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Feisty filaments: actin dynamics in the red blood cell membrane skeleton

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STRUCTURED ABSTRACT

Purpose of review—The purpose of this review is to discuss recent advances and unsolved questions in our understanding of actin filament organization and dynamics in the red blood cell (RBC) membrane skeleton, a two-dimensional quasi-hexagonal network consisting of $(\alpha_1\beta_1)_2$-spectrin tetramers interconnecting short actin filament-based junctional complexes.

Recent findings—In contrast to the long-held view that RBC actin filaments are static structures that do not exchange subunits with the cytosol, RBC actin filaments are dynamic structures that undergo subunit exchange and turnover, as evidenced by monomer incorporation experiments with rhodamine-actin and filament disruption experiments with actin-targeting drugs. The malaria-causing parasite, *Plasmodium falciparum*, co-opts RBC actin dynamics to construct aberrantly branched actin filament networks. Even though RBC actin filaments are dynamic, RBC actin filament lengths are highly uniform (~37 nm). RBC actin filament lengths are thought to be stabilized by the capping proteins, tropomodulin-1 and αβ-adducin, as well as the side-binding protein tropomyosin, present in an equimolar combination of two isoforms, TM5b (Tpm1.9) and TM5NM1 (Tpm3.1).

Summary—New evidence indicates that RBC actin filaments are not simply passive cytolinkers, but rather dynamic structures whose assembly and disassembly play important roles in RBC membrane function.

Keywords

erythrocyte; actin polymerization; spectrin; tropomodulin-1; tropomyosin

INTRODUCTION

The mammalian red blood cell (RBC) is highly specialized, exhibiting characteristic biconcave shape and containing ~360 mg/ml hemoglobin for O_2 delivery and CO_2 clearance [1]. RBCs are highly deformable yet mechanically stable, withstanding large shear stresses in central arteries and traversing capillaries smaller than their diameter in peripheral tissues. RBCs lack intracellular organelles and a transcellular cytoskeleton; instead, the robust

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CONFLICTS OF INTEREST

None.

mechanical properties of RBCs are imparted by the spectrin-actin membrane skeleton, a quasi-hexagonal cytoskeletal network of long and flexible $(\alpha_1\beta_1)_2$-spectrin tetramers interconnecting short actin filament (F-actin) junctional complexes, or "nodes," forming a two-dimensional lattice beneath the bilayer [2,3]. Recent data have challenged the long-standing view of RBC actin filaments as static "spot-welds" passively crosslinking adjacent $(\alpha_1\beta_1)_2$-spectrin tetramers. Here, we explore how the RBC membrane skeleton has served as a model system to understand spectrin-actin network organization and actin dynamics in diverse cell types. We then discuss recent advances in understanding RBC actin dynamics and length regulation in the membrane skeleton.

THE RBC MEMBRANE SKELETON: KEY CONCEPTS

Beneath the RBC plasma membrane, a quasi-hexagonal network of $(\alpha_1\beta_1)_2$-spectrin tetramers interconnects short F-actin nodes, establishing a two-dimensional lattice of "horizontal interactions" [2]. This network is tethered to the bilayer via "vertical interactions" mediated by ankyrin-B, an adaptor protein linking $(\alpha_1\beta_1)_2$-spectrin to Band3 [3], an abundant integral transmembrane protein that forms heteromultimeric complexes with other transmembrane glycoproteins (e.g, glycophorin-C, Rh, Duffy, Kell, XK, Glut1) [4,5]. Although purified RBC $(\alpha_1\beta_1)_2$-spectrin tetramers can extend into linear structures ~190 nm in length *in vitro* [6], the observed end-to-end distance of an $(\alpha_1\beta_1)_2$-spectrin tetramer *in situ* is considerably lower (~35–100 nm) [7–10], forming a folded configuration amenable to extension during RBC deformation (Fig. 1A). End-to-end lengths of $(\alpha_1\beta_1)_2$-spectrin tetramers vary widely due to conformational variability in the α_1- and β_1-spectrin polypeptides [6,11,12], but the F-actin nodes are relatively rigid, with highly uniform lengths of ~37 nm [2], orders of magnitude less than F-actin's persistence length (~17–18 μm) [13,14]. Uniformity of RBC actin filament length enables ~6 $(\alpha_1\beta_1)_2$-spectrin attachments/filament, essential for quasi-hexagonal symmetry [2] (Fig. 1B), but five or seven attachments/filament are occasionally observed in negatively-stained spread membrane skeletons [15,16], suggesting $(\alpha_1\beta_1)_2$-spectrin attachment/detachment events [17]. Unexpectedly, branched $(\alpha_1\beta_1)_2$-spectrin strands, indicative of higher-order oligomers (i.e., hexamers and octamers), associated with bent actin filaments, have also been observed [10], further contributing to the heterogeneity and complexity of the membrane skeleton. Clearly, numerous questions regarding $(\alpha_1\beta_1)_2$-spectrin structure and dynamics in the RBC membrane skeleton still remain [18], but here we focus on the RBC actin filament nodes.

Uniformity of RBC actin filament lengths arises from regulation of actin polymerization and depolymerization by actin-binding proteins. In addition to binding $(\alpha_1\beta_1)_2$-spectrin, RBC actin filaments are stabilized along their lengths by two tropomyosin isoforms, TM5b (Tpm1.9) and TM5NM1 (Tpm3.1), and capped at their pointed and barbed ends by two tropomodulin-1 (Tmod1) molecules and an αβ-adducin heterodimer, respectively [2]. Additional RBC actin-binding proteins include protein 4.1R, which enhances β_1-spectrin-F-actin binding, and dematin (protein 4.9), which bundles F-actin and/or enhances $(\alpha_1\beta_1)_2$-spectrin-F-actin binding [2]. Notably, RBC actin filaments provide additional sites of spectrin-actin network tethering to the bilayer [2,5]. For example, in addition to capping barbed ends, αβ-adducin binds Band3 [19], while protein 4.1R binds glycophorin-C [20,21] and Band3 [22]. Thus, RBC actin filaments can simultaneously form "vertical interactions"

with the membrane via αβ-adducin-Band3, 4.1R-Band3, and 4.1R-glycophorin-C binding in conjunction with "horizontal interactions" via $(\alpha_1\beta_1)_2$-spectrin-F-actin binding. Additional tethering interactions have also been proposed [5].

The architecture of the spectrin-actin membrane skeleton was deciphered in RBCs, but spectrin-actin networks are actually versatile "building blocks" controlling membrane curvature, mechanics, and microdomain formation in diverse cells. For example, Tmod3- and αβ-adducin-capped actin filaments are essential for normal membrane skeleton organization and morphology of polarized epithelial cells [23,24]. A recent study indicates that dynamic rearrangements enable the membrane skeleton to actively "patrol" lateral epithelial membrane domains, inhibiting endocytosis and preventing membrane internalization and loss [25]. F-actin stability in the membrane skeleton is also essential for membrane morphology and physiological function in the hexagonally packed fiber cells of the ocular lens [26], sarcoplasmic reticulum of skeletal muscle [27,28], and demarcation membrane system of megakaryocytes [29,30]. Of note, recent advances in super-resolution fluorescence imaging have enabled identification of an unusual spectrin-actin membrane skeleton in axons of hippocampal neurons [31,32]. In this structure, adducin-capped actin filaments are organized into periodic rings encircling the axonal circumference, with $(\alpha_2\beta_2)_2$-spectrin tetramers connecting successive F-actin rings [31]. Axonal F-actin rings exhibit periodicity of ~180–190 nm [31], almost identical to the length of a fully extended spectrin tetramer [6], suggesting that spectrin tetramers may serve as molecular rulers governing spacing of successive rings. Even with this unconventional layout, the axonal membrane skeleton requires normal F-actin stability to establish its architecture [32], as in RBCs and other aforementioned cell types [23,24,26,27,30,33].

DYNAMIC CHARACTERISTICS OF RBC ACTIN FILAMENTS

A long-standing assumption is that RBC actin filament nodes are static structures that do not undergo subunit exchange with free monomers (Fig. 1C). However, recent data have suggested that this is not the case, i.e., that dynamic actin subunit exchange occurs between the cytosol and actin filaments in the RBC membrane skeleton (Fig. 1D,E). Indeed, actin has been visualized in the RBC cytosol via immunogold labeling and electron microscopy of ultra-thin cryosections of intact RBCs [34]. Early estimates of the cytosolic actin concentration in RBCs yielded ~0.24 μM, based on the ability of actin monomers (G-actin) to inhibit DNAse-I [35]. A more recent estimate of the cytosolic actin concentration in human RBCs yielded ~0.36 μM—50% higher than the previous estimate—based on western blotting of actin in Triton-X-100-extracted membrane skeletons vs. cytosolic fractions [36]. Even with this seemingly high cytosolic actin concentration, the overwhelming majority of human RBC actin (~96.3%) is F-actin in RBC membrane skeleton, while only a small minority (~3.7%) of RBC actin is cytosolic G-actin [36].

The presence of G-actin in the RBC cytosol is necessary, but insufficient, for dynamic actin subunit exchange. The first direct evidence for polymerization and depolymerization of RBC actin filaments came from human RBCs infected with the malaria-causing parasite, *Plasmodium falciparum*, wherein RBC actin filaments are completely disassembled and reassembled into an aberrant dendritic cytosolic network to facilitate export of virulence

factors [34,37]. It is unknown whether *P. falciparum* depolymerizes F-actin throughout the membrane skeleton (Fig. 1D) by exporting parasite-derived F-actin-disassembly factors (e.g., cofilin/ADF [38,39] and/or gelsolin), or by activating RBC-endogenous disassembly factors. The latter scenario seems likelier, as peptides corresponding to F-actin-disassembly factors have been detected by mass spectrometry and proteomic analysis of both cultured human erythroblast-derived reticulocytes [40] and mature human RBCs [41,42]. Interestingly, hemolysates from RBCs with the mutant hemoglobins, HbSC (sickle cell trait hemoglobin) or HbCC constrain actin filament length *in vitro* and inhibit *P. falciparum*-induced RBC actin filament remodeling *in vivo* [34], potentially contributing to these hemoglobins' ability to protect against malaria [43].

Nucleation of dendritic F-actin networks requires Arp2/3 complex [44,45], but, as with the F-actin-disassembly factors described above, it remains unclear whether *P. falciparum* exports Arp2/3 complex into the RBC or co-opts RBC-endogenous Arp2/3 complex [41,42]. The function of RBC-endogenous Arp2/3 complex in normal RBC homeostasis has yet to be demonstrated, but it is likely related to the function of Hem-1, a hematopoietic-cell-specific member of the large pentameric WAVE (Wiskott-Aldrich syndrome verprolin-homologous protein) heterocomplex and Arp2/3 activator, which is present in mature RBC lysates along with other WAVE complex components (WAVE1, WAVE2, and Abi2) [46]. Hem-1-null mice have hemolytic anemia with abnormal RBC shapes, reduced RBC lifespan, and aberrant F-actin and membrane skeleton protein composition, suggesting that Hem-1 is required for RBC membrane skeleton assembly and/or stability [46,47]. Notably, WAVE complex activation of Arp2/3-mediated F-actin assembly depends on WAVE activation by Rac GTPases [48], and Rac1/Rac2 GTPase-deficient mouse RBCs exhibit abnormal shapes, reduced deformability, and membrane skeleton disorganization [49].

Recently, we directly observed RBC actin subunit dynamics by visualization of rhodamine-actin (rho-actin) incorporation into resealed human RBC ghosts. Rho-actin localizes to discrete puncta across the ghost membrane within 30 minutes, consistent with dynamic incorporation of rho-actin subunits into foci or "hotspots" in the membrane skeleton (Fig. 1E) [36]. These foci may represent permanent specialized microdomains within the membrane skeleton or transient structures reflecting a particular structural state of F-actin leading to localized filament assembly/disassembly events. Rho-actin incorporation can be blocked by treatment with 0.5 μM cytochalasin-D (CytoD), an inhibitor of barbed-end subunit exchange (Fig. 2A), indicating that barbed-end exchange mediates rho-actin incorporation into the RBC membrane skeleton [36]. A barbed-end-mediated mechanism for subunit exchange is consistent with a soluble G-actin concentration of ~0.36 μM [36]—halfway between the barbed-end and pointed-end critical concentrations of 0.1 μM and 0.6 μM, respectively [53]. Barbed-end assembly of rho-actin subunits may occur coincidently with pointed-end disassembly of endogenous subunits, resulting in treadmilling [54], or exogenous rho-actin subunits may exchange with endogenous subunits at barbed ends. The numbers of rho-actin foci assembling within 30 minutes were not determined, but visual inspection indicates at least an order of magnitude fewer foci than the 30,000–40,000 actin filaments per RBC [2,36,55]. Thus, only few filaments are dynamic at any instant, or, alternatively, all filaments may be dynamic but only infrequently. Future work will examine appearance, disappearance, kinetics and trajectories of these rho-actin foci by time-lapse

imaging and computational analysis of intact RBCs, using techniques analogous to those developed for fluorescent speckle microscopy of actin filaments in migrating cells [56,57].

In another approach, we labeled F-actin in intact human RBCs with a fluorescent jasplakinolide derivative (SiR-Jasp) [58] and investigated F-actin mobility by fluorescence recovery after photobleaching (FRAP). RBC F-actin has a mobile fraction of ~30% and fluorescence recovery half-time of ~2.5 minutes [36]; however, in this context, "mobility" likely involves both subunit assembly/disassembly (filament-level phenomena) and lateral movements of filaments (network-level phenomena). Inhibiting barbed-end assembly by CytoD treatment reduces the mobile fraction of F-actin from ~30% to ~25% [36], indicating that either ~1/6th of F-actin mobility can be explained by barbed-end assembly/disassembly, and/or that barbed-end assembly influences lateral movements of whole filaments. The mechanism for this crosstalk is unclear, but one possibility is that barbed-end assembly may influence $(\alpha_1\beta_1)_2$-spectrin-F-actin binding, such that barbed-end inhibition by CytoD treatment may alter the architecture of the $(\alpha_1\beta_1)_2$-spectrin-F-actin lattice. Indeed, barbed-end assembly is expected to require transient dissociation of the barbed-end cap, αβ-adducin, which would then weaken $(\alpha_1\beta_1)_2$-spectrin-F-actin interactions, since αβ-adducin recruits $(\alpha_1\beta_1)_2$-spectrin to F-actin [59,60].

Barbed-end regulation is critical for RBC physiology. Mice lacking α- or β-adducin have compensated hemolytic anemia with osmotically fragile, insufficiently deformable, and spherocytic RBCs, similar to hereditary spherocytosis [61–63], but neither RBC actin dynamics nor filament lengths have been studied. However, another barbed-end capping protein (CP, termed EcapZ) translocates from the RBC cytosol to the membrane skeleton in these mice [62,63], where it presumably caps adducin-deficient filament barbed ends [64], suggesting possible mechanisms for α- and β-adducin-null RBC phenotypes. For example, aberrant EcapZ-capped RBC actin filaments may have different dynamic properties than normal αβ-adducin-capped actin filaments [36], and EcapZ likely lacks αβ-adducin's ability to bind Band3 [19] or recruit $(\alpha_1\beta_1)_2$-spectrin to F-actin [59,60]. A role for αβ-adducin in F-actin regulation has also been suggested by analysis of Rac1/Rac2-GTPase-null RBCs, which exhibit increased αβ-adducin phosphorylation, altered $(\alpha_1\beta_1)_2$-spectrin:F-actin ratios, and membrane skeleton disruption [49]. This agrees with *in vitro* observations that phosphorylation of αβ-adducin reduces its ability to cap barbed ends [65] and recruit $(\alpha_1\beta_1)_2$-spectrin to F-actin [65,66].

Dynamic F-actin assembly also contributes to RBC physiology. Treatment of human RBCs with latrunculin-A (LatA), a drug that drives actin depolymerization by sequestering G-actin (Fig. 2B), results in ~2-fold-increased soluble actin, whereas treatment with jasplakinolide (Jasp), a drug that drives actin polymerization by stabilizing F-actin (Fig. 2C), results in ~60%-decreased soluble actin [36]. These effects are consistent with dynamic filaments capable of polymerization and depolymerization. Importantly, disruption of RBC actin filaments with LatA or Jasp significantly alters the RBC membrane mechanics, with both LatA and Jasp treatment increasing the membrane deformability of human RBCs, determined by shorter RBC passage times through microfluidic channels [36]. Surprisingly, LatA and Jasp have differential effects on spontaneous membrane fluctuations ("flickering"), with LatA treatment increasing the variance of flickering amplitudes and Jasp

treatment decreasing the variance [36]. Hence, RBC actin filament polymerization and depolymerization have differential effects on different aspects of RBC membrane mechanics. It is unclear whether the effects of LatA and Jasp on RBC cell mechanics are solely attributable to altered actin filament polymerization and depolymerization, or whether creation or elimination of $(\alpha_1\beta_1)_2$-spectrin attachments secondary to actin filament assembly/disassembly also plays a role.

How might actin dynamics drive RBC mechanical behaviors? Because such a small minority (~3.7%) of RBC actin is cytosolic [36], a ~2-fold increase in soluble actin induced by LatA or a ~60% decrease induced by Jasp does not imply wholesale restructuring of the RBC actin filament network. Rather, LatA- and Jasp-induced polymerization and depolymerization events most likely localize to foci capable of rho-actin incorporation [36], as described above. The classic “Brownian ratchet” theory postulates that actin polymerization near a lipid bilayer can rectify Brownian motion of G-actin and produce directed force against the bilayer [67,68]. Since RBC actin filament subunits exchange via barbed-end dynamics [36], Brownian ratchet forces may be generated where barbed ends within dynamic hotspots face toward the RBC membrane. However, analyses of actin filament orientation within the RBC membrane skeleton [69,70] have not assessed whether barbed or pointed ends are preferentially oriented towards the membrane. In the future, differential fluorescent labeling of barbed vs. pointed filament ends [71] followed by three-dimensional super-resolution imaging [72] of intact cells will enable such an analysis.

LENGTH REGULATION OF RBC ACTIN FILAMENTS

It seems paradoxical that RBC actin filaments can undergo dynamic subunit exchange while maintaining a uniform length of ~37 nm throughout the membrane skeleton. Length is not an intrinsic property of F-actin; when purified actin is polymerized to steady state *in vitro*, actin filaments assume an exponential distribution of lengths, with an abundance of short filaments and fewer long filaments [73,74]. Thus, RBC actin filaments must achieve their uniform *in vivo* lengths through concerted actions of actin-binding proteins. The barbed and pointed ends of RBC actin filaments are capped by αβ-adducin and Tmod1, respectively, which inhibit association and dissociation of actin subunits from their respective filament ends [75,76]. The mechanisms by which actin-capping proteins control filament lengths have primarily been elucidated in striated muscle cells, where actin (thin) filaments form antiparallel arrays within a sarcomere, with barbed ends anchored to Z-lines at the sarcomere periphery and pointed ends demarcating the H-zone at the sarcomere center. Like RBC actin filaments, sarcomeric thin filaments are long-lived cytoskeletal assemblies with precisely regulated and highly uniform lengths and actin-capping proteins at both ends [55]. However, sarcomeric thin filaments are much longer than RBC actin filaments (~1000 nm vs. ~37 nm, respectively [55]), and have barbed and pointed ends in spatial register. Thus, localizations of the barbed and pointed ends of sarcomeric thin filaments, and thin filament lengths, can be visualized and distinguished with conventional fluorescence microscopy.

In rho-actin-injected cardiac myocytes, subunits incorporate at both barbed and pointed thin filament ends [77]. CytoD treatment has no effect on thin filament length [77], indicating that barbed-end stability is dispensable for length regulation. However, overexpression of

GFP-Tmod1 shortens thin filaments [77], while antibody inhibition of Tmod1's pointed-end capping activity elongates thin filaments [78], indicating that pointed-end stability is essential for length regulation, with the extent of pointed-end capping by Tmod1 inversely related to lengths [79]. The extent to which these principles of length regulation extend to RBC actin filaments remains unclear. However, FRAP analyses of sarcomeric thin filaments in cultured muscle cells have identified an F-actin mobile fraction of ~25% and fluorescence recovery occurring within minutes [77,80,81], similar to RBCs [36], indicating similar kinetics of actin mobility despite markedly different cytoskeletal architectures.

To study the role of pointed-end stability in RBC actin filament length regulation, we examined Tmod1-null mice, which exhibit mild spherocytic elliptocytosis with osmotically fragile and inadequately deformable RBCs [33]. Tmod1-null RBCs exhibit compensatory appearance of Tmod3 [33], a Tmod isoform that is normally absent in mature RBCs and, instead, has critical functions during terminal erythroblast differentiation and enucleation [82]. In Tmod1-null RBCs, Tmod3 is present at ~20% of wild-type Tmod1 levels, with no changes in actin or other membrane skeleton protein levels [33]. Since *in vitro* experiments with purified Tmods demonstrate that Tmod3's pointed-end capping activity is equivalent to that of Tmod1 [83], Tmod1-null RBCs represent a model of ~80% depletion of pointed-end capping activity, rather than complete ablation of pointed-end capping activity. Negative-staining electron microscopy reveals that average RBC actin filament lengths are unchanged in Tmod1-null RBCs, but the uniformity of lengths is disturbed, with emergence of both shorter and longer filament populations, leading to enlarged and variably sized membrane skeleton fenestrations (pores), likely due to improper assembly and/or instability of $(\alpha_1\beta_1)_2$-spectrin attachments [33]. This is distinct from Tmod1 perturbation studies in striated muscle, which reveal uniform actin filament lengthening upon Tmod1 depletion [78,79,84,85], with the caveat that the light microscopic methods applied to striated muscle can only detect changes in average thin filament length within a sarcomere, and not changes in the length of any individual filament. Additional studies are required to determine whether Tmod1-deficient RBCs actin filaments have altered dynamics, i.e., efficiency of rho-actin incorporation and responses to CytoD, LatA, or Jasp.

Another regulator of actin filament pointed-end stability is tropomyosin, which binds along actin filament sides and inhibits pointed-end depolymerization [86]. Ektacytometry experiments have demonstrated that Mg^{2+}-free (tropomyosin-extracted) ghosts exhibit more rapid time-dependent decay in deformability than control Mg^{2+} (tropomyosin-containing) ghosts when subjected to constant shear flow [87], indicating that tropomyosin can regulate RBC membrane stability via its influence on RBC actin dynamics [36]. This effect is tropomyosin isoform-specific, as reconstitution of tropomyosin-extracted RBC actin filaments with purified RBC tropomyosin but not skeletal muscle tropomyosin restores normal membrane stability [87]. The two tropomyosin isoforms in RBCs, TM5b and TM5NM1, are present in an equimolar ratio (V.M. Fowler, unpublished data), and each rod-like tropomyosin molecule extends along most of the length of an RBC actin filament, possibly acting as a molecular ruler dictating filament length [2,55]. The failure of skeletal muscle tropomyosin to functionally substitute for RBC tropomyosin [87] supports this molecular ruler model, as skeletal muscle tropomyosins are "long" high-molecular-weight tropomyosins [88] that would extend beyond the ends of RBC actin filaments, unlike TM5b

and TM5NM1, which are “short” low-molecular-weight tropomyosins [88]. Moreover, RBC tropomyosins interact with Tmod1 at the actin filament pointed end [89–91], enhancing Tmod1’s pointed-end capping activity [83] and defining structural scaffolding for RBC actin filaments. Whether RBC tropomyosin also interacts with αβ-adducin at the actin filament barbed end remains unknown, but the observation that mice lacking α- or β-adducin exhibit reduced tropomyosin levels in RBCs [62,63] suggests such an interaction, although it may be indirect.

The distribution and functional significance of TM5b and TM5NM1 in RBCs are unclear. Two dimeric tropomyosin molecules are associated with each RBC actin filament [2], but it remains unknown whether TM5b and TM5NM1 form homo- or heterodimers. If they form homodimers, it remains unknown whether the homodimers segregate into exclusively TM5b- or TM5NM1-containing filaments, exist in hybrid TM5b/TM5NM1-containing filaments, or a combination thereof. TM5b binds F-actin more strongly than TM5NM1 *in vitro* [83] and protects F-actin more effectively against depolymerization [92], suggesting that TM5b may outcompete TM5NM1 early in RBC membrane skeleton assembly and form exclusively TM5b-containing actin filaments, leaving compositionally distinct TM5NM1-containing filaments to assemble at later time-points. However, this is speculative, since the diversity of actin-binding proteins associated with RBC actin filaments *in vivo* may alter the isoform-specific affinities of RBC tropomyosins for F-actin. Microscopic analyses with isoform-specific tropomyosin antibodies are required to assess the spatial distribution of RBC tropomyosins *in vivo*.

A broader puzzle is how RBC actin filament length uniformity is specified during membrane skeleton assembly [93]. RBC actin filaments may be nucleated and assembled into short 37-nm-long filaments *de novo*, or, alternatively, RBC actin filaments may first be assembled into overlong filaments that are then pruned into proper-length filaments. The latter hypothesis appears likelier, given that tropomyosin promotes elongation *in vitro* [86], and levels of membrane skeleton-associated RBC tropomyosin decrease during reticulocyte maturation, with no accompanying changes in Tmod1 [94]. Moving forward, detailed microscopic analysis of F-actin and actin-binding protein localization and dynamics during erythroblast differentiation and reticulocyte maturation, in wild-type and gene-targeted mice, will help elucidate the origins and regulation of uniform RBC actin filament lengths.

CONCLUSION

The studies discussed here demonstrate that the RBC field should abandon its long-held assumption that RBC actin filament nodes are static cytolinkers. Rather, RBC actin filaments exhibit sophisticated and finely tuned dynamic properties, which enable RBC actin filaments to exchange actin subunits with the RBC cytosol while maintaining uniform lengths and mechanically fortifying the RBC membrane. Thanks to recent advances in F-actin drugs, detection, and imaging, we can now interrogate the composition, architecture, dynamics, and physiology of RBC actin filaments in unprecedented detail, ensuring that the RBC membrane skeleton will continue its productive tenure as the paradigmatic membrane skeleton.

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KEY POINTS

- The red blood cell (RBC) membrane skeleton consists of $(\alpha_1\beta_1)_2$-spectrin tetramers interconnecting short actin filaments in a two-dimensional quasi-hexagonal network beneath the lipid bilayer.
- RBC actin filaments dynamically exchange subunits with the cytosol during normal RBC homeostasis and are remodeled into an aberrantly branched network in malaria parasite-infected RBCs.
- Uniform RBC actin filament lengths (~37 nm) and stability are coordinately regulated by actin-capping proteins, tropomodulin-1 and αβ-adducin, and two tropomyosin isoforms, TM5b (Tpm1.9) and TM5NM1 (Tpm3.1).

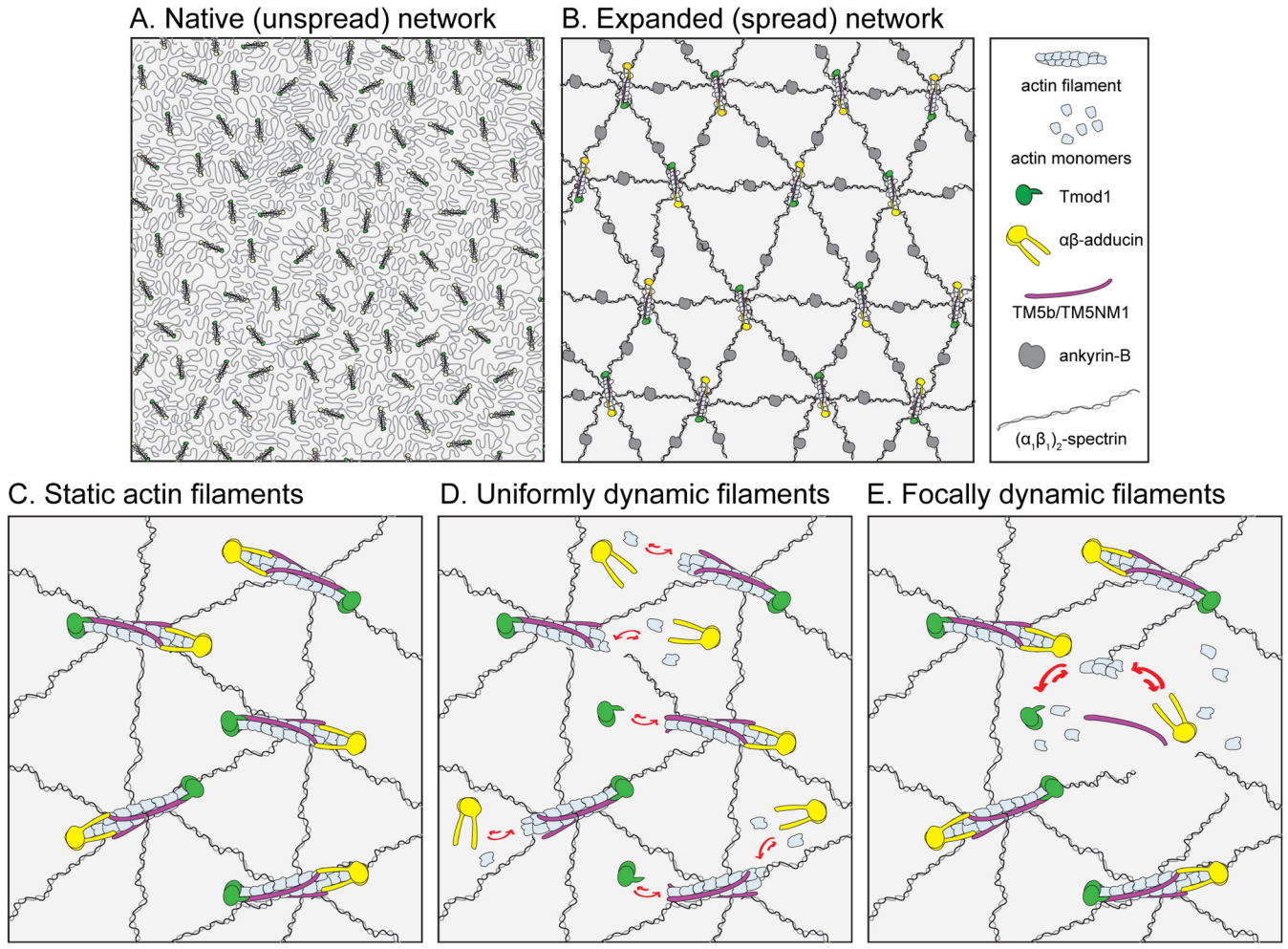

Figure 1. Membrane skeleton organization and models of actin dynamics in RBCs

(A) In the native (unspread) RBC membrane skeleton, folded $(\alpha_1\beta_1)_2$-spectrin tetramers form a two-dimensional network interconnecting short actin filament nodes, which are capped by Tmod1 and αβ-adducin and stabilized along their sides by TM5b and TM5NM1. (B) When the $(\alpha_1\beta_1)_2$-spectrin network is expanded (spread), its quasi-hexagonal symmetry is revealed. Ankyrin-B molecules tether the $(\alpha_1\beta_1)_2$-spectrin network to the bilayer between actin filament nodes. (C) The now-obsolete "static network" model presumes that the actin subunits comprising the actin filament nodes do not exchange with cytosolic G-actin. (D) The "uniformly dynamic network" model presumes that most or all of the filaments can partially assemble and disassemble actin subunits and exchange them with cytosolic G-actin. (E) The "focally dynamic network" model presumes that a subset of filaments can completely assemble and disassemble, while the others remain static. (Panels A and B are adapted from reference [2].)

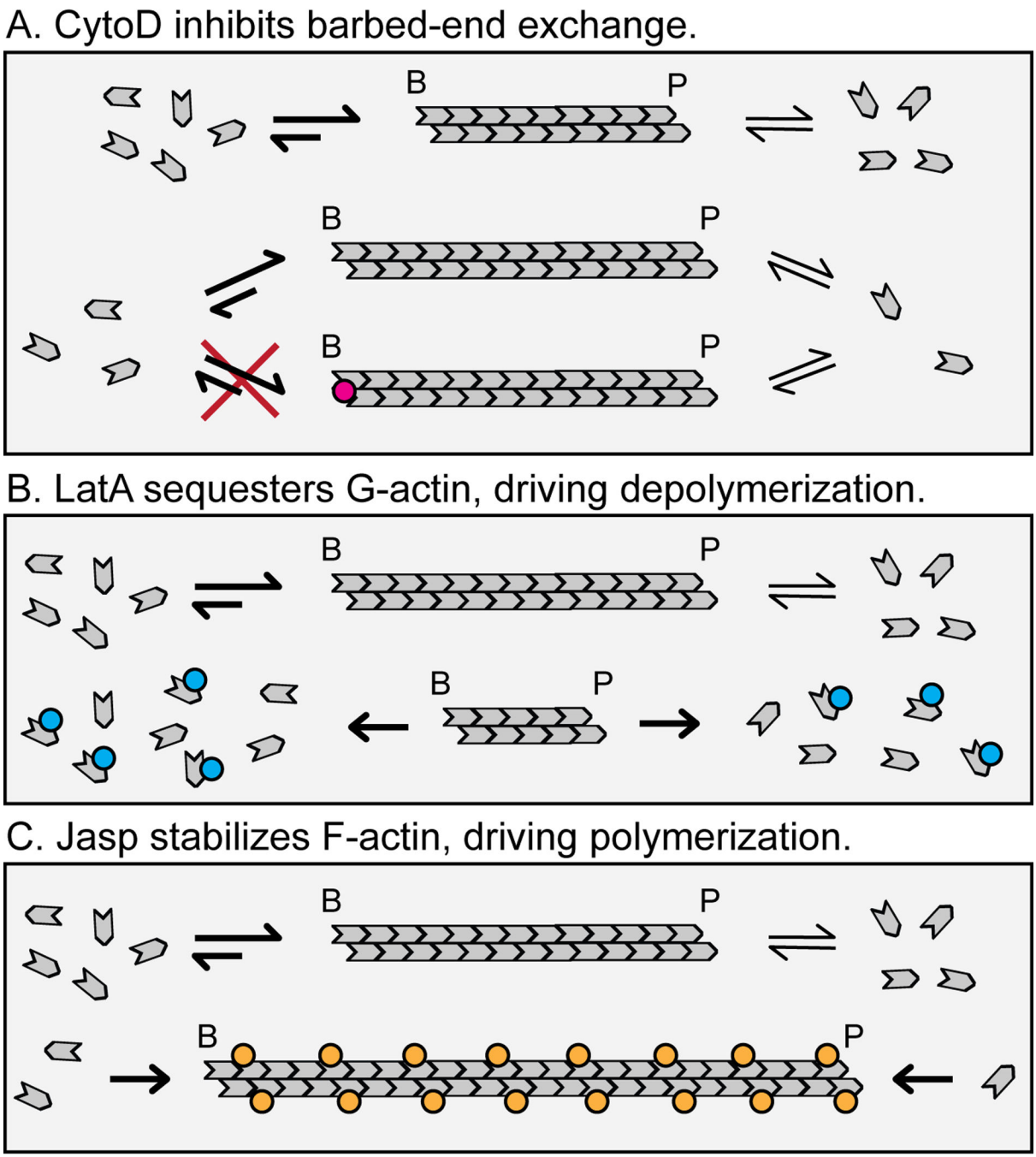

Figure 2. Effects of actin-disrupting drugs on actin filament assembly

(A) CytoD inhibits barbed-end assembly of actin monomers. (B) LatA sequesters actin monomers, driving the F:G-actin balance toward the G-actin state [50]. LatA function requires that the actin filament be dynamic (i.e., capable of exchanging subunits with the G-actin pool). (C) Jasp stabilizes actin filaments, driving the F:G actin balance toward the F-actin state. The depicted distribution of Jasp along the actin filament is speculative and does not reflect known distributions or stoichiometries of Jasp required for filament stabilization *in vitro* or *in vivo*. Jasp competes with phalloidin for F-actin binding *in vitro* [51], and one

phalloidin molecule can bind each subunit within F-actin under saturating conditions [52]. Thus, Jasp may recognize the same F-actin binding site as phalloidin with a similar stoichiometry, but this has not been proven.