Dynactin Subunit p150\textsuperscript{Glued} Is a Neuron-Specific Anti-Catastrophe Factor

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Abstract

Regulation of microtubule dynamics in neurons is critical, as defects in the microtubule-based transport of axonal organelles lead to neurodegenerative disease. The microtubule motor cytoplasmic dynein and its partner complex dynactin drive retrograde transport from the distal axon. We have recently shown that the p150\textsuperscript{Glued} subunit of dynactin promotes the initiation of dynein-driven cargo motility from the microtubule plus-end. Because plus end-localized microtubule-associated proteins like p150\textsuperscript{Glued} may also modulate the dynamics of microtubules, we hypothesized that p150\textsuperscript{Glued} might promote cargo initiation by stabilizing the microtubule track. Here, we demonstrate in vitro using assembly assays and TIRF microscopy, and in primary neurons using live-cell imaging, that p150\textsuperscript{Glued} is a potent anti-catastrophe factor for microtubules. p150\textsuperscript{Glued} alters microtubule dynamics by binding both to microtubules and to tubulin dimers; both the N-terminal CAP-Gly and basic domains of p150\textsuperscript{Glued} are required in tandem for this activity. p150\textsuperscript{Glued} is alternatively spliced in vivo, with the full-length isoform including these two domains expressed primarily in neurons. Accordingly, we find that RNAi of p150\textsuperscript{Glued} in nonpolarized cells does not alter microtubule dynamics, while depletion of p150\textsuperscript{Glued} in neurons leads to a dramatic increase in microtubule catastrophe. Strikingly, a mutation in p150\textsuperscript{Glued} causal for the lethal neurodegenerative disorder Perry syndrome abrogates this anti-catastrophe activity. Thus, we find that dynactin has multiple functions in neurons, both activating dynein-mediated retrograde axonal transport and enhancing microtubule stability through a novel anti-catastrophe mechanism regulated by tissue-specific isoform expression; disruption of either or both of these functions may contribute to neurodegenerative disease.

Introduction

Microtubules are dynamic, polarized polymers of tubulin that serve as tracks for long-distance transport in eukaryotic cells. In neurons, transport along microtubules is especially important yet particularly vulnerable to disruption, as these cells are long-lived and postmitotic with elongated axonal processes that can extend up to a meter [1]. There is accumulating evidence that axonal transport is disrupted in multiple neurodegenerative diseases, including amyotrophic lateral sclerosis and Huntington’s disease [2]. In neurodegeneration, defects in microtubule dynamics may precede transport defects [3].

The rate-limiting step in microtubule formation is nucleation from soluble tubulin, which in the canonical pathway is catalyzed by γ-TuRC enriched at the centrosome [4]. However, in large, postmitotic cells like neurons, noncentrosomal nucleation may be particularly important [5–7]. Following nucleation, the dynamics of polymerization and depolymerization are strongly influenced by microtubule-associated proteins (MAPs). In particular, a spatially specialized group of MAPs that localize to the microtubule plus end, the plus end-tracking proteins (+TIPs), are ideally poised to modulate dynamics in cells [8].

One of these +TIPs is dynactin [9], a large complex that binds and activates cytoplasmic dynein [10,11] and also associates with microtubules through its dimeric p150\textsuperscript{Glued} subunit [12]. p150\textsuperscript{Glued} has two alternatively spliced microtubule-binding domains at its N-terminus: a cytoskeleton associated protein glycine-rich (CAP-Gly) domain [13], followed by a serine-rich basic domain [14–16]. The p150\textsuperscript{Glued} microtubule-binding N-terminus is dispensable for most dynein-mediated organelle transport [16–19]. However, we and others have recently shown that it is specifically required for efficient transport initiation from the distal axon in neurons [18,19].

Because p150\textsuperscript{Glued} is specifically enriched at the microtubule plus end, we hypothesized that it might modify microtubule dynamics. Here, using solution assays and direct visualization of microtubule dynamics using TIRF microscopy, we show that p150\textsuperscript{Glued} promotes microtubule formation by binding both to...
Author Summary

Microtubules are polymers of tubulin that undergo successive cycles of growth and shrinkage so that the cell can maintain a stable yet adaptable cytoskeleton. In neurons, the microtubule motor protein dynein and its partner complex dynactin drive retrograde transport along microtubules from the distal axon towards the cell body. In addition to binding to dynein, the p150Glued subunit of dynactin independently binds directly to microtubules. We hypothesized that by binding to microtubules, p150Glued might also alter microtubule dynamics. We demonstrate using biochemistry and microscopy in vitro and in cells that p150Glued stabilizes microtubules by inhibiting the transition from growth to shrinkage. We show that specific domains of p150Glued encoded by neuronally enriched splice-forms are necessary for this activity. Although depletion of p150Glued in nonpolarized cells does not alter microtubule dynamics, depletion of endogenous p150Glued in neurons leads to dramatic microtubule instability. Strikingly, a mutation in p150Glued known to cause the neurodegenerative disorder Perry syndrome abolishes this activity. In summary, we identified a previously unappreciated function of dynactin in direct regulation of the microtubule cytoskeleton. This activity may enhance generic microtubule stability in the cell, but could be especially important in specific areas of the cell where dynactin and dynein are loaded onto microtubules.

microtubules and to soluble tubulin. Both the CAP-Gly and basic domains are required for tubulin-binding in vitro. The full-length isoform encoding both these domains in tandem is primarily expressed in neurons, so we hypothesized that this pro-polymerization activity might be a neuron-specific function of p150Glued. Accordingly, we find that in epithelial cells depleted of p150Glued there is no effect on microtubule dynamics, while in primary neurons, we observed a significant increase in catastrophe upon depletion of p150Glued that was specifically rescued by expression of the neuronal isoform. Finally, we find that a mutation in p150Glued causative for Perry syndrome, a lethal Parkinson’s syndrome, inhibits the anti-catastrophe activity. Thus, the novel neuron-specific anti-catastrophe activity described here may facilitate microtubule stabilization in neurons. We speculate that disruption of this function may contribute to neurodegeneration.

Results

To investigate the dynein-independent effects of dynactin on microtubule dynamics, we designed N-terminal recombinant polypeptides truncated before the dynein binding site within the first p150Glued coiled-coil domain (CC1, Figure 1A) [20]. Since CC1 is also required for endogenous dimerization of p150Glued, we replaced this domain with a short, well-characterized GCN4 coiled-coil [21]. In order to assay the effects of dimerization on microtubule dynamics, we also generated a corresponding construct lacking the dimerization domain, but including both the N-terminal CAP-Gly and basic microtubule-binding domains.

To characterize our recombinant polypeptides, we performed glutaraldehyde cross-linking and hydrodynamic analysis, and confirmed that p150 Nt-GCN4 dimerized as expected (Figure S1A). Both the monomeric and dimeric constructs were soluble and monodisperse following purification (Figure S1B). Hydrodynamic analysis also indicated that both polypeptides are highly elongated (Rr/Rmin>1.9, Figure S1B), consistent with previous electron microscopy images of dynactin [22] that indicate that the p150Glued dimer projects outward from the Arp1 filament that forms the base of the dynactin complex. We measured the relative affinities of both the dimeric and monomeric constructs for microtubules, and found that both bound to paclitaxel-stabilized microtubules with moderate affinities (Kd of 340 nM and 890 nM, respectively, Figure S1C), within the typical range for CAP-Gly proteins [23].

p150Glued Dimer Potently Promotes Microtubule Formation

Previous work from our lab suggested that the N-terminus of p150Glued promotes bulk microtubule formation [24]. To confirm and extend this result, we first compared the activities of the dimeric p150 Nt-GCN4 and the monomeric p150 Nt constructs using in vitro assembly assays. Only the dimeric construct induced a large increase in light scattering, a measure of increased microtubule polymerization (Figure 1B).

We also compared the activities of these constructs to that of the well-characterized neuronal MAPs tau (Tau23) and doublecortin (DCX-GFP) (Figures 1B and S1D), both of which have been shown to promote the formation of microtubules, a function that appears defective in the setting of disease [25,26]. The increase in light scattering induced by 1 μM p150 Nt-GCN4 was similar in magnitude to the increase induced by addition of 10 μM DCX-GFP, and approximately 4-fold more than the increase induced by 2 μM Tau23 (Figure 1B). Thus, p150, like these classical neuronal MAPs, enhances the polymerization of microtubules from soluble tubulin. In contrast, the microtubule-binding motor domain of kinesin-1 (rKin430-GFP) did not promote the formation of microtubules (Figure 1B), indicating that this effect is not a nonspecific one characteristic of all microtubule-binding proteins.

Since microtubule bundling, as well as microtubule polymerization, can lead to increases in light scattering signal, we pelleted the reaction mixtures and analyzed the resulting microtubule pellets by SDS-PAGE (Figure S1E). This analysis confirmed that p150 Nt-GCN4 induces a large increase in tubulin polymerization. Additionally, we tested to ensure that increased turbidity from protein aggregation was not responsible for the light scattering signal; when the purified p130Nt-GCN4 is incubated in the absence of tubulin, light scattering remained low and constant (Figure S1F).

As a final control, because the fusion of a His-affinity tag has been reported to disrupt the activity of another plus-tip protein, EB1 [27], we compared our His-tagged p150 constructs to equivalent Strep-tagged constructs, as well as to the activity of an untagged construct purified by ion exchange chromatography (Figure S2A). The untagged construct was identical to the His-tagged construct on size exclusion chromatography (Figure S2B), and the activities we measured were identical (Figure S2C, S2D), indicating that the promotion of microtubule polymerization by dimeric p150 is not affected by the nature of the N-terminal purification tag.

Though previous work concluded that monomeric p150 constructs could influence microtubule polymerization at high concentrations [28,29], in our assay, monomeric p150 Nt only marginally increased light scattering as compared to dimeric p150 Nt-GCN4 (Figure 1B); nor did we see increased tubulin in polymer in sedimentation assays as compared to buffer controls (Figure S1E). Thus, we find that dimerization of p150Glued is required for robust pro-polymerization activity in vitro.

p150Glued Enhances Microtubule Polymerization Rates and Inhibits Microtubule Catastrophe

We hypothesized that p150Glued, which localizes to microtubule plus-ends in the cell [9,16], might, like other +TIPS [8], influence
the parameters of dynamic instability. In the absence of MAPs, microtubule dynamic instability is characterized by periods of slow polymer growth ending in catastrophe and rapid depolymerization; following rescue, microtubule growth resumes by addition of new tubulin subunits at the plus end. To test the hypothesis that p150Glued alters these parameters, we used total internal reflection fluorescence (TIRF) microscopy to directly observe microtubule polymerization from pre-formed GMPCPP-stabilized microtubule seeds (Figure 1C) [30,31].

Addition of tubulin alone to pre-formed, stabilized seeds induced characteristic periods of slow growth, with transitions to rapid shrinkage following a catastrophe (Figure 1D). In contrast, addition of a low (100–200 nM), physiological concentration of p150 Nt-GCN4 [32] to the assay led to more rapid microtubule growth (Figure 1D). The polymerization rate increased more than 2-fold at 200 nM p150 Nt, or p150 Nt-GCN4. (E) Polymerization rates and (F) catastrophe frequencies from seeded assembly shows that p150 Nt-GCN4 promotes polymerization and inhibits catastrophe. All error bars represent SEM of three or more independent experiments. Statistical testing was performed with a two-tailed t test. *p < 0.05; **p < 0.01. For (E), p < 0.01 for all conditions compared to control.

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Figure 1. p150Glued promotes microtubule formation. (A) Schematic depicting endogenous full-length p150Glued and dimeric and monomeric constructs. (B) Light scattering traces for recombinant polypeptides or buffer control incubated with tubulin show that the dimeric p150 Nt-GCN4 and neuronal MAPs tau23 and DCX-GFP robustly promote microtubule assembly, while monomeric p150 Nt and rKin430-GFP do not. (C) TIRF elongation assay. As shown in the schematic at top, rhodamine-labeled tubulin is polymerized from biotinylated Alexa488-labeled GMPCPP microtubule seeds. Montage below shows free tubulin assembly (red) from a stabilized seed (green) in the absence of p150. Arrowhead identifies the microtubule plus-end. (D) Kymograph plots showing representative examples with buffer, 200 nM p150 Nt, or p150 Nt-GCN4. (E) Polymerization rates and (F) catastrophe frequencies from seeded assembly shows that p150 Nt-GCN4 promotes polymerization and inhibits catastrophe. All error bars represent SEM of three or more independent experiments. Statistical testing was performed with a two-tailed t test. *p < 0.05; **p < 0.01. For (E), p < 0.01 for all conditions compared to control.

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p150Glued Modifies Microtubule Dynamics Independently of EB1

Previous studies with a monomeric p150Glued CAP-Gly polypeptide lacking the basic domain suggested that EB1 was required for p150Glued to modify microtubule dynamics [28,29]. However, native p150Glued is dimeric, and our dimeric p150 Nt-GCN4 construct has potent effects on microtubule dynamics in the absence of EB1. However, we wondered if the addition of EB1 to our assays might further modulate the effects we observed.

Using analytical size exclusion chromatography, we confirmed that, as expected [28,33,34], p150 Nt-GCN4 forms a stable complex with recombinant full-length EB1 (Figure S3A–B). However, the ability of p150Glued to promote microtubule formation appears to be independent of this interaction; the addition of EB1 caused a minor decrease on the pro-polymerization activity of p150Glued (Figure S3C, S3E) and did not affect its ability to inhibit microtubule catastrophe (p > 0.5, Figure S3D, S3F).

These data confirm that p150Glued has an intrinsic ability to modify microtubule dynamics and, further, that p150Glued inhibits the inherent pro-catastrophe effects of EB1 in vitro.

p150Glued Catalyzes Microtubule Nucleation

We have shown that p150 Nt-GCN4 acts as a pro-polymerization and anti-catastrophe factor in vitro, acting on preformed microtubules. However, we suspected that p150 Nt-GCN4 might also promote microtubule nucleation because we observed a
decrease in the lag time for microtubule formation that was inversely dependent on the p150 Nt-GCN4 concentration (Figure 2A, 2B).

We designed a TIRF assay to directly test this hypothesis. We specifically oriented the p150 N-terminal microtubule-binding domains toward the microscopy chamber by immobilizing 250 nM p150 polypeptides on the coverslip via an antibody to the C-terminal His-tag (Figure 2C). We then washed the chamber to remove unbound p150, and perfused in 3.5 μM soluble tubulin, which is below the critical concentration for spontaneous microtubule nucleation.

Consistent with our hypothesis, in the presence of dimeric p150 Ni-GCN4, we observed the appearance of many short microtubules within 2 min (Figure 2D). We did not observe nucleation when monomeric p150 Ni was immobilized in the chamber (Figure 2D). When we reduced the amount of p150 Ni-GCN4 on the chamber surface so that we could observe the nucleation of individual filaments, we observed growth from both the microtubule minus- and plus-ends (Figure 2E). This suggested to us that p150 was not promoting nucleation via a template-and-capping mode like γ-TuRC [4], but instead might stabilize an oligomeric tubulin species, similar to the action of doublecortin [6], or by binding directly to soluble tubulin dimers.

**p150Glued Binds to Soluble Tubulin Dimers**

p150Glued is well-characterized as a MAP, but we hypothesized that it might affect microtubule dynamics by also binding to soluble tubulin through its CAP-Gly domain. The CAP-Gly domains of other structurally related proteins, such as CLIP-170 and tubulin binding cofactors B and E, have been shown to bind soluble tubulin [23]. We used analytical size exclusion chromatography to assay complex formation. To preclude microtubule nucleation, we performed all experiments at 4°C.

When both p150 Ni-GCN4 and p150 Ni were incubated with tubulin, we observed a pronounced shift in the elution peak, indicating complex formation (Figure 3A, Figure S4A–B). We varied the molar ratio of p150:tubulin until we no longer observed uncomplexed species [35]. In this way, we found that p150 Ni-GCN4 formed a stable complex with tubulin in a 1:1 ratio (Figure S4C,E–F). However, we observed a tail trailing the elution peak of the 1:1 p150 Ni-GCN4:tubulin complex (Figure 3A), which could indicate an exchange process between free p150 Ni-GCN4 and tubulin during the elution. In agreement with this possibility, we found that when we incubated excess tubulin with p150 Ni-GCN4 to encourage saturated binding, the elution peak shifted further to the left (Figure 3A, Figure S4H–I), indicating that a larger complex had formed, and suggesting that p150 Ni-GCN4 might promote microtubule nucleation by transiently binding multiple tubulin subunits and encouraging the formation of a stable seed.

We know that the acidic C-terminal domain of tubulin is important in the binding of p150Glued to microtubules [33,36]. To determine if p150Glued also binds to soluble tubulin dimers via their C-terminal tails, we cleaved these tails from tubulin using the protease subtilisin (Figure S4J–K) [37,38] and again assayed for complex formation via size exclusion chromatography. After cleavage, we could not detect a tubulin-p150 complex (Figure 3B), confirming that the p150 CAP-Gly domain interacts with the C-terminus of tubulin dimers in solution.

This orientation might leave the p150 basic domain free to stabilize the complex through electrostatic interactions. In support of this model, we found that when we eluted the tubulin-p150 Ni-GCN4 complex under increasing ionic strength, which should disrupt such interactions, the relative abundance of the complex...
was decreased at the expense of free tubulin (Figure 3C). This suggests that while either the CAP-Gly or the basic domains are sufficient to bind microtubules [14–16], both domains may be necessary for p150Glued to bind tubulin dimers and promote microtubule assembly.

**Neuron-Specific p150Glued Isoform Stabilizes Microtubules**

p150Glued is alternatively spliced in a tissue-specific manner with expression of full-length p150 containing both the CAP-Gly and basic domains restricted to the nervous system (Figure 4A) [15,16]. The two predominant p150 spliceforms expressed in vivo lack either the CAP-Gly domain or the basic domain. p153 is an isoform that, like p150, is enriched in the nervous system; it arises from an alternative start site and thus lacks the CAP-Gly domain and exon 5 [39]. In nonneuronal tissues, all or part of the basic domain is spliced out [15,16]. Thus we were able to use physiologically relevant splice forms to test our model.

We produced recombinant dimeric polypeptides recapitulating p135 (p135 Nt-GCN4) and p150 lacking exons 5, 6, and 7 (the majority of the basic region, Δ5–7 Nt-GCN4) (Figure 4A and Figure S5A–D). We found that when we co-incubated p135 Nt-GCN4 (which lacks the CAP-Gly domain) with tubulin, we did not observe complex formation by size exclusion chromatography (Figure 4B). This was unsurprising given the accepted role of CAP-Gly domains in binding to microtubules [23], and the role we establish here for p150Glued in promoting microtubule assembly. For the red trace, maximum absorbance is scaled to the complex peak. (B) Size exclusion chromatograms for p150 Nt-GCN4 incubated with tubulin and eluted at increasing ionic strength indicate that electrostatic interactions between tubulin and the p150 basic domain stabilize the p150-tubulin complex because progressively reduced complex formation is observed.

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**p150Glued Regulates Neuronal Microtubule Dynamics**

To determine how the cellular role of p150Glued corresponds to the biochemical activities we measured, we first depleted p150Glued from COS7 cells (Figure 5A), a primate cell line derived from kidney that should not express significant levels of the neuronal isoform [15]. We transfected the cells with low levels of GFP-EB3 to visualize the growing microtubule plus end, and examined microtubule dynamics in an unbiased manner using the PlusTipTracker software package [40]. Under conditions in which >95% of endogenous p150Glued was depleted (Figure 5A), we did not observe an appreciable effect on either the average displacement of EB3-GFP comets before microtubule catastrophe or on microtubule polymerization velocities (Figure 5B–C, p>0.1, and unpublished data). We obtained similar results in HeLa cells, another epithelial cell line.

We next investigated the effect of p150Glued depletion on microtubule dynamics in mammalian neurons, which express the full-length isoform which includes both the CAP-Gly and basic domains that we have identified as necessary for productive tubulin binding. Interestingly, though the CAP-Gly domain is a conserved feature of p150Glued, the basic domain appears to be specifically evolved in organisms with complex, dynamic microtubule cytoskeletons. It is absent in yeasts, but is apparent in protists, filamentous fungi, and vertebrates, with mammalian taxa expressing particularly basic regions downstream of the CAP-Gly domain (Figure S6A–B, Dataset S1) [41].

Next, we visualized microtubule dynamics using GFP-EB3 expressed at low levels in mouse dorsal root ganglion neurons (DRGs; Figure 5E,F). Because of the high levels of tubulin in mammalian neurons, we quantitated microtubule dynamics manually using kymograph analysis (Figure 5G). When p150Glued was depleted by ~80% (Figure 5D), we did not observe significant differences in either polymerization rates or the number of EB3-GFP comets compared to scrambled RNAi-treated cells (unpublished data). However, we noted that when p150Glued was depleted, there was a significant decrease in the distance GFP-EB3 comets traveled before catastrophe (Figure 5G,H), indicating a significant increase in the catastrophe frequency (Figure 5J). For example, compare the comets that traveled at least 5 µm before catastrophe [black] compared to those that travelled less than 2 µm (magenta) as shown in Figure 5H.

To confirm that this was a specific effect of p150Glued knockdown, we quantitated dynamics in RNAi-treated neurons rescued with either the full-length neuronal p150 isoform, or with p135, which lacks the N-terminal CAP-Gly domain. We observed that rescue of neurons with a plasmid encoding RNA-resistant
p150 significantly reduced the catastrophe frequency as compared to RNAi-treated cells (Figure 5I,J). Wild-type dynamics were not completely restored, likely because we did not fully restore endogenous expression levels with the rescue plasmid (Figure S6C). In contrast, transfection with the p135 isoform, which lacks the N-terminal CAP-Gly domain, did not rescue defective polymerization dynamics in primary neurons (Figure 5I,J). Importantly, we found that transfection with the D5–7 construct, the nonneuronal splice form that lacks the basic domain, also did not rescue the defective polymerization dynamics induced by depletion of endogenous p150Glued (Figure 5I,J). Thus, in strong agreement with our in vitro results, these observations indicate that the tandem CAP-Gly and basic domains of p150 are required to generate anti-catastrophe activity in neurons.

A Parkinson Disease Mutation Abolishes p150Glued Activity

Mutations in the CAP-Gly domain of p150Glued have been found to cause multiple neurodegenerative diseases, including Perry syndrome, a disease characterized by Parkinsonism, weight loss, hypoventilation, and depression. While human disease-associated mutations in p150Glued have been expressed throughout the body, these mutations induce pathologies only in the nervous system [2]. Thus, we hypothesized that the tissue-specific activity of p150Glued described above might be disrupted in this disease.

To test this hypothesis, we focused on the Q74P mutation (Figure S7A) [42]. The Q74P mutation has been shown to disrupt both the microtubule and EB1-binding activities of the p150 CAP-Gly domain but to have only a modest effect on overall protein stability [19,43]. To obviate any disease-induced aggregation, we expressed and purified recombinant Q74P Nt-GCN4 immediately before experimentation using a final gel filtration step to exclude higher order oligomers (Figure S7B, S7C), and confirmed proper dimerization by glutaraldehyde cross-linking (Figure S7D). In contrast to wild-type p150 Glued-Nt-GCN4, we found that Q74P Nt-GCN4 was defective in inhibiting microtubule catastrophe in vitro (Figure 5K). In primary neurons, we found that transfection with a plasmid expressing Q74P could not restore normal microtubule dynamics, in contrast to the rescue seen with the wild-type p150Glued construct (Figure 5L,M).

Discussion

Here we show that p150Glued promote microtubule formation in vitro by catalyzing nucleation, increasing the polymerization rate, and inhibiting catastrophe. These activities require dimerization and are dependent on the ability of p150Glued to form a stable complex with tubulin through interactions with both the N-terminal CAP-Gly and basic domains. In primary neurons, we observe that the dominant effect of p150Glued on microtubule dynamics is the suppression of catastrophe (Figure 6). Finally, we determine that a single point mutation within the CAP-Gly domain of p150Glued causative for a fatal familial form of Parkinson disease, known as Perry Syndrome, leaves p150Glued...
Figure 5. p150Glued stabilizes microtubules in neurons. (A) Depletion of p150Glued in COS7 cells by siRNA (KD) relative to control cells treated with scrambled (Scram) oligonucleotides; upper panel is a Western blot probed with a monoclonal antibody to p150Glued, and lower panel is Coomassie staining to show equal protein loading. (B) Representative rainbow-coded maximum intensity projections show that the overall microtubule architecture and dynamics are not perturbed in COS7 cells depleted of p150Glued relative to control cells. (C) PlusTipTracker quantitation of EB3-GFP comet velocities shows that knockdown does not alter parameters of microtubule dynamics, including distance to catastrophe, a measure of the catastrophe frequency ($p > 0.1$). (D) Western blot showing knockdown of p150Glued in DRG neurons (KD), relative to control neurons or COS7 cells treated with scrambled oligonucleotide. Note the differential splice forms of p150Glued expressed in neurons relative to COS7 cells. (E) Space-filling fluorescence in representative neurites used to investigate microtubule dynamics in primary DRG neurons. (F) Rainbow-coded maximum intensity projections of selected EB3-GFP comets demonstrate microtubule dynamics in DRG neurons. (G) Kymographs of GFP-EB3 comets in cultured DRG neurons treated with either scrambled or p150Glued RNAi reveal that in neurons, p150Glued inhibits catastrophe. (H) Camera lucida tracing of the kymographs in panel G to indicate polymerization events persisting greater than 5 μm (black) and less than 2 μm (magenta). (I) Kymographs of GFP-EB3 comets in neurons depleted of endogenous p150Glued by RNAi and rescued with either full-length p150, Δ5–7, or the neuron-specific alternative splice form p135. (J) Analysis of microtubule dynamics in scrambled control neurons, and neurons depleted of endogenous p150Glued with or without...
unable to promote microtubule assembly either in vitro or in neurons.

Dynactin was originally identified as a large protein complex that supported dynein-mediated vesicle transport [32,44]. The N-terminus of the 150 kDa subunit binds microtubules independently of dynein [12], and increases the processivity of dynein in vitro [14,45]. Recently, it has been demonstrated that the microtubule-binding N-terminus of p150Glued is dispensable for organelle localization and vesicular motility in nonneuronal cells [16,17,19]. However, the microtubule-binding CAP-Gly domain of dynactin is required for efficient transport initiation from the distal axon in neurons [18,19]. A plus end-localized pool of p150Glued may serve to load dynein onto the microtubule [19,46]. However, biochemical analyses and immunolocalization suggest that a large proportion of dynactin may in fact not be in complex with dynein [19,32,47,48]. Enriched at the plus end, this population of dynactin would be perfectly poised to affect microtubule dynamics.

Our data suggest a mechanism whereby p150Glued could modify microtubule dynamics. Recently, it has been suggested that the kinetics of tubulin association and dissociation with the microtubule plus end may be much faster than previously appreciated [49]. This makes it increasingly plausible that one mode whereby MAPs alter microtubule dynamics is by modulating the off-rate of tubulin subunits from microtubule plus ends. Since p150Glued can bind both to microtubules and to soluble tubulin, and because dimerization appears necessary for p150Glued to robustly modify dynamics, we speculate that p150Glued may be acting in this capacity by binding to both microtubules and tubulin at the same time, decreasing the off-rate and inhibiting catastrophe, enabling efficient initiation of dynein-mediated retrograde runs (Figure 6).

Interestingly, this mechanism is distinct from the mode by which cytoplasmic dynein independently functions to inhibit catastrophe [50]. Areas of the distal neuron where both cytoplasmic and dynactin are localized could be sites of particularly robust microtubule stabilization.

The regulation of these microtubule-modifying abilities of p150Glued may be multifactorial. We have shown that the basic region is necessary for the modification of microtubule dynamics by p150Glued, likely by ensuring a stable complex with the distributed acidic nature of tubulin. The basic region is also serine- and threonine-rich, and has been shown to be the target of phosphorylation by regulatory kinases [51,52], which might further modulate the p150-glutamin interaction during mitosis, or during development. p150Glued also binds to CLIP-170 [24,28,34,53], which could further modify the behavior of p150Glued in the cell.

In vivo, only the p150Glued isoform expressed in neurons includes both the full CAP-Gly and basic domains that we have shown are rescue with resistant constructs of p150 or p135 and Δ5–7. Bars represent mean of comet parameters from multiple cells on multiple days ± SEM. Statistical testing was performed via t test with correction for multiple comparisons. *** p<0.001. (K) In vitro analysis of catastrophe rates demonstrates that the Perry syndrome-associated mutation Q74P p150 Nt-GCN4 does not inhibit microtubule catastrophe (p>0.5), as compared to the wild-type p150NT-GCN4 construct. (L, M) Kymographs and quantitation of GFP-EB3 comets in cultured DRG neurons depleted of endogenous p150Glued and rescued with either wild-type or Q74P p150Glued reveal that mutant p150 is defective in inhibiting microtubule catastrophe in neurons.
necessary to modify microtubule assembly dynamics. We have recently shown that young, developing neurons depleted of p150Glued are morphologically normal [19]. In fact, profound depletion of both EB1 and EB3, which should effectively disrupt plus-end targeting, has no gross effects on neurite outgrowth [19]. It may be that, as recent evidence suggests, only as neurons age, and their processes lengthen and elaborate, does the centrosome lose its function as a microtubule organizing center and microtubule dynamics become particularly reliant on plus-end regulation [5,7]. It is perhaps telling that human patients with the Q74P p150Glued mutation do not show disease onset until the fifth decade of life [42]. More broadly, microtubule dynamics may alter in aging or degenerating neurons, as suggested from studies of cells from patients with sporadic Parkinson’s and Alzheimer’s disease [54–56].

In summary, we have identified and characterized a novel role for p150Glued in the tissue-specific stabilization of microtubules, and implicated defects in neurodegeneration. Further studies to disentangle the effects of the mutation on axonal transport and microtubule stability in neurons will be required to clarify the pathogenesis involved.

Experimental Procedures

Protein Purification

Amino acids 1–210 of human p150Glued (DCTN1, NM_004082.4) were inserted into pET-29a (Novagen) with a short linker (AAAAADPPVAT) before the C-terminal 6x-His tag. Dimeric constructs contained a GCN4 sequence (VMKQLEDK-VEELSKNYHLENEVARLKLLVGE) [21] before the linker and 6x-His. Δ5–7 dimer was deleted for exons 5–7 (RGLKPKKAPTARTTRRPK). The p135 dimer encodes an N-terminal MMRQ (replicating the endogenous expressed (RGLKPKKAPTARKTTTRRPK). The p135 dimer encodes a GCN4 sequence (VMKQLEDK-VEELSKNYHLENEVARLKLLVGE) [21] before the linker and 6x-His. Δ5–7 dimer was deleted for exons 5–7 (RGLKPKKAPTARTTRRPK). The p135 dimer encodes an N-terminal MMRQ (replicating the endogenous expressed plastid splicing form) appended to p150Glued AA 139–210. Q74P Nt-GCN4 was constructed from the p150 Glu NC-GCN4 backbone and previously used plasmids [19]. The tag-less p150Glued construct was similar to 1–210-GCN-His, but lacked the C-terminal His-tag. The Strep-tagged construct was made by replacing the C-terminal His-tag by PCR. C-terminal GFP-tagged constructs were expressed from pET-29a with EGFP-6x-His. Dimeric constructs were diluted to 0.5 μM and the p150 monomer was diluted to 1 μM in BRB20 with 125 mM NaCl and 1 mM DTT and incubated on ice with 0.5% glutaraldehyde (Thermo) for 2 h. The reactions were quenched with an equal volume of 1 M glycine, denatured, and analyzed by SDS-PAGE and colloidial Coomassie staining (Invitrogen).

Glutaraldehyde Cross-Linking

Recombinant polypeptides were thawed and incubated at the molar ratio indicated in the text with 30 μM tubulin or mock-incubated for 10' on ice, then loaded into a 50 μL loop with 80 μL final volume, and injected onto a Superdex 200 10/300 GL column pre-equilibrated with BRB20 (pH 7.1) supplemented with 35 mM KCl and 10 mM imidazole, or indicated concentrations of KCl for Figure 3C. Polypeptides were eluted isocratically at 4°C and 0.4 mL/min in the equilibration buffer and collected in 250 μL fractions. The column was calibrated with gel filtration molecular weight standards (ferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, and carbonic anhydrase) from Sigma. The void volume was at ~7.5 mL. We did not detect any material in the void for any experiments.

Subtilisin cleavage was performed for the indicated times at room temperature in a 1:500 mass ratio to tubulin after Knippling et al. [30] and Gupta et al. [59].

Light Scattering Assay

Assay was performed in BRB80 supplemented with 35 mM KCl, 5% glycerol (√/√), 1 mM DTT, 1 mM GTP, and 1 mg/mL casein (to prevent nonspecific adsorption) mixed with 20 μM tubulin in a 96-well half area UV transparent plate (Corning) on ice. The plate was read in kinetic absorption mode at 340 nm in a SynergyMx plate reader (BioTek) that had been prewarmed to 37°C. The heat quench in this setup is relatively slow, and we found that it corresponded to the limit of the exponential fit in Figure 2C.

TIRF Microtubule Elongation Assay

Experiments were conducted in flow cells (~8 μL in volume) constructed using slides and silanized coveslips (Amersham...
Biosciences) attached with double-sided adhesive tape and bordered with vacuum grease. The flow cell was coated with 25% monoclonal anti-biotin (Clone BN-54, Sigma) and then blocked with 5% pluronic F-127 (Sigma). The chamber was then washed with 1 mg/mL casein in BRB80, and 6.25 μg/mL freshly thawed double-cycled GMPCPP (Jenna) [30] microtubule seeds were then introduced in the flow cell. These seeds were labeled 1:1:33 with biotin and Alexa488 (Cytoskeleton Inc). The chamber was then washed with 1 mg/mL casein in BRB80. Polymerization was initiated by introducing polymerization buffer (as described previously [31]) with 7.5 µM tubulin labeled 1:50 with rhodamine tubulin (Cytoskeleton). The final buffer composition was BRB80 supplemented with 28 mM KCl and 3.75% glycerol (v/v). For the experiments with high concentrations of EB1, the final buffer composition was BRB80 supplemented with about 50 mM KCl and 5% glycerol. Imaging was performed at 37°C.

**TIRF Microtubule Nucleation Assay**

Flow constructs were as above coated with 15% anti-tetraHis (Qiagen). The chamber was blocked with 5% pluronic F-127. p150 constructs were adsorbed to the chamber at 250 nM, and unbound constructs were removed with copious washing with BRB80 supplemented with 1 mg/mL BSA. Imaging was initiated and nucleation buffer perfused consisting of 3.5 µM tubulin in BRB80 supplemented with 1 mg/mL BSA, 2.5 mM GTP, 50 mM DTT, 140 mM glucose, glucose catalase/oxygen antidote system, and 0.25% F-127. Imaging was performed at 37°C.

**Microtubule Binding Assays**

Unlabeled tubulin was polymerized at 5 mg/mL in BRB80 and 1 mM GTP and stabilized with 20 μM Taxol on the day of the experiment. Increasing concentrations of microtubules were incubated at 37°C for 20 min with 0.4 µM dimeric constructs or 0.8 µM monomeric construct, and centrifuged at 100 k rpm in the TLA 120.1 rotor (Beckman) at 37°C for 20 min. The supernatant and the pellet were then separated, denatured, and analyzed by SDS-PAGE and densitometry. For the binding assays done with C-terminal GFP-tagged constructs, microtubules were incubated with 0.1 µM constructs and the depletion of protein from the supernatant monitored by fluorimetry. Primary antibody for Western blotting was against the CAP-Gly domain of p150Glu (BD Biosciences).

**Live Cell Imaging and Analysis**

Isolation, culture, nucleofection, and imaging of DRG neurons was performed as done previously [19]. GFP-EB3 comets were imaged in both the mid- and distal axons, taking care to image only cells that were expressing at appropriately low levels. Kymographs were constructed and quantitated in ImageJ, using the Multiple Kymograph plugin (EMBL Heidelberg). The x-axis displacement of the comet we computed as the distance until catastrophe, while the slope of the line that the comet makes was taken as the polymerization velocity. COS7 cells were cultured, and transfected as described previously [16], and imaged as for neurons above. Cells expressing appropriate levels of GFP-EB3 were quantitated using the PlusTipTracker software package [40] using the following tracking parameters: search radius of 3–15 pixels; minimum subtract length of three frames; maximum gap length of five frames; maximum shrinkage factor (relative to growth speed) of 1.5; maximum angle of 30 degrees forward and 10 degrees backward; and fluctuation radius of 1.0 pixels.

The sequences of the siRNA oligonucleotides against p150Glu are GACUUCACCCCCUUUGAUUAA and CGAGCCUCACCA-GAGACCUG. The corresponding scrambled sequences are GATCCTTTACGTTCACAA and CCAUCGCAUUCC-GACCCAG, respectively [16,60].

**Supporting Information**

**Dataset S1 Analysis of nonmetazoan p150Glu N-termini.** The Homo sapiens p150Glu CAP-Gly was used as the query to perform a standard protein blast on the indicated organism. The results were then manually curated, and the core CAP-Gly domain in the listed sequence (highlighted in yellow) identified using the Conserved Domain Database. The p150Glu CC1 was then identified using Coils [http://embnet.vital-it.ch/software/COILS_form.html, highlighted in magenta] and acidic and basic amino acids (red and blue respectively) highlighted using Protein Colourer ([http://www.ebi.ac.uk/cgi-bin/protcol/enzyme/ProteinColourer.pl](http://www.ebi.ac.uk/cgi-bin/protcol/enzyme/ProteinColourer.pl)).

**Figure S1 Characterization of recombinant dimeric and monomeric p150 polypeptides.** (A) Coommassie staining demonstrates purity of recombinant polypeptides used in experiments, and at right, dimerization of p150 Nt-GCN4 confirmed by glutaraldehyde cross-linking. (B) Hydrodynamic analysis of the p150 Nt-GCN4 and p150 Nt confirms monodispersion and suggests elongated shape. From left: mean elution volume ± SEM for at least three separate column runs; expected molecular mass from the primary amino acid sequence; apparent molecular mass from the calibrated column; calculated Stokes radius from the calibrated column; minimal Stokes radius of a perfectly spherical protein of the expected molecular mass; ratio of Rs/Rmin (Globular 1.2–1.3; moderately elongated 1.5–1.9; highly elongated >2.0) [61]. (C) Microtubule pelleting curves for 100 nM C-terminal GFP-tagged constructs incubated with increasing concentrations of Taxol-stabilized microtubules shows both constructs bind microtubules with moderate affinity. Percent binding was determined fluorimetrically from the fraction of construct in the pellet. Below, validation of fluorimetry by Western blotting the supernatants for p150. (D) Coomassie staining of recombinant polypeptides used in experiments. (E) Pellets of assembly reactions from Figure 1B confirm microtubule formation. (F) Light scattering traces for recombinant p150 proteins with and without tubulin confirm that measured turbidity does not result from protein aggregation at the elevated temperatures used in the assay. All error bars indicate the SEM for three or more independent experiments.

**Figure S2 Characterization of untagged p150 Nt-GCN4 or p150 Nt-GCN4-Strep.** (A) Coomassie-stained or immunoblot for His-tag demonstrate purity of recombinant proteins and confirm absence of tag. (B) Analytical size exclusion chromatography of p150 Nt-GCN4-His and corresponding untagged construct. (C) Light scattering traces for buffer control or recombinant polypeptides incubated with 20 μM tubulin and warmed to 37°C confirm that the ability of p150 to promote microtubule formation does not result from excess charge on the His-tag. Note that since they otherwise would be indistinguishable, the traces for the 1 μM His- or Streptagged p150 Nt-GCN4 alternate. (D) Light scattering traces for buffer control compared to varying concentrations of untagged p150 Nt-GCN4.

**Figure S3 p150Glu modifies microtubule dynamics independently of EB1.** (A) Coomassie staining of purified full-length EB1 used in experiments. (B) Size exclusion chromatograms for EB1 and p150 Nt-GCN4 run alone, or pre-incubated in a 1:2 ratio indicate that they form a stable complex. (C, D) Polymerization
rates and catastrophe frequencies from seeded assembly shows that 200 nM p150 Nt-GCN4 promotes polymerization and inhibits catastrophe independently of 25 nM EB1; p<0.01 for all conditions compared to control. (E, F) Polymerization rates and catastrophe frequencies from seeded assembly for varying higher concentrations of EB1 with or without 200 nM p150 Nt-GCN4 confirm that p150 increases the microtubule polymerization rate independently of EB1 and damps the intrinsic pro-catastrophe activity of EB1. (G, H, I) Size exclusion chromatographs and corresponding fractions subjected to SDS-PAGE and coomassie staining suggest that at similar molar ratios, soluble p150 can complex with either tubulin or EB1.

Figure S4 Characterization of complex formation between the p150 Nt and tubulin. (A) Size exclusion chromatograms for p150 Nt run alone or pre-incubated with tubulin reveal that p150 forms a stable complex with tubulin. (B) Hydrodynamic analysis of the p150 Nt-GCN4 and p150 Nt complexes with tubulin suggests the formation of a globular complex. From left: mean elution volume ± SEM for at least three separate column runs; expected molecular mass from the primary amino acid sequence; apparent molecular mass from the calibrated column; calculated Stokes radius from the calibrated column; minimal Stokes radius of a perfectly spherical protein of the expected molecular mass; ratio of Rs/Rmin (Globular 1.2–1.3; moderately elongated 1.5–1.9; highly elongated >2.0) [61]. (C–I) Individual SEC fractions for the indicated conditions were subjected to SDS-PAGE and colloidal coomassie staining. (J) Subtilisin was incubated in a 1:350 weight ratio with tubulin at room temperature for the indicated times, then inactivated with 4 mM PMSF, and heat denatured. Twenty minutes cleavage was chosen for further experiments because separation of the β-tubulin band has plateaued and internal cleavage products have begun to form. (K) Size exclusion chromatograms of mock-treated tubulin or subtilisin-treated tubulin.

Figure S5 Characterization of recombinant Δ5–7 Nt-GCN4 and p135 Nt-GCN4 representing endogenous isoforms. (A) Coomassie staining of purified polypeptides used in experiments. (B) Coomassie stain of the recombinant dimers in the absence or presence of 0.5% glutaraldehyde shows that the mutant construct is similar to the wild-type construct. From left: mean elution volume ± SEM for at least three separate column runs; expected molecular mass from the primary amino acid sequence; apparent molecular mass from the calibrated column; calculated Stokes radius from the calibrated column; minimal Stokes radius of a perfectly spherical protein of the expected molecular mass; ratio of Rs/Rmin (Globular 1.2–1.3; moderately elongated 1.5–1.9; highly elongated >2.0) [61]. (E, F) Size exclusion chromatograms for EB1 and Δ5–7 Nt-GCN4 or p135 Nt-GCN4 run alone or pre-incubated in a 1:2 ratio indicate that Δ5–7 Nt-GCN4, which contains the CAP-Gly domain, but not p135 Nt-GCN4, which does not, can form a stable complex with EB1.

Figure S6 Characterization of p150Glued role in modulating microtubule dynamics in cells. (A) Bioinformatic analysis of p150Glued homologues in diverse metazoans characterizes the basicity downstream of the CAP-Gly domain. Using Homo sapiens p150Glued as a search term, PSI-BLAST was performed to identify as many metazoan homologues as possible [62]. Then, full-length cDNA or, to avoid biases due to splicing, genomic DNA computationally spliced using GENSCAN [63], was pairwise aligned using Clustal Omega [64], and peptide characteristics computed using ExPASy ProtParam [65]. (B) Basicity versus divergence time plot indicates that over metazoan evolution, the region downstream of the p150Glued CAP-Gly has evolved progressively more basicity. Divergence times calculated using TimeTree [66]. (C) GFP-EB3 comet distance to catastrophe was analyzed using kymograph analysis on a per-neuron basis to control for variable rescue transfection efficiency.

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Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: JEL AJM ELFH. Performed the experiments: JEL AJM MT. Analyzed the data: JEL AJM. Contributed reagents/materials/analysis tools: MT. Wrote the paper: JEL ELFH.

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