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Profilin choreographs actin and microtubules in cells and cancer

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Abstract

Actin and microtubules play essential roles in aberrant cell processes that define and converge in cancer including: signaling, morphology, motility, and division. Actin and microtubules do not directly interact, however shared regulators coordinate these polymers. While many of the individual proteins important for regulating and choreographing actin and microtubule behaviors have been identified, the way these molecules collaborate or fail in normal or disease contexts is not fully understood. Decades of research focus on Profilin as a signaling molecule, lipid-binding protein, and canonical regulator of actin assembly. Recent reports demonstrate that Profilin also regulates microtubule dynamics and polymerization. Thus, Profilin can coordinate both actin and microtubule polymer systems. Here we reconsider the biochemical and cellular roles for Profilin with a focus on the essential cytoskeletal-based cell processes that go awry in cancer. We also explore how the use of model organisms has helped to elucidate mechanisms that underlie the regulatory essence of Profilin in vivo and in the context of disease.

Keywords

Profilin; Profilin-1; Profilin-2; Actin; Microtubules; Cytoskeletal crosstalk; Motility; Cell division

1. INTRODUCTION: CELLULAR INFRASTRUCTURE CONSTRUCTED FROM CYTOSKELETAL BUILDING BLOCKS

Actin and microtubules bestow useful properties to cells including: paths for transport, shape, and the forces that propel and steer movement (Fig. 1). The actin and microtubule cytoskeletons are traditionally studied as separate entities, restricted to specific cellular regions, and regulated by different suites of binding proteins unique for each polymer. Mounting genetic and pharmacological evidence suggests a rich degree of functional

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AUTHOR CONTRIBUTIONS

J.L.H.-R. downloaded and analyzed the transcript data from the National Cancer Institute Genomic Data Commons (https://portal.gdc.cancer.gov; Grossman et al., 2016). M.L.P. and J.L.H-R designed the Figs. J.H., M.L.P., and J.L.H-R wrote portions of this work.

CONFLICTS OF INTEREST

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crosstalk and physical interactions between both systems, ultimately manifesting in the normal function of fundamental cellular processes, e.g., motility, adhesion, intracellular transport, wound healing, phagocytosis, and cell division (Coles and Bradke, 2015; Dogterom and Koenderink, 2019; Etienne-Manneville, 2004; Li and Gundersen, 2008; Rodriguez et al., 2003). Many proteins likely to be involved in actin-microtubule interactions have already been identified and characterized with regard to either actin or microtubules alone, owing to pioneering work in genetically tractable model organisms (Chang and Martin, 2009; Coles and Bradke, 2015; Dogterom and Koenderink, 2019; Etienne-Manneville, 2004; Gardiner and Marc, 2011; Prokop et al., 2013; Roeles and Tsiavaliaris, 2019; Slater et al., 2017). However, the detailed mechanisms that underlie cytoskeletal synergy and that go wrong in cancer and disease are just starting be investigated (Akhmanova and Steinmetz, 2010; Coles and Bradke, 2015; Dogterom and Koenderink, 2019; Mitchison and Kirschner, 1984a, 1984b; Rodriguez et al., 2003; Salmon et al., 2002). Here we focus on Profilin as a regulator of actin and microtubules in biochemical assays, and cancer-relevant cell processes and signaling pathways.

Actin filaments and microtubules share many properties. All cells contain both polymers in subunit-based and polarized filament forms (Fig. 1, inset). New assembly of either polymer is kinetically unfavorable and requires a template to organize polymer growth. For actin: de novo assembly requires a seed of three to four monomers that can further polymerize into helical two-stranded filaments with a diameter ~ 7 nm (Barshop et al., 1983; Cooper et al., 1983; Courtemanche, 2018; Oda et al., 2016; Sept and McCammon, 2001; Skruber et al., 2018; Svitkina et al., 1997). For microtubules: spontaneous microtubule assembly can occur in vitro with high concentrations of tubulin and time (Caudron et al., 2000; Desai and Mitchison, 1997; Fygenson et al., 1995; Job et al., 2003; Nogales, 2001; Roostalu and Surrey, 2017). In cells the critical concentration required for microtubule assembly far exceeds the amount of available free tubulin (estimated ~ 20 μM) (Fygenson et al., 1995; Voter and Erickson, 1984; Wieczorek et al., 2015). Thus, cellular microtubule assembly requires a stable template (i.e., a ring-shaped complex that mimics microtubule dimensions or a severed microtubule) to ultimately form a ~ 25 nm cylinder held together through the lateral contact of ~ 13 parallel protofilaments (this number varies depending on cell source and experiment) (Chaaban and Brouhard, 2017; Kollman et al., 2010; Roostalu and Surrey, 2017; Wieczorek et al., 2015). Protofilaments are formed from αβ-tubulin heterodimers that intrinsically self-assemble in a head-to-tail fashion and impart structural polarity to the microtubule, with an exposed β-subunit pointing outward (Alushin et al., 2014; Löwe et al., 2001; Mitchison, 1993; Nogales, 2001; Nogales et al., 1998). Cells employ a plethora of actin and microtubule nucleation promoting factors to overcome these barriers to nucleation at specific times and locations (for actin: the Arp2/3 complex, Formins, Ena/Vasp, Spire; for microtubules: γ-Turc, XMAP215) (Chesarone et al., 2010; Courtemanche, 2018; Kollman et al., 2010; Kovar et al., 2006; Krause et al., 2003; Machesky et al., 1999; Moritz et al., 2000; Mullins and Pollard, 1999; Mullins et al., 1998; Oakley et al., 2015; Pollard, 2007; Popov et al., 2002; Pruyne et al., 2002; Quinlan et al., 2005; Sagot et al., 2002; Thawani et al., 2018). Shared actin and microtubule nucleation proteins can also link dynamic cytoskeletal behaviors in cells and reconstituted systems (Chang, 2000; Colin et al., 2018; Elie et al., 2015; Gaillard et al., 2011; Henty-Ridilla et al., 2016; Inoue et al., 2019; Kita et al., 2019;

Lewkowicz et al., 2008; Plessner et al., 2019; Prezel et al., 2018; Szikora et al., 2017). For example, Profilin can block actin filament assembly rendering building blocks sterically or nucleotide assembly incompetent (De La Cruz et al., 2000; Skruber et al., 2018) and it can also stabilize parameters of microtubule growth at concentrations below the normal critical concentration required for assembly (Henty-Ridilla et al., 2017).

Many cellular actin and microtubule structures like filopodia or the mitotic spindle apparatus are not long lived. Actin and microtubules each display periods of growth (although on different time scales) and rapid disassembly. Dismantling either cytoskeletal polymer is part of a recycling process required for polymer growth to occur again, elsewhere. Many disassembly factors recognize the nucleotide state of actin (ATP, ADP-Pi, or ADP) or microtubules (GTP, GTP-Pi, or GDP) for their function, which can result in a targeted destruction of “aged” regions of polymer (Berro et al., 2010; Brieher, 2013; Manandhar et al., 2018; Margolis, 1981; Pollard and Borisy, 2003). The role of actin or microtubule disassembly factors in coordinating both cytoskeletal polymers have not been extensively studied. However, Profilin promotes actin filament disassembly by sequestering actin monomers and sterically blocking actin assembly (Carlier et al., 1993; Pantaloni and Carlier, 1993). Compared to actin filaments, microtubules have an additional intrinsic disassembly/recycling property (i.e., dynamic instability) that results in the co-existence of growing and shrinking microtubules (Burbank and Mitchison, 2006; Erickson and O’Brien, 1992; Mitchison and Kirschner, 1984a,b; Zhang et al., 2015). Catastrophe events, denoted by the stochastic switch from periods of microtubule growth to rapid depolymerization, may or may not be rescued. Thus, dynamic instability may provide an example of how physical linkages between actin and microtubules disengage. In microtubule dynamics assays Profilin did not have a significant effect on these parameters of microtubule disassembly (Henty-Ridilla et al., 2017). However, because many parameters of actin and microtubule dynamics are concentration dependent, control of either subunit pool through assembly-disassembly dynamics may ultimately influence the coordinated behaviors manifested during diverse cell processes.

In cancers, the unchecked dynamics (nucleation, assembly, disassembly, and crosstalk) of either actin or microtubules results in abnormal cell division, aberrant cell morphology or size, and invasive cell migration (Bae et al., 2009; Ding et al., 2014; Hall, 2009; Mouneimne et al., 2012; Roy and Jacobson, 2004; Tang et al., 2015). Many tumor suppressors (APC, N- and E-cadherin, neurofibromin) interact with both microtubules and actin and have been proposed to disrupt epithelial-mesenchymal transitions (EMT), epithelial positioning, and many aspects of cell migration and division (Hernandez and Tirnauer, 2010; Juanes et al., 2017, 2019). Thus, Profilin is posed as a convergence point linking the cytoskeleton and the signaling pathways that regulate cancer onset, progression, and severity in several tissues. Below we explore the biochemical and cellular facets of Profilin in coordinating actin and microtubules in normal cells, cancer, and lessons learned from unique model organisms.

2. THE PROFILIN ORIGIN STORY

Profilin was first isolated and crystalized from calf spleen extracts as an unrecognized protein present in a 1:1 complex with monomeric actin (Fig. 2A) (Carlsson et al., 1976a).

This low molecular weight contaminant was later characterized as the first actin monomer binding protein—remarkable because the hypothesis that a large pool of unpolymerized actin could exist in muscle or non-muscle cells was an emerging idea at the time (Carlsson et al., 1976b, 1977; Tilney, 1976). The association of this unknown protein was sufficient to fully explain the presence of a monomeric actin state in various cell extracts (Carlsson et al., 1977). Profilin was proposed to be a rapidly reversible storage solution, as sequestered actin monomers could be rapidly interconverted between different polymerization states to promote new actin filament formation to adapt to changing cell requirements (Carlsson et al., 1976b; Tilney, 1976). To date, no eukaryotic cells have been described that do not contain actin or Profilin, and both proteins are among the most abundant proteins on Earth (Beck et al., 2011; Dominguez and Holmes, 2011).

Profilins are small (12–15 kDa), well conserved, and highly abundant (Carlsson et al., 1976b; Pollard et al., 2000; Witke, 2004; Witke et al., 1998). They are essential in all forms of life from *Archaea* to eukaryotes, and even some viruses (Akıl and Robinson, 2018; Zaremba-Niedzwiedzka et al., 2017). Profilins exist as a single gene in many organisms (yeast, *Drosophila*, *Acanthamoeba*, certain viruses, *Toxoplasma*) and as several isoforms in others (*Arabidopsis*, *Chlamydomonas*, humans, worms, *Dictyostelium*). The greatest number and diversity of Profilin isoforms comes from *Maize* (10; and 2 additional non-annotated sequences); however, more diversity may be possible in higher ploidy phytozome genomes (Bao et al., 2011). The role of Profilin as a major regulator of actin assembly is broadly conserved in each of these systems (Akıl and Robinson, 2018; Di Nardo et al., 2000; Dominguez and Holmes, 2011; Witke, 2004; Zaremba-Niedzwiedzka et al., 2017). Most Profilins have highly conserved actin-, poly-*L*-proline (PLP)-, and lipid-binding regions, which impart the potential to interact with a variety of additional cytoskeletal regulating factors (Dominguez and Holmes, 2011; Haarer and Brown, 1990; Kaiser et al., 1999; Kovar et al., 2000; Krishnan and Moens, 2009; Thorn et al., 1997; Vinson et al., 1998).

There are four Profilin isoforms present in humans with ~ 33–95% similarity in amino acids (Fig. 2B–C). Depending on cell type, the estimate and measured amount of Profilin in mammalian cells is between 50 and 200 μM, which is generally considered sufficient or even excessive to the concentration of actin monomers in non-muscle cells (Funk et al., 2019; Kaiser et al., 1999; Lämmermann and Sixt, 2009; Vargas et al., 2017). Profilin-1 and Profilin-2 are ubiquitously expressed and the most abundant Profilin homologs in mammalian cells, whereas Profilin-3 and Profilin-4 proteins and transcripts have only been detected in the testes and kidneys (Behnen et al., 2009; Fagerberg et al., 2014; Mouneimne et al., 2012; Witke, 2004; Witke et al., 1998). Profilin-1 is the best characterized isoform, which is a ligand for over 128 binding partners and plays roles in actin assembly, nuclear transport and DNA repair, phase-separation, and as a tumor suppressor in diverse cancers (Burke et al., 2014; Henty-Ridilla and Goode, 2015; Hurst and Welch, 2011; Percipalle and Vartiainen, 2019; Posey et al., 2018; Rotty et al., 2015; Stüven et al., 2003; Suarez et al., 2015; Suarez and Kovar, 2016; Wittenmayer et al., 2004).

Finally, while much focus (> 2000 research articles) has illuminated the role of Profilin isoforms with regard to cellular processes that require proper actin dynamics for function, evidence suggests Profilin isoforms can regulate properties of microtubule dynamics through

direct and indirect mechanisms in cells and reconstituted assays (see below) (Henty-Ridilla et al., 2017; Holzinger et al., 2000; Nejedla et al., 2016; Witke et al., 1998). Both Profilin-1 and Profilin-2 have been localized to microtubule structures in cells. Profilin-2 has been found on mitotic spindles and asters during mitosis, and Profilin-1 remains present on the sides of microtubules in mouse melanoma cells following cytoplasmic extraction (Di Nardo et al., 2000; Nejedla et al., 2016). Together these findings suggest an intriguing and emerging role for Profilin isoforms in regulating actin-microtubule crosstalk mechanisms (Henty-Ridilla et al., 2017; Holzinger et al., 2000; Pinto-Costa and Sousa, 2019; Wu et al., 2012). Such interactions and isoform differences may ultimately dictate a protective or malignant function in many pathologies including allergies, cardiovascular disease, Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease, Alzheimer's Disease, diabetes, and lung, breast, and colorectal cancers (Ali et al., 2016; Alvarado et al., 2014; Bae et al., 2009; Boopathy et al., 2015; Burnett et al., 2008; Caglayan et al., 2010; Chakraborty et al., 2014; Coumans et al., 2014; Hauser et al., 2010; Henty-Ridilla et al., 2017; Horrevoets, 2007; Janke et al., 2000; Kim et al., 2015; Kooij et al., 2016; Lee et al., 2005; Roy and Jacobson, 2004; Santos and Van Ree, 2011; Shao et al., 2008; Tang et al., 2015; Wittenmayer et al., 2004; Wu et al., 2012; Zoidakis et al., 2012).

3. BIOCHEMISTRY OF PROFILIN IN ACTIN AND MICROTUBULE DYNAMICS

Competitive interactions underpin all biological processes ranging from individual molecules to ecological scales. Profilin is a champion of navigating the biochemical competition to ultimately regulate actin and microtubule dynamics, lipid and receptor interactions at the membrane, and diverse aspects of cancer signaling. Attempts to engineer a completely functional tagged version of Profilin to assess interactions between > 100 cellular binding partners or actin monomers have been extremely challenging (Funk et al., 2019; Melak et al., 2017; Plastino and Blanchoin, 2018; Skruber et al., 2018, 2020; Vitriol et al., 2015). Conventional fluorescent tags (~ 30 kDa) are twice as large as Profilin (~ 15 kDa). Tagging Profilin on the C-terminal end disrupts PLP-binding and using the N-terminus or an exposed loop can reduce Profilin-actin binding interactions (Nejedla et al., 2017; Wittenmayer et al., 2000). Direct labeling approaches for detecting single molecules of Profilin require four to five mutations and can result in aggregation (Henty-Ridilla et al., 2017; Vinson et al., 1998). As a consequence, most of what we know about Profilin has been determined from biochemical investigations of purified proteins, rather than cellular observations.

Cellular actin exists in several forms that are generated and maintained by different classes of regulatory proteins: straight filaments, bundles, branched networks, and globular monomers (Blanchoin et al., 2014; Michelot and Drubin, 2011; Svitkina, 2018; Svitkina and Borisy, 1999). Based solely on biochemical principles (the critical concentration for assembly is 0.1 μM), and estimates of the total cellular concentration of actin (~ 20–200 μM) and Profilin (20–200 μM), actin in cells predominately exists as polymerized filaments (Dominguez and Holmes, 2011; Funk et al., 2019; Gordon et al., 1977; Lodish et al., 2000; Pollard, 1986; Pollard et al., 2000). Actin filaments are constantly remodeled, assembled,

destroyed, and recycled to reflect changing cell requirements in response diverse stimuli (Henty-Ridilla et al., 2013; Pollard, 2016). To achieve such actin dynamics, cells maintain a substantial stash of monomeric actin (~ 50–90% of the total actin depending on cell source), buffered by sequestering proteins that include Profilin and Thymosin-β4 (Tβ4) (Carlsson et al., 1977; Dominguez and Holmes, 2011; Pollard et al., 2000; Safer et al., 1991; Skruber et al., 2018). It has been tempting to speculate that most monomeric actin in cells exists in a 1:1 complex with Profilin because the concentration is commonly reported as near equimolar with actin, Profilin effectively out-competes Tβ4 for binding actin monomers, and structurally inhibits new actin filament polymerization (Blanchoin et al., 2014; Carlier et al., 1996; Carlsson et al., 1976b; Cooper et al., 1984; Dominguez and Holmes, 2011; Funk et al., 2019; Goldschmidt-Clermont et al., 1992; Kaiser et al., 1999; Vinson et al., 1998). Although the activities of Profilin sum to an attractive explanation for the requirement of nucleation factors to stimulate actin assembly, the balance between free actin monomers and those bound to Profilin, Tβ4, or other cellular ligands is not well characterized and extremely challenging to determine (Aroush et al., 2017; Henty-Ridilla et al., 2017; Plastino and Blanchoin, 2018; Skruber et al., 2018). Further, the availability of cellular actin monomers also fluctuates due to mechanisms of actin filament turnover and the presence of suites of disassembly proteins that synergize with Profilin to convert actin filaments into a polymerizable or sequestered monomeric state (Aroush et al., 2017; Blanchoin et al., 2000; Blanchoin and Pollard, 1998; Brieher, 2013; Bubb et al., 2003; Didry et al., 1998; Pollard, 2016; Pollard and Borisy, 2003; Pollard et al., 2000; Yarmola et al., 2001).

Profilin has a profound effect on actin polymerization in other ways besides sequestering actin including recharging actin monomers with ATP for new filament assembly, interactions with signaling lipids and proline-rich proteins (Chou and Pollard, 2019; Machesky and Pollard, 1993; Merino et al., 2018; Schlüter et al., 1997). Only two proteins are known to catalyze nucleotide exchange on actin monomers, Cyclase-Associated Protein (CAP) and Profilin. Historically Profilin has been depicted as the key driver of actin monomer recycling because it is able to bind the “aged” ADP-bound actin monomers that are released from depolymerizing actin filaments (Bertling et al., 2007; Goldschmidt-Clermont et al., 1992; Kotila et al., 2018; Mockrin and Korn, 1980; Vinson et al., 1998; Wolven et al., 2000). New evidence suggests that CAP can do this more efficiently than Profilin in mammalian systems, and there are organisms where Profilin is not known to catalyze this nucleotide exchange (Kotila et al., 2018; Ono, 2013). Profilin also has strikingly different affinities for monomers and for the growing ends of polymerizing actin filaments, and this property permits the efficient dissociation of Profilin from the ends of growing filaments (Courtemanche and Pollard, 2013; Funk et al., 2019; Jégou et al., 2011). Profilins have high affinity for binding PIP lipids which are conveniently located in the cellular membranes that polymerizing actin filaments push against to generate forces, create protrusions, and drive endocytosis (Dürre et al., 2018; Lu and Pollard, 2001). Interactions between PIP lipids and Profilin facilitate the release of Profilin-bound actin monomers for actin assembly (Lassing and Lindberg, 1985; Lu and Pollard, 2001; Ostrander et al., 1999; Schlett, 2017). All Profilins are known to bind some form of PIP lipid alone or in complex with actin monomers, with the exact PIP binding site defined by ~ 11 positively charged amino acids (Lu and Pollard, 2001; Sohn et al., 1995; Vinson et al., 1993). It is difficult to compare the

exact affinities of Profilin for various PIP lipids across studies because different experimental methods (i.e., PIP compositions, ionic strength buffers, and wash steps) have been used and each of these variables drastically influences PIP binding (Shirey et al., 2017).

Profilin can reliably recognize and jointly bind, actin monomers and proline-rich motifs (i.e., PLP) present in many cytoskeletal regulatory proteins including: Formins, Ena/VASP, WASP/VCA-domain activators of the Arp2/3 Complex, and Drebrin (Chang et al., 1997; Evangelista, 1997; Evangelista et al., 2002; Ferron et al., 2007; Gertler et al., 1996; Higgs and Pollard, 1999; Mammoto et al., 1998; Miki et al., 1998; Reinhard et al., 1995; Rodal et al., 2003; Suetsugu et al., 1998). The ability to simultaneously bind actin monomers and PLP motifs endows Profilin with strong regulatory powers over actin polymerization and the actin monomer pool. Profilin-Formin interactions are mediated by PLP tracks present in all Formin proteins (Paul et al., 2008). Profilin interactions with specific Formin PLP tracks can strongly enhance the elongation and nucleation phases of actin assembly with micromolar to millimolar affinities (Chang et al., 1997; Courtemanche, 2018; Funk et al., 2019; Horan et al., 2018; Paul and Pollard, 2008; Perelroizen et al., 1994; Petrella et al., 1996; Sagot et al., 2002; Sherer et al., 2018; Watanabe et al., 1997; Zweifel and Courtemanche, 2020). The number and lengths of PLP tracks vary with each Formin, but each track competes to bind Profilin-bound actin monomers which ultimately increases the probability and speed actin monomers will be added to the growing actin filament in the correct orientation (Courtemanche and Pollard, 2012; Horan et al., 2018; Sherer et al., 2018; Zweifel and Courtemanche, 2020). Profilin-bound actin monomers hinder branched actin filament assembly mediated by the Arp2/3 Complex which prefers unbound actin monomers for assembly (Burke et al., 2014; Mullins et al., 1998; Rodal et al., 2003; Rotty et al., 2015; Skruber et al., 2018, 2020; Suarez et al., 2015; Suarez and, Kovar, 2016). Contrary to this mechanism, actin assembly mediated by Formin proteins requires Profilin and is significantly enhanced with Profilin-bound actin monomers (Burke et al., 2014; Chang et al., 1997; Evangelista et al., 2002; Funk et al., 2019; Henty-Ridilla and Goode, 2015; Kovar et al., 2003; Neidt et al., 2009; Romero et al., 2004; Rotty et al., 2015; Skruber et al., 2018, 2020; Suarez et al., 2015; Suarez and Kovar, 2016). Thus, competition between different actin nucleation systems has led the popular idea that Profilin tunes specific forms of actin assembly depending on the concentration of *active* nucleation proteins present (Rotty et al., 2015; Skruber et al., 2020; Suarez et al., 2015). Profilin-Formin isoform pairs in worms can further tune these activities (Neidt et al., 2009), which may have important implications in systems with higher numbers of Formin and Profilin isoforms present.

While much attention has focused on the role of Profilin in regulating actin dynamics, Profilin is also capable of regulating microtubule polymers and actin-microtubule crosstalk. In one of the first comprehensive studies comparing Profilin isoforms, tubulin and microtubule-associated proteins were first identified as ligands of Profilin-1 and Profilin-2 from affinity chromatography of mouse brain extracts (Witke et al., 1998). Profilin directly binds to microtubule sides (K_D = ~ 11 μM) through specific amino acids in sites adjacent to the actin-binding surface on Profilin, and this microtubule binding activity is sensitive to the presence of actin monomers when both cytoskeletal elements are present in equal concentrations (Henty-Ridilla et al., 2017). In cells, Profilin resides on spindle and astral

microtubules during mitosis and influences microtubule dynamics (Di Nardo et al., 2000; Henty-Ridilla et al., 2017; Nejedla et al., 2016). Some microtubule effects may be indirectly mediated through interactions between Profilin and Formin proteins that can also bind to microtubules (Bender et al., 2014; Nejedla et al., 2016; Pinto-Costa and Sousa, 2019; Szikora et al., 2017). At present there is not a simple assay to assess whether endogenous Profilin influences microtubule dynamics through direct mechanisms in cells. However, based on biochemical observations, cellular concentrations, estimates of the size of the Profilin-bound actin monomer pool, and relevant protein affinities, it is very likely that a pool of free “unbound” Profilin exists in the cytoplasm of mammalian cells and is available to bind microtubules and additional ligands at physiological concentrations (Fig. 3) (Henty-Ridilla et al., 2017; Henty-Ridilla and Goode, 2015; Plastino and Blanchoin, 2018).

4. ROLE OF PROFILIN ISOFORMS IN CANCER

Humans have four Profilin isoforms, with Profilin-1 commonly accepted as is the most ubiquitous and abundant isoform in almost all tissues and cell types (Fig. 4A) (Behnen et al., 2009; Fagerberg et al., 2014; Mouneimne et al., 2012; Witke, 2004; Witke et al., 1998). Thus, the majority of cellular and biochemical studies have focused on the activities of Profilin-1. Profilin-3 transcripts are virtually absent from all tissues except kidneys where transcripts are 83-fold less abundant than Profilin-1 (Fig. 4A). Profilin-4 transcripts are more abundant than Profilin-3 across tissues except kidneys, but are still much less abundant than either Profilin-1 or Profilin-2 isoforms (Fig. 4A). The only known location where Profilin-1 is not the most predominate isoform is in neuronal-derived tissues and cells. Here, Profilin-2 proteins and transcripts have been measured ~ 5-fold more abundant than Profilin-1, although the exact mechanisms that underlie this distinct distribution are still not fully elucidated (Fig. 4A) (Gareus et al., 2006; Mouneimne et al., 2012; Witke et al., 1998). There are two alternatively spliced versions of Profilin-2 (designated 2a and 2b) differing by nine amino acids in the C-terminal region and an extended patch of aromatic resides (Gieselmann et al., 2008; Lambrechts et al., 1997; Nodelman et al., 1999)Both. splice variants of Profilin-2 have similar affinities for actin but differ in binding other ligands (Nodelman et al., 1999; Witke et al., 1998). Profilin-2a is the predominant form, whereas Profilin-2b is restricted to very limited tissues (Lambrechts et al., 2006). While Profilin-1 and Profilin-2 have similar effects on many biochemical properties pertaining to actin dynamics including nucleotide exchange and binding phosphatidylinositol (PIP) lipids, Profilin-2 has a five-fold lower binding affinity for actin monomers and has higher affinity for EVL and VASP PLP-containing ligands (Gieselmann et al., 2008; Mouneimne et al., 2012). Thus far, Profilin-2 has been studied as a regulator of actin dynamics in PLP ligand binding assays.

Several studies have suggested the intriguing and controversial idea that perturbations to Profilin-1 and Profilin-2 have opposing phenotypes in several cancers (Baraniskin et al., 2012; Cui et al., 2016; Janke et al., 2000; Mouneimne et al., 2012; Wittenmayer et al., 2004; Zhang et al., 2018; Zoidakis et al., 2012). In short, elevated levels of Profilin-1 are correlated with a tumor suppressive effect and reduced metastasis in breast, lung, colorectal, bladder, esophageal, and thyroid cancers, whereas elevated levels of Profilin-2 produce higher metastatic behaviors (Janke et al., 2000; Jiang et al., 2017; Mouneimne et al., 2012; Wittenmayer et al., 2004; Zhang et al., 2018; Zou et al., 2007). There are inconsistencies to

this dichotomy, however, and no satisfying explanation reconciling the differences between these studies has been reached beyond differences arising from tissue-derived or cell line specific phenotypes and sample sizes used.

To attempt to clarify whether Profilin isoform levels are correlated with tumor development and/or metastasis across diverse cancers, we analyzed all RNAseq transcript data in "Projects" that contained normal tissue and primary tumor samples from cancer patients currently available in the National Cancer Institute Genomic Data Commons (https://portal.gdc.cancer.gov; Grossman et al., 2016) (Fig. 4). In most of the tissues examined (i.e., bladder, breast, esophagus, head and neck squamous cell (HNSC), lung, prostate, thymus, and uterus), both Profilin-1 and Profiln-2 transcript levels were elevated in primary tumors compared to normal tissues (Fig. 4B and C). The second most common trend observed was that primary tumor transcripts displayed decreased Profilin-1 transcripts and elevated Profilin-2 transcripts compared to normal tissues (i.e., adrenal gland, cervix, colon, pancreas, and skin) (Fig. 4B and C). The only measurement where both Profilin isoforms decreased was in stomach primary tumors (Fig. 4B and C). We did not observe any transcript-based trends between Profilin isoforms for metastatic and reoccurring tumors, although this data was much less abundant and not available for all the tissues investigated above. The most spectacular observation was in brain tumors—in normal tissue Profilin-2 transcripts are ~ 5-fold more abundant than Profilin-1; however, in primary tumors Profilin-1 transcripts outnumber Profilin-2 by 2.7-fold (Fig. 4B and C). Combined this results in an over 12-fold relative increase Profilin-1 transcripts in brain tumors!

These observations and previous studies may raise the exciting possibility that cellular ratios of Profilin-1 and Profilin-2 underlie the behavioral differences between cancers (Fig. 4D). In general terms the ratio of Profilin-1 to Profilin-2 transcripts in primary tumors decreases (Fig. 4D), but this is not simply the result of a change in Profilin-1 transcripts (i.e., Profilin-1 transcripts can be reduced, Profilin-2 transcripts can become elevated, or combination of both may occur) (Fig. 4B and C). We also explored whether patient survival was connected to changes in Profilin transcripts or ratios from this data, but no specific correlation was observed. However, this observation has several confounding factors that should be considered including: the similarities and differences in patient treatment plans, age, gender, and progression at diagnosis. Finally, while transcript levels are relatively easy to obtain or measure, they do not always reflect the amount of protein present and available for cellular activities, particularly those associated with regulating the cytoskeleton. Future studies will likely have to measure these parameters in specific cell types and circumstances.

5. PROFILIN IN CELL DIVISION

Cancer in the most basic sense is disordered or uncontrolled cell division instigating changes in the rate cells divide, the activity of cell cycle regulators and signals, or inhibition of normal cell maintenance/death. In a cancer-free context, the details underling the dynamics of the mitotic spindle have been the subject of intense scrutiny for literally hundreds of years (McIntosh and Hays, 2016). Changes in cell architecture supported by the actin and microtubule cytoskeletons is a normal requirement to progress through the cell cycle. Some of the most dramatic cytoskeletal reorganizations are triggered by Cyclin complexes which

alter the dynamics of motor proteins organizing the mitotic spindle, Rho GTPases, and proteins that facilitate actin-microtubule interactions (Blangy et al., 1995; Böttcher et al., 2009; Jiang et al., 2015; Kita et al., 2019; Miller, 2011; Plessner et al., 2019; Pollard and Wu, 2010; Ubersax et al., 2003; Yamashiro et al., 1991). Much information that clarifies the roles for microtubules and many signaling factors is known, however functions of actin (and regulatory partners) in cell division have been more elusive and are just starting to emerge. For example, many tools used for visualizing cytoskeletal proteins in mitotic spindles were considered technically limiting and challenging for actin filaments, despite plentiful descriptions of its presence associated with the mitotic spindle (Cande et al., 1977; Gawadi, 1971; Herman and Pollard, 1979; Sanger, 1975). While the classic and most characterized roles for actin in cell division pertain to generating the forces required for cytokinesis, additional studies demonstrate that actin is important for positioning the spindle and spindle pole separation (Miller, 2011; Pelham and Chang, 2002; Rosenblatt et al., 2004; Théry et al., 2005; Toyoshima and Nishida, 2007; Watanabe et al., 2008), extensively reviewed: (Pollard and O'Shaughnessy, 2019). Recent observations show that there are dynamic populations of actin and actin-microtubule-associated structures localized to the mitotic spindle, and these structures reorganize as cells advance through the mitotic phase (Kita et al., 2019; Plessner et al., 2019). Actin nucleation proteins, microtubule-associated proteins, and Profilin are proposed mediators of these dynamics, but the detailed mechanisms of how they may go wrong in cancer are not clear (Henty-Ridilla et al., 2016, 2017; Ishizaki et al., 2001; Kita et al., 2019; Nejedla et al., 2016; Plessner et al., 2019; Roth-Johnson et al., 2014; Wade, 2007).

The role for Profilin in the cell cycle has been difficult to discern, complicated by its affinity for a plethora of signaling molecules and actin and microtubule regulation proteins. Classic genetics has demonstrated that Profilin-1 is essential for generating the cytokinetic ring, cell survival, and division in many organisms (Chang et al., 1997; Kandasamy et al., 2002; Kovar et al., 2003; Polet et al., 2006; Severson et al., 2002; Vidali et al., 2007; Witke et al., 2001). Further, in systems where multiple isoforms of Profilin are available, the loss of the most ubiquitous Profilin cannot be fully rescued for cell cycle effects with the other isoforms (Polet al., et al., 2006; Witke et al., 2001). Specialized mouse cells lacking Profilin-1 displayed morphological defects and aberrant actin filament distributions but were able to complete mitosis albeit on a slower timescale than normal cells (Böttcher et al., 2009). These phenotypes were unable to be rescued with Profilin point mutants deficient for binding actin or Formin proteins. These results may indicate cell dependent differences in Profilin function but may suggest the involvement of actin-independent functions of Profilin.

In addition to organizing microtubules, centrosomes organize a network of actin filaments generated by the Arp2/3 complex. Intriguingly, increasing density or crosslinking of actin filaments correlates with a reduction in microtubules in vitro and at centrosomes in cells (Colin et al., 2018; Farina et al., 2016, 2019; Inoue et al., 2019; Ricketts et al., 2019). Hence, the centrosome is a coordinator of actin and microtubules, and in light of biochemical studies elucidating the role of Profilin with actin nucleation promoting factors and microtubules, this relationship may be indirectly regulated through Profilin (Burke et al., 2014; Funk et al., 2019; Rotty et al., 2015; Skruber et al., 2020; Suarez et al., 2015). In sum, the complete functions of Profilin in cell division remain unclear. Some roles are likely directly related to Arp2/3- or Formin-mediated actin filament assembly or in regulating

microtubule dynamics, and some may go beyond including generating the forces for cytokinesis (Chang et al., 1997; Kita et al., 2019; Kovar et al., 2003; Nejedla et al., 2016; Plessner et al., 2019; Severson et al., 2002).

6. PROFILIN IN CELL MOTILITY AND METASTASIS

The migration of cells is a complex biological process that requires the reorganization of actin, microtubules, membrane receptors, lipids, and the cell-matrix. The loss of proper cell migration has profound effects on neuronal pathfinding, development, wound healing, and overactive migration is a classic hallmark of metastasis and ultimately responsible for distributing tumorigenic cells to sites in the body where they do not normally belong. Cells initiate movements by extending lamellar membrane protrusions driven by physical forces produced by assembling actin filaments that push on the membrane surface. Traction is produced from directionally elongated focal adhesion sites and actin stress fibers to propel the cell forward. Meanwhile contractile forces produced by actin-myosin stress fibers retract the back of the cell as it advances onward (Pollard and Borisy, 2003). Less is known about microtubules or crosstalk between actin and microtubules in this process, however pharmacological evidence demonstrates that perturbing either system alters motile behaviors (Coles and Bradke, 2015; Dogterom and Koenderink, 2019; Etienne-Manneville, 2004; Rodriguez et al., 2003). There are also numerous connections between microtubules and focal adhesion complexes as well as integrin-based extracellular matrix adhesions (Borisy et al., 2016; Bouchet and Akhmanova, 2017; Bouchet et al., 2016; Dziezanowski et al., 1980; Euteneuer and Schliwa, 1984; Kaverina and Straube, 2011; Kaverina et al., 1998; Rodionov et al., 1998; Wittenmayer et al., 2004; Wittmann and Waterman-Storer, 2001).

Motility can be recapitulated on a bead in vitro or in genetically tractable motile organisms like *Listeria* from a core set of proteins including actin, the Arp2/3 Complex, Capping Protein, Cofilin, and Profilin (Loisel et al., 1999; Pantaloni et al., 2001; Pollard and Borisy, 2003; Theriot et al., 1992, 1994; Tilney et al., 1992; Wiesner et al., 2003). Actin filaments at the leading edge are mostly formed by the Arp2/3 Complex oriented with the faster growing end oriented outward (Pollard and Borisy, 2003; Rouiller et al., 2008; Small, 1988; Small and Celis, 1978; Svitkina, 2018; Svitkina and Borisy, 1999; Svitkina et al., 2003; Symons and Mitchison, 1991). Growing filaments are capped relatively quickly at short lengths and are therefore mechanically suited to generate/sustain sufficient forces to propel the cell forward (Akin et al., 2008; Blanchoin et al., 2000; Mogilner and Oster, 1996). Biochemical evidence demonstrates that the presence of Profilin-bound actin inhibits branched actin assembly, that Profilin is required for nucleotide exchange to assemble new actin filaments, and that amounts of free Profilin can compete for the faster growing end of actin filaments with Capping Proteins, Formins, and other ligands (Bubb et al., 2003; Cooper et al., 1984; Dos Remedios et al., 2003; Kaiser et al., 1999; Mockrin and Korn, 1980; Mullins et al., 1998; Pernier et al., 2016; Rotty et al., 2015; Skruber et al., 2020; Suarez et al., 2015; Vinson et al., 1998). There are still many questions that underlie the behavior of actin and microtubule networks at the leading edge of crawling cells. How do cells assemble and rearrange cytoskeletal polymers so quickly? Why do cells expend so much energy incessantly constructing and disassembling these proteins? What detailed roles do the five

minimal proteins perform in cells, can they be visualized, and how do they go awry in disease?

7. CELL SIGNALS CONVERGING ON PROFILIN

Many interconnected signaling pathways and feedback loops contribute to cell homeostasis and respond in disease, particularly in cancer. This labyrinth of signals commonly challenges the development of therapeutics, especially when target molecules exhibit high sensitivity to a diversity of ligands spanning pathways on very rapid timescales. Several signaling pathways converge on Profilin in cancer (Fig. 5), however whether or not these pathways use Profilin in its roles as a regulator of the cytoskeleton are not always clear. In addition, specific modifications to Profilin (usually phosphorylation) directly impact actin dynamics, but the identity and timing of signals and kinases regulating these modifications in disease remain unknown.

The Transforming Growth Factor Beta (TGFβ) pathway is essential in development, regulating cell growth and differentiation, apoptosis, and is a common place where signals go astray in diverse cancers, ultimately driving the epithelial to mesenchymal transition (EMT) of cancer cells and permitting invasive migratory behaviors (Moustakas and Heldin, 2008). TGFβ ligands bind cell receptors which recruit and phosphorylate signal transducing transcription factors (SMADs) to mediate downstream responses. While Profilin-1 has no reported effect on TGFβ signals, increased Profilin-2 protein correlates with SMAD2/3 signals, reducing Profilin-2 or SMAD2/3 levels correlated with tumor suppression in mice, and an early spike in TGFβ activity in a luciferase assay was reduced (Tang et al., 2015). Additional analysis revealed a downstream cytoplasmic interaction between Profilin-2 with HDAC1 that further reinforces SMAD nuclear activities by inhibiting HDAC1 (Fig. 5A) (Tang et al., 2015). The P13K/AKT/mTOR (Phosphatidylinositol 3-kinase/Protein Kinase B/mammalian Target of Rapamycin) intracellular pathway is one of the most commonly mutated in cancer (Melamed et al., 2019; Paplomata and O'Regan, 2014). The overexpression of Profilin-2 in head and neck cancer cell lines increased cell proliferation, and these cells displayed higher levels of phosphorylation for AKT and downstream effectors including β-catenin (Zhou et al., 2019). Therefore, Profilins are an important link in the web of cancer signaling pathways that require the cytoskeleton for function.

Protein phosphorylation is widely used to regulate biological functions. Phosphorylation is the only known category of post-translational modification of Profilin, and it can occur at several amino acid sites to regulate actin-based activities (Alkam et al., 2017). In cells, Profilin-1 can be targeted for phosphorylation by Protein Kinase C (PKC) at S137 downstream of P13K signals, and also by Rho-associated Kinase-1 (ROCK1) downstream of GTP-signals, and is linked to promoting metastasis and invasion in breast cancer cells (Fig. 5B) (Hansson et al., 1988; Rizwani et al., 2014; Sathish et al., 2004; Shao et al., 2008; Singh et al., 1996; Yang et al., 2017; Yao et al., 2014). In biochemical assays phosphomimetic mutations at this site indicate that actin monomers do not bind this modification of Profilin as efficiently compared to wild-type versions (Shao et al., 2008). Profilin-1 can also be phosphorylated at Y129, which is present in the actin binding site of the protein and unsurprisingly reduces the binding capacity of Profilin-1 for monomeric actin (Fan et al.,

2012). In cells this modification occurs by Src kinase initiated through a Vascular Endothelial Growth Factor Receptor Kinase 2 (VEGFR2) signaling cascade (Fan et al., 2012; Simons and Schwartz, 2012). The Y129 phosphorylation has also been linked to the progression of glioblastoma by forming a complex with the Von Hippel-Lindau protein that prevents the degradation of hypoxia induced factor 1 alpha (HIF-1α), ultimately driving the vascularization of tumors and cancer progression (Fig. 5C) (Fan et al., 2014). The impacts of phosphorylation on Profilin-2 have not been as extensively investigated. Using an in silico approach, 14 potential phosphorylation sites on the Profilin-2a protein have been identified, seven of which were biochemically characterized: Y29, S71, S76, Y78, S129, Y133, and S137 (Walter et al., 2020). Phosphorylation of Profilin-2 at S71, S76, or S129 disrupted actin-binding activities, and intriguingly phosphorylation of S76 was able to stimulate the elongation phase of actin polymerization (Walter et al., 2020). Lastly, to use post-translational modifications as effective molecular switches, the cellular balance between the phosphorylated and dephosphorylated states of Profilin must be maintained. To date Protein Phosphatase-1 (PP1) is the only known kinase to effectively dephosphorylate Profilin-1, specific to amino acid S137 (Shao and Diamond, 2012). Whether PP1 can perform this role at other phosphorylation sites in Profilin-1 or Profilin-2 or if other kinases contribute to these functions has not been fully explored.

8. PROFILIN IN IMMUNE SYSTEM RESPONSES

How do cancer cells avoid detection or eradication by the immune system? Can targeting the host immune responses contribute to better treatment outcomes? The presence of inflammatory immune cells in human tumors and innate (receptor-ligand interactions) and adaptive immune responses (phagocytic macrophages) contribute to the progression of cancer by inducing immunosuppression, stimulating cancer proliferation and metastasis (Palucka and Coussens, 2016). Reports have investigated the contribution of Profilin in the innate and adaptive immune responses elicited by different microbes, but these responses in the context of cancer or cancer recovery have not been explored. In the human adaptive immune system, actin and microtubules are essential for migration, phagocytosis, cell secretion, and cell-cell interactions (Mostowy and Shenoy, 2015; Pfajfer et al., 2018; Wickramarachchi et al., 2010). Although only disruptions to actin dynamics were investigated, cells without Profilin-1 or Profilin-2 fail to perform actin-microtubule mediated phagocytosis in macrophages (Coppolino et al., 2002; Kim et al., 2012). In Cytotoxic T Lymphocytes (CTLs) Profilin negatively regulates the exocytosis of lytic granules, which may suggest an enhanced ability to both eliminate tumor cells and increase the migration and invasion of these “helpful” immune cells (Schoppmeyer et al., 2017). In other immune cells (e.g., dendritic cells, neutrophils) Profilin-1 protein levels were higher than cancer cell lines (HT1080 and B16F10) and Profilin-2 was only detected in the dendritic cell line used (Funk et al., 2019). Presumably these findings suggest a role for Profilin in ameboid migratory behaviors controlled by the actin and microtubule cytoskeletons but the exact mechanisms have not been fully elucidated (Lämmermann and Sixt, 2009).

9. TARGETING PROFILIN AS A CANCER THERAPEUTIC

The timing of the cell cycle, the morphology of cells and tissues, and the direction and speed of cellular movements are essential processes regulated by the broad actions of cellular actin and microtubule dynamics. As a consequence, compounds (synthetic and natural) that disrupt cytoskeletal dynamics are among the most widely utilized chemotherapeutics available. These properties also cause treatments to be extremely toxic to patients. With the critical nature of microtubules in cells, drugs that target microtubule dynamics are some of the most effective therapeutics available (Mukhtar et al., 2014). For example, one of the first compounds that targeted the cytoskeleton to treat cancer was Taxol, which stabilizes microtubules and effectively arrests cell division in breast, ovarian, lung, prostate, blood, and many other cancers (Fife et al., 2014; Weaver, 2014). Existing pharmaceutical agents target actin dynamics (i.e., latrunculins, cytochalasins, jasplakinolides), however these compounds are indiscriminately toxic to numerous organs, cardiac, and muscle function in addition to cancerous tumors (Bonello et al., 2009). The development of compounds that target actin and microtubule regulatory proteins are even more rare, but come with the advantage of a targeting specific features of cytoskeleton dynamics (i.e., cytoskeletal assembly, disassembly, stabilization, turnover, or motor protein dynamics) or potentially actin-microtubule crosstalk. Compounds targeting the microtubule-associated proteins Tau or the kinesin Eg5 are excellent at inducing mitotic arrest and limiting tumor proliferation but frequently fail as a clinical monotherapy due to their acute specificity (Chan et al., 2010; Engelke et al., 2016; Hancock, 2014; Milic et al., 2018; Pan et al., 2017; Smith et al., 2013; Sturgill et al., 2016). Other small-molecule screens targeting actin assembly identified inhibitors for the Arp2/3 complex, N-WASP, Tropomyosins, and Formins, although recent evidence questions the specificity of some of these molecules in cells (Bolger-Munro et al., 2019; Hetrick et al., 2013; Isogai et al., 2015; Nolen et al., 2009; Peterson et al., 2004; Rizvi et al., 2009; Sellers et al., 2020; Stehn et al., 2013).

Profilin may represent an effective therapeutic target to fight diverse cancers due to its roles in cytoskeletal regulation and position in cancer signaling cascades. Intriguingly hyperactive Profilin-1 in signaling pathways can lead to precocious apoptosis and resistance to several chemotherapeutics, while silencing Profilin-1 can reduce tumor growth in vivo (Frantzi et al., 2016; Zou et al., 2010). Small molecule screens have revealed compounds that mitigate breast cancer-induced changes in Profilin expression and migration and two small molecules that prevent the interaction of Profilin with actin monomers have been identified (C1 and C2) (Gau et al., 2018; Joy et al., 2014). In biochemical assays C1 and C2 obstruct Profilin-actin binding at high concentrations (50–100 μM), supporting more total actin polymerization than control assays conducted in their absence (Gau et al., 2018). In cells these compounds slowed endothelial cell migration, proliferation, and inhibited angiogenesis (Gau et al., 2018). Do these small molecules also target the actin binding affinity of other Profilin isoforms and actin? Further biochemical and cellular characterization of C1 and C2 with regard to cancer signaling, phosphorylation state, lipid-binding, or microtubule effects may provide valuable mechanistic insights for using these molecules to treat diseases.

10. TALES FROM DIVERSE MODEL SYSTEMS

Many mechanisms underlying protein function are conserved across evolutionary scales. The natural course of disease can sometimes take a lifetime to manifest (evolutionary lifetime is shorter in some organisms). Model organisms can quickly develop a disease or its symptoms allowing researchers to study links between genetic factors, aberrant protein functions and cellular processes on a much shorter time frame. Studying the diversity of protein homologs could lead us down unexpected paths illuminating new therapeutics or elucidating new molecular connections that can be exploited with new treatment approaches. Profilins are evolutionarily conserved in all forms of life where it has been investigated (including *Archaea*, bacteria, viruses, and eukaryotes) and this provides an exceptional opportunity to take advantage of model organisms to study its role as a regulator of actin and microtubule dynamics in disease.

There is much to learn about cancer not only by observing the differences between normal biology and how normal biology goes wrong, but also from how organisms use the same or similar biology in unique ways. Developmental regimes in the *Drosophila*, zebrafish, and *C. elegans* model systems are similar to cancer progression and metastasis in that they require the same tools for execution: cytoskeletal dynamics, cell migration, and cell division. The simplest mechanism explaining the connection of the development of each these model organisms with cancer is that that the loss or misregulation of Profilin is linked to failures in actin assembly and microtubule dynamics. Intriguingly these dynamics are not restored by other Profilin isoforms in vertebrates and many eukaryotes (Cooley et al., 1992; Kovar et al., 2000; Lai et al., 2008; Müssar et al., 2015; Polet et al., 2006; Reeve et al., 2005; Verheyen and Cooley, 1994; Witke et al., 2001). Profilin is critical for maintaining the mesh of actin bundles keeping *Drosophila* nurse cells (in oocytes) intact (Ghiglione et al., 2018; Verheyen and Cooley, 1994). In addition to reduced viability, flies with reduced Profilin levels possess a weakened microtubule spindle apparatus, a less contractile actomyosin cytokinetic ring, and over-proliferative somatic cells (Giansanti et al., 1998; Giansanti and Fuller, 2012; Shields et al., 2014; Verheyen and Cooley, 1994). Zebrafish and *C. elegans* require Profilin for the growth of neuronal cells, neuronal maturation, myelination, and muscle development (Ehler, 2018; Kooij et al., 2016; Kwak et al., 2013; LeCorgne et al., 2018; Majesky, 2007; Meyers, 2018; Murk et al., 2009; Polet et al., 2006; Roth et al., 1999; Yuan et al., 2018). Profilin from *C. elegans* is required for anterior-posterior establishment, DNA positioning and abscission during mitosis, and to optimally regulate Formin-mediated actin polymerization through specific Formin-Profilin isoform pairs (Davies et al., 2018; Neidt et al., 2009; Panzica et al., 2017; Severson et al., 2002). Zebrafish undergo extensive cell migration phases during development that require key regulators of actin for normal execution. Profilin is required for the completion of gastrulation, endothelial cell proliferation, neural cord development, and establishing heart progenitor cell lineage (Ding et al., 2006; Lai et al., 2008; Yuan et al., 2018). Thus, Profilin-mediated development has historically revealed fresh perspectives for the underlying mechanisms that explain how Profilin goes rogue in cancer from these organisms.

Several viral Profilin homologs effectively bind mammalian actin monomers but with weaker affinity and actin nucleotide exchange (Blasco et al., 1991; Butler-Cole et al., 2007;

Machesky et al., 1994; Moreau et al., 2017, 2020). Neither *Vaccinia* nor *Ectromelia* homologs bind PLP regions (Butler-Cole et al., 2007; Machesky et al., 1994). Intriguingly, *Vaccinia* Profilin binds phosphoinositide (PIP) lipids with higher affinity than human Profilin-1, and Profilin from *Ectromelia* does not bind PIP lipids but directly interacts with other actin regulatory proteins like tropomyosin for function (Butler-Cole et al., 2007; Machesky et al., 1994). *Apicomplexa* are extremely susceptible to actin-polymerizing and depolymerizing agents (Baum et al., 2006; Gordon and Sibley, 2005). *Toxoplasma gondii* Profilin binds and sequesters actin monomers, and loss of *Toxoplasma* Profilin prevents parasite replication and host invasion by disrupting host and parasite actin dynamics (Plattner et al., 2008; Skillman et al., 2012). Genetic, immunological, structural, and cell biological studies have further demonstrated *Toxoplasma* Profilin is important in pathogen-host interactions initiated through interleukins and toll-like receptors from both host and parasite (Denkers, 2010; Kucera et al., 2010; Plattner et al., 2008; Yarovinsky et al., 2005). Similar observations converging on Profilin and actin dynamics in innate immunity have been investigated in chytrid fungi and plants (Babik et al., 2014; Cao et al., 2016; Qiao et al., 2019; Sun et al., 2018). Thus, actin regulation by Profilin proteins has been important in the "evolutionary arms race" between hosts and diverse microbes.

The role of cilia in cancer signaling and cell cycle regulation with regard to the microtubule cytoskeleton has been studied extensively (Fabbri et al., 2020; Goetz and Anderson, 2010; Golemis et al., 2018; Higgins et al., 2019). Emerging evidence demonstrating the involvement of actin in ciliary formation and development from the tractable model system *Chlamydomonas reinhardtii* demonstrate important new forms of ciliary regulation that challenge long-held ideas suggesting actin or actin-microtubule crosstalk is necessary for normal ciliary assembly, motility, and signaling. *Chlamydomonas* is an excellent model system for studying the duality of the actin and microtubule cytoskeletons in disease. It features two easily accessible cilia that behave and are regulated by mechanisms virtually identical to mammalian forms and has yielded important insights into human diseases and developmental disorders including primary ciliary dyskinesia (PCD), polycystic kidney disease (PKD), *situs inversus*, and numerous ciliopathies (Harris, 2001; Pazour and Witman, 2009; Wase et al., 2019). *Chlamydomonas* actin structures depend on specific actin architectures and localizations to execute diverse cell processes (Christensen et al., 2019; Craig et al., 2019; Detmers, 1985; Detmers et al., 1983; Harper et al., 1992; Jack et al., 2019; Kovar et al., 2001; Onishi et al., 2016; Piperno and Luck, 1979; Wilson et al., 1997). This includes cilia which were historically studied with microtubules as the predominant cytoskeletal polymer in ciliary assembly (Avasthi et al., 2014; Jack et al., 2019; Kovar et al., 2001; Tai et al., 1999). *Chlamydomonas* Profilin is found throughout the organism including ciliary structures (Kovar et al., 2001). *Chlamydomonas* Profilin binds actin, can inhibit aspects of Arp2/3-mediated actin assembly and enhance actin assembly through Formins. However, this Profilin is unique—it does not recycle nucleotides on actin monomers, it very potently inhibits spontaneous filament nucleation, it caps the fast-growing ends of actin filaments 5- to 10-fold more efficiently than other homologs, and seems to protect a specific actin isoform (IDA5) from degradation (Christensen et al., 2019; Courtemanche and Pollard, 2013; Kovar et al., 2001; Onishi et al., 2016; Pernier et al., 2016). Competitive interactions between Profilin and diverse actin assembly factors may ultimately dictate timing and

dimensions of assembled actin in these cells. In addition, a single Profilin regulates two very different actin isoforms (IDA5 and NAP1). The occupation of actin filament ends by Profilin may limit the role for Profilin on ciliary microtubules or liberate shared actin-microtubule regulators to orchestrate linked cytoskeletal behaviors. Thus, *Chlamydomonas*, is uniquely situated to elucidate foundation mechanisms concerning the role of Profilin in actin assembly and as a facilitator of actin-microtubule crosstalk.

Yeast model systems are genetic powerhouses that have been indispensable in developing a "parts list" and interactome for many complex pathways and cellular processes. Many foundational studies dissecting Profilin-mediated cytoskeletal dynamics come from yeast, including the discoveries that: Profilin facilitates nucleotide exchange with Srv2/CAP (Amberg et al., 1995; Lila and Drubin, 1997; Ono, 2013; Wolven et al., 2000); Profilin interacts with essential actin assembly factors during cell division (Chang et al., 1997); Profilin synergizes with Formin proteins to promote actin polymerization (Pruyne et al., 2002; Sagot et al., 2002); and that Profilin dictates actin structure (straight or branched filaments) by regulating the actin monomer pool (Suarez and Kovar, 2016). The ability to precisely engineer yeast coupled with a rapid life cycle are unparalleled for dissecting mechanisms in vivo. In one compelling example, the interaction and mechanism of how Profilin binds to PLP motifs was dissected by introducing 87-point mutations into yeast Profilin (Lu and Pollard, 2001). Similar approaches have been used to quickly assess the viability and role of disease-specific Profilin variants in human disease (Figley et al., 2014). Further, yeast-based technologies have been utilized in drug discovery screens, to produce anti-cancer drugs, and personalized cancer therapies (Ferreira et al., 2019).

Profilins and many of their biochemical activities are conserved across evolution—features that provide an exceptional opportunity to employ model organisms in studying the roles of Profilin as a regulator of actin and microtubule dynamics in disease. A major drawback of conventional mammalian systems in such endeavors is the inability to resolve the fine details of cytoskeletal dynamics in vivo by the standard microscopy techniques used in many screens (i.e., interactor, small-molecule therapeutics, localization) and even fewer in living organisms. Traditional single-molecule attempts to visualize individual actin filaments require injecting fluorescently tagged actin polymers or though FRAP/photoconversion methods that track fiducial marks on preformed actin filaments or bundles (Dovas et al., 2011; McGrath et al., 1998; Wang, 1984; Waterman-Storer and Salmon, 1998). Plant model systems (*Arabidopsis* and *Physcomitrella*) may be the only model systems where dynamics of individual actin filaments have been measured in cells due to the presence of sparse cytoskeletal arrays that afford high-resolution on fast scales (ms) (Augustine et al., 2011; Staiger et al., 2009). Other model systems are approaching this resolution for actin and microtubules through creative combinations of traditional approaches (i.e., microinjection, FRAP, photoactivation), super-resolution imaging modalities, genetically-encodable fluorescently-stable single-molecule tags/tools, and gene-editing technologies (Aumeier et al., 2016; Fritzsche et al., 2017; Funk et al., 2019; Huang et al., 2008; Rust et al., 2006; Skruber et al., 2020; Tas et al., 2017; Vignaud et al., 2020; Vitriol et al., 2015).

11. OPEN QUESTIONS AND FUTURE DIRECTIONS

Profilin is a much more complicated and elegant molecule than suggested by its defining role as a sequestering protein. Profilin was first identified in the 1970s and since then thousands of publications have described and defined its mechanisms regulating the actin cytoskeleton. Recent studies suggest that Profilin still has secrets to share regarding how tubulin, microtubules, and cytoskeletal proteins are regulated, how Profilin is uniquely positioned to choreograph the cytoskeleton during essential cell processes, and the snafus that cause diseases including cancer, neurodegeneration, cardiovascular decline, allergies, and many more. We have still not discerned complete molecular mechanisms that connect Profilin to these processes. When it comes to the role of Profilin the details are truly important.

Clearly actin regulation is an essential function of Profilin, but what other roles do Profilin proteins fulfill? Some versions of Profilin contain extended structural regions, and although these regions do not alter Profilin-actin binding, point mutations there correlate with a loss of parasite motility and force generation (Kursula et al., 2008; Moreau et al., 2020; Nodelman et al., 1999; Qiao et al., 2019; Sun et al., 2018). Some structurally distinct eukaryotic Profilins form oligomers (dimers, trimers, and tetramers) that can reduce actin assembly mediated by Formins by reducing the affinity of Profilin for PLP and also obstructing Profilin-actin binding sites along Formin homology domains (Qiao et al., 2019; Sun et al., 2018). Profilin oligomers are also present in mammalian systems and are suggested to play important roles in disease (specifically neurodegeneration and immune responses), although the detailed mechanisms that underlie the physiological function and formation of these higher-order Profilin configurations are far from clear (Babich et al., 1996; Korupolu et al., 2009; Mares-Mejía et al., 2016; Posey et al., 2018).

Does a free Profilin pool exist in cells? Studies have consistently reported similar concentrations of actin and Profilin in diverse cell types and species. While these calculations are appealing to explain Profilin function with regard to actin monomers, they often fail to account for the presence of actin filaments or the affinity of Profilin for other ligands (i.e., Ena/VASP, Formins, activators of the Arp2/3 Complex, microtubules). Thus, with the assumption that 50–90% of cellular actin is polymerized into filaments (Funk et al., 2019; Pollard et al., 2000), at least half the total amount of Profilin may be available for functions beyond binding actin monomers or remaining unbound as a free Profilin pool. Does the presence of Profilin isoforms further complicate these interactions? Human Profilin isoforms 1–3 each bind actin, PIP lipids, and PLP albeit with different affinities, and whether the isoforms can be used interchangeably for these processes is unclear (Behnen et al., 2009; Lambrechts et al., 2006; Michaelsen et al., 2010). Profilin-2 binds PLP residues more strongly, but actin less efficiently, than Profilin-1 (Lu and Pollard, 2001; Vinson et al., 1998). Do these properties translate into more or less proficient Formin-mediated assembly? These interactions might slow Formin-mediated actin polymerization because this version of Profilin doesn’t bind actin well. On the other hand, these circumstances might aid Formin proteins in finding cytoplasmic actin monomers and more efficiently releasing Profilin from the polymerizing actin filament. Additionally, neuronal tissues are known to have more Profilin-2 than Profilin-1 and more tubulin than other tissues in the body (Witke et al.,

1998). Although not explicitly tested, does this suggest that Profilin-2 is a better regulator of microtubule dynamics than Profilin-1? If so, this may further elucidate some of the complex details that underlie how cellular actin and microtubule dynamics are linked. Intriguingly, the testes-specific Profilin-4 isoform does not bind actin or PLP stretches, and mouse studies have demonstrated that each additional isoform of Profilin does not fully rescue the effects of Profilin-1. This suggests that there may be distinct tissue-specific roles for Profilin that go beyond actin assembly and define even more interactions that compete for cellular Profilin (Behnen et al., 2009; Polet et al., 2006; Witke et al., 1998).

Do we really understand the role of Profilin in cancer? Some have tried to bin specific Profilin isoforms as indicators of cancer prognosis with Profilin-1 behaving as a tumor suppressor and Profilin-2 suggesting malignancy, but there are multiple lines of evidence to contradict both of these statements. Data available from one of the most comprehensive databases quantifying RNA transcripts in cancers suggests that Profilin-1 and Profilin-2 RNA transcripts become elevated in most (but not all) cancers (Fig. 4). Is Profilin a good target for chemotherapies or to reinforce for better patient recovery? Regardless, to develop an effective pharmaceutical target we need to understand the exact timescales and mechanisms of Profilin function that are disrupted in disease and whether inhibiting one of them (i.e., actin binding) is enough. What if the role of Profilin goes beyond regulating cytoskeletal dynamics? Some reports suggest Profilin may play mysterious roles unrelated to the cytoskeleton in the nucleus (i.e., nuclear import/export and signaling), however if and how these studies link to cancer has not been elucidated (Holzinger et al., 2000; Lederer et al., 2005; Söderberg et al., 2012; Stüven et al., 2003). Alternatively, perhaps Profilin-facilitated post-translational modification of actin goes askew in cancer, ultimately leading to changes to specific forms of actin assembly and cell migratory behaviors (e.g., more actin N-terminal-acetylation decreases cell migration, and the formation of filopodia and lamellipodial protrusions) (Rebowski et al., 2020). Model organisms are powerful tools in this regard and can help to elucidate new and underexplored roles of Profilin in these and cancer-relevant cell processes.

Finally, an increasing number of recent studies link specific cancers directly to proteins that form or regulate the formation of biomolecular condensates (Chen et al., 2019; Kamagata et al., 2020). Although Profilin does not phase-separate on its own, it is found in many cellular condensates and can regulate their size and dynamics (Ghosh et al., 2019; Molliex et al., 2015; Posey et al., 2018). To date no studies have explored whether Profilin contributes to the pathological role of biomolecular condensates in cancer, however understanding exactly how Profilin regulates these processes could be a valuable asset in the development of cancer pharmaceutical agents and therapies.

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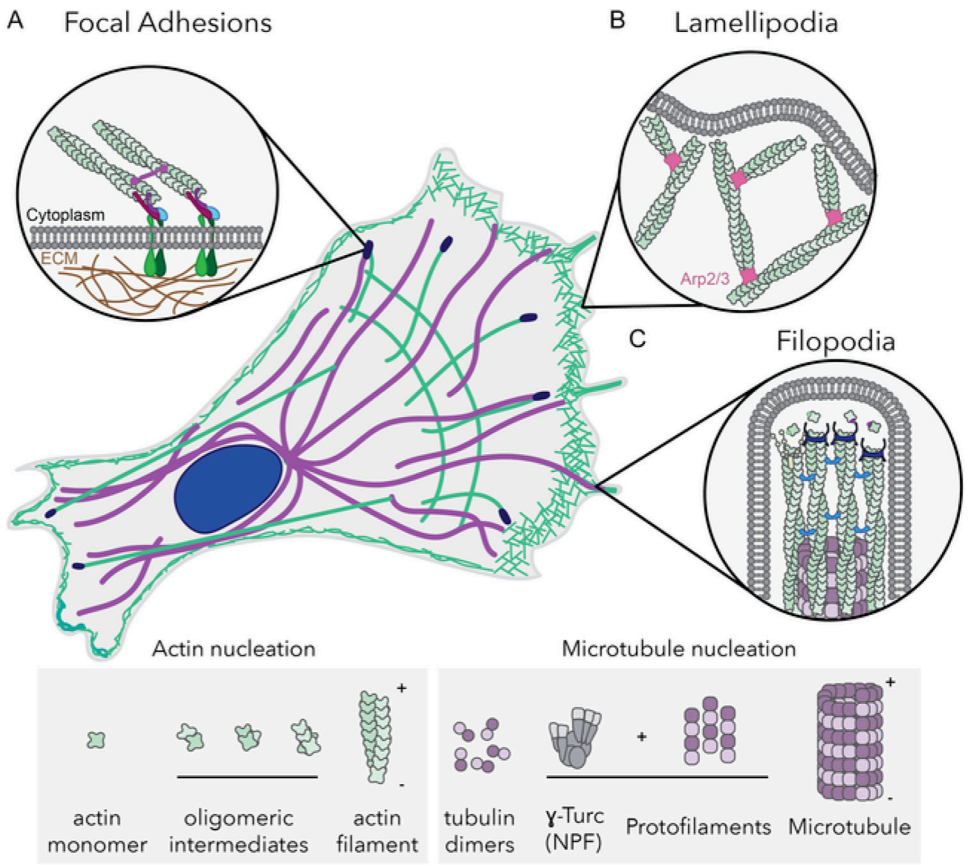
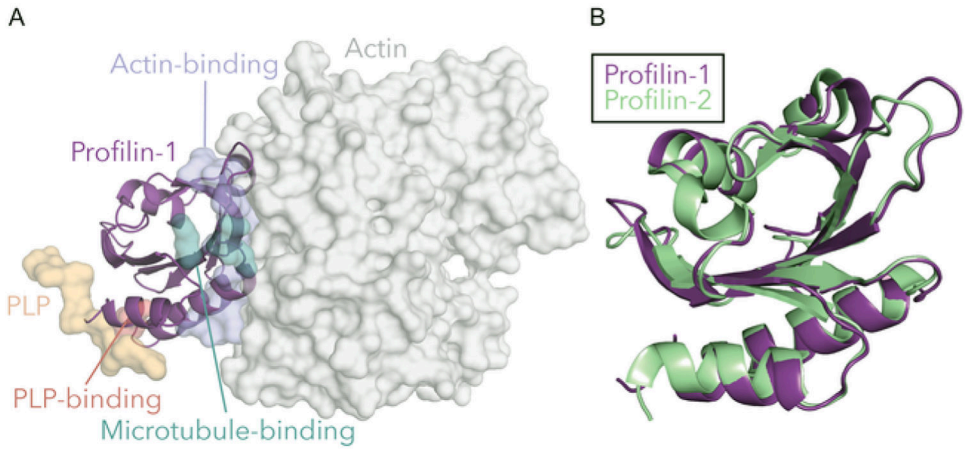


Fig. 1.

Cellular actin and microtubule architectures. Cartoon of actin (green) and microtubule (purple) structures in a motile cell. (A) Diagram of actin contact with focal adhesion proteins including: Integrins (green), Vinculin (purple), Talin (red), and Paxillin (blue). Actin filaments are bundled by α-actinin (magenta). (B) Leading edge lamellipodial actin branched actin structures are generated by the Arp2/3 complex. (C) Formins (navy) and Ena/VASP (yellow) elongate straight filaments bundled by Fascin proteins (cyan) in filopodial structures. Inset: Diagram of nucleation steps for actin and microtubules. NPF, nucleation promoting factor. +, the faster growing end of actin or microtubules. −, the slower growing end of actin or microtubules.



C

	1	2a	2b	3	4
Profilin-1	100	75	78	61	45
Profilin-2a	75	100	95	58	33
Profilin-2b	78	95	100	61	34
Profilin-3	61	58	61	100	38
Profilin-4	45	33	34	38	100

Fig. 2.

Structural Features of Profilin. (A) View of Profilin-1 (purple) interacting with actin (gray) and with the poly-L-proline region of VASP (yellow). Important Profilin residues for interactions with actin (blue), microtubules (teal), and poly-L-proline (PLP) peptides (orange) are highlighted. Structures modeled using PDBIDs 2PAVE and 2BTF (Ferron et al., 2007; Schutt et al., 1993). (B) Profilin-1 (purple) and Profilin-2 (green) structures are very similar (RMSD = 0.3), as evident from the aligned structures. Structures modeled using PDBIDs 1FIK and 1D1J (Nodelman et al., 1999). (C) Table of amino acid similarity from BLASTp alignments for Human Profilin isoforms.

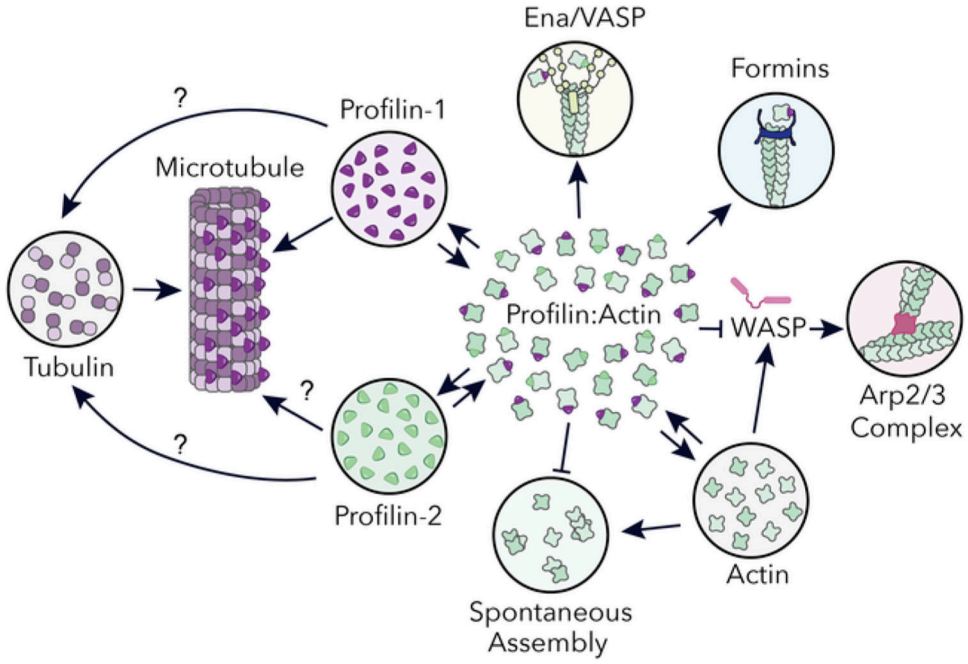


Fig. 3.

Competition for Profilin Between Cellular Ligands Dictate the Types of Cellular Cytoskeletal Structures Formed. Cartoon model for the distribution of Profilin to actin, microtubules, or regulatory ligands (Formins, Ena/VASP, the Arp2/3 Complex). Based on biochemical principles, free Profilin pools likely exist in cells. Direct interactions between isoforms of Profilin and tubulin are hypothesized but not yet directly confirmed (Henty-Ridilla et al., 2017; Nejedla et al., 2016; Pinto-Costa and Sousa, 2019; Witke et al., 1998).

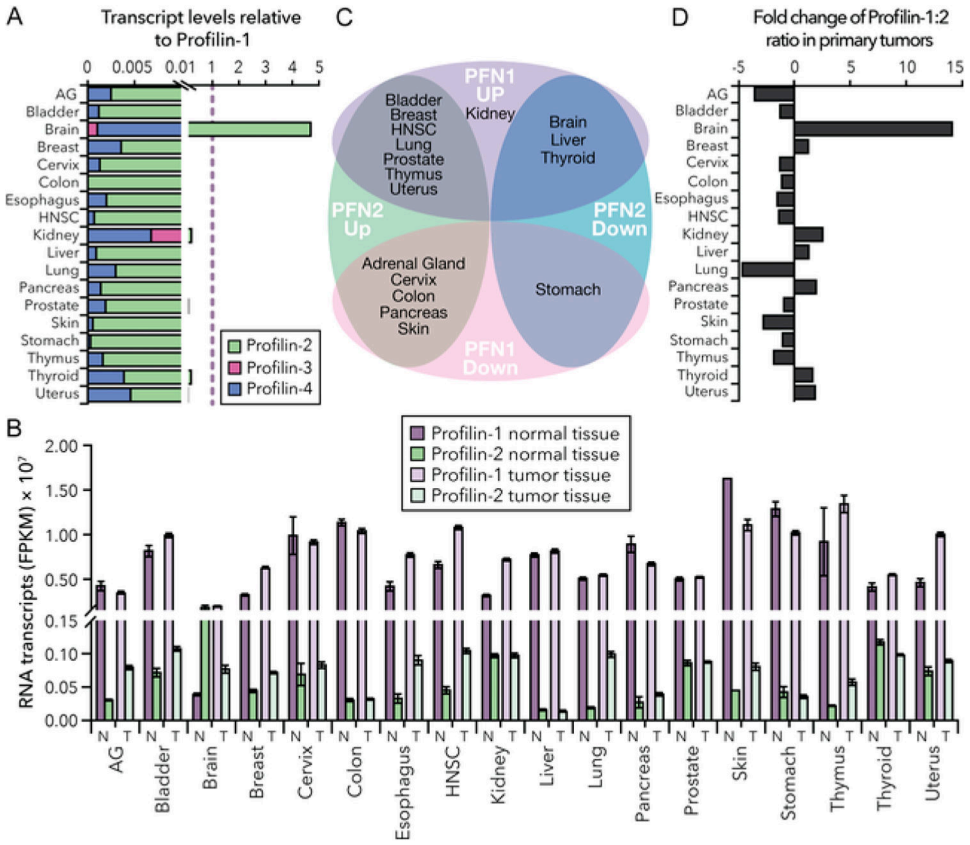


Fig. 4.

Profilin Isoform Transcripts in Normal and Tumor Tissues. (A) Transcript levels for Profilin-2, Profilin-3, and Profilin-4 relative to the most ubiquitous expressed isoform, Profilin-1. We normalized the means of all available RNAseq transcript data in “Projects” that contained normal tissue samples from cancer patients (n = 1–215) currently available in the National Cancer Institute Genomic Data Commons (https://portal.gdc.cancer.gov; Grossman et al., 2016). The purple dotted line represents Profilin-1 levels in each tissue shown. AG, adrenal gland. HNSC, head neck squamous cells. (B) Mean RNA transcripts of Profilin-1 and Profilin-2 obtained from the database in (A) for normal and tumor tissues in fragments per kilobase exon model per million mapped reads (FPKM), (n = 1–1191). Error bars, SEM. (C) A Venn diagram summarizing the changes found in (B) for Profilin-1 (PFN1) and Profilin-2 (PFN2) transcripts in tumors compared to normal tissues. (D) Fold change in the Profilin-1:2 ratio in primary tumors. All data was downloaded and analyzed from https://portal.gdc.cancer.gov on 10 April 2020 (data release 23.0). All transcript data was sorted by tissue source across “Project” databases with the exception of HNSC which was is presented a mix of tissue sources.

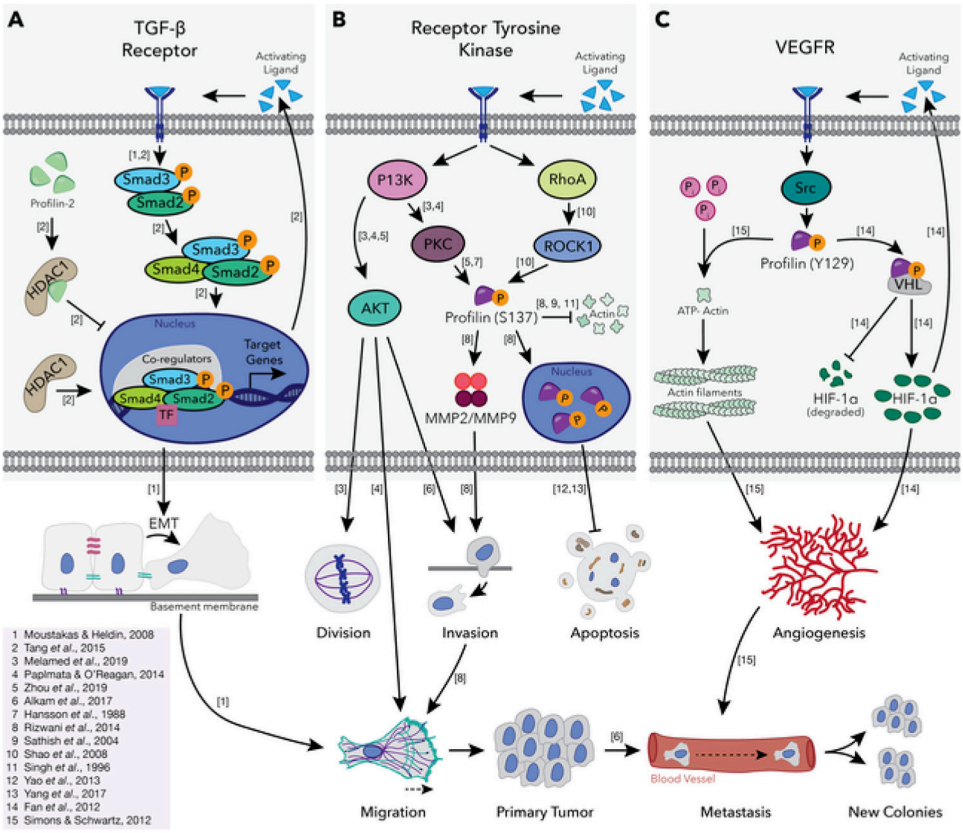


Fig. 5.

Signaling Pathways Converge on Profilin. (A) Upon stimulation by an activating ligand the TGFβ signaling pathway activates Smads which stimulate the epithelial to mesenchymal transition (EMT). Profilin-2 has an inhibitory effect on this pathway by preventing HDAC1 signals to the nucleus. TF, transcription factor. (B) Upon activation by diverse receptor tyrosine kinases the P13K/TOR/AKT pathway ultimately stimulates cell proliferation, invasion, and migration. Phosphorylation of Profilin-1 (S137) loses the ability to bind actin monomers and becomes translocated to the nucleus and inhibits cell death pathways. (C) Activation of the VEGFR pathway leads to the phosphorylation of Profilin-1 (Y129), stimulating angiogenesis. Phosphorylated Profilin-1 (Y129) enhances nucleotide exchange on actin monomers, ultimately increasing overall actin filament polymerization. Numbers present throughout the figure are references (purple box) for particular signaling steps.