulates Rho-GTP levels in the cleavage furrow (Manukyan et al., 2015), the protrusion localization sequence is needed for p190 to localize at the leading edges of actin and for negative regulation of p190, cortactin binds the protrusion localization sequence and is required for p190 targeting the protrusions (Binamé et al., 2016).
9. P122: Rho GAP, localizes at the ends of actin stress fibers and focal adhesion points. (Kawai et al., 2005)
10. Myr: Rho GAP: reduces the amount of actin stress fibers when it is overexpressed. (Müller et al., 1997)
11. ARHGap18: activity of RhoA is suppressed and stress fiber formation is disrupted when ARHGap18 is overexpressed, lack of ARHGap18 enhanced stress fiber formation and induced rounding of cells. Crucial for the control of cell shape, spreading, and migration. (Maeda et al., 2011)
12. Tiam: Cdc42 and rac GEF: for cell migration (Lawson and Ridley, 2018), the Par complex including Par6 controls cell polarity partly by recruiting Tiam1 and Tiam1 promotes local Rac1 activation and cytoskeletal remodeling (Narayanan et al., 2013)
13. Trio rac GEF: signals Rac to regulate cytoskeletal rearrangements necessary for growth cone migrations. (Steven et al., 2005)
14. BCR and ABR: BCR rac GEF/GAP and ABR rac and Cdc42 GAP work together, overexpressing ABR accelerates Cdc42 inactivation and causes a two times increase in Cdc42 turnover (Burkel et al., 2012). ABR separates Rho and Cdc42 (Schwayer et al., 2016). The only GAP to date that negatively regulates Rac GTPase function. (Koh et al., 2001)
15. Pix GEF: involved with Cdc42 and Rac GTPases, localize eta1PIX to the cell edges and does PIX dimerization. (Koh et al., 2001)
16. Beta-chimaerin: Rac GAP, activated by lipid second messenger diacylglycerol through their C1 domain upon activated tyrosine kinase receptors which restricts Rac signaling. (Griner et al., 2010)
17. Alpha-chimaerin: Rac GAP, suppresses rac1 at the apical membrane. (Yagi et al., 2012)
18. P190 Rac GAP: activated Rac1 binds p190. (Bustos et al., 2008)

19. racGAP50c: specifies the site of cleavage. Anillin and racGAP50c are dependent on each other, without anillin racGAP50c loses its association with equatorial cortex and cytokinesis fails. (Gregory et al., 2008). Binds with Ect2 but at a different place than RacGAP50 binds with Anillin (Piekny and Maddox, 2010). Anillin C-terminus binds RacGAP50's N-terminus. RacGAP50 is the term for it in Drosophila, but this GAP is also known as MgcRacGAP in mammals or Cyk-4 (Piekny and Maddox, 2010).
20. RacGAP1: when mutated cells stops dividing, is component of central spindle complex (Warga et al., 2016)
21. Intersectin-1: Cdc42 GEF, Intersectin-1 is recruited to the virus before any actin base motility. Cdc42, intersectin-1, and WASP function together in a feed-forward loop to promote vaccinia-induced actin polymerization. (Humphries et al., 2014)
22. Intersectin-2: Cdc42 GEF, localizes to centrosomes and regulates Cdc42 activation during epithelial morphogenesis. Silencing Intersectin-1 or Cdc42 disrupts the correct orientation of the mitotic spindle which indicates there may be a direct relationship between these two. (Rodriguez-Fraticelli et al., 2010)
23. Fgd1: Cdc42 GEF, proline rich N-terminal region, two PH domains, FYVE-finger domain, involved in signaling and subcellular localization. (Estrada et al., 2001)
24. P21 GAP: involved with Cdc42 and rac GTPases, p21 activated kinases (PAKs) bind to active Cdc42 and Rac1. (Sahai and Marshall, 2002)
25. Bem3p: inhibit Cdc42, Cdc42 GAP, found after bud emergence after septin's job is finished. (Knaus et al.2007)
26. Rga1p and Rga2p: Cdc42 GAP, phosphatidylethanolamine and phosphatidylserine stimulate GAP activity towards Cdc42. (Saito et al., 2007)

<u>Proteins Effecting GEFs and GAPs</u>

1. Anillin: involved in scaffolding that recruits structural and signaling constituents of the contractile ring (Schwayer et al., 2016). Localizes to epithelial cell-cell junctions, promotes stable cleavage furrow in cytokinesis (Reyes et al., 2014).
2. Septins: involved in scaffolding that cross-link actin filaments and link ring to plasma membrane either directly or through anillin (Schwayer et al., 2016). Have variable N-terminal and C-terminal region but a conserved core (Caviston et al., 2003). Cdc42 GTPase cycling is involved in septins-ring formation (Gladfelter et al., 2002).
3. Cortactin: role in cellular migration and invasion, promotes actin polymerization, prominent maker of lamellipodia but not essential for formation. Activates Arp2/3, stabilizes actin branches, recruited by Rac activation (Kirkbride et al., 2011).
4. ROCK: as a kinase it affects actomyosin contraction, transformation, and transcription. As a scaffold it affects actin polymerization, transcription, and microtubules. Activation of ROCK can lead to loss of cell-cell junctions, required in endothelial and in migrating cells to move through vascular endothelium, assembly integrins at focal adhesions (Sahai and Marshall, 2002)
5. PRK1/PKN: effects RhoA/C as a Kinase, endocytosis, MAPK activation, transcription, cytokinesis. Effects RhoB as a kinase, EGFR trafficking, motility, and cell adhesion. (Sahai and Marshall, 2002). Without PRK/PKN cells have late cytokinesis which is similar in Ect2 depleted cells, suggesting PRK is regulated by Ect2 (Schmidt et al., 2007)
6. Kinectin: scaffolding protein, effects RhoG, microtubule binding. (Sahai and Marshall, 2002; Vignal et al., 2001)
7. PAK1,2,3: effect Rac 1,2,3 as a kinase, JNK activation, and transformation. Effect Cdc42 as a kinase, JNK activation and transformation. (Sahai and Marshall, 2002) Cdc42p GEF works with PAK to do symmetry breaking polarization (Kozubowski et al., 2008)
8. MLK2,3: effects Rac 1,2,3 as a kinase, JNK activation (Sahai and Marshall, 2002)

9. Par6: effects Rac 1,2,3 as a kinase, cell polarity, transformation (Sahai and Marshall, 2002)
10. P67PHOX: effects Rac 1,2,3 as an enzyme, NADPH oxidation, transcription(Sahai and Marshall, 2002). Regulates NADPH oxidation by stimulating Vav1. (Ming et al., 2007)
11. IQGAP1,2: Effects Rac 1,2,3 as a scaffold/GAP, adherens junctions (Sahai and Marshall, 2002)
12. POSH: is a scaffold, effects Rac 1,2,3 in JNK activation and transcription (Sahai and Marshall, 2002)
13. IRSp53: scaffold, effects Rac 1,2,3 in actin polymerization (Sahai and Marshall, 2002). Complexes with Tiam1 to direct specificity of Rac-mediated actin cytoskeleton development (Connolly et al., 2005)
14. N-WASP, WASP: effects Cdc42, scaffolding, and actin polymerization (Sahai and Marshall, 2002)
15. MRCK1,2: effects Cdc42, kinase, and actin organization (Sahai and Marshall, 2002)
16. Moesin: effects microvilli structure, cell polarity. Regulates cortical actin cytoskeleton. (Sherrard and Fehon, 2015)

<u>Other</u>

1. Crumbs: transmembrane protein which functions in apical polarity and epithelial integrity. Regulates apicobasal polarity in Drosophila and vertebrates, regulates stability of Adherens junctions (Sherrard and Fehon, 2015)
2. Adherens Junction: cell junction whose cytoplasmic face is linked to actin cytoskeleton. Crucial for epithelial adhesion and barrier function.(Niessen, 2007)
3. Par proteins: regulate polarity (Pichaud, 2018)m required for asymmetric cell division (Goldstein and Macara, 2007). Par3/Par6/aPKC complex is part of par proteins (Chen and Zhang, 2013)
4. Juxtamembrane domain: promotes adherens junction formation. (Niessen, 2007)
5. Atypical protein kinase C (aPKC): serine/threonine kinase and required for apico/basal cell polarity (Suzuki, 2006)
6. Actin: a protein that provides internal mechanical support, tracks for movement of materials, and aids cell locomotion (Ladt et al., 2016; Pollard and Cooper, 2009)
7. Myosin: fibrous protein that works with actin to make contractile filaments and aid cell locomotion (Lodish et al., 2000)
8. Rho GTPases: molecular switches that regulate a variety of functions in all cells with GTP activating them and GDP inactivating them (Jaffe and Hall, 2005)
9. Guanine Nucleotide Exchange Factors (GEFs): catalyze the exchange of GDP for GTP, activate RHO GTPases. (Jaffe and Hall, 2005)
10. GTPase activating proteins (GAPs): stimulate intrinsic GTPase activity to inactivate switch in RHO GTPases. (Jaffe and Hall, 2005)
11. Guanine Nucleotide dissociation inhibitors (GDIs): block spontaneous activation of RHO GTPases, in cytoplasm they hold into GTPases to keep inactive. (Jaffe and Hall, 2005)
12. Formins: protein polymerizing actin, create long singular actin, actin nucleator (Schwayer et al., 2016)
13. Arp2/3: Actin nucleator, create branched actin, activated by WASp/scar proteins (Amann and Pollard, 2000; Schwayer et al., 2016)
14. Zyxin: A protein that localizes at focal adhesion of actin cytoskeleton (Yoshigi et al., 2005).
15. mDia: a formin involved with Rho GTPases, stabilizes microtubules, contains essential FH1 domain (Jaffe and Hall, 2005)
16. Cofilin: sever actin filaments to create uncapped barbed ends for actin polymerization. (Jaffe and Hall, 2005)
17. WASP-family verprolin-homologous protein (WAVE): scaffolds that link upstream signals to the activation of the ARP2/3 complex, leading to a burst of actin polymerization (Takenawa and Suetsugu, 2007)

18. Wiskott-Aldrich syndrome (WASPs): roles in signaling and cytoskeletal structure. (Pollitt and Insall, 2009)
19. JNK proteins: activated by 2 MAP kinases, member of MAPK family, regulate important physiological processes including neuronal functions, immunological actions, and embryonic development, cytoskeletal protein dynamics, and cell death/survival pathways (Zeke et al., 2016)
20. Mitogen activated protein kinases (MAPK): conserved family of serine/threonine protein kinases involved in a variety of fundamental cellular processes such as proliferation, differentiation, motility, stress response, apoptosis, and survival.(Cargnello and Roux, 2011)
21. Profilin: binds actin monomers and prevents their association with pointed ends but not with barbed end (Amann and Pollard, 2000)
22. Lamellipodia: pleat-shaped protrusions are cell edges, implicated in cell migration (Hall, 1998; Symons, 1996).
23. Filopodia: actin rich surface protrusions. (Hall, 1998)
24. Rhotekin: binds active RhoA to regulate signaling (Piekny and Glotzer, 2008)
25. Capz: actin barbed end capping protein. (Caldwell et al., 1989)