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Nucleotide binding to ARL2 in the TBCD ARL2 β -tubulin complex drives conformational changes in β -tubulin

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Abstract

Microtubules are highly dynamic tubulin polymers that are required for a variety of cellular functions. Despite the importance of a cellular population of tubulin dimers, we have incomplete information about the mechanisms involved in the biogenesis of $\alpha\beta$ -tubulin heterodimers. In addition to prefoldin and the TCP-1 Ring Complex, five tubulin-specific chaperones, termed cofactors A-E (TBCA-E), and GTP are required for the folding of α - and β -tubulin subunits and assembly into heterodimers. We recently described the purification of a novel trimer, TBCD•ARL2• β -tubulin. Here, we employed hydrogen/deuterium exchange coupled with mass spectrometry to explore the dynamics of each of the proteins in the trimer. Addition of guanine nucleotides resulted in changes in the solvent accessibility of regions of each protein that led to predictions about each's role in tubulin folding. Initial testing of that model confirmed that it is ARL2, and not β -tubulin, that exchanges GTP in the trimer. Comparisons of the dynamics of ARL2 monomer to ARL2 in the trimer suggested that its protein interactions were comparable to those of a canonical GTPase with an effector. This was supported by the use of nucleotide binding assays that revealed an increase in the affinity for GTP by ARL2 in the trimer. We conclude that the TBCD•ARL2• β -tubulin complex represents a functional intermediate in the β -tubulin folding pathway whose activity is regulated by the cycling of nucleotides on ARL2. The co-purification of guanine nucleotide on the β -tubulin in the trimer is also shown, with implications to modeling the pathway.

Graphical Abstract

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Author Contributions: JWF conducted many of the experiments, analyzed all data, and wrote the paper. DG and SJN designed, performed and, along with BDP, analyzed the HDX experiments. PRG coordinated HDX analyses and contributed to writing of the manuscript. ERW designed, performed and analyzed data shown in Figure 2. EAO provided critical analysis of HDX data and manuscript. RAK coordinated the study and wrote the paper with JWF.

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Keywords

small GTPase; tubulin; hydrogen-deuterium exchange; conformational dynamics; conformational change; ARF-like 2 (ARL2); tubulin-specific chaperone D (TBCD); guanine nucleotide binding

Introduction

Microtubules are polymers of $\alpha\beta$ -tubulin heterodimers, which can be highly dynamic or stable ultrastructural components, and are present in all eukaryotic cells. They are essential during cell division, forming mitotic spindles that resolve chromosomes to mother and daughter cells. In other parts of the cell cycle, microtubules serve as tracks along which organelles traffic, which is particularly important in highly polarized cells such as neurons. They are also core to the axonemes present in both sensory and motile cilia and flagella. Several genes encode each α - or β -tubulin subunit (*e.g.*, see Lewis, et al [1]), resulting in diversity in composition, with functional consequences that are incompletely understood. Tubulins can also be post-translationally modified to alter the dynamics of microtubules (*e.g.*, acetylation, phosphorylation, tyrosination). Tubulins/microtubules are also the target of several anti-tumor agents, *e.g.*, the taxanes and Vinca alkaloids [2]. Despite their importance to cells and in the clinic, we lack a detailed understanding of the mechanisms regulating the formation of the key component, the $\alpha\beta$ -tubulin heterodimer.

Tubulins are among the most abundant proteins in every cell, often reaching levels of ~5% of total cell protein. Despite, or perhaps because of, that abundance a complex series of biosynthetic steps is required to support tubulin folding and dimer assembly. Investigations into the mechanisms of tubulin heterodimer biogenesis have been ongoing for almost 50 years now. Most of these studies have depended upon assays involving *in vitro* transcription/translation and [³⁵S]methionine-radiolabeling of (typically beta-) tubulin using reticulocyte lysates. The quaternary state of tubulin in such assays is inferred from the migration of different radiolabeled products in non-denaturing acrylamide gels [3–5]. Though not always fully appreciated, the interpretations of data generated from such assays were always complicated by the poorly understood role(s) of proteins contributed by the reticulocyte lysates to the folding and assembly of $\alpha\beta$ -tubulin. In addition, only small amounts of

intermediates were generated, and these were often unstable, making definitive characterization of the components impractical. Despite these limitations, such assays were foundational and identified roles for two chaperone complexes and five other proteins that are required for tubulin heterodimer biogenesis [4, 6, 7].

Many proteins, particularly abundant ones like α - and β -tubulin, first interact with the hexameric chaperone complex, termed prefoldin, upon exiting the ribosome [7]. They are then transferred to the cytosolic chaperonin, TCP-1 Ring Complex (TriC or CCT), as the next step in their folding process [4, 8]. Tubulins then uniquely interact with five tubulin-specific co-chaperones, termed cofactors A-E, in a series of interactions first described by Tian et al. [5, 9]. Upon exit from the CCT complex, α -tubulin has been found to already be bound to GTP and thus is folded into a functional state at least in this regard [10]. It has been inferred that perhaps β -tubulin is similarly liganded, though direct evidence for this subunit is lacking. This foundational work provided the first model for the steps in the tubulin-folding pathway, but aspects of it are challenged by more recent studies [11, 12]. A detailed model for tubulin folding that withstands rigorous testing would include the roles played by each of the required components, allow the generation of key biochemical reagents for multiple studies, and generate novel opportunities for therapeutic development. Such a model requires the ability to generate each component in a functional state that would then allow reconstitution of the formation of the $\alpha\beta$ -tubulin dimer. One roadblock to this goal has been the lack of a soluble, stable preparation of the largest component in the folding pathway, tubulin-specific chaperone D (TBCD, also termed Cofactor D), as it is insoluble in bacteria and poorly expressed or unstable in other expression systems. This is in marked contrast to the other four cofactors, A-C and E [13]. We recently described the use of human embryonic kidney (HEK293T/17) cells to overexpress and purify human or bovine TBCD and showed it to be soluble and stable [11]. That study identified a number of novel complexes containing TBCD and strong physical connection to the regulatory GTPase, ARL2.

Links between ARL2, tubulin biogenesis and specifically to TBCD first came from genetic studies. Screening *S. cerevisiae* for altered sensitivity to benomyl, a microtubule poison, identified the yeast orthologs of TBCD and ARL2, *CIN1* and *CIN4*, respectively [14, 15]. Similar screens pulled out *Alp1* and *Alp41* in *S. pombe* [16, 17], and *TTN1* and *TTN5* in *A. thaliana* [18, 19], encoding orthologs of TBCD and ARL2, respectively. When combined with the results from the Cowan lab showing direct effects on tubulin folding, there is compelling evidence for TBCD in this pathway. Other screens found mutations in ARL2 orthologs in *C. elegans* [20], *T. brucei* [21], and *D. melanogaster* [22] that further support a role for this regulatory GTPase in tubulin biology. However, ARL2 was never identified in the earlier tubulin folding assays. This is likely because ARL2 is present in reticulocyte lysates and its small size (~20 kDa) would result in its migration at the dye front in the low percentage acrylamide gels required in the analysis of the large protein complexes that include tubulins.

In addition to their roles in tubulin biogenesis, there are also data supporting roles for TBCD and ARL2 acting at distinct sites and pathways; e.g., at centrosomes [23–25] and the cell surface [26, 27]. Also, a spate of recent reports describe point mutations in TBCD found in

patients with links to early-onset encephalopathy [28–31] and intractable seizures [32]. A subset of these mutations were specifically shown to disrupt formation and stability of the TBCD•ARL2• β -tubulin complex [29], which we have recently purified and characterized [11].

ARL2 is a member of the ARF family and RAS superfamily of GTPases, which are typically found as monomers in cytosol. In contrast, ARL2 was first purified from bovine tissues as a dimer of TBCD•ARL2 [33]. We recently showed that in most cells and tissues a species of ~200 kDa is the predominant form of ARL2 and TBCD and that it represents the TBCD•ARL2• β -tubulin trimer [11]. The TBCD•ARL2 dimer purified from bovine brain was inactive in the tubulin folding assay and displayed the unique property of binding GDP but not GTP [33]. To allow more detailed functional and structural studies, we developed an expression system in mammalian cells capable of generating milligram amounts of TBCD complexes [11]. This study found that only when co-expressed with ARL2 does TBCD purify as a monodisperse species, a 1:1:1 complex of TBCD•ARL2• β -tubulin. The presence of β -tubulin in the novel TBCD•ARL2• β -tubulin trimer clearly distinguishes it from the TBCD•ARL2 dimer and strongly implicates it playing a role in tubulin folding.

Small GTPases are single domain proteins that bind and hydrolyze GTP in a highly regulated fashion to alter signaling through transient interactions with effectors, modulators, and often membranes [34–39]. The binding of guanine nucleotides typically stabilize the folded structures of the GTPase such that the apoproteins are unstable and rarely detected. This is not true of ARL2 as it displays relatively weak affinity for guanine nucleotides [40], yet is stable as either monomer or trimer. Thus, ARL2 provides an unusual opportunity to compare structural features of the apoprotein to the liganded forms.

A common feature of RAS superfamily GTPases is the presence of a highly conserved set of motifs, termed G motifs, which provide direct sites of interaction with bound nucleotides [41]. For example, the G1 motif (aka Walker A motif) is the consensus GXXXXGK(S/T) and interacts with phosphates of the bound nucleotide. The G3 motif (DXXGQ) includes the glutamine that is critical to GTP hydrolysis. Another distinguishing feature of regulatory GTPases are the two switches, I and II, as the conformation of each of these is altered upon exchange of GTP for GDP, resulting in increased affinity for effectors and modulators. ARL2 contains the canonical G motifs and switches I/II, but unlike other families within the RAS superfamily, ARF family members, including ARL2, have an N-terminal extension that forms an amphipathic α -helix that is also critical to function. The N-termini are commonly found to serve as a functional switch III in that the conformation is sensitive to the bound nucleotide and residues within it make direct contacts with effectors and modulatory proteins [42–44]. In contrast, β -tubulin is considered an atypical GTPase as it does not share many of the common motifs that are highly conserved among regulatory GTP-binding proteins [45]. However, there is a high level of conservation of the residues involved in nucleotide binding among tubulin isoforms, as well as between α - and β -tubulin subunits [46]. GTPase cascades (*i.e.*, increased activity of one GTPase leading to activation of another GTPase in the same pathway, typically through the recruitment of the guanine nucleotide exchange factor (GEF) for the next GTPase as well as GTPase activating protein (GAP) for the earlier acting one) are increasingly common, particularly in membrane traffic

[36, 47–49]. However, it is very uncommon, perhaps unique, for a regulatory GTPase, like ARL2, to play a fundamental role in the folding or biogenesis of another GTPase.

The current model for tubulin folding [5, 6] proposes that the final step involves the TBCD•TBCE• α -tubulin• β -tubulin tetramer binding TBCC, resulting in release of the folded $\alpha\beta$ -tubulin heterodimer and disassembly of other components. Due to its large size (133 kDa) and predicted, predominantly α -helical structure [50], TBCD is proposed to act as a scaffold onto which other components assemble. A challenge to aspects of this model has come from studies in which co-expression in bacteria of the yeast orthologs revealed the formation of a trimer of TBCD(CIN1p)-ARL2(CIN4p)-TBCE(PAC2p) [12]. Our studies using human proteins purified from HEK cells failed to identify the homologous complex, though we did report the co-purification of our TBCD•ARL2• β -tubulin trimer with sub-stoichiometric amounts of TBCE only after overexpression of the GST-TBCD, ARL2, and TBCE. Thus, differences between yeast and mammals likely exist in the affinities for different subunit interactions resulting in the formation or stability of different protein complexes. In the current study, we focused on the TBCD•ARL2• β -tubulin trimer in efforts to better understand the role(s) of the regulatory GTPase and of GTP binding to the processes of tubulin folding and protein complex assembly/disassembly.

Results

We recently described the identification and purification of a trimeric complex of TBCD•ARL2• β -tubulin [11]. We co-express human GST-TBCD and ARL2 in human embryonic kidney (HEK) cells, followed by purification by affinity chromatography using glutathione conjugated Sepharose beads, as described under Materials and Methods. The GST tag can be removed using the engineered TEV protease cleavage site, and the trimer is further purified by gel filtration in a Superdex 200 column to remove the TEV, GST and minor contaminants. The complex elutes as a symmetrical peak off this column and at the appropriate position for a ~200 kDa complex. The trimeric complex is stable at 4 °C for at least one week, remains soluble after freezing and thawing, and runs as a sharp band of ~200 kDa in blue native gels. All of this is consistent with the TBCD•ARL2• β -tubulin trimer being a well-behaved, stable species amenable for further biochemical analyses.

We used hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS) to monitor solvent exchange of amide hydrogens of each protein in the trimer and compared results after the complex was pre-incubated with saturating amounts of GDP or GTP γ S, or when no nucleotide was added. Alterations in HDX reflect changes in the environment of the corresponding amides within the protein. Increased protection to exchange suggests an increased number or strength of hydrogen bonds, often characterized as a decrease in solvent accessibility, whereas decreased protection would suggest a decrease in hydrogen bonding (*i.e.*, increased solvent accessibility). We used the same approach to compare ARL2 monomer to ARL2 in the trimer, again under different nucleotide-bound states. This allowed additional comparisons to be made and, because ARL2 is among the very few regulatory GTPases that are stable in solution as an apoprotein, allowed us to monitor effects of nucleotide binding on protein structure.

Conformational dynamics of TBCD in the TBCD•ARL2• β -tubulin complex are only minimally affected by the addition of guanine nucleotides

Current models of tubulin folding portray TBCD as a scaffold onto which other components bind, based more on its relatively large size (133 kDa) than on any functional data. With the exception of *in silico* predictions [50] and low-resolution electron microscopy data [12], there are almost no structural data to support this proposed role for TBCD. A large roadblock to structural studies of TBCD has been the lack a recombinant expression system capable of producing large amounts of soluble, stable TBCD. We have recently described the purification of three novel TBCD complexes [11], with TBCD•ARL2• β -tubulin being the most stable. To date, neither our lab nor others has achieved high level purification of monomeric TBCD and our analyses of the protein in cell and tissue lysates [11] suggest it may only be stable in cells when complexed with other proteins. The TBCD-promoted folding of β -tubulin is proposed to ultimately produce functional $\alpha\beta$ -tubulin heterodimers that contain a non-exchangeable nucleotide binding site in the α -tubulin and an exchangeable site in the β -tubulin [51]. However, it is currently unknown at which point in the folding process the β -tubulin becomes able to bind guanine nucleotides, though evidence suggests it may be while bound to, or upon release from, CCT [4, 10, 52, 53]. Thus, we performed conformational dynamics analyses of the purified trimer with no added nucleotides and after pre-incubation with either GDP or GTP γ S, using hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS).

While TBCD itself does not bind guanine nucleotides, we applied differential HDX-MS to assess changes in TBCD solvent exchange that may result from the binding of GDP or GTP γ S to other components in the trimer. Purified TBCD•ARL2• β -tubulin (10 μ M) was incubated with a 10-fold molar excess (100 μ M) of GDP, GTP γ S, or no nucleotide for at least 1 hour on ice before analysis by HDX-MS. We incubated the samples in D₂O at 4 °C and collected six time points over the course of an hour (10, 30, 60, 300, 900, and 3600 seconds), as described under Materials and Methods. For each time point, solvent exchange was slowed by addition of low pH buffer and each sample was then subjected to on-line pepsin digestion. The peptic peptides were separated by RP-HPLC, coupled to an ESI source and the ions for each peptide were mass measured using a high resolution, high mass accuracy mass spectrometer, as described under Materials and Methods. HDX Workbench software [54] was used to calculate the deuterium uptake over time for each peptide. TBCD sequence coverage was ~92%, with data generated for many overlapping peptides throughout the protein. The average percent deuterium uptake was calculated for all peptides whose sequence was verified by tandem mass spectrometry.

To directly compare the effect that the addition of nucleotide had on TBCD solvent exchange, the uptake profiles for the no added nucleotide, GDP, and GTP γ S pre-incubated samples were subtracted from one another to generate three differential HDX datasets that portray the *change* in average deuterium uptake between the different samples. For example, to determine the effect that addition of GDP had on TBCD deuterium exchange, we subtracted the data for the no added nucleotide (apo) condition from that of the sample incubated with GDP and plotted the differences. Thus, a region of the protein that has reduced deuterium exchange after addition of GDP yields a negative value, and this region

would be considered stabilized in terms of conformational mobility. Conversely, a positive value indicates an increase in the rate of deuterium exchange, or increased conformational mobility, occurred after addition of the nucleotide. To extract putative single amide resolution of the HDX data, average values were determined for each residue of the protein; *e.g.*, those for TBCD are shown in the graph in Figure 1. Dotted lines indicate regions of the protein that were not covered in the MS-MS analyses. It is apparent that only quite modest changes in the levels of deuterium exchange were observed for TBCD when comparing the three conditions. However, we observe a few localized regions that exhibit moderate changes ($\pm 15\%$) in deuterium exchange. Note that in each figure that displays HDX data we label as “apo” the samples that were incubated in the absence of excess guanine nucleotides, even though we later (see below) show that the β -tubulin in the trimer co-purifies with nucleotides bound. When comparing the no added nucleotide (apo) and GDP samples (apo $\rightarrow$ GDP; blue line), the tendency is a slight increase in solvent exchange upon inclusion of GDP, indicative of an increase in the conformational flexibility of the protein. In contrast, the apo $\rightarrow$ GTP γ S (red line) and GDP $\rightarrow$ GTP γ S (green line) comparisons show a general decrease in solvent exchange, indicative of stabilization of the protein. While the changes observed are subtle, they are statistically significant, and these data indicate that the addition of GDP or GTP γ S has opposing effects on the conformational dynamics (*i.e.*, backbone mobility) of TBCD in the TBCD•ARL2• β -tubulin complex. We interpret these results as evidence that there are conformational changes within TBCD that are induced by nucleotide binding to the complex. As TBCD is not the species responsible for binding the nucleotide, the observed changes are interpreted as allosteric (*i.e.*, driven by nucleotide binding to one or both of the other proteins). Thus, these data are consistent with TBCD acting as a scaffold on which other proteins assemble, and perhaps support changes in their tertiary structure. We next examined conformational dynamics of the β -tubulin in the trimer, again by comparisons between samples incubated with different nucleotides.

Addition of guanine nucleotides to the TBCD•ARL2• β -tubulin complex results in conformational changes in β -tubulin that do not map onto regions involved in guanine-nucleotide binding

We performed differential HDX analyses of β -tubulin in the TBCD•ARL2• β -tubulin complex after the addition of GDP, GTP γ S, or no nucleotide. The graphical representation of the differential HDX data describing the transitions between the three nucleotide-bound states are shown in Figure 2A. Upon incubation with GDP (apo $\rightarrow$ GDP) there was a moderate increase in deuterium exchange throughout the entire β -tubulin protein, indicating a decrease in the conformational stability of the protein (Figure 2A, blue line). In stark contrast, addition of GTP γ S to the complex resulted in almost no changes in the levels of deuterium exchange seen in β -tubulin (Figure 2A, red line), suggesting that the conformational dynamics of β -tubulin in the trimer are very similar between the no added nucleotide and GTP γ S-incubated states. These findings are further highlighted in Figure 2B, where the differential HDX data were converted to a heat map and overlaid onto a three-dimensional representation of β -tubulin. Decreases in deuterium exchange are represented in shades of blue, increases in yellow/red, and no significant change ($<5\%$) in white, as indicated in the scale below the structures. Areas shaded grey are indicative of regions of the protein that were not covered by the MS analyses ($\sim 86\%$ sequence coverage of β -tubulin in

all assays). As clearly shown by the HDX perturbation map, the addition of GDP or GTP γ S caused no observable decrease in solvent exchange around the nucleotide-binding pocket of β -tubulin (bound GDP is shown in magenta). Ligand binding typically leads to a localized decrease in deuterium exchange (protection to solvent exchange), specifically around the ligand-binding pocket (*e.g.*, see below). These results are inconsistent with β -tubulin being the species that binds GDP or GTP γ S in the TBCD•ARL2• β -tubulin trimer. Interestingly, when we compared the GDP- and GTP γ S-bound states (GDP→GTP γ S), there was an overall decrease in deuterium exchange observed throughout β -tubulin. This shows that when GTP γ S replaces GDP in the TBCD•ARL2• β -tubulin complex, we see a decrease in solvent exchange, or an increase in protein stability, for β -tubulin. As we hypothesize that β -tubulin is not binding nucleotide during the pre-incubation of the trimer, we interpret these results as evidence of an allosteric effect on the conformation of β -tubulin that is differentially altered by the binding of GDP or GTP γ S to the only other protein capable of doing so, ARL2. However, this is surprising as we previously found that a closely related complex, TBCD•ARL2 cannot bind GTP [33].

Binding of guanine nucleotides to the trimer results in alterations in solvent accessibility of nucleotide binding motifs within ARL2

We completed the comparisons of effects of added guanine nucleotides on HDX of each protein in the trimer by analyses of ARL2. As described above, we analyzed the changes in deuterium uptake for ARL2 in the trimer by plotting the average values per residue (Figure 3A) and showing a structural overlay of the corresponding heat map data (Figure 3B). The addition of GDP (apo→GDP) or GTP γ S (apo→GTP γ S) to the TBCD•ARL2• β -tubulin trimer resulted in large localized stabilizations, or decreased exchange, particularly at the conserved nucleotide binding motifs G1, G4 and G5 of ARL2. In contrast, we did not see a change in solvent exchange around the G3 motif after the addition of GTP γ S, or in the comparison of GDP→GTP γ S. We speculate that this may be due to a strong stabilization of G3 and switch II/G2 of ARL2 that result from interaction with TBCD• β -tubulin. As can be clearly seen in Figure 3B, the addition of nucleotide to the TBCD•ARL2• β -tubulin complex results in highly localized decreases in deuterium exchange that are centered around the nucleotide binding pocket of ARL2. These results are consistent with the conclusion that ARL2 in the trimer can bind either nucleotide, GDP or GTP γ S. Note that this is in contrast to the TBCD•ARL2 dimer purified from bovine brain that binds GDP but not GTP (GTP γ S) in radioligand binding assays (29).

HDX-MS analysis of ARL2 monomer reveals large changes at consensus GTP-binding motifs

ARL2 is notable among the ARF family GTPases as it has relatively low affinity for guanine nucleotides and is stable as an apoprotein. These characteristics allow us to study effects of binding either GDP or GTP γ S without interference from pre-bound GDP. We overexpressed and purified human ARL2 from HEK cell lysates using anion-exchange and size exclusion chromatography, as described previously [55] and in Materials and Methods. To determine the effects of nucleotide addition on the conformational dynamics of monomeric ARL2, we again performed differential HDX-MS analyses comparing the three nucleotide-bound states and graphed the average change in deuterium uptake per residue (Figure 4A), as described

above. The addition of GDP to ARL2 (apo→GDP) resulted in large protection to deuterium uptake at the conserved nucleotide-binding motifs G1 and G4 (defined here as residues 23–30 and 125–128, respectively; see Figure 4A, blue line). These results are consistent with the well-established roles that these motifs play in nucleotide binding. Adding GTP γ S to ARL2 (apo→GTP γ S) showed similar changes in the G1 and G4 motifs to those seen in the apo→GDP comparison (Figure 4A, red line). The addition of GDP or GTP γ S also caused increased solvent exchange around the effector binding loops, switches I and II (residues 37–50 and 67–79, respectively). Because these switches are directly involved in binding to effectors and/or modulators of the GTPase, it is expected that they become more conformationally mobile upon binding GTP γ S. The binding of GTP γ S also caused a stabilization, not seen in apo→GDP, around the G3 motif (residues 66–70), which is also involved in interactions with the gamma phosphate of GTP and contains the glutamine residue required for hydrolysis.

The GDP→GTP γ S comparison is relevant to the actions of this GTPase in biology as it represents the activation process. This comparison (Figure 4A, green line) reveals that the largest increase in protein stabilization (*i.e.*, decrease in deuterium uptake, protection to solvent exchange) was localized around the G3 motif. We generated the heat map that was overlaid onto a previously published crystal structure of ARL2 [42]. The heat map data corresponding to the changes in deuterium uptake between each of the three comparisons made (apo→GDP, apo→GTP γ S, and GDP→GTP γ S) are shown in Figure 4B. As highlighted in these HDX perturbation map structures, addition of GDP or GTP γ S to ARL2 results in the stabilization of the protein in regions concentrated around the nucleotide binding pocket (bound GTP shown in magenta), consistent with a typical protein-ligand interaction. These results are quite similar to what was observed for ARL2 in the trimer and support our interpretation that ARL2 in the trimer binds both GDP and GTP γ S.

Formation of the TBCD•ARL2• β -tubulin complex results in distinct changes in the conformational dynamics of ARL2

Comprehensive HDX analysis and comparisons between monomers and oligomers can help identify sites of protein-protein interactions, using a differential deuterium exchange profile, as well as ligand binding sites. A protein-protein interaction interface will result in increased intermolecular hydrogen bond formation, overall stabilization of the complex, and reduced solvent accessibility, resulting in decreased solvent exchange. Unfortunately, ARL2 is the only component of our TBCD•ARL2• β -tubulin trimer that we can purify as a monomer in sufficient quantities to make such a comparison. Thus, we compared solvent access to residues in ARL2 as a monomer and trimer, with a goal of identifying regions/residues involved in protein-protein interactions.

We compared the changes in deuterium uptake by ARL2 between the monomeric and trimeric species for each of the three forms (ARL2 with no added nucleotide, ARL2•GDP, and ARL2•GTP γ S) and the average values per residue are shown in Figure 5A. Though the magnitude of the effects differed, the differential uptake profiles for ARL2 in the trimer were largely conserved between the different liganded states. This can be seen in the overall similarities in the three graphs in Figure 5A, or when the differences are mapped onto the

three-dimensional model of ARL2•GTP (Figure 5B). Note that because no atomic structures are available for apo-ARL2 or ARL2•GDP the structure shown in every case is that of ARL2 bound to GTP and an effector (not shown), and thus are not intended to represent the actual structures of monomer or ARL2 in the trimer. Rather, we show them to highlight where in the folded protein the differences are occurring. Most obviously, there was a complete protection to deuterium exchange at the N terminus of ARL2 in the trimer compared to the monomer. This is interpreted as strong evidence that the N-terminus is involved in binding to TBCD• β -tubulin. The N-terminal, amphipathic helix of ARF family members act like a third GTP-binding sensitive switch region and, specifically, structures of ARL2 bound to effectors document such a role in effector interactions [42, 43]. As highlighted by the structural overlay of the HDX perturbation map data (Figure 5B), the N-terminal helix is positioned away from the main body of the protein, making it readily available for interaction with effector proteins. The next most altered regions of ARL2 in this comparison are switch II and the C-terminus (residues ~174–184). While switch II is also known to be directly involved in binding to protein partners, no such role has previously been identified for the C-terminus. In contrast, residues 21–50 had increased solvent exchange in the trimer, in comparison to the monomer. This region includes the G1/G2 motifs and switch I, with the effect on the latter reversed upon binding of GDP or GTP γ S. Thus, these data are consistent with ARL2 interacting with TBCD• β -tubulin via two regions previously shown to be involved in ARL2-effector interactions [42, 43]; *i.e.*, the N-terminal helix and switch II, and possibly a novel role for the C-terminal helix. The only destabilization seen in all three liganded states was localized to the G1 region, indicating that ARL2 interaction with TBCD• β -tubulin causes this conserved nucleotide-binding motif to become more conformationally flexible.

The TBCD•ARL2• β -tubulin complex binds/exchanges both GDP and GTP

Based upon the HDX-MS data described above, we predicted that ARL2 in the trimer retains the ability to bind/exchange guanine nucleotides. And in contrast to the TBCD•ARL2 dimer [33], we believe it is capable of binding either GDP or GTP (GTP γ S). We first used the classical filter trapping assay, as described under Materials and Methods, to determine the guanine-nucleotide binding properties of the trimer, with comparisons to the ARL2 monomer. The trimer was incubated with the slowly hydrolyzable GTP analog, [35 S]GTP γ S, at 30 °C and at various times the reaction was stopped by dilution into ice cold buffer containing 10 mM MgCl₂, to slow nucleotide dissociation [55]. Rapid filtration on BA85 nitrocellulose filters that retain protein and bound nucleotides allowed determination of radioligand binding by scintillation counting (Figure 6), with all samples analyzed in duplicate. The TBCD•ARL2• β -tubulin trimer binds GTP γ S, reaching a plateau within 5–10 min that is stable for at least 30 min. Human ARL2 monomer, exogenously expressed and purified from *E. coli* or HEK cells, bound GTP γ S to similar stoichiometries, ranging between 0.15–0.25 pmol GTP γ S bound/pmol protein in different replicates. The finding that equimolar concentrations of ARL2 monomer and TBCD•ARL2• β -tubulin trimer bind equal amounts of GTP γ S is consistent with the trimer being a 1:1:1 complex, though small differences would go undetected using this metric.

To test the model that it is ARL2 in the trimer that is binding guanine nucleotides in the binding assay, we compared the ability of the TBCD•ARL2• β -tubulin trimer to that containing a point mutant of ARL2, ARL2[T30N], that has been shown to be deficient in binding guanine nucleotides [40]. By analogy to ARF1[T31N] [56, 57] and RAS[S17N] [58–61], ARL2[T30N] is thought to act as an apoprotein in a dominant negative fashion in cells by mimicking the transition state while bound to its GEF, to prevent activation of endogenous ARL2. Co-expression of human GST-TBCD and ARL2[T30N] allowed us to purify the GST-TBCD•ARL2[T30N] • β -tubulin trimer with comparable purity and abundance as when wild type ARL2 was used [11]. In contrast to the trimer containing wild type ARL2, TBCD•ARL2[T30N] • β -tubulin has almost completely lost the ability to bind GTP γ S (Figure 6) in this radioligand binding assay. We interpret these results as consistent with the previous data, indicating that ARL2 is the species in the trimer that binds added guanine nucleotides.

As differences have been noted between the human and bovine orthologs of TBCD [23, 62], we also co-expressed bovine GST-TBCD with human ARL2 in HEK cells (bovine and human ARL2 are 98% identical in primary sequence, with only 3 conservative differences). Bovine TBCD behaved comparably to the human ortholog and was purified from HEK cells as the trimer upon co-expression with ARL2 [11]. We found no clear differences between the two trimers, made up of bovine or human TBCD, in their ability to bind [³⁵S]GTP γ S in the filter trapping assay (Figure 6).

We interpret these data as evidence that ARL2 in the trimer binds GTP γ S, but it is also possible that the ARL2 is dissociating under the conditions used in the binding assay and is binding nucleotide as a monomer. To test this we pre-incubated the trimer with GDP or GTP γ S, using the same reaction conditions described above, and attempted to resolve subunits in each of two different ways: blue native PAGE or ultrafiltration. The former showed that the trimer remained an intact complex with all three components migrating to the same position in the gel after incubation with GDP or GTP γ S, though nucleotide dissociation could have occurred during electrophoresis. More convincingly, we found that continual exposure to GDP or GTP γ S before and during ultrafiltration was able to completely resolve a ~24 kDa contaminant that co-purifies on glutathione-Sepharose beads (predicted to be an endogenous HEK cell GST [63]) but resulted in no losses in ARL2.

Should subunit dissociation be occurring upon binding of a guanine nucleotide, it might be expected to alter the stability of one or more of the proteins. Thus, we also analyzed effects of nucleotide addition on the stability of the TBCD•ARL2• β -tubulin complex using a Thermofluor assay (Figure 7). We incubated the purified trimer with a 10-fold molar excess of GDP, GTP γ S or no nucleotide and then added to each solution the fluorescent dye, SYPRO Orange. The dye binds to hydrophobic regions and alters its fluorescent properties upon doing so. We varied the temperature and determined the fluorescence under each condition to determine the melting points, defined as the half-maximal change in fluorescence (Figure 7). In each case we observed curves displaying a single, gradual change in fluorescence, consistent with coordinated unfolding/denaturation. We found no apparent differences in the melting temperature when the TBCD•ARL2• β -tubulin trimer was pre-incubated without nucleotide, with GDP, or with GTP γ S (41.1 °C, 41.1 °C, and 40.4 °C,

respectively). Altogether these data support the conclusion that the trimeric complex is capable of binding GDP or GTP γ S stably, and doing so does not result in substantial dissociation of subunits.

β -tubulin in the trimer co-purifies with nucleotide bound

We describe compelling evidence above that ARL2 in the trimer binds both GDP and GTP *in vitro*, and that the HDX data appear to be inconsistent with β -tubulin doing so. The fact that α -tubulin has previously been shown already to have GTP bound upon release from the CCT complex [10] prompted us to examine whether the β -tubulin in our trimer may also purify with pre-bound nucleotide(s). We determined the nucleotide content in solution after heat denaturation (5 min at 95 °C) and removal of proteins from the trimer and ARL2 monomer. We used a strong anion exchange column and HPLC to resolve guanine nucleotides and compared peak volumes to that of nucleotide standards, as described under Materials and Methods. As a positive control, we assayed for nucleotide co-purifying with bacterially expressed ARF6. This yielded values of 0.3 mol GDP/mol ARF6, performed in duplicate from one preparation. Previous evidence from other ARF isoforms are consistent with ARFs co-purifying with GDP. However, when we treated our pure GTP standard the same as our protein samples, including buffer and boiling, we found ~5% of the GTP had been converted to GDP. And given that some of the protein preparations analyzed had been stored at -80 °C for months, during which time additional hydrolysis of GTP may have occurred, we do not believe it is safe to draw hard conclusions as to the identity of the guanine nucleotide co-purifying, though do maintain that the stoichiometries determined are sound.

As ARL2 is known to have weak affinities for guanine nucleotides [40], we expected the purified monomer preparation to have little or no co-purifying nucleotides. As predicted, ARL2 had no detectable GDP or GTP in our HPLC analyses after heat denaturation and removal of protein. In contrast, the purified trimer was found to co-purify with ~0.39 mol GDP/mol trimer. Three independently purified preparations of trimer were analyzed and yielded values of 0.19, 0.29, and 0.70 mol GDP/mol trimer, suggesting a large variability in the fractional occupancy. The lack of co-purifying nucleotides with ARL2 monomer is suggestive that it is the β -tubulin in the trimer that has GDP bound, but we wanted to further test this conclusion. As described above, we were able to purify trimer that contains ARL2[T30N] instead of wild type ARL2 so next analyzed the nucleotide content of this trimer as purified from HEK cells. Two independently purified preparations of the mutant trimer yielded the same result, 0.46 mol GDP/mol trimer. Thus, we found very similar amounts of GDP to co-purify with trimer regardless whether wild type or the nucleotide-binding deficient ARL2[T30N] mutant was included and no nucleotide co-purifying with ARL2 monomer. We interpret this as evidence that the co-purifying nucleotide is bound to the β -tubulin in the trimer.

ARL2 in the trimer is predisposed to the rapid and higher affinity binding of activating nucleotides

The observations that ARL2 remains bound in the trimer after binding different ligands and that its interaction with other subunits involves many of the same regions that are used in

binding biological effectors suggest that TBCD• β -tubulin may be acting like an effector of ARL2. Such a model would represent a novel GTPase-effector interaction as these are typically envisioned as promoted in a GTP-dependent fashion and rapidly reversed upon hydrolysis or dissociation of GTP. A prediction from this model is that ARL2 in the trimer has a higher affinity for GTP than does ARL2 monomer, based upon the conformational selection model of protein-ligand interactions [64]. That is, the binding of ARL2 in the trimer limits its ability to sample different conformations, perhaps promoting an induced fit that mimics the GTP-bound or effector binding conformation.

As a test of this idea, we performed real-time nucleotide binding assays using the non-hydrolyzable GTP analog, 5'-[β , γ -imido]triphosphate (Gpp(NH)p), labeled with the *N*-methylanthraniloyl (mant) fluorophore. We incubated TBCD•ARL2• β -tubulin or ARL2 (2 μ M each) with 1 μ M mant-Gpp(NH)p and changes in fluorescence were measured at room temperature using a BioTek Synergy 4 plate reader equipped with an injector system to allow monitoring of rapid kinetics. After 20 min, excess (100 μ M) unlabeled nucleotide was added to the reaction mixture, and the fluorescence intensity was measured for an additional 20 min to allow determination of off-rates (Figure 8). We normalized the resulting data to the percentage of maximal signal to allow ready comparisons of the binding curves (Figure 8A) and summarize the results in the table shown in Figure 8B. The TBCD•ARL2• β -tubulin trimer binds mant-Gpp(NH)p more rapidly than does the monomer (Figure 8A, B). In order to determine kinetic rate constants and calculate apparent affinities, we fit the data to mathematical models using GraphPad Prism software, as described in Materials and Methods. Though TBCD•ARL2• β -tubulin and ARL2 show similar dissociation rates for mant-Gpp(NH)p ($k_{\text{off}} = 0.047 \pm 0.001 \text{ min}^{-1}$ and $0.076 \pm 0.001 \text{ min}^{-1}$, respectively), the trimer has a ~10-fold increase in the rate of association, compared to ARL2 ($k_{\text{on}} = 1.4 \times 10^6 \pm 1.1 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ and $1.3 \times 10^5 \pm 3.0 \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$, respectively). Together, these data reveal that ARL2 has a ~18-fold higher affinity for mant-Gpp(NH)p when in the trimer than as a monomer ($K_{\text{D(app)}} = 33 \text{ nM}$ and 590 nM , respectively). Note that every binding experiment included a no protein control and the values generated by this control were subtracted from the experimental data to yield "specific binding". As a result, the non-specific binding was set to 0 in the GraphPad analysis. The interpretation of the data shown in Figure 8A would be consistent with a very rapid on-rate for the trimer, such that more than 50% of the maximal binding was observed at the first time point. However, we also considered an alternate interpretation of these earliest time points. That is, the initial burst of binding could represent a different type of non-specific binding, resulting from interaction of the ligand with the protein outside of the nucleotide binding site. Though we consider this unlikely, we analyzed these same data by setting the non-specific binding to have no constraint in the non-linear regression analysis. The result is on-rates for TBCD•ARL2• β -tubulin and ARL2 of $3.9 \times 10^5 \pm 3.9 \times 10^4$ and $1.2 \times 10^5 \pm 4.8 \times 10^3$, respectively (see Table in Fig. 8B). The off-rates for the trimer and ARL2 were calculated to be 0.14 ± 0.009 and 0.079 ± 0.001 , respectively. Using this alternate method, the derived apparent affinity for the trimer ($K_{\text{Dapp}} = 360 \text{ nM}$) is still higher than that for the monomer ($K_{\text{Dapp}} = 660 \text{ nM}$), though the difference is quite a bit less than the 18-fold difference described earlier.

Due to the very fast rates of nucleotide binding, particularly for the trimer, the numbers shown here are likely underestimates of the true association constants. Together, these data

support a model in which ARL2 binding to TBCD• β -tubulin results in stabilization of ARL2 into a conformation that more readily binds GTP, yet canonical GTPase-effector interactions, the release or absence of bound nucleotide does not de-stabilize the GTPase-effector interaction.

Discussion

Genetic screens in multiple model organisms have identified orthologs of ARL2 and TBCD as sharing similar links to tubulins and microtubule dependent processes [15–19, 65]. Our own earlier studies [33] identified physical interaction between ARL2 and TBCD yet suggested that the dimer was inactive in tubulin biogenesis. Other data were interpreted as evidence that ARL2 and TBCD act in an opposing fashion to regulate a proposed “tubulin destruction” pathway in which binding of ARL2 or TBCD to β -tubulin was mutually exclusive [66]. In contrast, we interpret the data provided above as strong support for a new model (see Figure 9) in which ARL2 is an obligate component in the tubulin folding pathway. We conclude that it is GTP binding to ARL2 that drives the pathway forward, toward the ultimate release of the nascent $\alpha\beta$ -tubulin heterodimer. The actions of ARL2 in our model are consistent with it acting as a canonical regulatory GTPase and for TBCD to serve as an effector of ARL2, though with both canonical and unique properties. The binding of GTP to ARL2 is increased markedly when present in the trimer and the N-terminal helix and switch II are each strongly implicated in the binding to TBCD• β -tubulin, the same two regions that make direct contacts with two other known effectors and for which x-ray crystal structures are available [42, 43]. Our data further suggest that the actions of the TBCD•ARL2• β -tubulin trimer might more accurately be termed a tubulin heterodimerization pathway as both the α - and β -tubulin are already folded sufficiently to bind guanine nucleotides and require only dimerization for stabilization of each subunit.

We recently described the use of HEK cells to express human or bovine TBCD, either alone or with co-expression of other components of the tubulin-folding pathway, and purified three novel protein complexes that each contained TBCD [11]. Only one of those appeared to be a monodisperse complex with stoichiometric components, the TBCD•ARL2• β -tubulin trimer, which we analyze in much more detail here. The binding of ARL2 to TBCD is highly specific in that other ARF family members, specifically the closest paralog ARL3, could not bind to TBCD under these conditions, in contrast to previously reported results [67]. Additionally, no α -tubulin was present in the trimer [11], consistent with the trimer playing a role in the canonical tubulin folding pathway upstream of association with α -tubulin. In that earlier study we also identified the TBCD•ARL2• β -tubulin trimer as the most abundant quaternary state for both ARL2 and TBCD in a number of cells and tissues, though clearly not all [11]. Surprisingly, we observed differences between mammals in the quaternary state of ARL2 and TBCD, with human and bovine brain homogenates displaying a larger molecular weight species than the ~200 kDa TBCD•ARL2• β -tubulin trimer seen in murine brain homogenates. Further, a different stable complex containing the yeast orthologs of TBCD, TBCE, and ARL2 has been purified from bacteria over-expressing each component [12], providing further support for species differences in the steady state distribution of quasi-stable protein complexes in the folding pathway. We believe that the high degree of conservation in primary sequences of the proteins involved, their antiquity and essential

nature of several of them are all consistent with the predicted conservation of the steps involved in tubulin biogenesis. However, differences in the levels of expression of different components, in the half-lives of different complexes, or potentially in post-translational modifications of specific components are predicted to result in differences in the steady state distribution of the different protein complexes seen in different organisms and tissues.

We summarize our model for the role of ARL2 in tubulin biogenesis in Figure 9, highlighting the focus of the current study, the TBCD•ARL2• β -tubulin trimer, in the boxed step at the top of the diagram. Characterization of the nucleotide-binding properties of the TBCD•ARL2• β -tubulin complex is inherently confounded by the presence of two guanine nucleotide-binding proteins. We used HDX-MS as a means of monitoring changes in protein structure resulting from the binding of GDP or GTP. We conclude that it is the ARL2 in the trimer that binds guanine nucleotides in our *in vitro* binding assays, based largely upon the results of the HDX experiments. Specifically, the differential HDX data reveal clear and consistent changes in the solvent accessibility of three of the five conserved G-motifs that are directly involved in binding nucleotides in ARL2 (Figure 3). When the differences are mapped onto the structure of ARL2 it is readily apparent that those large changes are in regions that encircle the nucleotide binding site, consistent with the binding of a ligand to that site. In marked contrast, while changes in the differential rates of deuterium uptake are observed in β -tubulin when comparing the different nucleotide bound states, those changes are throughout the protein and show no bias toward the nucleotide binding site (Figure 2). We interpret this finding as evidence that the β -tubulin in the trimer is not binding guanine nucleotides in our binding assays or upon *in vitro* incubation with added nucleotides prior to HDX analyses. We cannot exclude the possibility that the β -tubulin is binding/exchanging guanine nucleotides, but that its interaction with TBCD (or ARL2) prevents changes in conformation that would be detected by HDX. However, we consider this very unlikely, particularly as the ARL2 is demonstrating clear evidence of binding nucleotides. Rather than the β -tubulin binding guanine nucleotides during our *in vitro* incubations, we conclude that the changes in HDX in β -tubulin result from ligand binding to ARL2 causing allosteric changes in β -tubulin. As depicted in our model (Figure 9), exchange of GTP for GDP on ARL2 causes conformational changes in each component in the trimer and these changes are predicted to promote later associations with TBCE, α -tubulin, and TBCC with progression toward release of nascent $\alpha\beta$ -tubulin.

That it is the ARL2 in the trimer that binds guanine nucleotides *in vitro*, and not β -tubulin, is further supported by our radioligand and fluorescence binding assays. The trimer was found to bind [35 S]GTP γ S in the filter trapping assay with very similar kinetics and stoichiometry as ARL2 monomer (Figure 6). But when the nucleotide binding deficient mutant, ARL2[T30N], was instead present in the complex, this binding was all but absent. The simplest interpretation of these data is that it is the ARL2 in the trimer that is responsible for the bulk, or perhaps all, of the binding detected in this assay. And because β -tubulin is present at equal amounts in both wild type and mutant ARL2 trimers, we conclude that β -tubulin is not binding radioligand. Thus, together the HDX and radioligand binding assays demonstrate that it is the ARL2 in the trimer that can bind or exchange guanine nucleotides, and this activity results in allosteric changes in the solvent accessibility of specific regions of β -tubulin and TBCD (Figure 9, top). Interestingly, the possibility that another GTP binding

protein, other than a tubulin, was responsible for the GTP-dependence of the folding process was speculated upon in earlier studies [68].

Almost 50 years ago it was shown that $\alpha\beta$ -tubulin co-purifies with bound GTP and that the dimer has both exchangeable and a non-exchangeable guanine nucleotide binding sites [69, 70]. Much more recent structure work has revealed the details and reasons why the binding site on α -tubulin is non-exchangeable and that on β -tubulin is exchangeable [51, 71]. Studies of the tubulin folding and assembly processes showed that α -tubulin binds GTP early, likely while still bound to the CCT complex, and that doing so promotes stability of the folded protein [10, 52, 70]. In contrast, we could find no published data demonstrating the stage at which GTP bound to β -tubulin, though the binding was inferred to be perhaps as early as the CCT bound step, from studies of the folding process [52, 68]. Our ability to generate mg amounts of a purified intermediate in the folding process, the TBCD•ARL2• β -tubulin trimer, allowed us to assay for the presence of pre-bound guanine nucleotides. We found values to vary substantially but every trimer analyzed was found to co-purify with 0.2 – 0.7 moles GDP/mole trimer. This result is consistent with earlier predictions that β -tubulin may bind guanine nucleotides while bound to the CCT complex but certainly shortly thereafter, while bound to TBCD. While our analyses identified only GDP, and no GTP, in these studies we caution against over-interpretation as hydrolysis may have occurred during purification, storage, or analyses. Thus, we conclude that like α -tubulin, β -tubulin binds guanine nucleotide(s) early in the folding process and doing so likely helps stabilize the β -tubulin throughout, but does not exchange them in our *in vitro* conditions.

Though we conclude that the β -tubulin is not binding guanine nucleotides in our binding assays, we do believe its structure is altered in response to the ARL2 binding ligands. The binding of GTP to ARL2 caused moderate decreases in deuterium exchange across nearly the entire β -tubulin sequence, as compared to the GDP-bound state. The increased solvent protection is not localized to specific regions of the protein, suggesting perhaps a concerted conformational change of the β -tubulin while still bound in the complex. The predominant factor in decreased, or slowed, hydrogen/deuterium exchange is the hydrogen bond, specifically the strengthening of existing H-bonds or the formation of new H-bonds. Because we see a decrease in solvent exchange (*i.e.*, increased hydrogen bonding) throughout β -tubulin, we conclude that exchange of GDP for GTP on ARL2 causes an increase in the stability of β -tubulin, consistent with its progression toward folding into a more native state.

The 133 kDa TBCD in the trimer did not display large changes in deuterium exchange upon incubation with nucleotides. There were a few localized changes that occurred after the addition of GDP or GTP, suggesting that nucleotide binding (to ARL2) can induce conformational changes that result in altered conformational dynamics of TBCD. It also appears that the conformational changes are nucleotide-specific as the addition of GDP or GTP had generally opposing effects, with GDP causing a few moderate increases in deuterium exchange and GTP causing slight decreases in exchange. We conclude that these changes in TBCD structure are consistent with it playing a scaffolding role in binding ARL2 and β -tubulin, allowing nucleotide binding and promoting a more stable conformation, respectively.

In addition to the effects of nucleotide binding, a stable preparation of ARL2 monomer permitted comparisons of the protein monomer vs in the trimer, which is not currently feasible for the other components of the complex. Mammalian ARL2 can be readily purified from bacteria as a soluble, stable and active monomer [55, 72]. However, in efforts to minimize potential differences resulting from bacterial expression, we also purified ARL2 monomer from HEK cells after overexpression of the human protein. ARL2 has a relatively low affinity for nucleotide [40] and was assumed for some time to purify in the nucleotide-free state, unlike most other regulatory GTPases that purify with GDP bound and are unstable as apoproteins [73, 74]. We now provide evidence that confirms the absence of co-purifying guanine nucleotides with ARL2 monomer. This allowed us to directly compare the apo and nucleotide-bound states in our HDX studies, which to our knowledge has not been reported previously for a regulatory GTPase. Upon comparing the changes in conformational dynamics between ARL2 in the trimer (Fig. 3A) and the monomer (Fig. 4A) we observed more changes in hydrogen bonding in the monomer, presumably as a result of its relative lack of constraint and greater access to solvent. Some of the changes observed upon binding nucleotides are present in both forms of ARL2. For example, the G1 and G4 motifs that interact with the β -phosphate and guanine base, respectively, show the greatest decrease in exchange, consistent with those motifs being directly involved in ligand binding. Data are missing for the G5 motif in the monomer, but this region responds similarly to G4 in the trimer and would be expected to do so as well in the monomer based on its role in contacting the guanine base. The G3 motif responds similarly in each case with somewhat larger changes seen in the monomer. Interestingly, both switch I and II display much greater solvent accessibility in the monomer, consistent with their roles as ligand-sensitive loops capable of binding effectors and modulators. The fact that these changes are absent in the ARL2 in the trimer is consistent with them already being engaged in protein-protein interactions.

By comparing the HDX profiles of ARL2 monomer and ARL2 in the trimer (Fig. 5), we were able also to identify regions of ARL2 that we propose to be involved in the binding of ARL2 to TBCD• β -tubulin. The N-terminal, amphipathic helix of ARF family members has been proposed to function as a third switch region as a result of its dramatic changes in structure associated with activation of the protein. In some family members (including the ARFs and ARL1) the N terminus is myristoylated and GTP binding results in increased translocation onto membranes, with the N terminus being directly involved in membrane insertion [75–78]. Though ARL2 is not acylated, its N terminus is directly involved in interaction with downstream effectors, based upon both detailed structural studies of protein complexes and the loss of interactions and functions resulting from its deletion [11, 42, 43]. Consistently, the N-terminus shows the most dramatic decrease in deuterium exchange when comparing the monomer and trimer forms of ARL2, strongly indicating a direct role in protein-protein interaction. The HDX data also suggest that switch II, and possibly the C-terminus, are involved in direct interaction with TBCD• β -tubulin. This potential direct role of the C-terminus in protein interactions should serve as a caution to the use of epitope tagging or other ARL2 fusion proteins, as highlighted previously for the ARFs [79]. We previously showed that a mutation in switch I, ARL2[F50A], caused a loss of the ability of ARL2 to co-purify with TBCD• β -tubulin [11]. However, the data shown here only show a

moderate protection to solvent exchange around switch I. Interestingly, switch I is the only region of ARL2 that shows a difference between nucleotide-bound and apo states, perhaps a further indication of different nucleotide-dependent conformations of the TBCD•ARL2• β -tubulin complex.

We also found changes in both on- and off-rates of guanine nucleotide binding to ARL2 monomer, compared to ligand binding when ARL2 is complexed to TBCD• β -tubulin that result in the trimer having higher apparent affinity for mant-Gpp(NH)p than monomer (Figure 8). The fact that the TBCD•ARL2• β -tubulin trimer bound both GDP and GTP γ S, in contrast to the previously characterized TBCD•ARL2 dimer purified from bovine brain which bound only GDP [33], led to several novel interpretations. Although we found no evidence of a TBCD•ARL2 dimer being present in HEK cells after expression of GST-TBCD with or without ARL2 over-expression, the fact that we were able to purify such a dimer from bovine brain is strong evidence of its presence as a stable species in mammals. This leads us to propose in our model (Figure 9) that it is the binding of β -tubulin to the TBCD•ARL2 dimer (left side of Figure 9; going from step 4 to 1) that forms our trimer and changes the ARL2 into a form that can bind GTP. In our model we show the source of the β -tubulin to be coming from TBCA, though this is inferred from previously published work [6]. By analogy to canonical regulatory GTPase biology, this may suggest that the β -tubulin is acting as a GEF for the ARL2 in the dimer, though clearly an atypical one. In contrast to the canonical actions of a GEF, the ARL2 is not released upon binding the activating ligand, GTP γ S.

Rather, we believe that TBCD• β -tubulin may be acting as an effector of ARL2. This conclusion is based upon the observations that the binding of GTP to ARL2 results in a “downstream” effect upon its binding partner resulting in biological changes. Perhaps more convincing is the observation that ARL2 in the trimer may have as much as an 18-fold higher affinity for GTP (mant-Gpp(NH)p) than does ARL2 monomer (Figure 8B). While we typically think of GTP binding as leading to an increase in the affinity of the GTPase for effector(s), it is also formally true that the binding of the two proteins leads to an increase in the affinity for the activating ligand, GTP, as seen here. Regardless the model one chooses to use in thinking about GTP binding to a regulatory GTPase (*e.g.*, “lock and key”, “induced fit” or “conformational selection” [64]), this result is predicted by and consistent with a GTPase: effector interaction. Thus, ARL2 displays both canonical and non-canonical features as a regulatory GTPase in this pathway. Whether other GTPases act homologously will be interesting to discover. Because ARL2 has other essential actions in other parts of the cell, notably effects on mitochondrial morphology and motility, ATP production, as well as centrosomal processes [80–84], it is interesting that the vast majority of it is complexed with TBCD and not readily released upon nucleotide exchange. We speculate that release of ARL2 from the trimer may be a regulated process with the potential to link its disparate functions in cells. Such a system would allow functional communication between tubulin biogenesis, ATP generation, cell division, and perhaps other essential cell functions. We have previously coined this “higher order signaling” [81] as it offers a means to integrate otherwise distinct processes through regulatory GTPases.

The model shown in Figure 9 is intended to emphasize steps 1–2, as this is where our data provide new insights. We also note above that previous data from our lab argue for the existence of a TBCD•ARL2 dimer and we infer that it is the precursor of the trimer. This is in part supported by the observation that almost all ARL2 found in cytosol is present in complex with TBCD [11]. The other steps in the cycle of tubulin heterodimer assembly shown in our model come from published work from other laboratories [5, 6, 66, 85] and is based upon solid biochemical data, but would also benefit from more rigorous testing.

We [23] and others [62, 66, 86] have also previously reported that over-expression of TBCD (or TBCE) in cultured mammalian cells results in the loss of polymerized microtubules in what has been termed the tubulin destruction pathway. This effect of TBCD can be prevented by co-expression with ARL2. This led to the conclusion that ARL2 and β -tubulin compete for a common binding site on TBCD, which of course is inconsistent with the discovery of the trimer that is the focus of the current work. There is currently no evidence supporting a role for ARL2, or any other proteins other than TBCE, in the biogenesis of α -tubulin after its release from the CCT complex. Whether or not the tubulin destruction pathway is a biologically relevant system or an artifact of protein over-expression is an important, but incompletely answered, question. We previously purified a different trimer, TBCD• β -tubulin• α -tubulin, though it ran in a high molecular weight smear in blue native gels that prevents further analyses [11]. This result does appear to confirm that TBCD can bind the tubulin heterodimer and possibly microtubules, which may well lead to a change in the stability of the microtubule. It will be important to examine further whether TBCD or ARL2 may play such a role in microtubule dynamics.

In summary, we have found that the TBCD•ARL2• β -tubulin trimer purifies with bound guanine nucleotides that are predicted to be bound to the β -tubulin but that do not exchange in standard nucleotide binding assays. In contrast, the ARL2 in the trimer does bind both GDP and GTP (GTP γ S or mant-Gpp(NH)p), and upon binding GTP causes allosteric changes in the structures of the other components in the trimer. This is interpreted as direct evidence that ARL2 is an obligate component in what has been termed the tubulin folding pathway and that the ARL2 is acting as a canonical regulatory GTPase, though with some unique details. Because we found that the β -tubulin in the trimer purifies with guanine nucleotide(s) bound, we further propose that the actions of the five tubulin specific co-chaperones, TBCE, are more likely to be in assembling the tubulin heterodimer than in folding from an unfolded state. Our previous purification of TBCD•ARL2 as a dimer that cannot bind GTP [33] is consistent with the binding of β -tubulin acting to change the nucleotide binding properties of the ARL2, specifically promoting GTP binding, which we hypothesize then allosterically alters β -tubulin structure into one that promotes binding to α -tubulin. Finally, there is steadily increasing evidence this work, our previous study [11], and a recent publication [87], that reveal important species differences in tubulins and microtubules that need to be considered when testing hypotheses regarding the complex folding or polymerization pathways.

Materials and Methods

Cell Culture

Human embryonic kidney (HEK293T/17) cells were obtained from the ATCC and grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 2 mM glutamine at 37 °C in the presence of 5% CO₂. Cells are not used beyond passage number 50.

Plasmids

Plasmids directing expression of human ARL2 and ARL2[T30N] in pcDNA3.1 [88] and pET3C [72] vectors were described previously. We obtained a plasmid containing human TBCD (NP_005984.3) from ATCC (clone MGC-1583) and the full-length open reading frame was moved into the pLEXm-GST vector (gift from James Hurley, NIH) using NotI and XhoI sites to generate an N-terminal, GST-tagged construct (GST-TBCD). We also cloned full-length bovine TBCD (NP_776619.1; Source Biosciences) into the same sites. The human and bovine constructs have a TEV protease cleavage site between the GST and TBCD, which results in a 12 amino acid residual extension at the N terminus of TBCD after cleavage.

Recombinant Protein Expression/Purification

Human ARL2 was expressed in BL21(DE3) *E. coli* and purified as previously described [55]. For recombinant mammalian expression of GST-TBCD and ARL2, HEK293T/17 (HEK) cells were transfected as previously described [11]. Briefly, HEK cells were transfected at 70–90% density using polyethylenimine (PEI; Polysciences, catalog no. 24765–2) at a 1:3 ratio (DNA:PEI). In all transfections and co-transfections, plasmids were used at 1 µg DNA/mL medium. After 48 hours, cells were harvested and the pellets either lysed immediately for purification or flash frozen in liquid nitrogen and stored at –80 °C for later use. GST-TBCD complexes were purified from HEK cell lysates as previously described [11]. Briefly, HEK cell pellets were resuspended in lysis buffer (25 mM HEPES, 100 mM NaCl, 1 mM DTT, 1% CHAPS, pH 7.4, supplemented with protease inhibitor cocktail (Sigma, catalog no. P2714)) and incubated at 4 °C for 15 min, with gentle rotation. Lysates were clarified by centrifugation at 14,000×*g* for 30 min (S14). Recombinant ARL2 was purified from HEK cells as previously described for purification from bacteria [55]. For purification of GST-TBCD complexes, we incubated the S14 with glutathione-conjugated Sepharose beads (GE, catalog no. 17075601) overnight at 4 °C, with gentle rotation. The sample was poured onto a chromatography column (Bio-Rad, catalog no. 7311550), washed with 5 column volumes of wash buffer (25 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.4), and eluted in wash buffer supplemented with 20 mM glutathione (EMD Millipore, catalog no. 3541), adjusted to pH 8.0. For removal of the GST tag, 1% (w/w) TEV protease was added to the sample and incubated overnight at 4 °C. We further purified the sample by size exclusion chromatography (Superdex 200), and then pooled and concentrated the appropriate fractions.

Nucleotide Binding Assays

Binding of [^{35}S]GTP γ S to ARL2 or TBCD complexes was assayed using the nitrocellulose filter trapping method, as previously described [55]. Nucleotide binding kinetics were determined in a solution-based assay using 5'-[β,γ -imido]triphosphate (Gpp(NH)p), fluorescently labeled with *N*-methylantraniloyl (mant). Using an automatic injector, mant-Gpp(NH)p (1 μM) was added to purified proteins (2 μM) diluted in binding buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 2.5 mM MgCl_2 , pH 7.4), and fluorescence intensity (Ex 340, Em 460) was measured every 10 s at room temperature using a BioTek Synergy 4 plate reader. After 20 min, excess (100 μM) unlabeled Gpp(NH)p was added to the reaction and the fluorescence intensity was again measured every 10 s. For each individual assay, we obtained a baseline measurement for 2 min prior to injection of nucleotide to account for intrinsic fluorescence of the protein; the average value of the baseline signal was subtracted from every point measured throughout the assay. A no protein control was included in each assay and subtracted to account for the background fluorescence of the mant-labeled nucleotides. We imported the resulting datasets into GraphPad Prism software for graphing and kinetics analyses. Nonlinear regression was used to fit an association/dissociation model to the data, from which on- and off-rates, and corresponding apparent affinities, were determined. The curve fitting was performed in GraphPad using two different approaches to account for non-specific binding in the assays. The non-specific binding constraint was either set to equal "0" or was set to have no constraint. All binding assays (radioligand and mant) were performed at least twice for each of the proteins and protein complexes shown and typically with independently prepared protein preparations.

Guanine Nucleotide Quantification

ARL2 monomer and GST-TBCD•ARL2• β -tubulin or GST-TBCD•ARL2[T30N]• β -tubulin trimers were each purified from HEK cells, as described above. ARF6 was purified from bacteria, as described previously [55]. Each protein preparation was in 25 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.4. Purified proteins (240–1000 pmol) were heat denatured at 95 °C for 5 minutes and proteins subsequently removed by centrifugation at 14,000 x g for 30 minutes. The supernatants were analyzed for nucleotide content using a Partisil 10 SAX column (Whatman; 25 cm x 4.6 mm) run on a Shimadzu LC-20AT liquid chromatograph at a fixed flow rate of 1 mL/min. Samples (50–100 μL) were applied, the column was washed for 5 min in 75% Buffer A (25 mM Tris-HCl, pH 8) and 25% Buffer B (25 mM Tris-HCl, 1M NaCl, pH 8) and then a 20 min gradient to 100% Buffer B was applied, followed by a final 5 min wash in 100% Buffer B. Baseline resolution was achieved between GDP and GTP samples, with retention times of 22 and 27 min, respectively. Nucleotides were detected using a SPD-20A UV/Vis detector (Shimadzu) measuring at 253 nm. GDP and GTP standards (50, 250, 500, 750, 1000, and 1500 pmol) were analyzed to generate a standard curve using the areas under each peak. The GDP standard curve had an R-value of 0.99 and was used to quantify the GDP in the protein samples, as no GTP was detected in any of the protein samples. At least two samples were analyzed for each protein, and technical replicates produced variations of $\leq 5\%$ for each of the samples analyzed. We typically analyzed at least two separately prepared samples of each protein or protein complex and averaged the results, showing the range.

Thermal Denaturation (Thermofluor) Assay

The Thermofluor (or Differential Scanning Fluorescence) Assay, used to analyze protein thermal stability, was performed as previously described [89–91]. Briefly, purified TBCD•ARL2• β -tubulin (5 μ M) was pre-incubated with a 10-fold molar excess of GDP, GTP γ S or no nucleotide for one hr on ice. MgCl₂ was included at a final concentration of 1 mM to facilitate nucleotide binding. Immediately prior to analysis, 2 μ L of a 1:100 dilution of SYPRO Orange dye (Sigma, catalog no. S5692) was added to 20 μ L of each sample. Fluorescence intensity (Ex 488 nm, Em 603 nm) was monitored, as a measure of thermal denaturation, using a StepOnePlus Real-Time PCR System (ThermoFisher, catalog no. 4376600) every 2 min over the range of 25–55 °C (0.5 °C/min temperature gradient). We fit the resulting curves to a Boltzmann sigmoidal equation using GraphPad Prism software to determine the melting temperature (inflection point) for each sample. Data represent the averages of two experiments for each sample.

Hydrogen/Deuterium Exchange Coupled with Mass Spectrometry (HDX-MS)

TBCD•ARL2• β -tubulin and ARL2 monomer were purified from HEK cells, as described above. Purified proteins (10 μ M) were pre-incubated in 100 μ M GDP, GTP γ S or no nucleotide with 1 mM MgCl₂ for at least 1 hr on ice prior to HDX analyses. Solution phase amide HDX was performed with a fully automated system as previously described [92]. Briefly, 5 μ L of 10 μ M sample (monomer or trimer) was diluted to 25 μ L with D₂O-containing wash buffer (described above) and incubated at 4 °C for 10, 30, 60, 300, 900, or 3600 s. Following on-exchange, back-exchange was minimized and the protein denatured by dilution to 50 μ L in a low pH and low temperature buffer containing 0.1% (v/v) TFA in 3 M urea (held at 4 °C). Samples were then passed across an immobilized pepsin column (prepared in house) at 50 μ L/min (0.1% (v/v) TFA, 15 °C). The resulting peptides were trapped on a C8 trap cartridge (Hypersil Gold, Thermo Fisher). Peptides were then gradient eluted from 4% (w/v) CH₃CN to 40% (w/v) CH₃CN, 0.3% (w/v) formic acid over 5 min at 4 °C across a 1 X 50-mm C18 HPLC column (Hypersil Gold, Thermo Fisher) and electrosprayed directly into an Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher). Peptide ion signals were confirmed if they had a MASCOT score of 20 or greater and had no ambiguous hits using a decoy (reverse) sequence in a separate experiment using a 60 min gradient. The intensity-weighted average m/z value (centroid) of each peptide's isotopic envelope was calculated with software developed in house [54] and corrected for back-exchange on an estimated 70% recovery and accounting for the known deuterium content of the on-exchange buffer. HDX analyses were performed in triplicate, with single preparations of each purified protein/complex. The intensity weighted mean (m/z) centroid value of each peptide envelope was calculated and subsequently converted into a percentage of deuterium incorporation. This is accomplished determining the observed averages of the undeuterated and fully deuterated spectra and using the conventional formula described elsewhere [93]. Statistical significance for the differential HDX data is determined by an unpaired t-test for each time point, and is integrated into the HDX Workbench software [54].

Data Rendering

In order to visualize the differential exchange data for each of the comparisons made, we calculated the average percentage of deuterium uptake for each sequenced peptide following 10, 30, 60, 300, 900, and 3600 s of on-exchange, and then we subtracted the average percentage of deuterium uptake for the corresponding sample (*i.e.*, GDP – apo, GTP γ S – apo, GTP γ S – GDP, and trimer – monomer). We then used a residue averaging approach to obtain better resolution of the data. For each residue, the deuterium incorporation values and peptide lengths from all overlapping peptides were assembled. A weighting function was applied in which shorter peptides were weighted more heavily and longer peptides were weighted less. Each of the weighted deuterium incorporation values were then averaged to produce a single value for each amino acid. The initial two residues of each peptide, as well as prolines, were omitted from the calculations. This approach is similar to that previously described [94]. These single residue values were plotted using GraphPad Prism software. The data were also color coded based on magnitude and direction, and the resulting heat map data were overlaid onto crystal structures of the proteins, where available. A previously published structure for ARL2 (PDB ID: 3DOE [42]) and a homology model for human β -tubulin (based on PDB ID: 3RYC [95]) were used. For each of these structures, the bound nucleotide (GTP for ARL2 and GDP for β -tubulin) was included in the figure (shown in magenta) to highlight the nucleotide-binding pocket of each protein.

Reproducibility and Statistical Analyses

All nucleotide binding and thermal denaturation assays described have been independently repeated at least twice. HDX analyses were performed in triplicate, with single preparations of each purified protein/complex. Statistical significance for the differential HDX was determined as described above.

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Abbreviation used in the text include

GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
Gpp(NH)p	5'-[β , γ -imido]triphosphate
GST	glutathione S-transferase
HDX-MS	Hydrogen/Deuterium exchange coupled with mass spectrometry
mant	<i>N</i> -methylantraniloyl
TBCD	tubulin-specific chaperone D

TBCE tubulin-specific chaperone E

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Highlights

- The assembly of the $\alpha\beta$ -tubulin heterodimer is a poorly understood process.
- We purified a novel complex of TBCD/ARL2/ β -tubulin.
- The purified trimer has distinct nucleotide binding characteristics.
- We conclude that the cycling of nucleotide on ARL2 regulates dimer assembly.

Figure 1. The molecular dynamics of TBCD in the TBCD•ARL2• β -tubulin complex are minimally affected by nucleotide binding to the trimer

We analyzed purified TBCD•ARL2• β -tubulin (10 μ M) by HDX-MS before (apo) and after pre-incubation with GDP or GTP γ S (100 μ M each). The average percent of deuterium exchange over 1 hr was determined for each sample, and datasets were then compared to determine the change in deuterium uptake between each of the nucleotide-bound states. For each of the comparisons, the average percent change in deuterium uptake was determined and plotted for each residue in the TBCD sequence. Blue lines show the result of subtracting values from the apo-trimer from those of the GDP-bound trimer. Red lines show differential data of GTP γ S minus the apo-complex and green lines for the GTP γ S minus GDP data. Dotted lines indicate regions of the protein not covered in the mass spectrometry analysis. Positive values indicate an increase in deuterium uptake and negative values indicate a decrease in uptake.

Figure 2. The binding of guanine nucleotides to TBCD•ARL2• β -tubulin causes moderate changes throughout the β -tubulin protein

Purified TBCD•ARL2• β -tubulin (10 μM) was pre-incubated with a 10-fold molar excess of GDP, GTP γ S or no nucleotide (apo) and analyzed by HDX-MS, as described in the legend to Figure 4. **(A)** The change in the average percent of deuterium uptake among the three samples was determined per residue of β -tubulin. Asterisks above the graph indicate regions of the protein involved in direct interaction with nucleotide, based upon published structures. Dotted lines indicate regions of the protein not covered by MSMS. **(B)** Differential data were converted to heat map form and overlaid onto a homology model for human β -tubulin (based on PDB ID: 3RYC). The nucleotide-binding pocket is shown in magenta. Gray represents regions not sequenced by mass spectrometry. N, amino terminus. C, carboxyl terminus.

Figure 3. Binding of guanine nucleotides by the TBCD•ARL2• β -tubulin trimer results in highly localized changes in deuterium uptake around the nucleotide binding pocket of ARL2

Purified trimer (10 μM) was pre-incubated with 100 μM GDP, GTP γ S or no nucleotide (apo) before being analyzed by HDX-MS. **(A)** The changes in deuterium uptake at each residue of ARL2 were compared among the three samples. Dotted lines indicate regions not covered by MSMS. **(B)** The differential deuterium exchange data were overlaid onto an ARL2 structure (PDB ID: 3DOE). The nucleotide binding site is highlighted in magenta. Gray indicates regions not covered by mass spectrometry sequencing. N, amino terminus. C, carboxyl terminus.

Figure 4. Nucleotide binding to ARL2 monomer results in distinct changes to the G motifs and switch regions of the protein

ARL2 monomer was overexpressed and purified from HEK cells, as described in Materials and Methods. Purified ARL2 (10 μM) was pre-incubated with 100 μM GDP, GTP γ S or no nucleotide (apo) before analysis by HDX-MS. **(A)** The change in average percent of deuterium uptake was determined among the three samples at each residue. Dotted lines indicate regions not covered by mass spectrometry. Shown above the graph are the locations of conserved nucleotide binding motifs, G1–G4, the two canonical switches (Sw I/II), and α -helices and β -strands, based upon published structures. **(B)** We converted the differential deuterium exchange data to a heat map and overlaid it onto a previously published crystal structure of ARL2 (PDB ID: 3DOE). The nucleotide-binding site is shown in magenta. Gray indicates regions not sequenced by mass spectrometry. N, amino terminus. C, carboxyl terminus.

Figure 5. Comparing ARL2 monomer to the TBCD•ARL2• β -tubulin trimer reveals putative sites of protein-protein interaction

ARL2 monomer or TBCD•ARL2• β -tubulin trimer were prepared and analyzed by HDX-MS, as described in previous legends and under Materials and Methods. **(A)** The changes in deuterium exchange were determined per residue for ARL2 by subtracting the average rate of deuterium exchange at each residue in the monomer from the same residue in the trimer after incubation with no nucleotide (apo; blue line), GDP (red line), or GTP γ S (green line). Dotted lines indicate regions not sequenced by mass spectrometry. **(B)** The differential exchange data were overlaid onto an ARL2 crystal structure (PDB ID: 3DOE) to highlight the localized changes in deuterium uptake. The nucleotide binding pocket is highlighted in magenta. Gray indicates regions not covered by mass spectrometry sequencing. N, amino terminus. C, carboxyl terminus.

Figure 6. TBCD•ARL2•β-tubulin trimer and ARL2 monomer bind GTP

Human GST-TBCD was co-expressed with human ARL2 (green circles) or ARL2[T30N] (orange diamonds) in HEK cells and affinity purified as trimers, as described under Materials and Methods. Bovine GST-TBCD•ARL2•β-tubulin was similarly purified (blue triangles) for assay. We expressed and purified ARL2 monomer (red boxes) from BL21(DE3) cells. TBCD complexes and ARL2 monomer (1 μM each) were incubated with 10 μM [³⁵S]GTPγS at 30 °C and nucleotide binding was determined at various time points, using the filter trapping assay. Samples were analyzed in duplicate. *Bt*, *Bos taurus*.

Figure 7. Nucleotide binding does not alter the thermal stability of the TBCD•ARL2• β -tubulin complex

We used the ThermoFluor assay to determine the thermal stability of purified TBCD•ARL2• β -tubulin after the addition of GDP (red squares), GTP γ S (green triangles) or no nucleotide (apo; blue circles). Samples were mixed with SYPRO Orange dye and the fluorescence was measured every 2 min for 1 hr, while the temperature was increased 0.5°C/min. Melting points (T_m) were determined as the half-maximal change in fluorescence.

Figure 8. The TBCD•ARL2• β -tubulin trimer has a higher affinity than does ARL2 for guanine nucleotides

Kinetics of nucleotide binding to TBCD•ARL2• β -tubulin (red circles) or ARL2 monomer (green circles) were determined by monitoring changes in fluorescence of mant-Gpp(NH)p in a solution-based assay. (A) We incubated TBCD•ARL2• β -tubulin trimer or ARL2 (2 μ M each) with 1 μ M mant-Gpp(NH)p and the fluorescence intensity was measured at room temperature for 20 min, at which point excess (100 μ M) unlabeled Gpp(NH)p was added and monitoring of fluorescence continued for another 20 min. Data were normalized to percent maximal signal. (B) On- and off-rates were determined, using GraphPad Prism software, and to calculate apparent affinities ($K_{D(app)}$) and R^2 values. Solid lines represent the modeled curves generated when no constraint was set for non-specific binding. (NS = no constraint). Rates and apparent affinities were also determined using the same data but fixing the zero time point to zero (NS = 0). *S.E.*, standard error. *NS*, no constraint.

Figure 9. Model for the role of ARL2 as an obligate component in the cofactor-mediated folding and assembly of the αβ-tubulin heterodimer

ARL2 is proposed to play an integral role in regulating the folding and production of native tubulin dimers. **1.** Partially folded β-tubulin polypeptides are transferred from TBCA to a complex of TBCD•ARL2-GDP. **2.** The binding of β-tubulin promotes binding of GTP on ARL2, which in turn facilitates the progression of β-tubulin to different folded state, presumably one more conducive to binding of α-tubulin. **3.** Association of TBCE•α-tubulin and TBCC brings the tubulin subunits together and results in formation of the cofactor/tubulins super complex. **4.** GTP hydrolysis on ARL2 promotes the dissociation of TBCE and TBCC with predicted release of the native αβ-tubulin heterodimer and TBCD•ARL2. The TBCD•ARL2-GDP dimer is predicted to remain intact, based their previously established co-purification, and ready to accept another β-tubulin polypeptide for continuation through the folding cycle.