Tyrosination of α -tubulin controls the initiation of processive dynein–dynactin motility

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Abstract

Post-translational modifications (PTMs) of α/β -tubulin are believed to regulate interactions with microtubule-binding proteins. A well-characterized PTM involves in the removal and re-ligation of the C-terminal tyrosine on α -tubulin, but the purpose of this tyrosination–detyrosination cycle remains elusive. Here, we examined the processive motility of mammalian dynein complexed with dynactin and BicD2 (DDB) on tyrosinated versus detyrosinated microtubules. Motility was decreased ~fourfold on detyrosinated microtubules, constituting the largest effect of a tubulin PTM on motor function observed to date. This preference is mediated by dynactin's microtubule-binding p150 subunit rather than dynein itself. Interestingly, on a bipartite microtubule consisting of tyrosinated and detyrosinated segments, DDB molecules that initiated movement on tyrosinated tubulin continued moving into the segment composed of detyrosinated tubulin. This result indicates that the α -tubulin tyrosine facilitates initial motor–tubulin encounters, but is not needed for subsequent motility. Our results reveal a strong effect of the C-terminal α -tubulin tyrosine on dynein–dynactin motility and suggest that the tubulin tyrosination cycle could modulate the initiation of dynein-driven motility in cells.

Keywords dynein; microtubule; post-translational modification; tyrosination

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Membrane & Intracellular Transport

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Introduction

Cytoplasmic dynein is the predominant minus-end-directed microtubule motor in living cells (Allan, 2011; Vallee *et al*, 2012; Roberts *et al*, 2013). In metazoans, dynein is not strongly processive on its own, and long-range directional movement requires dynein's association with the large dynactin complex, which is mediated by one of a number of coiled-coil cargo-specific adaptor proteins (McKenney

et al, 2014; Schlager *et al*, 2014). Recent structural studies suggest that the docking of dynein's tail domain into dynactin's actin-related 1 (Arp1) filament may re-orient the dynein motor domains for productive motility (Urnavicius *et al*, 2015). Additionally, dynactin contains its own microtubule-binding domain, located at the N-terminus of the p150^{Glued} subunit (hereafter termed p150) (Schroer, 2004), but the role of this domain in dynein motility remains unclear (Culver-Hanlon *et al*, 2006; Kim *et al*, 2007; Dixit *et al*, 2008; Kardon *et al*, 2009; Ayloo *et al*, 2014; Tripathy *et al*, 2014).

In addition to activation via an allosteric mechanism, dynein's motor activity may also be regulated by the microtubule track. The unstructured C-terminal tail domains (CTTs) of both α - and β -tubulin are subjected to several types of PTMs, including the addition and removal of branched chains of glutamate and glycine residues, and the cyclical removal and addition of the terminal tyrosine on α -tubulin. These PTMs and genetic tubulin isoforms have been proposed to create a “tubulin code” that could modulate interactions with molecular motors or other microtubule-binding proteins (Janke & Bulinski, 2011; Garnham & Roll-Mecak, 2012; Janke, 2014; Roll-Mecak, 2015; Yu *et al*, 2015). In support of this idea, our previous work revealed certain selective differences in processivity and/or velocity of several kinesin motors on microtubules composed of different CTT modifications. However, the movement of yeast cytoplasmic dynein (which does not require dynactin for processive motility) revealed little selective preference for any of the microtubule substrates tested (Sirajuddin *et al*, 2014).

Here, we utilize *in vitro* reconstitution to dissect the role of the CTT domains and tubulin PTMs on the motility of mammalian dynein complexed with dynactin and the adaptor protein BicD2 (DDB). We show the α -tubulin CTT and in particular its C-terminal tyrosine are important for DDB motility.

Results

DDB motility requires the CTT on α -tubulin but not β -tubulin

We previously reported that microtubules (MTs) treated with subtilisin, which removes the CTTs of both α - and β -tubulin, are poor substrates for single molecule DDB motility *in vitro* (McKenney *et al*, 2014). To parse the roles of the individual α - and β -tubulin

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CTT domains in DDB motility, we utilized a recombinant expression system in which human CTTs were fused onto the structural core of *S. cerevisiae* tubulin; the recombinant tubulin was purified by Ni-NTA affinity chromatography with a hexahistidine tag placed within a loop on α -tubulin that faces the MT lumen (Sirajuddin *et al*, 2014). For these assays, we used the CTTs corresponding to human tubulin isoforms TUBA1A and TUBB2A, which are common isoforms expressed in mammals. The DDB complex was purified from porcine brain; a SNAPf tag was fused to the truncated recombinant BicD2 so that the movement of the DDB complex could be followed by total internal reflection fluorescence (TIRF) microscopy (McKenney *et al*, 2014). Single molecules of DDB moved processively on MTs polymerized from recombinant TUBA1A/TUBB2A tubulin (hereafter referred to as WT tubulin) with a velocity similar to that reported previously on mammalian MTs (Fig 1A and B; McKenney *et al*, 2014; Schlager *et al*, 2014).

Next, we prepared recombinant tubulin lacking either the α - or β -tubulin CTT. DDB motility was assayed in chambers containing the Δ -CTT MTs as well as control WT MTs (each labeled with ~1% incorporation of fluorescently labeled and biotinylated porcine brain tubulin). We observed a dramatic decrease (~85%) in the number of processive DDB molecules on MTs lacking the α -CTT compared to WT MTs in the same chamber (Fig 1A and C, Movie EV1). Strikingly, the frequency of processive DDB movement on MTs lacking the β -CTT was only modestly reduced (~20%) compared to WT MTs (Fig 1A and C, Movie EV1). While deletion of the α -CTTs strongly suppressed DDB movement, the few complexes that did move on these MTs did so at a similar velocity as WT MTs (Fig 1B). As previously reported, a subset of DDB complexes (~30%) displayed only diffusive back-and-forth motion on the MT with no obvious directional bias (Fig 1A, arrow; McKenney *et al*, 2014). Similar to processive complexes, the number of diffusive DDB complexes was dramatically reduced by deletion of the α -CTT domain, but not the β -CTT domain (Fig 1A and D). Thus, the α -CTT, but not the β -CTT domain, plays a major role in both processive and diffusive DDB interactions with the MT lattice.

We next swapped the positions of the CTTs, such that the α -tubulin CTT was placed on β -tubulin and vice versa (see Materials and Methods). Strikingly, we observed a strong decrease in processive complexes, as well as a moderate reduction in diffusive movement on these MTs, despite the presence of the α -CTT on the β -tubulin core (Fig 1A, C and D, Movie EV1). These results demonstrate that DDB motility along the MT lattice requires the stereospecific location of the α -tubulin CTT.

Analysis of the MT-binding domains of dynein and dynactin

Dynein–dynactin–BicD2 contains two defined MT-binding domains: one located within the dynein motor domain, and the other on the p150 subunit of dynactin. We investigated how each of these MT-binding domains interacts with the CTTs. We tested our previously characterized recombinant GST-dimerized human dynein motor domain (GST-hdyn) and a truncated dimeric construct of the neuronal isoform of p150 that contains the CAP-Gly and basic domains, both of which have been shown to interact with MTs (Culver-Hanlon *et al*, 2006; Trokter *et al*, 2012; Ayloo *et al*, 2014; Duellberg *et al*, 2014; McKenney *et al*, 2014; Tripathy *et al*, 2014).

In the presence of ATP, GST-hDyn bound transiently, but did not move processively on our recombinant MTs, similar to previous reports on mammalian MTs (Trokter *et al*, 2012; McKenney *et al*, 2014; Torisawa *et al*, 2014). Fluorescent motors transiently binding to the MT surface generate large pixel intensity variation over background noise. To more readily observe any lattice preferences in the transient binding events of GST-hDyn to the MTs, the standard deviation of the fluorescence intensity for each pixel in the image over the entire time series was calculated and projected onto a single image, as a standard deviation (SD) map (Cai *et al*, 2007, 2009). The SD map revealed that GST-hDyn binding to microtubules with a deleted α - or β -tubulin CTT was similar to wild-type MTs (Fig 1E). Quantification of the total GST-hDyn fluorescence on the MT via projection of the maximum intensity pixel values over the whole time series also revealed that motor binding was not significantly diminished by the loss of either CTT (Fig 1G). These results are consistent with structural studies suggesting that dynein binds to the MT lattice at the interface of the α/β -tubulin dimer and does not make contact with the tubulin CTTs (Mizuno *et al*, 2004; Carter *et al*, 2008; Redwine *et al*, 2012; Uchimura *et al*, 2015). While mammalian dynein's non-processive interaction with the MT was not affected by loss of the tubulin CTTs in our assay, previous studies reported that removal of the CTTs leads to a decrease in processive run-lengths for yeast dynein (Redwine *et al*, 2012; Sirajuddin *et al*, 2014), as well as for mammalian dynein attached to plastic beads (Wang & Sheetz, 2000).

The p150 construct also bound to the recombinant MT lattice and displayed bidirectional and diffusive motility as previously described (Culver-Hanlon *et al*, 2006; Ayloo *et al*, 2014; Tripathy *et al*, 2014). In contrast to GST-hDyn, p150 binding was strongly diminished by removal of the α -CTT, and somewhat less so by removal of the β -CTT (Fig 1F and G). Thus, the p150 MT-binding domain, similar to DDB, displays a requirement for the presence of the α -CTT. However, in contrast to DDB, p150 binding also decreased by more than 50% after removal of the β -CTT.

The C-terminal tyrosine of α -tubulin is critical for DDB motility

Because of the strong requirement for the α -CTT in DDB motility, we turned our attention to PTMs specific to this domain. One of the first and most prominent PTMs to be discovered is the cyclical removal and re-addition of the terminal tyrosine residue on the α -CTT (Arce *et al*, 1975; Hallak *et al*, 1977; Gundersen *et al*, 1984). Interestingly, the N-terminal CAP-Gly domain of p150 has been previously shown to recognize the –EEY/F motif at the C-terminus of α -tubulin (Peris *et al*, 2006; Wang *et al*, 2014). Thus, we wanted to test whether this tyrosine residue plays an important role in the DDB-MT interaction.

We assayed DDB motility on recombinant tubulin in which we genetically deleted the terminal tyrosine residue on the α -CTT (detyrosinated MTs). We note that tubulin purified from yeast does not contain other types of PTMs that are commonly found in tubulin purified from mammalian sources (e.g., polyglutamylation, polyglycylation, acetylation), and budding yeast lack the ligase that is needed to post-translationally add tyrosine to the C-terminus of α -tubulin. Strikingly, DDB movement was ~fourfold more frequent on MTs containing tyrosinated α -CTT than on detyrosinated MTs placed in the same chamber (Movie EV2, Fig 2A and B (note that data for $\Delta\alpha$ -CTT are re-plotted from Fig 1C for comparison)). Maps

Figure 1. Role of tubulin tail domains in DDB movement.

- A** Representative kymographs showing tetramethylrhodamine (TMR)-labeled DDB movement on each type of recombinant MT lattice, as indicated. Scale bars are 5 μ m and 25 s. Pink arrowhead marks a diffusive DDB complex. See also Movie EV1.
- B** Quantification of DDB velocity on MT lattices lacking each of the tubulin CTTs ($n \geq 100$ DDB complexes per condition from at least three independent experiments). No statistical difference was observed between WT and mutant MTs ($P \geq 0.1$, unpaired t -test). Error bars represent SD.
- C, D** Quantification of the number of processive (**C**) or diffusive (**D**) DDB complexes relative to WT MTs in the same chamber ($n \geq 10$ MTs quantified for each condition from at least three independent experiments). Processive (unidirectional motion exceeding 0.5 μ m) and diffusive (back-and-forth motion) events were scored in a field of view and then divided by the lengths of MTs and time, thus normalizing to a value of number of motility events per μ m MT per s. Error bars represent SD.
- E, F** TIRF images of 1 nM TMR-labeled GST-hDyn (2 mM ATP) (**E**), or 0.3 nM TMR-labeled p150 (**F**) molecules (green) bound to either $\Delta\alpha$ -CTT (top), or $\Delta\beta$ -CTT MTs (bottom). Pink arrows show mutant MTs in both the raw, and SD map images, highlighting differences in binding between WT and mutant MTs that are apparent in the SD map. Right, standard deviation maps from an entire time sequence reveal ensemble binding and dissociation events that lead to variations in pixel intensities (see Materials and Methods). Scale bars are 5 μ m.
- G** Quantification of mean fluorescence intensity (arbitrary units) per μ m MT for GST-hDyn (2 mM ATP) or p150 bound to the indicated mutant MT relative to WT MTs in the same chamber. Image shows first frame from time series. The intensity of GST-hDyn was quantified from maximum intensity projections of the entire time sequence due to transient binding of GST-hDyn to the MT ($n \geq 10$ MTs quantified for each condition from at least three independent experiments). Error bars represent SD. P -value (unpaired t -test) of $\Delta\alpha$ -CTT vs. $\Delta\beta$ -CTT MTs = 0.5208 for GST-hDyn, and ≤ 0.0001 for p150.

of the pixel standard deviation over the whole acquisition series confirmed that DDB bound much less frequently on detyrosinated MTs than on tyrosinated MTs (Fig 2A). Similarly, the diffusive population of DDB was strongly reduced on detyrosinated MTs compared to tyrosinated MTs (Fig 2C (note that data for $\Delta\alpha$ -CTT are re-plotted from Fig 1D for comparison)). We next polymerized MTs containing variable amounts of tyrosinated α -tubulin and quantified the frequency of processive DDB movement (see Materials and Methods). This analysis revealed that maximal DDB motility is achieved when 50% of the MT lattice contains tyrosinated α -tubulin, while ~75% of full motility is achieved at 30% tyrosination (Fig 2D). In summary, these results demonstrate that the C-terminal tyrosine on α -tubulin is necessary for a robust DDB-MT interaction.

We next examined how the C-terminal α -tubulin tyrosine affects the dynein- and p150-MT interactions individually. GST-hDyn showed no preference for binding to tyrosinated or detyrosinated MTs (Fig 2E and F), which is consistent with the above findings (Fig 1E–G), and previous reports for mammalian and yeast dynein (Carter *et al*, 2008; Redwine *et al*, 2012; Sirajuddin *et al*, 2014; Uchimura *et al*, 2015). In striking contrast, removal of the terminal tyrosine nearly abolished the interaction of p150 with the detyrosinated MTs (Fig 2E and F). We conclude that in the absence of other tubulin PTMs, tyrosination of the α -CTT is critical for both DDB motility and p150 binding, but not for dynein binding. These results show that the MT-binding requirements of p150 correlate with those for DDB movement along the MT lattice.

To probe the role of other tubulin isotypes and PTMs in conjunction with the α -CTT tyrosination state in DDB motility, we turned to tubulin purified from porcine brain, which is a composite of many tubulin isotypes and PTMs. Treatment with the enzyme carboxypeptidase A (CPA) specifically removed the α -CTT tyrosine without affecting other PTMs (Fig 3A) (Webster *et al*, 1987b). Strikingly, CPA treatment reduced the number of processively moving DDB molecules on digested MTs by ~50% compared to WT MTs in the same chamber (Fig 3B and C). Examination of both GST-hDyn and p150 binding to CPA MTs showed CPA treatment had little effect on the binding of GST-hDyn, but strongly reduced the binding of p150 to the MT (Fig 3D and E), likely due to the loss of interaction between p150's CAP-Gly domain and the α -CTT –EEY/F motif. Thus, removal of the α -CTT tyrosine, in the background of mixed tubulin isotypes and PTMs, reduces both DDB movement and p150 binding.

To examine whether the p150 CAP-Gly domain is directly required for p150's preference for tyrosinated MTs, we purified a

neuron-specific isoform of p150 that lacks the N-terminal CAP-Gly domain (termed p135), but retains a portion of the adjacent basic domain that also bind MTs (Fig 4A) (Tokito *et al*, 1996; Culver-Hanlon *et al*, 2006). At similar protein concentrations, much less p135 protein bound along MTs compared to p150, consistent with its previously reported lower affinity for MTs (Fig 4B; Lazarus *et al*, 2013). In contrast to p150, p135 did not bind preferentially to tyrosinated MTs (Fig 4B and C), revealing that the CAP-Gly domain dictates p150's preferential interaction with tyrosinated MTs. Our results suggest that recognition of the α -tubulin tyrosine by the CAP-Gly domain of p150 plays a key role for DDB motility, even in the background of a heterogeneous mixture of tubulin isotypes and other PTMs.

Initiation, but not the continuation of processive DDB motility, requires α -tubulin tyrosination

Having established that tyrosination of the α -CTT is critical for both p150 binding and DDB movement, we next sought to investigate how p150 may regulate DDB motility. Dynactin has been proposed to tether dynein to the MT surface during processive motility (King & Schroer, 2000; Culver-Hanlon *et al*, 2006; Ross *et al*, 2006; Ayloo *et al*, 2014). We reasoned that if this model were correct, we should observe a cessation of processive movement when a DDB complex moving processively along a tyrosinated stretch of a MT encountered a detyrosinated section of a MT. To create such a bipartite MT lattice, stabilized MTs composed of either tyrosinated or detyrosinated α -tubulin were joined end-on-end through a spontaneous annealing reaction (Rothwell *et al*, 1986). This resulted in single bipartite MTs containing localized zones of tyrosinated and detyrosinated tubulin (Fig 5A). We then observed the behavior of processive DDB complexes as they traversed a junction between a tyrosinated and detyrosinated MT segment. DDB molecules usually initiated movement on tyrosinated regions of a chimeric microtubule, as described earlier (Fig 2). Surprisingly, the majority of processive DDB complexes moved uninterrupted through the tyrosinated to detyrosinated MT boundary and continued long processive movements along the detyrosinated section of the MT (59.2%, Fig 5B pink arrow, Movie EV3), while the remaining DDB complexes either stalled at the junction or dissociated from the MT. In contrast to this behavior, the subset of diffusive DDB complexes never traversed the junction and often accumulated there (Fig 5B, yellow arrow). Similarly, the p150

Figure 2. Requirement of α -tubulin tyrosination for processive DDB movement.

- A TIRF image of TMR-labeled DDB molecules (green) bound to either tyrosinated (red) or detyrosinated (blue) MTs. A standard deviation map of the whole time sequence is shown, revealing the preferential association of DDB with tyrosinated MTs versus detyrosinated MTs (pink arrow). Representative kymographs of DDB movement are shown from each type of MT present in the same chamber. Scale bars are 5 μ m and 25 s. See also Movie EV2.
- B, C Quantification of the number of processive (B) or diffusive (C) DDB complexes per μ m MT per s on detyrosinated MTs relative to tyrosinated MTs in the same chamber ($n \geq 20$ MTs quantified for each condition from three independent experiments). Error bars represent SD. Data from $\Delta\alpha$ -CTT MTs (Fig 1) are re-plotted for comparison.
- D Quantification of the frequency of processive DDB molecules as a function of the amount of tyrosinated tubulin incorporated into the lattice normalized to fully tyrosinated MTs. Data points from two independent experiments are shown.
- E TIRF images of 0.3 nM TMR-labeled p150 (top) or 1 nM TMR-labeled GST-hDyn (bottom) molecules (green) bound to either tyrosinated (red) or detyrosinated (blue) MTs. Right, standard deviation maps of protein binding over an entire time sequence and kymographs from either tyrosinated or detyrosinated MTs are shown. Scale bars are 5 μ m and 25 s. Mutant MTs in TIRF images and SD maps are highlighted for comparison (pink arrows).
- F Quantification of mean fluorescence intensity (arbitrary units) per μ m MT for TMR-labeled GST-hDyn (2 mM ATP) or p150 bound to the indicated mutant MT relative to WT MTs in the same chamber. The intensity of GST-hDyn was quantified from maximum intensity projections of the entire time sequence due to transient binding of GST-hDyn to the MT ($n \geq 10$ MTs quantified for each condition from at least three independent experiments). Error bars represent SD.

Figure 2.

Figure 3. Role of tyrosination in DDB movement on brain MTs.

- A** Immunoblot of brain tubulin with or without carboxypeptidase A (CPA). Note that CPA specifically removes the α -tubulin C-terminal tyrosine without affecting other PTMs.
- B** TIRF image of TMR-labeled DDB molecules (green) on either WT (red) or CPA-treated (blue) pig brain MTs. Standard deviation map of the entire time sequence shows DDB preference for WT MTs and kymographs from either WT or CPA treated are shown. Scale bars are 5 μ m and 25 s. A CPA MT is highlighted in both the TIRF image and SD map for comparison (pink arrow).
- C** Quantification of the number of processive DDB complexes per μ m MT per s on the CPA-treated MTs, relative to WT MTs in the same chamber ($n \geq 7$ MTs of each type quantified from at least three independent experiments). Error bars represent SD.
- D** TIRF images of 0.3 nM TMR-labeled p150 (top) or 1 nM TMR-labeled GST-hDyn (bottom) molecules (green) bound to either WT (red), or CPA-treated MTs (blue). Right, standard deviation maps of protein binding over an entire time sequence and kymographs from either WT or CPA treated are shown. Scale bars are 5 μ m and 25 s for p150, and 5 μ m and 12 s for GST-hDyn.
- E** Quantification of mean fluorescence intensity (arbitrary units) per μ m MT for TMR-labeled GST-hDyn (2 mM ATP) or p150 bound to CPA-treated MTs relative to WT MTs in the same chamber. The intensity of GST-hDyn was quantified from maximum intensity projections of the entire time sequence due to transient binding of GST-hDyn to the MT ($n \geq 10$ MTs quantified for each condition from at least three independent experiments). Error bars represent SD.

Figure 4. Role of the p150 CAP-Gly domain in recognition of α -tubulin tyrosination.

- A** Schematic of the p150 constructs used. The p135 isoform lacks the CAP-Gly domain, but retains part of the adjacent basic domain (red) and coiled-coil domains (blue). Right, Coomassie blue stained gel of the purified p150 and p135 proteins. Molecular weight markers are indicated.
- B** TIRF image of either p150 (0.3 nM), or p135 (0.3 or 6 nM) bound to either WT (red), or CPA-treated porcine MTs (blue). Note: no binding of p135 is observed at comparable concentrations to p150 indicating the lower binding affinity of this isoform. Proteins were visualized by fluorescence from the GFP tag fused to each construct. Scale bars are 5 μ m.
- C** Still images from a TIRF time series of p135 (green) binding to either WT (red), or CPA-treated porcine MTs (blue). Right, standard deviation map of p135 binding shows no preference for either type of MT, and quantification of mean fluorescence intensity (arbitrary units) per μ m MT for p135 bound to CPA-treated MTs relative to WT MTs in the same chamber ($n \geq 10$ MTs quantified for each condition from three independent experiments). Scale bars are 5 μ m.

construct bound and diffused nearly exclusively along the tyrosinated section of MT and rarely crossed the boundary to a detyrosinated section of MT (Fig 5C). Because the diffusive population of DDB complexes behaved similarly to p150 with respect to the tyrosinated boundary, we speculate that the diffusive DDB complexes are bound to the MT exclusively through the p150 subunit of dynactin.

We further confirmed these results using annealed MT lattices composed of sections of porcine tubulin differentially treated with CPA to remove the α -CTT tyrosine. Consistent with the results described above for engineered yeast tubulin, we observed that 78% of processive DDB complexes were able to traverse the boundary from WT tubulin into a section of CPA-treated tubulin

(Fig 5D). This percentage is only slightly lower than that observed for processive DDB complexes that traversed the annealed boundary between two WT MTs without pausing (93.5%, Fig 5E). We also created a single annealed MT composed of untreated and subtilisin-digested porcine tubulin, which removes both CTTs (Δ -CTT) and largely abolished DDB motility (McKenney *et al*, 2014). Our results show that a subset of processive DDB complexes that bound initially to the untreated tubulin section of the MT crossed the boundary and continued processive motility on the Δ -CTT section of MT (41.1%, Fig 5F). As p150 cannot interact with Δ -CTT MTs (Lazarus *et al*, 2013; McKenney *et al*, 2014), we reason that p150 does not act as a continuous MT tether during processive movement of DDBs on Δ -CTT MTs. From these data, we conclude that the interaction of p150 with the C-terminal tyrosine of α -CTT is required for the initiation of processive DDB motility, but not its continuous movement.

Discussion

We have found that the initiation of processive motility by dynein–dynactin is greatly enhanced by the interaction of the CAP-Gly domain of p150 with the C-terminal tyrosine of α -tubulin; however, the subsequent continuation of processive motility does not require this interaction. These results, in conjunction with other recent findings, help to formulate a model for how dynactin might regulate motility of mammalian dynein. Previous work has indicated that p150 in the isolated dynactin complex may not interact with MTs (Kardon *et al*, 2009; McKenney *et al*, 2014), likely due to a folded-back, autoinhibited state that places the CAP-Gly domain close to the Arp1 backbone of dynactin (Tripathy *et al*, 2014; Urnavicius *et al*, 2015). However, the interaction of dynein with an adapter protein (e.g., BicD2) may release this inhibition and allow p150 to interact with MTs (Urnavicius *et al*, 2015). In fact, in the DDB complex, p150 may provide the dominant means of initiating the first encounter with the MT. Evidence for this is that dynein alone interacts well with detyrosinated MTs, while the entire DDB complex shows a strong preference for tyrosinated over detyrosinated MTs.

The initiation of the p150–MT encounter may provide time or steric space for both microtubule-binding domains of dynein to engage with the MTs; once this occurs, our data suggest that the p150–MT interaction is no longer required. Dynactin–BicD2, however, may serve an additional allosteric role in orienting the two motor domains of dynein homodimer to facilitate processive movement (Chowdhury *et al*, 2015; Urnavicius *et al*, 2015). These results and model also might explain the roles for two isoforms of p150 in neuronal tissues—the p150 isoform discussed above and the p135 isoform lacking the CAP-Gly domain (Tokito *et al*, 1996). Dynactin molecules with either isoform may be equally effective in inducing the allosteric change of the dynein homodimer, but p135-containing complexes would have a lower initiation rate of motility (McKenney *et al*, 2014) and lack a preference for tyrosinated MTs.

How might the preference of dynein–dynactin for tyrosinated α -tubulin influence cargo transport *in vivo*? In cells, many cargos bind plus-end-directed kinesin and minus-end-directed dynein

motors simultaneously, resulting in often salutatory, bidirectional motion. Interestingly, several *in vivo* studies have suggested that certain kinesin motors preferentially move on detyrosinated MTs *in vivo* (Cai *et al.*, 2009; Konishi & Setou, 2009; Kaul *et al.*, 2014; Barisic *et al.*, 2015). *In vitro*, the processivity of kinesin-2 is also enhanced ~twofold on detyrosinated MTs (Sirajuddin *et al.*, 2014). The preference of dynein–dynactin for tyrosinated MTs and kinesins for detyrosinated MTs could, in principle, enable the post-translational modification of the microtubule to bias the direction of transport. For example, a cargo with a mixture of dyneins and kinesins (Soppina *et al.*, 2009; Hendricks *et al.*, 2010; Rai *et al.*, 2013) could be biased toward the periphery (MT plus-end) if interacting with a heavily detyrosinated MT, or conversely toward the cell center (MT minus-end) if it encounters a tyrosinated MT. Another function of this tyrosination preference might involve in regulating *in vivo* motility through interactions of p150 with microtubule plus-end-binding proteins. Previous studies have found that the p150 CAP-Gly domain also recognizes the C-terminal –EEY/F motifs on EB1 and CLIP-170 (Lansbergen *et al.*, 2004; Honnappa *et al.*, 2006; Weisbrich *et al.*, 2007; Duellberg *et al.*, 2014), which might provide an additional mechanism for facilitating the initiation of dynein motility at the plus-ends of MTs (Lloyd *et al.*, 2012; Moughamian & Holzbaur, 2012).

In summary, our results provide strong support for the “tubulin code” hypothesis in regulating the interaction of motor proteins with MTs. Our observations of DDB preference for tyrosinated MTs constitute the largest effect of PTMs on motor activity *in vitro* reported to date (Sirajuddin *et al.*, 2014; Barisic *et al.*, 2015). An upcoming challenge will be to decipher how motor preferences for

Figure 5. α -Tubulin tyrosination is required for the initiation, but not continuation of processive DDB movement.

- A** Schematic showing the assembly of a chimeric MT lattice created by end-to-end annealing of stabilized MTs. The MTs are differentially fluorescently labeled prior to annealing.
- B** Example of a chimeric MT generated from recombinant yeast tubulin containing tyrosinated (red) and detyrosinated (blue) sections. Most TMR-labeled DDB molecules (green) move processively through the annealed junction between the two types of MT lattice (65%, $n = 836$ processive complexes, 65 MT-MT junctions, three independent experiments). Diffusive DDB molecules (white arrow) are primarily observed on the tyrosinated section of the MT and do not diffuse across the boundary. Right, video frames showing a processive DDB complex (pink arrowhead) moving across the junction (yellow arrowhead) of tyrosinated and detyrosinated MT. The (+) and (–) signs denote MT polarity inferred from the directionality of DDB movement. Scale bars are 5 μ m and 25 s. See also Movie EV3.
- C** TIRF images of low (0.3 nM) and high (10 nM) concentrations of TMR-labeled p150 (green) bound to chimeric yeast MTs containing tyrosinated (red) and detyrosinated (blue) sections of MT. Representative kymographs below show diffusive behavior of p150 molecules on the MT. Note that p150 rarely diffuses into the detyrosinated section of MT, even at high concentrations. Scale bars are 5 μ m and 10 s.
- D, E** TIRF images and kymographs of annealed WT and CPA-treated porcine MTs (D), or annealed WT MTs (E). The majority of DDB complexes (78%, $n = 960$ processive complexes, 45 MT-MT junctions, three independent experiments) move processively across the boundary between WT and CPA MTs (D). Note that TMR-labeled DDB complexes (green) in (E) traverse the annealed boundary without pausing (93%, $n = 677$ processive complexes, 45 MT-MT junctions, three independent experiments), suggesting that the annealed junction does not affect DDB motility. Scale bars are 5 μ m and 25 s.
- F** TIRF image and kymograph from a chimeric MT composed of WT (red) and subtilisin-treated porcine MTs (blue). Note some DDB complexes (pink arrows) traverse the boundary and continue moving along the subtilisin-treated section of the MT (41%, $n = 709$ processive complexes, 56 MT-MT junctions, three independent experiments). Scale bars are 5 μ m and 25 s.

certain MT tracks translate into controlling the frequency and/or directionality of cargo transport in living cells.

Materials and Methods

Recombinant tubulin, polymerization, and chimeric microtubules

Recombinant yeast tubulin with human C-terminal tails (chimeric yeast core-human CTT tubulin heterodimer) contains an internal hexahistidine tag located on the α -tubulin subunit and was expressed and purified as described earlier (Sirajuddin *et al.*, 2014). The tail-swapped tubulin used here was constructed by replacing the α -CTT with β -CTT and vice versa. For the yeast alpha-tubulin core fused to TUBB2A CTT, the 19 amino acid peptide DATADEQ GEFEEEEGEDEA from TUBB2A was genetically fused to yeast alpha-tubulin after amino acid 420. Similarly, the 13 amino acid peptide SVEGEGEEGEEY from TUB1A was genetically fused to yeast beta-tubulin after amino acid 426. The purified recombinant tubulin heterodimer (~1–2 mg/ml concentration) was stored in BRB80 buffer (80 mM Pipes pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA) and 200 μ M GTP at -80°C . For each day of assays, the recombinant tubulins were polymerized overnight at 30°C , in the presence of 2 mM GTP with 5 μ M epothilone-B (Sigma). All the polymerized

microtubules contain ~1:250 and ~1:100 ratio of biotinylated and fluorescently labeled (Alexa-488 or -640) porcine brain tubulin, respectively. The percentage ratios of tyrosinated microtubules reported in Fig 2D were prepared by mixing the following molar ratios of tyrosinated and detyrosinated recombinant tubulin: 0.05:1, 0.1:1, 0.5:1, and 1:1 representing 5, 10, 25, and 50% tyrosinated microtubules, respectively.

Porcine brain tubulin was purified according to standard methods (Castoldi & Popov, 2003). To polymerize brain MTs, purified brain tubulin was first incubated with 1 mM GTP at 37°C for 10 min, followed by the addition of 20 μ M taxol for an additional 20 min. All polymerized MTs were purified further by centrifugation at 22,000 *g* for 10 min over a 25% sucrose cushion made in BRB80 buffer containing 10 μ M paclitaxel for porcine MTs or 5 μ M epothilone-B for yeast MTs before use in TIRF assays. The carboxypeptidase A (CPA)-treated porcine brain tubulin protocol was adapted from (Webster *et al*, 1987a). Briefly, 12 μ g/ml CPA (Sigma) was incubated with tubulin (12.5 mg/ml) and 1 mM GTP for 20 min at 37°C, followed by the addition of 20 μ M taxol for an additional 20 min. This was the lowest concentration of protease that completely remove the signal by Western blotting with an antibody specific for tyrosinated form of tubulin. The digestion was stopped by the addition of 10 mM DTT, and the CPA enzyme was removed by centrifugation of the MTs over a 25% sucrose cushion as described above. The subtilisin-treated MTs were prepared as described earlier (McKenney *et al*, 2014). Chimeric microtubules were prepared by mixing equal parts of two species of polymerized and stabilized microtubules, labeled with different fluorescent dyes, and incubated overnight at 30°C for recombinant yeast MTs or room temp for porcine brain MTs.

Antibodies

Antibodies used were as follows: anti- α -tubulin DM1A (T9026, Sigma), anti-tyrosinated tubulin (T9028, Sigma), anti-detyrosinated tubulin (ab48389, Abcam), anti-Delta2 tubulin (AB3203, Millipore), and anti-polyglutamated tubulin GT335 (Adipogen). Western blots were visualized using a LiCor Odyssey system.

Purification of dynein–dynactin–BicD2 (DDB) complex and p150 constructs

Recombinant strepII-SNAPf-BicD2 and p150-sfGFP-SNAPf-strepII were purified from bacteria as previously described (McKenney *et al*, 2014). Human p135 cDNA was obtained from the mammalian gene collection (Dharmacon, GE, GenBank accession number BC071583.1). A construct encoding the first 413 amino acids of human p135, followed by an sfGFP-SNAPf-strepII cassette, was constructed similarly in a pET28 backbone (McKenney *et al*, 2014). Proteins were expressed from bacteria grown in LB media and induced with 1 mM IPTG for 18 h at 18°C. All bacterially expressed proteins were purified by affinity chromatography on StrepTactin resin (GE Life Sciences) followed by gel filtration on a Superose 6 column (GE Life Science). SNAPf-BicD2 was purified and gel-filtered in buffer A (30 mM Hepes pH 7.4, 50 mM K-acetate, 2 mM Mg-acetate, 1 mM EGTA, 10% glycerol), with protease inhibitor cocktail (Promega) and 2 mM DTT. Both p150 and p135 constructs were purified in Buffer B (50 mM Tris-Base pH 8.0, 150 mM K-acetate,

2 mM Mg-acetate, 1 mM EGTA, 10% glycerol), with protease inhibitor cocktail and 2 mM DTT. The purified proteins were then gel-filtered on a Superose 6 column equilibrated in buffer A. Peak fractions were pooled, concentrated on Amicon filters (Millipore), and flash-frozen in LN₂.

Recombinant SNAPf-GST-hDyn protein was prepared using the Bac-to-Bac baculovirus system (Invitrogen) as previously described (McKenney *et al*, 2014). The purified protein was labeled with 10 μ M SNAP-Cell TMR-Star (NEB) while bound to the StrepTactin resin during purification. The protein was subjected to a cycle of MT binding and release by ATP to select for active motors. Briefly, motors were bound to an excess of stabilized porcine MTs in BRB80 buffer with 10 μ M taxol. MTs were pelleted at 60,000 *g* for 10 min at room temperature. Bound motors were released by re-suspension of the MT pellet in BRB80 with 10 μ M taxol and 10 mM ATP. MTs were pelleted again as before, and the eluted motors were frozen in LN₂ after the addition of 20% sucrose and 1 mg/ml BSA as cryoprotectants.

The DDB complex was prepared by adding recombinant strepII-SNAPf-tagged BicD2 (N-terminal construct encompassing amino acids 25–400) to high-speed porcine brain lysates as previously described (McKenney *et al*, 2014). The DDB complexes were fluorescently labeled with excess SNAP-Cell TMR-Star dye (NEB) during purification as described (McKenney *et al*, 2014), and aliquots of eluted DDB were flash-frozen in LN₂ and stored at –80°C. We note that freezing the complex leads to an apparently larger percentage of diffusive complexes in our assays (~15% for unfrozen versus ~30% for frozen).

Microscopy experiments and quantification

Glass chambers were prepared by acid washing as previously described (Tanenbaum *et al*, 2013). Polymerized microtubules were flowed into streptavidin adsorbed flow chambers and allowed to adhere for 5–10 min. After washing the excess unbound microtubules using assay buffer (30 mM Hepes pH 7.4, 50 mM K-acetate, 2 mM Mg-acetate, 1 mM EGTA, 10% glycerol, 0.1 mg/ml biotin-BSA, 0.2 mg/ml K-casein, 0.5% Pluronic F127, and an oxygen scavenging system (Aitken *et al*, 2008)), a motility mixture containing labeled DDB complex, p150, or recombinant GST-hDyn was then flowed in as described earlier (McKenney *et al*, 2014). Images were acquired using Micromanager software (Edelstein *et al*, 2010) controlled Nikon TE microscope (1.49 NA, 100 $\times$ objective) equipped with a TIRF illuminator and Andor iXon CCD EM camera. In the case of GST-hDyn, or DDB complex, 2 mM ATP was included in the buffer. Velocities were calculated from kymographs generated in ImageJ. For fluorescent intensity values, we used maximum intensity projections of time series to quantify GST-hDyn due to its transient binding to the MT. For p150 and p135, raw images were quantified due to these proteins longer bound lifetime on the MT. Standard deviation maps (Cai *et al*, 2009, 2010) were generated using the image stack Z-projection function in ImageJ. The statistical intensity variation of each pixel location from raw images of the acquired time series was plotted in the form of a single image using the ImageJ ZProjection tool and standard deviation projection type as described previously (Cai *et al*, 2009, 2010). For figure preparation, microscopy images were background subtracted using the ‘subtract background’ function in ImageJ with a rolling ball

radius of 50 pixels. Image contrast was linearly adjusted using ImageJ. Because the DDB molecules often traverse the entire microtubule lengths, we did not analyze the run-lengths of DDB motility. Statistical tests were performed in Graphpad Prism 5.0f.

Expanded View for this article is available online.

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Author contributions

RJM and MS conceived the project. RJM and MS performed experiments. RJM, MS, and WH analyzed data. RDV supervised the project, and RJM, MS, WH, and RDV wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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