Dynamics of Tpm1.8 domains on actin filaments with single-molecule resolution

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ABSTRACT Tropomyosins regulate the dynamics and functions of the actin cytoskeleton by forming long chains along the two strands of actin filaments that act as gatekeepers for the binding of other actin-binding proteins. The fundamental molecular interactions underlying the binding of tropomyosin to actin are still poorly understood. Using microfluidics and fluorescence microscopy, we observed the binding of the fluorescently labeled tropomyosin isoform Tpm1.8 to unlabeled actin filaments in real time. This approach, in conjunction with mathematical modeling, enabled us to quantify the nucleation, assembly, and disassembly kinetics of Tpm1.8 on single filaments and at the single-molecule level. Our analysis suggests that Tpm1.8 decorates the two strands of the actin filament independently. Nucleation of a growing tropomyosin domain proceeds with high probability as soon as the first Tpm1.8 molecule is stabilized by the addition of a second molecule, ultimately leading to full decoration of the actin filament. In addition, Tpm1.8 domains are asymmetrical, with enhanced dynamics at the edge oriented toward the barbed end of the actin filament. The complete description of Tpm1.8 kinetics on actin filaments presented here provides molecular insight into actin–tropomyosin filament formation and the role of tropomyosins in regulating actin filament dynamics.

INTRODUCTION

Actin is a highly conserved protein found in all eukaryotic cells. With the help of a myriad of actin-binding proteins (ABPs), actin filaments form extensive, highly dynamic networks that are associated with various structures and functions, including cell division, migration, intracellular transport, and cell–cell and cell–matrix adhesion (Pollard, 2016). Among the ABPs, tropomyosin is well known for its roles in the regulation and stabilization of actin filaments. There are multiple isoforms of tropomyosin (~40 in mammals), which are associated with functionally distinct populations of actin filaments (Gunning et al., 2015). Tropomyosin is thought to facilitate functional specialization by controlling the recruitment of specific sets of actin-binding proteins in an isoform–specific manner (Tojkander et al., 2011; Johnson et al., 2014; Gunning et al., 2015). Recent studies indicate that the majority of actin filaments in the cell are present as copolymers with tropomyosin (Meiring et al., 2018). Thus, to properly understand the variable functions and dynamics of actin filaments, it becomes crucial to elucidate the fundamental molecular interactions between actin and tropomyosin.

Each tropomyosin molecule is a parallel dimeric alpha-helical coiled coil that covers six or seven actin monomers, depending on whether the isoform is low or high molecular weight (Khaitlina, 2008); however, tropomyosin is able to polymerize along actin,
which drastically strengthens its binding affinity through avidity (Wegner, 1979; Wegner, 1980; Singh and Hitchcock-DeGregori, 2009). Tropomyosin molecules bind to the two strands of the double-helical actin filament, where they interact in a head-to-tail manner to form two continuous chains that wrap around the actin filament (Perry, 2001; Li et al., 2011; Khaitlina, 2015), as shown schematically in Figure 1B.

Cell-biological and biochemical techniques have been used extensively to identify different tropomyosin isoforms, their localization, and the corresponding ABPs they regulate (Bryce et al., 2003; Creed et al., 2011; Tojkander et al., 2011; Brayford et al., 2016; Gateva et al., 2017; Pathan-Chhatbar et al., 2017). Ensemble measurements (solution assays) have shown differences in the affinity and cooperativity of tropomyosin isoforms binding to actin and in their effect on actin assembly kinetics (Jancó et al., 2016). However, the self-assembly of tropomyosin on actin filaments is a highly stochastic and nonlinear process, which is difficult to resolve using ensemble measurements. Basic nucleation and growth models have been proposed (Vilfan, 2001) that are able to recapitulate observations from ensemble measurements (Wegner, 1979; 1980; Wegner and Walsh, 1981; Keiser and Wegner, 1985; Wegner and Ruhnau, 1988; Weigt et al., 1991). Parameterization from these models suggests that binding of tropomyosin to actin filaments involves a slow initial nucleation step followed by rapid elongation.

Recent developments in reconstituting actin filaments near surfaces for observation by time-lapse fluorescence microscopy have enabled the study of various processes regulating actin dynamics at a molecular level (Carlier et al., 2014; Jégou and Romet-Lemonne, 2016; Shekhar and Carlier, 2016), including the interplay between different tropomyosins and other ABPs (Hsiao et al., 2015; Schmidt et al., 2015; Skolnick et al., 2016; Christensen et al., 2017; Jansen and Goode, 2019). These studies demonstrate the power of this approach for dissecting individual steps of tropomyosin domain nucleation, elongation, and shrinkage on actin filaments, which will enable testing and refining our models of these processes. It remains largely unknown to what extent tropomyosin assembly differs between species and between isoforms. A dissection of the common and isoform-specific mechanisms governing the interplay of tropomyosin with actin and their relationship to function, especially in the context of the numerous cytosolic isoforms that are spatially and temporally regulated in mammalian cells (Gunning et al., 2005), will therefore require detailed studies of individual isoforms that are involved in different actin-mediated processes.

The human cytosolic low–molecular weight isoform Tpm1.8 is associated with stress fibers and lamellipodia, where it is involved in regulating the highly dynamic process of cell migration (Bryce et al., 2003; Brayford et al., 2016). It exhibits one of the strongest affinities for actin (Moraczewska et al., 1999), but its assembly and turnover on actin and how these properties relate to the dynamics of Tpm1.8-containing cellular actin structures remain unresolved. In this study, we used microfluidics and total internal reflection fluorescence (TIRF) microscopy to measure the dynamic interactions of Tpm1.8 molecules with preformed actin filaments in vitro at the single-filament and the single-molecule levels.

Our data provide a complete experimentally parameterized model of Tpm1.8 assembly and disassembly kinetics on actin, providing molecular insight into the interplay between the two polymer systems.

RESULTS

Microfluidics experiments allow direct observation of Tpm1.8 dynamics on individual actin filaments in real time

We used a combination of microfluidics and TIRF microscopy to characterize the fundamental molecular interactions underlying
tropomyosin binding to actin filaments, as shown in Figure 1A. We immobilized spectrin–actin seeds on the surface of a coverslip that formed the bottom of a microfluidic flow channel. The spectrin–actin seeds anchored actin filaments at their pointed ends and allowed the growth of actin filaments at the barbed ends. Each field of view typically contained 50–60 actin filaments (Supplemental Figure 1). After actin filaments were grown and aged to get ADP-F-actin, fluorescence Tpm1.8 was flowed into the channel and its binding to the actin filaments was directly imaged using TIRF microscopy. Throughout the acquisition, the filaments were kept aligned parallel to the surface by a constant flow of the solution. This approach allowed the proteins to bind freely to the filaments without any hindrance from the surface. After complete decoration of the filaments, the flow channel was washed with buffer and the dissociation of Tpm1.8 from the filaments was observed. We used recombinant Tpm1.8 fused at its N-terminus to an alanine–serine extension mimicking acetylation (Monteiro et al., 1994) and to mNeonGreen.

Figure 1B summarizes the main steps of the actin–tropomyosin interaction that were characterized in this study. The initial assembly intermediates of new tropomyosin domains were detected as the appearance of diffraction-limited dots of fluorescent Tpm1.8 molecules. In the range of concentrations used, multiple such events were detected at different times and locations over the filaments; the number of observable stable domains per filament increased with concentration (Supplemental Figure 2). These dots then elongated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gated in both directions until actin filaments (unlabeled) were fully gat
Sao et al., 2019); they also assemble on actin filaments in vitro (Gateva et al., 2017). To test whether the N-terminal fluorescent protein affected the kinetics of Tpm1.8 in our assays, we monitored the assembly of an equimolar mixture of tagged and untagged Tpm1.8, which yielded elongation rates similar to those observed above (Supplemental Table 1). The fluorescence intensity of actin filaments decorated with the equimolar mixture was 42% of the value for fully tagged Tpm1.8, that is, close to the value expected for equal incorporation of tagged and untagged Tpm1.8. Furthermore, elongation kinetics of the related isoform Tpm1.1 fused to a fluorescent protein or labeled with an organic fluorophore on an internal cysteine were similar to each other, and assembly showed the same general features as for mNeonGreen–Tpm1.8, including the asymmetry in elongation rates (Supplemental Figure 4). Although it is likely that N-terminal fusions affect some function of tropomyosins, our observations suggest that the fluorescent protein tag had only a minor effect on elongation kinetics and actin affinity in our in vitro assays.

Domain shrinkage rates determined from the slopes of signal decrease in the kymographs after washout were independent of the mNeonGreen–Tpm1.8 concentration used to decorate the filaments before washout, as expected (Supplemental Figure 6). Domain shrinkage was also asymmetric (Figure 2B); Release of Tpm1.8 was 1.85 times faster from the domain edge directed toward the barbed end (1.98 × 10−9 m·s−1) than from that directed toward the pointed end (1.07 × 10−9 m·s−1) of the actin filament. Overall, our single-filament data reveal that the polarity of the actin filament with faster growth at the barbed end is reflected by the asymmetrical kinetics of Tpm1.8 domains with faster growth at the C-terminal edge (Figure 2C), with potential implications for allowing Tpm1.8 domain elongation to keep up with actin filament growth (Supplemental Figure 7).

To determine the effective length of a single Tpm1.8 molecule (i.e., the length added to a domain by addition of a Tpm1.8 molecule), we measured the fluorescence intensity per unit length of actin filaments fully decorated with mNeonGreen–Tpm1.8 (Supplemental Figure 8) and related this value to the fluorescence intensity of a single mNeonGreen–Tpm1.8 (Figure 4E, green curve). Using these values, we then found that each Tpm1.8 molecule covered a length of ~33 nm on an actin filament (Supplemental Figure 8), corresponding to six actin monomers (Holmes et al., 1990; Dominguez and Holmes, 2011), as expected for low–molecular weight tropomyosin isoforms. The effective length can be used to convert the kinetic rates for association and dissociation determined from length changes in the fluorescence images into units of number of molecules per unit time (see Table 1).

Finally, we obtained estimates for the critical concentration for the elongation of Tpm1.8 (i.e., the concentration above which binding at domain edges occurs faster than dissociation) from the x-axis intercepts of the fit lines in Figure 2A. As the ratios between the elongation and shrinkage rates are the same for the two domain edges, the critical concentration is also the same toward the two ends of the actin filament (1.65 nM). As a result, there is no concentration where treadmilling (net growth at one domain edge and dissociation at the other) of Tpm1.8 occurs.

### Tpm1.8 decorates the two actin filament strands independently

One unanswered question is whether tropomyosin domains on opposite actin strands can influence each other. We took advantage of our ability to distinguish Tpm1.8 domain kinetics on the two strands to address this question. We measured the following four rates from the corresponding slopes in the kymographs (Figure 1C): 1) first and 2) second level of increase in intensity at the barbed end of the domain (blue and green arrow, respectively) and 3) first and 4) second level of increase in intensity at the pointed end of the domain (red and yellow arrow, respectively). The concentration dependence of these elongation rates (Figure 3A) confirmed the asymmetry toward pointed and barbed ends noted above. However, no significant differences between the first and second strands were observed in the elongation rates in either direction (barbed or pointed). A corresponding analysis of dissociation rates showed that these were the same on stretches of actin filament coated with a Tpm1.8 domain on one or both strands of the actin filament (Figure 3B). Thus, there was no difference in the Tpm1.8 kinetics for the two strands of the actin filament, suggesting that binding of Tpm1.8 to the ends of existing domains occurs independently on each of the two actin strands.

Next, we asked whether the presence of a Tpm1.8 chain on one strand of the actin filament could enhance nucleation of a domain on the opposite strand of the actin filament, as observed for the fission yeast tropomyosin Cdc8 (Christensen et al., 2017). To answer this question, we measured how frequently the second Tpm1.8 domain appeared opposite the first domain versus elsewhere on the actin filament. We also measured the length of the first domain (relative to the length of the actin filament) at the time of second domain appearance and determined the probability that the second

### TABLE 1: Summary of kinetic rate constants used for modeling.

| Rate constant | Description | Parameter value (length) | Parameter value (molecules) | Source |
|---------------|-------------|--------------------------|-----------------------------|--------|
| $B_I$         | Binding of an isolated Tpm to the actin filament | $4.55 \times 10^{11}$ m$^{-1}$s$^{-1}$ | $2.3 \times 10^{3}$ M$^{-1}$s$^{-1}$ | Figure 5C |
| $R_I$         | Release of an isolated Tpm from the actin filament | $4.125 \times 10^{-8}$ m$^{-1}$ | $1.25$ s$^{-1}$ | Figure 4F |
| $B_P$         | Binding to the domain edge at the pointed end | $0.64$ M$^{-1}$s$^{-1}$ | $2 \times 10^{7}$ M$^{-1}$s$^{-1}$ | Figure 2 |
| $R_P$         | Release from the domain edge at the pointed end | $1.07 \times 10^{-4}$ m$^{-1}$ | $0.033$ s$^{-1}$ | Figure 2 |
| $B_B$         | Binding to the domain edge at the barbed end | $1.19$ M$^{-1}$s$^{-1}$ | $3.6 \times 10^{10}$ M$^{-1}$s$^{-1}$ | Figure 2 |
| $R_B$         | Release from the domain edge at the barbed end | $1.98 \times 10^{-9}$ m$^{-1}$ | $0.061$ s$^{-1}$ | Figure 2 |
| $B_{BP}$      | Combined binding rate at both domain edges ($B_B+B_P$) | $1.83$ M$^{-1}$s$^{-1}$ | $5.6 \times 10^{7}$ M$^{-1}$s$^{-1}$ | Figure 2 |
| $R_{BP}$      | Combined release rate at both domain edges ($R_B+R_P$) | $3.05 \times 10^{-9}$ m$^{-1}$ | $0.094$ s$^{-1}$ | Figure 2 |

*a* Determined as a scaling factor in Figure 5C and expressed per unit length of actin filament.

*b* Calculated assuming that Tpm1.8 can bind to a naked actin filament at sites separated by an actin subunit (5 nm).

*c* Calculated assuming an effective length of 33 nm for the Tpm1.8 molecule.

*d* Obtained from Tpm1.8 domain length changes measured from kymographs.
nucleation would occur opposite the first Tpm1.8 domain by chance (Figure 3C). We found that nucleations were observed opposite the first Tpm1.8 domain in 25 of 198 kymographs (12.6%), which was not significantly higher than expected by chance (11.3%), suggesting that Tpm1.8 domains have no or minimal effect on second strand nucleation. Overall, we conclude that there is no evidence for the existence of indirect binding cooperativity for Tpm1.8 domain nucleation or elongation between the two strands.

Tpm1.8 domains grow and shrink one tropomyosin molecule at a time

Using single-molecule imaging, we resolved the kinetics of newly formed Tpm1.8 domains on actin filaments to distinguish whether Tpm1.8 undergoes binding and dissociation as monomers or multimeric species. We immobilized actin filaments via multiple (nonspecific) anchoring points to the surface (Figure 4Ai). This immobilization method provided a greater number of filaments per field of view and also facilitated detection of single molecules because the filaments were static (in contrast to filaments grown from a spectrin–actin seed, which show movement at the nontethered end). We then flowed low concentrations of mNeonGreen–Tpm1.8 (3, 4, or 5 nM) over the actin to measure initial binding events (appearing as diffraction-limited spots) and early steps of domain elongation (Figure 4Aii), but not full decoration of actin filaments. Fluorescence intensity traces were generated at the sites of initial binding and dissociation traces. To obtain a baseline for the fluorescence intensity traces of the first Tpm1.8 molecule to a naked region of actin filament), for subsequent positive steps (association), and for negative steps (dissociation) are overlaid with the single-molecule intensity distribution of Tpm1.8 molecules in Figure 4E. The strong similarity between all four distributions suggests that Tpm1.8 binds, elongates, and dissociates from actin filaments as a monomer.

The observations above confirmed that the first assembly intermediate consists of an isolated (or solitary) Tpm1.8 bound to actin. This complex is thought to be highly unstable, but its half-life is unknown. To obtain insight into this process, we analyzed distributions of dwell times of fluorescence signals corresponding to single molecules bound to the filament at a given location and obtained a lower limit estimate for the dissociation rate of an isolated mNeonGreen–Tpm1.8 molecule of 1.25 s⁻¹ (Figure 4F; see Supplemental Figure 10 for details).

Taken together, our observations suggest that all Tpm1.8 assembly and disassembly steps proceed in units of single molecules, so that the kinetics of binding and release from the ends of domains is independent of the length of the domains. Isolated tropomyosin molecules detach from the actin filaments at least an order of magnitude faster than from the edges of domains.
A model of domain appearance with a stable nucleus containing only two Tpm1.8 molecules is sufficient to recapitulate experimental data

The early stages of tropomyosin domain formation on actin filaments have been difficult to establish. Studies have hinted at an initial slow process of nucleation followed by a faster process of elongation (Wegner and Ruhnau, 1988; Weigt et al., 1991). However, it is still not known whether this “nucleus” consists of a minimum number of tropomyosins to be stable, and what the rate-limiting step of this reaction is. To address these questions, we compare experimental rates of domain appearance with theoretical predictions.

We obtained rates of domain appearance (nucleation events that proceed to form observable, elongating Tpm1.8 domains) by measuring the earliest time at which the first growing mNeonGreen–Tpm1.8 domain was detectable in the kymographs. For this analysis we considered only the appearance of the first domain per each filament, that is, when the entire filament was available for binding. We assumed that the probability of domain appearance per unit length per unit time is constant, that is, the time taken for the initial event should be inversely proportional to the length of the filament. Therefore, we multiplied the appearance time by the length of the filament and used this measure to generate survival curves of filaments that remain without Tpm1.8 domains. Exponential fits of these survival curves then provided experimental rates for domain appearance (Supplemental Figure 11), which increased nonlinearly with increasing Tpm1.8 concentration (Figure 5C, red data points).

The kinetic interactions observed in this work are summarized in Figure 6: reversible weak binding of the first tropomyosin molecule to an undecorated stretch of actin filament is followed by reversible addition of tropomyosin molecules to the edges of the growing domain. The domain appearance rate can be described as split into two components: 1) the binding of the first tropomyosin molecule (as a rate constant $B_1$) and 2) the probability of continued growth into a tropomyosin strand, that is, success of nucleation. Together, the domain appearance rate ($k_{DA}$) can be expressed as

$$k_{DA} = c \cdot B_1 \cdot S(c)$$

where $c$ is the tropomyosin concentration, $B_1$ is the on-rate constant of an isolated tropomyosin molecule binding to the actin filament, and $S(c)$ is the probability of continued growth into an observable tropomyosin domain, that is, successful nucleation.

To explore the interplay between the set of kinetic interactions described in Figure 6 and the probability of continued growth into an observable tropomyosin domain, we converted these interactions into a state space model where each state was denoted by the length of the tropomyosin domain and transitions between states are governed by the effective experimentally measured rates (see the Supplemental Information for details). Using this model, we calculated the probability of continued growth, $S(c)$, by multiplying all of the transition probabilities from the single tropomyosin state to an infinitely long domain, which gives

$$S(c) = \frac{c B_{BP} - R_{BP}}{R_1 + c B_{BP} - R_{BP}}$$

where $c$ is the tropomyosin concentration, $B_{BP}$ and $R_{BP}$ are the sums of binding and release rate constants, respectively, for the

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**FIGURE 4:** Single-molecule kinetics of Tpm1.8 on naked actin filaments and at the edges of diffraction-limited domains. (A) Assembly of mNeonGreen-Tpm1.8 on unlabelled actin filaments attached non-specifically to the surface. (i) TIRF image of actin filaments after complete decoration with mNeonGreen–Tpm1.8 (endpoint of the assembly process). (ii) TIRF image of actin filaments (unlabelled) with short mNeonGreen-Tpm1.8 domains that appeared shortly after injection of mNeonGreen-Tpm1.8 at a concentration of 3 nM. (B) Time-lapse images showing nucleation and growth of a diffraction-limited mNeonGreen-Tpm1.8 domain on an unlabelled actin filament. (C) Intensity trace of the mNeonGreen-Tpm1.8 segment shown in (B) recorded with an imaging frequency of 2 Hz with step fit (blue line) to identify times of addition/dissociation of Tpm1.8 molecules on the domain undergoing net elongation. (D) Combined rate constant for binding at both domain edges ($k_{BP}$) determined from step fitting of intensity traces of individual segments. The same rate constant determined from kymographs is shown for comparison. $N$ (number of positive step times) = 13504 (3 nM), 15002 (4 nM), 14895 (5 nM). Errors represent the 95% CI of the residual of the fits (E) Distribution of mNeonGreen-Tpm1.8 intensity (green, determined by the intensity of molecules sparsely adhered to a clean glass surface) overlayed with intensity distributions of the first positive step (purple, binding of a mNeonGreen– Tpm1.8 molecule to a stretch of naked actin filament), all positive steps (red; binding events) and negative steps (blue; dissociation events) obtained from the step fitting of intensity traces of individual mNeonGreen-Tpm1.8 domains. $N$ (number of events) = 12364 (single mNeonGreen–Tpm1.8 photobleaching), 4767 (first steps), 48168 (positive steps), 44641 (negative steps). (F) Estimated release rate ($R_1$) for an isolated mNeonGreen-Tpm1.8 molecule that binds to the actin filament (see Supplementary Figure 6 for details). $N$ (number of single molecule events) = 9213 (4 nM), 15850 (5 nM).
The domain appearance model is able to reproduce the non-linear increase in the nucleation rate with tropomyosin concentration, suggesting that the nucleation process is essentially governed by two sets of reaction rates for 1) binding/dissociation of the first tropomyosin to an undecorated section of an actin filament and 2) binding/dissociation of a tropomyosin molecule at the edges of a domain. We note that in this model the smallest domain at which the latter reaction rates become independent of domain length consists of only two tropomyosin molecules.

A limited set of kinetic parameters account for Tpm1.8 domain dynamics

To test whether the experimentally measured parameters are sufficient to reproduce all features of the observed tropomyosin kinetics, we combined the interactions investigated here (summarized in Figure 6A) into an experimentally parameterized stochastic model (see Table 1 for parameter values and the measurements from which they were derived). In this model, we assumed that independently nucleated tropomyosin domains anneal into a single chain when they have sufficiently elongated toward each other to form a contact. In reality, domains presumably only end up annealing into a single chain when they are in register with each other; that is, the gap between the two domains is a multiple of the length of the Tpm1.8 binding site (six actin subunits; Supplemental Figure 3). However, given how tightly Tpm1.8 binds, this simplification does not have a significant effect on the kinetics of filament decoration. The kymographs generated using the model at a range of Tpm1.8 concentrations resembled the experimental kymographs (Figure 6B) and faithfully reproduced elongation kinetics and domain appearance rates (Supplemental Figure 14). Overall, this comparison confirmed that the steps incorporated into our model were sufficient to predict the experimental kinetics of Tpm1.8 assembly.

**DISCUSSION**

Direct observation of Tpm1.8 assembly on actin filaments in vitro at the single-molecule and single-filament levels with unparalleled resolution enabled us to measure the assembly units and kinetics of Tpm1.8 domain appearance, elongation, and shrinkage. On the basis of our observations we propose the following pathway: all associations/dissociations steps occur in units of single (dimeric) tropomyosin molecules. A tropomyosin molecule in solution binds to actin in two main steps: (1) nucleation of our observations we propose the following pathway: all association/dissociation steps occur in units of single (dimeric) tropomyosin molecules. A tropomyosin molecule in solution binds to actin in two main steps: (1) nucleation of one or more molecules is governed by the elongation rate (c/BP-BP, where c is the Tpm concentration, Bp and BP are the rate constants and binding and release at both edges of the domain). The probability of each reaction is given by dividing each rate by the sum of the rates of reactions out of the relevant state. (B) Plot of the probability of domain appearance as a function of concentration calculated using the model in (A). The inset shows the low concentration regime of the curve. (C) Domain appearance rate measured experimentally (red dots) from single filaments kymographs (Supplementary Figure 7), and fitted with the nucleation model (blue curve) with the rate constant (BI) for binding of an isolated Tpm to the naked actin filament as the only free parameter, yielding a value of BI = 455 nm⁻¹ M⁻¹ s⁻¹.

**FIGURE 5:** Analysis of domain appearance. (A) A simplified model of the growth pathway for a newly formed domain. Single (isolated) Tpm molecules bound to actin are in state 1. Single molecules that dissociate move to the 0 state at the release rate, R1. Alternatively, a second molecule may bind to the isolated Tpm (1), moving it to state 2. Addition of Tpm to states containing one or more molecules is governed by the elongation rate (c/BP-BP, where c is the Tpm concentration, BP and BP are the rate constants for binding and release at both edges of the domain). The probability of each reaction is given by dividing each rate by the sum of the rates of reactions out of the relevant state. (B) Plot of the probability of domain appearance as a function of concentration calculated using the model in (A). The inset shows the low concentration regime of the curve. (C) Domain appearance rate measured experimentally (red dots) from single filaments kymographs (Supplementary Figure 7), and fitted with the nucleation model (blue curve) with the rate constant (BI) for binding of an isolated Tpm to the naked actin filament as the only free parameter, yielding a value of BI = 455 nm⁻¹ M⁻¹ s⁻¹.
suggests that this pronounced increase in stability is largely achieved upon binding of a second tropomyosin to the first (solitary) molecule on actin. That is, we propose that the minimal domain (or nucleus) with sufficient stability so that it can grow or shrink with approximately the same kinetics as much longer domains consists of just two molecules. This model is supported by the single-molecule data, where dissociation of domains with two or more tropomyosin molecules was not prominent. Indeed, a domain consisting of two Tpm1.8 molecules almost wraps around the actin filament, and it is tempting to speculate that shorter tropomyosins (such as those found in yeast) would require three molecules to form a stable domain. Nucleation occurs at all tropomyosin concentrations above the critical concentration (1.65 nM) and ultimately leads to the complete decoration of the actin filament as well as bidirectional domain growth leading to full decoration have been observed for mammalian Tpm1.1 produced recombinantly (Nicovich et al. 2016; Jancz et al. 2018) or purified along with other isoforms from muscle (Schmidt et al. 2015). These features have also been observed for fission yeast Cdc8 (Christensen et al. 2017; Palani et al. 2019) and nonmuscle Drosophila Tm1A (Hsiao et al. 2015). Interestingly, Tm1A preferentially nucleates domains on ADP-bound regions of the actin filament near the pointed end, which then elongate toward the pointed end (Hsiao et al. 2015). Other characteristics either have not been measured for other isoforms or show different behavior, such as binding to an actin filament may enhance binding of tropomyosin on the opposite strand, which could result in enhanced domain nucleation and/or elongation. We tested for the existence of this additional cooperativity in our data by comparing the elongation and shrinkage rates for the two tropomyosin chains, represented by the two levels of fluorescence intensity in the kymographs. From these measurements, we observed no difference in the kinetics for the two Tpm1.8 chains. We also observed no increase in domain nucleation frequency opposite of an existing Tpm1.8 strand. Taken together our observations indicate that Tpm1.8 binds independently on the two strands of actin filaments (Figure 3).

While the binding affinity of Tpm1.8 is the same at both edges of a domain, we discovered that the on/off-kinetcs are up to twofold faster at one edge than at the other, as previously observed (albeit to a lesser extent) for the yeast tropomyosin Cdc8 (Christensen et al. 2017). Tropomyosin chains are oriented on the actin filament so that N-terminus of tropomyosin is directed toward the pointed end while the C-terminus is directed toward the barbed end (Orzechowski et al. 2014; Figure 2C). The asymmetry in rates at opposing edges could result from an increased activation energy barrier for binding and dissociation at the domain edge with exposed N-terminus as compared with the domain edge with exposed C-terminus (Figure 2C). Such an increase in activation energy could arise if the N-terminus of a tropomyosin bound to actin were poorly accessible, for example, due to reduced conformational plasticity required for forming an overlap junction (Orzechowski et al. 2014). It should be noted that the N-terminal fluorescent protein tag used here can affect the molecular interactions between tropomyosins and between tropomyosin and actin, but the principal characteristics of mNeonGreen–Tpm1.8 kinetics, including asymmetry, were also observed for mixtures of tagged and untagged Tpm1.8 as well as for Tpm1.1 labeled with an organic fluorophore at the cysteine residue at position 190. The physiological relevance of the asymmetry in kinetics at opposing domain edges, if present for native tropomyosins in the cell, is unclear, but may be relevant for a potential role of Tpm1.8 in regulating actin dynamics (for example, in lamellipodia, Brayford et al. 2016) or its interplay with other actin-binding proteins.

Several features of mNeonGreen–Tpm1.8 assembly have been observed before with chemically labeled tropomyosin isoforms from a range of species. Nucleation of multiple domains on both strands of the actin filament as well as bidirectional domain growth leading to full decoration have been observed for mammalian Tpm1.1 produced recombinantly (Nicovich et al. 2016; Jancz et al. 2018) or purified along with other isoforms from muscle (Schmidt et al. 2015). These features have also been observed for fission yeast Cdc8 (Christensen et al. 2017; Palani et al. 2019) and nonmuscle Drosophila Tm1A (Hsiao et al. 2015). Interestingly, Tm1A preferentially nucleates domains on ADP-bound regions of the actin filament near the pointed end, which then elongate toward the pointed end (Hsiao et al. 2015). Other characteristics either have not been measured for other isoforms or show different behavior, such as binding...
cooperativity for tropomyosin domain nucleation on opposite strands on the actin filament (absent for Tpm1.8 but observed for Cdc8; Christensen et al. 2017).

In this work, we have characterized the molecular interactions of Tpm1.8 binding to actin. This provides a platform for unraveling how tropomyosin decoration of actin is controlled within human cells. Similar approaches can be applied to other isoforms of tropomyosin to further identify characteristics common to isoforms associated with similar functions. Further, the addition of regulatory proteins or drugs will allow identification of the specific molecular interaction being affected.

MATERIALS AND METHODS

Constructs

The coding sequence for a fusion protein of mNeonGreen and mRubyII Tpm1.8 was cloned into a pET28a(+) vector using the EcoRI and Xhol sites, and additional features were introduced using site-directed mutagenesis. Expression of the final construct yields a protein (referred to as mNeonGreen–Tpm1.8 and mRubyII–Tpm1.8) with an N-terminal His$_6$-tag followed by mNeonGreen and a peptide linker (GGGSGGGGSGTAS) fused to the N-terminus of Tpm1.8. The coding sequence for a fusion protein of mCherry and Tpm1.8 was cloned into a pET28a vector and an N-terminal His$_6$-tag followed by mCherry and a peptide linker (SGLRSGGGGSGGGSATAS) fused to the N-terminus of Tpm1.8.

Protein expression and purification

The fusion proteins mNeonGreen–Tpm1.8 and mRubyII–Tpm1.8 were expressed in Rosetta pLysS cells grown in 1 L of LB broth containing 50 μg/ml kanamycin at 37°C. The fusion protein mCherry–Tpm1.8 was expressed in BL21 DE3 star cells grown in 1 L of LB broth containing 0.1 mg/ml ampicillin at 37°C. Protein expression was induced with IPTG (1 mM) when the OD$_{600}$ reached 0.6 and the cells were grown for an additional 4 h. Cells were pelleted by centrifugation (F10S-6 × 5000 rotor, Sorvall RC6) at 8000 rpm for 10 min at 4°C. The cell pellet was stored at −80°C until use. The pellet was resuspended in lysis buffer (20 mM Tris-HCl pH 7.8, 500 mM NaCl, 1 mM NaN$_3$, 5 mM imidazole, 2 mM MgCl$_2$, 0.1% Triton X100, 2% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], and a Roche–Hitachi EDTA free protease inhibitor tablet) and sonicated (8 min, 15 s on, 15 s off). The lysate was centrifuged (R18A rotor, Hitachi VX22N) at 15,000 rpm for 60 min at 4°C. The supernatant was filtered using a 0.45-μm syringe filter and loaded onto a 5-ml Hi-Trap column (GE Healthcare Life Sciences) equilibrated with lysis buffer. The column was washed with 50 mL buffer containing 30 mM imidazole, 500 mM NaCl, 20 mM Tris, 2 mM MgCl$_2$, 1 mM NaN$_3$ at pH 7.8 followed by 75 mL buffer containing 100 mM imidazole. The protein was eluted using a buffer containing 250 mM imidazole. The fractions with the protein were pooled and further purified by size exclusion chromatography using a 16/60 superdex 75pg column (GE Healthcare Life Sciences) equilibrated with a buffer containing 10 mM Tris pH 7.8, 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 1 mM NaN$_3$, and 1% sucrose. The fractions containing protein were pooled, snap frozen in aliquots using liquid nitrogen, and stored at −80°C.

Microfluidics setup

Microfluidic devices with five channels (11 × 0.8 × 0.06 mm, L × W × H) were prepared from polydimethylsiloxane (PDMS) by replica molding and ports for tubing (inlet/outlet) were punched at the channel ends using a biopsy punch (diameter 0.7 mm). The device was washed with isopropanol and MilliQ water before use. Coverslips (Marienfeld superior No. 1.5H, 24 × 60 mm) were sonicated in filtered 100% ethanol for 30 min, washed with MilliQ water, sonicated in filtered 1 M NaOH for 30 min, and washed again with MilliQ water. The coverslips were blown dry under a stream of filtered nitrogen and then further dried at 70°C. The PDMS replica and the coverslip were treated with an air plasma for 3 min at 700 Torr. The channel device was assembled by pressing the PDMS replica onto the coverslip and annealing the device at 70°C for 4–5 h.

TIRF microscope setup

TIRF data were acquired using a custom-built TIRF microscope based around an ASI-ARAMM frame (Applied Scientific Instrumentation) with a Nikon 100× CFI Apochromat TIRF (1.49 NA) oil immersion objective. Lasers were incorporated using the NicoLase system (Nicovich et al. 2017). Images were captured on two Andor iXon 888 EMCCD cameras (Andor Technology Ltd) and 300-mm tube lenses were used to give a field of view of 88.68 × 88.68 μm at Nyquist sampling frequency (86 nm per pixel). On our system we typically use a power density of ∼1–3 W cm$^{-2}$ (measured at the objective with the laser beam normal to the surface of the coverslip).

Channel preparation and surface chemistry for single-filament experiments

Anchoring of filaments at the pointed end using spectrin–actin seeds.

Spectrin–actin seeds were prepared as described previously (Casella et al., 1986), diluted in phosphate-buffered saline (PBS) to a final spectrin–actin concentration of 30 nM, injected into the channel, and allowed to incubate for 4 min. The channel was washed with PBS and incubated with a solution of PLL-PEG (SuSoS AG) in PBS (1 mg/ml) for 30–60 min. The channel was connected to the syringe pump via the outlet tubing and washed with Buffer F (5 mM Tris-HCl pH 7.8, 100 mM KCl, 1 mM NaCl, 0.1 mM CaCl$_2$, 0.1% NaN$_3$, 10 mM DTT, 1 mg/ml bovine serum albumin [BSA], 1 mM MgCl$_2$, 0.2 mM EGTA). The channel was then filled with a BSA solution (10 mg/ml in PBS) and incubated for 5 min, followed by a wash with buffer F (0.8 μM, rabbit skeletal muscle actin, Hypermol E.K., Germany). A solution of G-actin in buffer F was flowed into the channel and actin filaments were grown from the surface-immobilized spectrin–actin seeds for 6 min. Actin was then flowed through the channel at its critical concentration (0.1 μM) for 15 min to prevent further polymerization or depolymerization and allow complete phosphate release.

Anchoring of filaments at the barbed end using gelsolin.

Channels were prepared as described above with the following modifications. The glass surface was passivated using PLL-PEG-biotin (1 mg/ml, >1 h). The surface was further passivated with BSA (5%, 10 min) and casein (Hammarsten Bovine, 5 mg/ml, 10 min). Neutravidin was injected (5 μg/ml, 10 min), followed by gelsolin (5 nM, 3 min, purified as described in Wioland et al. 2017). Unlabeled F-actin was prepolymerized (8 μM, >1 h), diluted to 0.8 μM, and injected in the microfluidic chamber to bind to surface-immobilized gelsolin.

Single-filament imaging of Tpm1.8 association and dissociation

A solution of mNeonGreen–Tpm1.8 in Buffer F (containing 0.1 μM actin to avoid depolymerization of actin filaments) was flowed through the channel at 30 μl/min and association on actin filaments attached to the glass surface was recorded by time-lapse TIRF imaging (488-nm laser, 20 mW, 30 ms exposure time) with a frame rate depending on the concentration of mNeonGreen–Tpm1.8. Dissociation was initiated by flowing Buffer F (containing 0.1 μM
actin) through the flow channel while recording TIRF images with a frame rate of 0.1 Hz.

Channel preparation and surface chemistry for single-molecule experiments
The channel was incubated with a solution of PLL-PEG (1 mg/ml in PBS) for 20 min, connected to the syringe pump via the outlet tubing, and washed with buffer F. A solution of 0.8 μM actin in Buffer F was flowed into the channel and incubated for 2 min. During this period, actin filaments polymerized in solution and adhered nonspecifically to the surface of the coverslip. A wash with Buffer F containing 0.1 μM actin was then given.

Single-molecule imaging of Tpm1.8 association and dissociation
A solution of mNeonGreen–Tpm1.8 (3, 4, or 5 nM) in Buffer F (containing 0.1 μM actin) was flowed through the channel and association on actin filaments attached to the glass surface was recorded by time-lapse TIRF imaging (488-nm laser, 45 mW, 50 ms exposure time). The length and frame rate of the acquisition were adjusted depending on the concentration to obtain a sufficient number of nucleation and elongation steps without significant photobleaching.

Image analysis at the level of single filaments
Elongation and dissociation rates. Kymographs of Tpm1.8 association and dissociation were generated using the FIJI kymograph plugin. The slopes of the kymographs were fitted using linear regression in Wolfram Mathematica to obtain elongation and dissociation rates. To determine the initial domain appearance rate, we assumed that the probability of binding anywhere on the filament is the same such that the time taken for the first nucleation to occur is inversely proportional to the length of the filament. Hence, the product of the time of the first nucleation event (t) and the length of the filament (l) is a constant. We plotted survival curves for the number of actin filaments without a nucleation event as a function of the t l x t constant, which decreased exponentially. Fitting these exponentials gave the nucleation half-lives for different concentrations of Tpm1.8.

Nucleation cooperativity analysis
The experimental frequency of a second-domain nucleation occurring opposite the first domain was determined from kymographs. For each kymograph, we also measured the fraction (f) of the filament covered (on one strand) by the first Tpm1.8 domain at the nucleation time of the second domain. The probability that the second nucleation would occur opposite the first domain is then given by f / (2 − f). The probability was further corrected for a slight bias in our data in detecting nucleation sites closer to the anchor site (possibly arising from actin filament dynamics and/or anchoring artefacts). Without this bias correction, the probability of spatial coincidence is 0.106 ± 0.02 (mean ± SD) for nucleation at random locations, which is not significantly lower than the experimental frequency (p = 0.16).

Image analysis at the single-molecule level
Image stacks were analyzed using the JIM Immobilized Microscopy Suite to extract intensity traces at sites corresponding to nucleation events (https://github.com/libbutsa/JIM-Immobilized-Microscopy-Suite). Intensity traces were divided by the intensity of a dimeric mNeonGreen–Tpm1.8 molecule (equal to twice the intensity measured for mNeonGreen). Step fitting of traces was achieved using the findchangepts function in Matlab with a threshold of 0.03. Histograms of the intensity step heights for nucleation (first step), elongation (subsequent positive steps), and dissociation (negative steps) of Tpm1.8 were generated using Wolfram Mathematica. Cumulative probability functions of the time before a positive/negative step were fitted with single-exponential decays to obtain elongation/dissociation rates, respectively. Dividing the association rates by the concentration gave the association rate constant.

Stochastic Model
The stochastic model was solved using a custom program written in Mathematica 10.2 (Wolfram). The script used the Gillespie algorithm to generate statistically correct trajectories (Gillespie, 1976, 1977). A list containing the ends of each tropomyosin domain was used to calculate the probability of each reaction. The code for this program is freely available at (https://github.com/libbutsa/Tropomyosin_Stochastic_Model).

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